UNIVERSITAT DE BARCELONA

Nutritional management of Myotonic Dystrophy type 1

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

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MARTA PASCUAL GILABERT 2022

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RECERCA, DESENVOLUPAMENT I CONTROL DE MEDICAMENTS

NUTRITIONAL MANAGEMENT OF MYOTONIC DYSTROPHY TYPE 1

Memòria presentada per Marta Pascual Gilabert per optar al títol de doctora per la Universitat de Barcelona

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Als meus pares

Al Sergi i a l'Èric

A tots els qui estimo

A la mare, *in memoriam*

Una dona forta, qui la trobarà? És més preciosa que les perles. El seu marit confia en ella: no enyorarà cap més tresor; en rebrà benestar i no malestar tots els dies de la vida. Ella es procura llana i lli, i les seves mans treballen amb delit. Com les naus d'un mercader, porta de lluny les provisions. És de nit i ja es lleva; prepara el menjar per als de casa i la feina a les criades. Es mira un camp i el compra, amb el fruit del seu treball planta una vinya. Es posa amb energia a la feina, fa anar els braços amb vigor. Comprova que els negocis marxen bé: en plena nit encara té encesa la llàntia. Les seves mans agafen el fus, buida que buida la filosa. Obre la mà als pobres, l'allarga als necessitats. Si neva no pateix pels de casa: doble abric porten tots ells. Ella mateixa es teixeix les flassades, es vesteix de porpra i de roba de fil. El seu marit és respectat en l'assemblea, quan té sessió amb els notables del país. Teixeix teles per a vendre, proveeix de cenyidors els marxants. Va vestida amb honor i dignitat, es riu del dia de demà. Parla amb sensatesa, els seus llavis instrueixen dolçament. Vetlla per la marxa de la casa, no menja el pa sense guanyar-se'l. Els fills van a felicitar-la, el marit en canta les lloances: «Hi ha moltes dones fortes, però tu les guanyes totes!» L'encís és enganyós, la bellesa s'esvaeix; la dona que venera el Senyor mereix de ser lloada. Reconeixeu-li el fruit del seu treball: per tot el que fa, rebrà pública lloança.

Proverbis 31:10-31

I quan la tempesta s'acabi, no sabràs com te n'has sortit ni com hi has pogut sobreviure. Ni tan sols sabràs del cert si s'ha acabat. Però una cosa és segura: quan en surtis no seràs el mateix que hi va entrar. Aquest és el sentit de la tempesta de sorra.

H. Murakami

Kafka a la platja

AGRAÏMENTS

Hi ha un proverbi africà que diu “Si vols anar ràpid vés sol, si vols arribar lluny vés acompanyat”. No sé si he arribat lluny, però sí sé que el camí l’he fet amb molt bona companyia. Per això, escriure aquestes dues pàgines ha estat probablement la part més difícil de tota la memòria de tesi, i no pas per no tenir clar a qui mencionar, sinó per voler trobar les paraules d’agraïment que millor puguin expressar el doll de sentiments que afloren en recordar a tots els qui m’han acompanyat fins aquí. Per això, vagin per endavant les meves més sinceres disculpes als qui pugui haver omès en escriure-les.

En primer lloc vull agrair als meus co-directors de tesi, el Dr. Josep Castells i el Dr. David Miguel haver dipositat la seva confiança en mi per dur a terme un projecte tan ambiciós i acompanyar-m’hi amb il·lusió i perseverança des del principi fins a la fi, malgrat totes les dificultats esdevingudes pel camí. Els estic immensament agraïda pels seus bons consells, dedicació i rigor que ben segur qualsevol tesi doctoral requereix, però que en el camp de les malalties minoritàries porta associat un compromís personal addicional.

També vull agrair a la Dra. María Carmen López Sabater, tutora d’aquesta tesi a la Facultat de Farmàcia i a la Dra. Marisa García, coordinadora del Programa de Doctorat en Recerca, Desenvolupament i Control de Medicaments de la UB, la seva amabilitat i acompanyament en tots els dubtes plantejats i les gestions necessàries per assolir les diverses fites del procés.

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A tot l’equip d’Inkemia, IUCT i Myogem Health Company, companys i companyes professionals, i a la vegada amics i amigues personals, un agraïment en majúscula per les dues dècades que hem viscut plegats tant intensament. Permeteu-me no posar els vostres noms, em fa por oblidar-me algú… No hi ha paraules per agrair tot el recolzament, respecte, confiança i estima que sempre vaig rebre de vosaltres, i que no sé si sempre vaig saber correspondre en la mateixa mesura, centrada i molt sovint abduïda com he estat en la feina i en els molts projectes que hi hem desenvolupat. M’agradaria fer una menció especial per la Paqui, en Javi, l’Héctor, en Marco, la Maria, la Carme, en Jesús i en Joan. Junts vam fer possible que la composició es transformés en formulació i la formulació en producte. I per als meus estimats *Drug Discoverers* pel seu suport en tot moment. Us estimo molt i sempre us duré en el cor!

Al grup de Genòmica Translacional de la Facultat de Ciències Biològiques de la Universitat de València, un agraïment per la feina feta en els models *in vitro* i *in vivo* de DM1. M’agradaria fer un reconeixement especial al seu líder, referent mundial en DM1, Prof. Rubén Artero, pels seus bons consells científics i tot el suport rebut a nivell personal per poder culminar la tesi.

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de l'inici del projecte. El seu recolzament va ser fonalmental per fer sortir aquesta recerca del laboratori i dur-la a prop de la comunitat de DM1. *Un abrazo, Antonio*.

Al Dr. Jordi Roma, amb qui vam iniciar el camí dels estudis clínics, un agraïment pels seus ensenyaments i la paciència amb que ens va anar guiant per un món tan complex i regulat.

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Als meus companys d'R+D en l'àmbit de l'alimentació, Alfonso i Alberto, en qui vaig trobar uns excel·lents professionals, un puntal personal i uns amics per sempre. *¡Muchas gracias chicos!*

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Als meus amics i amigues de sempre, pel seu recolzament malgrat la distància i la pandèmia, i en especial a la Montse. Tan de bo hagués pogut compartir aquests moments amb tu, bonica.

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Als meus pares, Jaume i Antonieta, simplement per TOT. Perquè absolutament res hagués pogut esdevenir ni tindria sentit sense vosaltres. Com agrair en tan poques línies l'amor incondicional que m'heu donat, els valors que m'heu ensenyat, els sacrificis, l'esforç i tot el suport que m'heu donat durant tota la vida? Si hi ha un AGRAÏMENT en majúscules, és per a vosaltres, pares estimats!

Al Sergi, com puc agrair tot el que has fet per mi d'ençà que ens vàrem conèixer a la Facultat de Química? Amic, marit i company de vida, has viscut amb mi els moments més dolços i més difícils als que he hagut de fer front, i certament t'has endut la pitjor part dels meus nervis, dubtes i angoixes. Sense tu, aquesta tesi mai hagués estat ni arribat al final. T'estimo molt!

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A tots i a totes, de tot cor, gràcies infinites!

ABSTRACT

Myotonic dystrophy type 1 (DM1), one of the more complex diseases already known, is the most common rare, multisystemic neuromuscular disease among adults, with no cure and limited pharmacological treatments. In the light of the ´Leave No-one Behind´ commitment behind Sustainable Development Goals, the nutritional management of DM1 may be a valuable strategy to improve the very limited quality of life of people living and suffering this minority disease.

Our previous background research suggested that caffeine might elicit some positive effects in DM1. Therefore, the present investigation aimed to evaluate the potential of methylxanthines in order to scientifically substantiate properly formulated food products based on such methylxanthines as a novel nutritional management approach in Myotonic Dystrophy type 1.

In this regard, the research disclosed herein comprises: technology surveillance on nutritional studies in DM1 and natural sources of methylxanthines; food content and consumption of methylxanthines of natural origin; the regulatory framework for food products, in particular, food supplements and food for special medical purposes; as well as the European requirements for launching of such food products on the market. To this end, the project required the complete research and development of the investigational food product, from the definition of the composition through *in vitro* and *in vivo* studies in DM1 models, to the definition of dosage and development of the dosage form, while carefully taking into consideration intellectual property constraints, such as patent protection status and freedom to operate studies.
Then, an observational clinical study evaluating the background nutritional habits in a cohort of adults with DM1 precedes the interventional clinical trial (study code NCT04634682) to measure the effect of the investigational food product on quality of life, fatigue and hypersomnia in adult patients with DM1.

The data shown herein suggest that the controlled and dosaged administration of the investigational food product could provide statistically significant improvements in decreasing excessive daytime sleepiness and increasing the 6-minute walking distance, at least in adult DM1 male. Excessive daytime sleepiness has been rated as one of the main aspects limiting the quality of life in DM1.

Although the present pilot study covered a limited data set, the obtained results encourage further clinical trials in larger cohorts, ideally to be performed beyond the COVID-19 pandemic era.

RESUM

La Distròfia Miotònica de tipus 1 (DM1), una de les malalties més complexes que es coneixen, és una malaltia rara neuromuscular i multisistèmica, la més freqüent en adults, sense cura i amb tractaments farmacològics limitats. En el marc de 'No deixar ningú enrere' associat als Objectius de Desenvolupament Sostenible, el maneig nutricional de la DM1 pot proporcionar una estratègia valuosa per millorar la tant limitada qualitat de vida de les persones que conviuen i pateixen aquesta malaltia minoritària.

La nostra recerca prèvia suggeria que la cafeïna podia induir alguns efectes positius en DM1. Per tant, la recerca actual tenia com a objectiu avaluar el potencial de la família de metilxantines per fonamentar científicament productes alimentaris basats en metilxantines, addientment formulats, com a una nova aproximació al maneig nutricional de la DM1.

En aquest sentit, la recerca que s'ha dut a terme inclou: vigilància tecnològica d'estudis nutricionals en DM1; fonts naturals de metilxantines; contingut i consum de metilxantines presents en fonts naturals alimentàries; el marc regulatori dels productes alimentaris, particularment, complements alimentosos i aliments per a usos mèdics especials; així com els requeriments regulatoris europeus necessaris per poder comercialitzar aquests productes. Per tal d'assolir els seus objectius, el projecte requeria de la recerca i desenvolupament complerts del producte en investigació, des de la definició de la seva composició a partir de resultats d'estudis en models *in vitro* and *in vivo* de DM1, a la definició de dosi i desenvolupament de la forma galènica, i a la vegada considerant especialment possibles limitacions en matèria de propietat intel·lectual i industrial, com ara l'estat de protecció en patents i estudis de llibertat operacional.

A continuació, l'estudi clínic observacional per avaluar els hàbits nutricionals de base en una cohort d'adults afectats per DM1 va precedir l'estudi clínic intervencionista (codi estudi a clinicaltrials.gov NCT04634682) per mesurar l'efecte del producte en investigació en la qualitat de vida, fatiga i somnolència diürna excessiva en malalts adults de DM1.

Els resultats assolits i descrits en aquesta memòria suggereixen que l'administració controlada i dosificada del producte en investigació pot proporcionar millores estadísticament significatives en la disminució de la somnolència diürna excessiva i un increment en la distància recorreguda durant sis minuts, almenys en homes adults afectats per DM1. La somnolència diürna excessiva s'ha considerat un dels principals factors que limiten la qualitat de vida en DM1.

Malgrat que aquest estudi pilot es va realitzar en un grup limitat de participants, els resultats obtinguts encoratgen dur a terme nous estudis clínics en cohorts més grans, idealment per dur a terme un cop superada la pandèmia de COVID-19.

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6MWT	6-Minute walk test
AAV	Adeno-associated virus
ACSA	*Agència Catalana de Seguretat Alimentària*
ADHD	Attention deficit hyperactivity disorder
AE	Adverse event
AEMPS	*Agencia Española de Medicamentos y Productos Sanitarios*
AESAN	*Agencia Española de Seguridad Alimentaria y Nutrición*
ALT	Alanine transaminase
APP	Amyloid beta precursor protein
AR	Adenosine receptor
ASEM	*Asociación Española de Enfermedades Neuromusculares*
ASO	Antisense oligonucleotide
AST	Aspartate transaminase
ATP	Adenosine triphosphate
ATP2A2	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase 2
BIN1	Bridging integrator 1
BMI	Body mass index
BOE	*Boletín Oficial del Estado*
BP	Bodily pain
Bw	Body weight
ca	*circa*
CACNA1S	Calcium channel CaV1.1
CAFF	Caffeine
CAG	Cytosine-adenine-guanine
Calc	Calculated
CAPN3	Calpain 3
Cas9	CRISPR associated protein 9
CCTG	Cytosine-cytosine-thymine-guanine
CEIm	*Comité Ético de Investigación con medicamentos*
CELF	CUG-BP1 and Elav-like protein family
CF	Caffeine
CFCA	Food consumption frequency questionnaire
cfu	Colony-forming unit
cGCP	current Good Clinical Practices
cGMP	current Good Manufacturing Practices
CI	Confident interval
CK	Creatinine
CK18	Cytokeratin-18 fragments
CLCN1	Muscle-specific chloride channel
CMD	Congenital myotonic dystrophy
CMO	Contract manufacturing organization
CNBP	CCHC-type zinc finger nucleic acid-binding protein

CNS	Central nervous system
COVID-19	Coronavirus disease 2019
CRF	Case report form
CRISPR	Clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CTG	Cytosine-thymine-guanine
CYP	Cytochrome
DM	*Dystrophia myotonica*
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMD	Duchene muscular dystrophy
DMPK	DM1 protein kinase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRI	Daily recommended intake
DTNA	Dystrobrevin-α
DTT	Dithiothreitol
DRV	Dietary reference value
ECG	Electrocardiogram
EFSA	European Food Safety Agency
EMA	European Medicines Agency
EOS	End of study
EOT	End of treatment
EP	European Pharmacopoeia
ESS	Epworth's sleepiness scale
EU	European Union
Exp	Experimental
FAM	6-Carboxyfluorescein
FBDG	Food-based dietary guidelines
FDA	Food Drug Administration
FIH	First-in-human
FIS	Fatigue impact scale
FISH	Hybridation buffer solution containing formamide and dextran sulfate
FS	Food supplement
FSMP	Food for special medical purposes
GABA	Gamma-aminobutyric acid
GGT	Gamma glutamyl transferase
GH	General health
GPAQ	Global Physical Activity Questionnaire
HDL	High-density lipoprotein
HED	Human equivalent dose
HRQoL	Health-related quality of life
HSA^{LR}	Human skeletal alpha-actin long repeat
IC	Informed consent
IFM	Indirect flight muscles

INCLIVA	*Instituto de Investigación Sanitaria*
IND	Investigational new drug
INQOL	Individualized Neuromuscular Quality of Life
INSR	Insulin receptor
IQR	Interquartile range
IQS	Institut Químic de Sarrià
IUCT	Institut Univ. de Ciència i Tecnologia
JORF	*Journal officiel de la République française*
LD50	Lethal dose 50
LDL	Low-density lipoprotein
MAPT	Microtubule-associated protein tau
MBNL	Muscleblind Like protein family
MBNL1	Muscleblind Like Splicing Regulator 1
MBNL2	Muscleblind Like Splicing Regulator 2
MCS	Mental component summary
MDF	Myotonic Dystrophy Foundation
MH	Mental health
MHLW	Ministry of Health, Labour and Welfare
MIFS	Modified impact fatigue scale
miRNA	Micro ribonucleic acid
MIRS	Muscular impairment rating scale
mRNA	Messenger ribonucleic acid
MRSD	Maximum recommended starting dose
MS	Metabolic syndrome
MTMR1	Myotubularin-related protein 1
MX	Methylxanthine
MYODM	Trade name of the investigational food product as food supplement
MYOM1	Myomesin 1
N	Population
NA	Non-applicable; non-available
NCE	New chemical entity
NCT	Number of the clinical trial
NMD	Neuromuscular disease
NMDAR1	*N*-methyl-*D*-aspartate receptor
NMPA	National Medical Products Administration
NP	Not published
NW	Normal weight
OMIM	Online Mendelian inheritance in man
OW	Overweight
PCS	Physical component summary
PDE	Phosphodiesterase
PF	Physical functioning
Pip6A-PMO	Peptide 6 A linked to phosphorodiamidate morpholino oligomer
PKM2	Pyruvate kinase M2
PROM	Primary outcome measure

PROMM	Proximal Myotonic Myopathy
QOL	Quality of life
RBFOX2	RNA Binding Fox-1 Homolog 2
RE	Role emotional
RNA	Ribonucleic acid
RNAi	RNA interference
RNase H	Bovine pancreatic ribonuclease
RP	Role physical
RTD	Ready to drink
RyR1	Ryanodine receptor
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SERCA1	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase 1
SERCA2	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase 2
SCN5A	α- subunit of the cardiac voltage channel NaV1.5
SD	Standard deviation
SDG	Sustainable Development Goals
SEEN	*Sociedad Española de Endocrinología y Nutrición*
SEM	Standard error of the mean
SF	Social functioning
SF-36	Short form 36 health survey questionnaire
SOS1	Son of sevenless homolog 1
TAMC	Total aerobic microbial count
TCH	Total cholesterol
TB	Theobromine
TG	Triglycerides
TNNT2	Cardiac troponin T2
TNNT3	Fast troponin T3
TTN	Titin
TYMC	Total yeast and mold count
USA	United States of America
UTR	Untranslated region
UVEG	*Universitat de València Estudis Generals*
UW	Underweight
VT	Vitality
w/w	Weight/weight ratio
WHO	World Health Organization
WT	Walking test
WT	Wild-type
YW	Yellow-white *Drosophila melanogaster* strain
ZASP (LDB3)	LIM Domain Binding 3
ZNF9	Zinc finger 9 gene

INTRODUCTION

Rare diseases

Since the concept 'rare disease' emerged for the first time in literature in 1867,[1] more than 300,000 publications have been published, as retrieved from PubMed.gov database as per May 2022.[2] However, currently there is still no universal definition of what a rare disease is, and surprisingly, different definitions are used by different jurisdictions around the world, mostly related to the prevalence concept,[3] *i.e.* the proportion of patients in a population who have a specific characteristic in a given time.

In general terms, a rare disease is a health condition that affects a relatively small number of people compared with other prevalent diseases (those more common, usual and widespread diseases) which affect the majority of the population.

In the US, through the pioneer Orphan Drug Act in 1983,[4] the FDA defined the term 'rare disease or condition' as any disease or condition which:

> (A) Affects less than 200,000 persons in the United States, or
>
> (B) Affects more than 200,000 in the United States and for which there is no reasonable expectation that the cost of developing and making available in the United States a drug for such disease or condition will be recovered from sales in the United States of such drug.

Following the US actions on rare diseases, in 1993 the Ministry of Health, Labour and Welfare (MHLW) in Japan launched the Orphan Product Development Support Program for those drugs intended for use in diseases affecting less than 50,000 patients in Japan (less than 3.9 per 10,000 individuals approximately), and for which there is a high medical need.[5]

In the European Union, a rare disease is defined as a life-threatening or chronically debilitating condition affecting not more than 5 in 10,000 persons. Formally, the concept of rare disease was used in the public health field in 2008,[6] although implicitly it first appeared in EU legislation in 1999.[7] The European Commission estimates that approximately 5,000-8,000 distinct rare diseases affect 6-8% of the EU population *i.e.* between 27 and 36 million European citizens.[8]

In China, there is no official legislation defining the term rare disease, and even there are no figures on the number of people with rare diseases, although some sources estimate that more than 20 million Chinese citizens could be affected by a rare disease.[9] Based on the latest definition suggested in 2021, in China 'a rare disease is defined as a condition with an incidence of less than 1/10,000 among newborns, a prevalence of less than 1/10,000, or an affected population of less than 140,000'.[10]

In Latin America, the situation is quite heterogeneous among the different countries. Mexico, Argentina, Chile and Colombia use the EU definition and prevalence of rare diseases. Brazil considers the prevalence to be not more than 65 in every 100,000. Peru has no official definition of a rare disease.[11]

In Africa, rare diseases' prevalence is understudied, as many challenges need to be addressed. Moreover, the needs of people living with rare diseases must also be balanced with basic needs, such as nutrition and communicable-disease prevention.[12]

It is estimated that around 7,000 rare diseases exist,[13] mostly from genetic origin and Mendelian inheritance, in all therapeutic areas including but not limited to neurological diseases, respiratory diseases, cancer (both solid tumours and haematological diseases), diseases of the heart, vascular diseases, bone and skin disorders, neuromuscular diseases, eye diseases, immunodeficiency, autoinflammatory and autoimmune diseases, endocrine conditions, kidney diseases, hepatological diseases, urogenital diseases, metabolic disorders, and the like, both in adult and children populations.

Extensive and updated high-quality information for each particular already classified rare disease, in terms of signs and clinic symptoms, molecular genetics and inheritance pattern, pathogenesis, diagnosis, clinical management and the like, can be found in OMIM[14] and Orphanet[15] databases. Overall, some authors pointed out that under a conservative approach, around 263–446 million persons could be affected globally by a rare disease at any point in time.[16]

Unfortunately, only less than 6% of rare diseases have some related approved treatment[17] (accounting for around 204 drugs with approval and/or marketing authorization in the FDA, EMA databases and/or China's NMPA databases as per June 2021),[18] highlighting the extraordinary inequity that exists for people living and suffering from a rare disease.

Worldwide, through orphan drug designation status, drug regulators offer incentives and market advantages to encourage pharmaceutical companies to research and develop medicines for the treatment, prevention or diagnosis of rare diseases, provided that certain criteria are met.[19,20]

Traditionally, the main therapeutic approaches include small molecules, drug repurposing, and biologics such as monoclonal antibodies, protein replacement therapies and oligonucleotides, which are considered the gold standard strategies to cure or treat diseases in general and rare diseases in particular.[21] Currently, some other more technologically challenging such as gene and cell therapies have started to change the conventional paradigm in rare diseases treatments[22] and arise as promising approaches to correct or replace a defective gene in the next forthcoming years, as envisioned by the interest of regulatory agencies and leading innovative pharmaceutical companies.[23]

Rare neuromuscular diseases

1.1. *Introduction to neuromuscular diseases*

Neuromuscular diseases (NMDs) are a broad and heterogeneous group of neurologic diseases that result from disorders or lesions in the peripheral nervous system, wherein the functioning of neural structures, including the motor nerves, neuromuscular junctions, or the muscles themselves are affected.[24,25] As a result, the global motor system is impaired to achieve its main goal: the production of body force and movement.

The origin of these chronic diseases may be found in inherited underlying defects in the genes that encode muscle proteins or due to defects in the nerves, as well as acquired traumas and injuries.

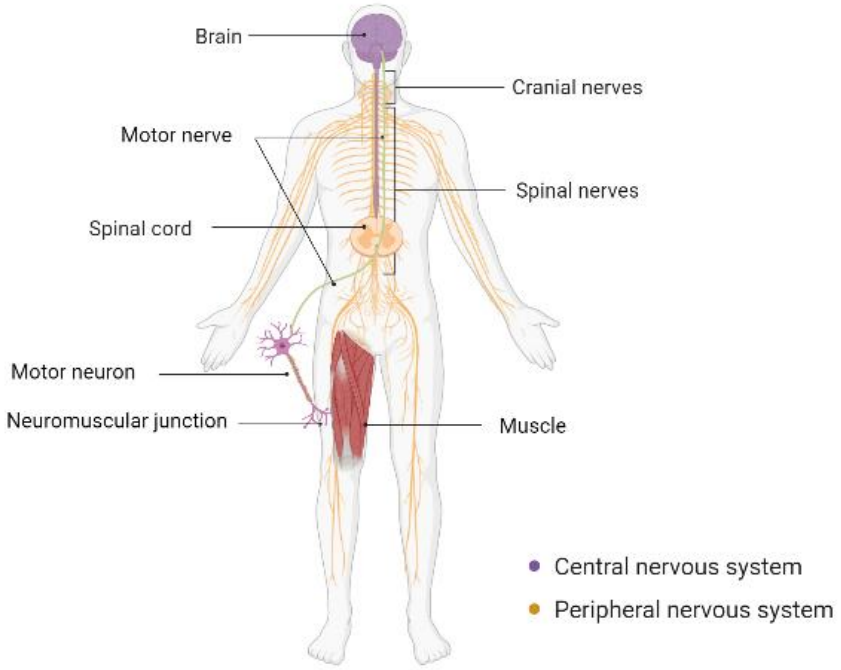

Figure 1: The motor system. *Adapted from* 'The major components of the nervous system', *by BioRender.com (2022)*

Motor nerves are bundles of motor neurons, which are a mosaic of cell types of neuronal cells located in the central nervous system covering a broad range of functions. Motor neurons are divided into upper motor neurons, those that connect the cerebral cortex with the spinal cord, and lower motor neurons, those that connect the brainstem and spinal cord with the effector muscles in the periphery. Upper motor neurons give initial instructions whereas lower motor neurons perform the above-mentioned instructions.[26] Particularly, spinal cord motor nerves are the longest known cell type, extending through several meters in mammals.[26]

The neuromuscular junction is the site where the motor neuron makes contact with the membrane of skeletal muscle fiber, in order to efficiently transmit the motor impulse to the muscle fiber and eventually induce force and movement.

Muscles, soft tissues formed by bundles of elongated cells with the ability to contract and produce movement, can be anatomically and functionally divided into three classes which are different in terms of structure, function and regulation:

- Cardiac muscles: those exclusively found in the heart, which are involved in its rhythmic contraction in order to pump blood through the circulatory system.
- Smooth muscles: those that control the diameter of blood vessels and the internal digestive and excreting organs, and that are involuntarily controlled.
- Skeletal muscles: those responsible for posture and movement, which are firmly attached to the skeleton by the tendons. Skeletal muscles are the most abundant muscle class, with around 639 different muscles in the human body. The function of skeletal muscles is to respond to neural control and efficiently transduce chemical energy (ATP) to force and movement.[26]

1.2. *Clinical symptoms of neuromuscular diseases*

Progressive muscle weakness is a central and the most common feature in neuromuscular diseases, as well as fatigue, muscle wasting, swallowing and breathing difficulties, and progressive loss of mobility.[27]

Usually, neuromuscular disorders main symptoms shared by other common central nervous system disorders[27] are:

- Weakness: is the main cause of disability in neuromuscular diseases, directly associated with the unpaired function of any component of the motor unit. Divided into proximal and distal weakness, according to the main parts of the body that are affected (from pelvic girdle, legs, feet, toes, to hands and fingers), weakness strongly impairs the mobility and autonomy of people suffering a neuromuscular disease.
- Fatigue: occurs related to weakness. Due to the damage in many muscle fibers, which are non-functional, the remaining non-damaged muscle fibers need to compensate for force, and often work at their metabolic limits. Fatigue could impair many different movements, from climbing a single step to trying to raise a leg.
- Sensory disturbances: include myalgia (muscle pain even at rest or with exertion), neuropathic pain, paresthesias (pins and needles sensation and tingling), dysesthesias (uncomfortable sensation particularly to touch various surfaces), hyperpathia (painful response to a non-painful stimulus), numbness, loss of sensation, imbalance.
- Reflexes: deep tendon reflexes assess the integrity of the motor system. In neuromuscular diseases, reflexes are depressed or even lost closely related to the degree of weakness or muscle atrophy.

However, beyond the above-mentioned common symptoms to some other CNS disorders, more particular symptoms which are more specific to neuromuscular disorders include abnormal muscle movements,[27] such as:

- Fasciculations: asynchronous twitches of groups or bundles of muscle fibers due to involuntary contractions of motor units.
- Myokymia: a form of continuous involuntary, repeated, 'wormlike' contractions of part of a muscle at rest, caused by spontaneous, repetitive firing of groups of motor unit potentials.
- Myotonia: muscle delayed relaxation or failure of muscle to relax immediately after stimulation or cessation of voluntary contraction.
- Muscle cramps: painful involuntary spasmodic muscular contractions involving part of or the entire muscle that last from seconds to minutes.
- Muscle atrophy: loss of skeletal muscle mass.
- Hypertrophy: increase in the volume of an organ or tissue due to the enlargement of its component cells.

1.3. *Classification of rare neuromuscular diseases*

Rare neuromuscular diseases are neuromuscular diseases that involve muscles, motor neurons, peripheral nerves or the neuromuscular junction, as previously disclosed, but for which their origin is related to genetic inheritance.

Single-gene diseases are often known as Mendelian diseases, in which the gene mutated or 'disease' allele is inherited and expressed under a dominant pattern (expressed when even one single copy of the mutation is present in the chromosomes) or recessive pattern (that requires two mutated copies for disease to develop).

As per 2021, more than 740 genes have been disclosed to be associated with monogenic neuromuscular diseases,[28] following different inheritance patterns. The gene table is continuously being updated as the knowledge of genetics increases and triggers deciphering the complexity of these diseases. However, beyond the single gene disorders and classic Mendelian pattern of inheritance, there are many other abnormalities originating from more complex origins, such as oligogenic and non-Mendelian inheritance patterns.[29] An illustrative classification of genetic neuromuscular diseases is disclosed in **Table 1**. In each case, a brief description and a representative but not exhaustive short list of related diseases per classification is provided.

The reason why we focused our attention and research particularly in myotonic dystrophy type 1 among such a substantial amount of rare diseases in general, and of rare neuromuscular diseases in particular, was because DM1 is the most frequent muscular dystrophy in adults and, due to its dominant nature compared to other genetic recessive diseases, which may be due to the lack of function of a gene, it was deemed more feasible to develop a pharmacological treatment for DM1. Moreover, at that time our group owned a proprietary compound library of new chemical entities (small molecules) that could be screened in the DM1 *in vitro* and *in vivo* models in collaborative drug discovery research programs. Boosted by that goal, the entire research program on DM1 was launched in our group.

Table 1: Classification of neuromuscular diseases (adapted from Muscular Dystrophy Association[30])

Classification	Description and related diseases
Motor neuron diseases[31]	Nerve cells called motor neurons (both lower and upper) progressively lose function, causing limb, bulbar and respiratory muscles they control to become weak and then gradually non-functional to death
	Amyotrophic lateral sclerosis
	Spinal-bulbar muscular atrophy
	Spinal muscular atrophy
Peripheral nerve diseases (peripheral neuropathies)[32]	In peripheral nerve diseases, the motor and sensory nerves that connect the brain and spinal cord to the rest of the body are affected, causing impaired sensations, movement and other body dysfunctions
	Charcot-Marie-Tooth disease
	Giant axonal neuropathy
Neuromuscular junction disorders[33]	Neuromuscular junction disorders result from the destruction, malfunction or absence of one or more key proteins involved in the transmission of signals between muscles and nerves
	Congenital myasthenic syndromes
	Lambert-Eaton myasthenic syndrome
	Myasthenia gravis
Mitochondrial diseases[34]	Mitochondrial diseases occur due to defective cellular energy production by the mitochondrial respiratory chain, which can affect any organ. The involvement of both muscle and nerve is common
	Friedreich's ataxia
	Mitochondrial myopathies
Ion channel diseases (channelopathies)[35]	Diseases associated with mutations in muscle voltage-gated sodium, potassium, calcium, chloride channels, and acetylcholine-gated channel cause a very wide range of renal, endocrine, bone, neurological, cardiac and skeletal muscle disorders.
	Andersen-Tawil syndrome
	Hyperkalemic periodic paralysis
	Hypokalemic periodic paralysis
	Myotonia congenita
	Paramyotonia congenita
	Potassium-aggravated myotonia
Myopathies[36]	Heterogeneous group of disorders affecting skeletal muscle originated by a dysfunction of the muscle fibers, that results in muscular weakness and pain
	Congenital myopathies
	Distal myopathies
	Endocrine myopathies
	Inflammatory myopathies
	Metabolic myopathies
	Myofibrillar myopathies
	Scapuloperoneal myopathy
Muscular Dystrophies[37]	Heterogeneous group of inherited diseases characterized by progressive wasting, weakness and degeneration of the skeletal muscles
	Becker muscular dystrophy
	Congenital muscular dystrophies
	Duchene muscular dystrophy
	Emery-Dreifuss muscular dystrophy
	Facioscapulohumeral muscular dystrophy
	Limb-girdle muscular dystrophies
	Oculopharyngeal muscular dystrophy
	Myotonic dystrophy Myotonic dystrophy type 1 (DM1) Myotonic dystrophy type 2 (DM2)

Myotonic dystrophy

1.4. *Background*

Myotonic dystrophy is a progressive multisystemic disorder, and the most common inherited form of muscle disease in adults.[37] Myotonic dystrophy has been classified as a muscular dystrophy within the neuromuscular disorders, since the main common affectations are myopathy (muscle weakness), myotonia (difficulties in relaxing muscles) and atrophy (muscle wasting that worsens over time). However, the complex mechanisms underlying myotonic dystrophy[38] is extended to the complex spectrum of the disease symptoms towards many other body systems that are also affected, such as the cardiovascular, respiratory, gastrointestinal, and cognitive, among others.[39] In this regard, myotonic dystrophy has been described as one of the more variable diseases found in medicine.[40] Beyond the muscular impairment, the central nervous system dysfunction extraordinarily limits the quality of life in DM1 patients.[41,42]

Figure 2 and **Table 2** illustrate the plethora of possible deleterious effects of myotonic dystrophy in the different body systems.[43] It is worth mentioning that not all the symptoms are necessarily simultaneously present in each single patient, but observed along the myotonic dystrophy community.

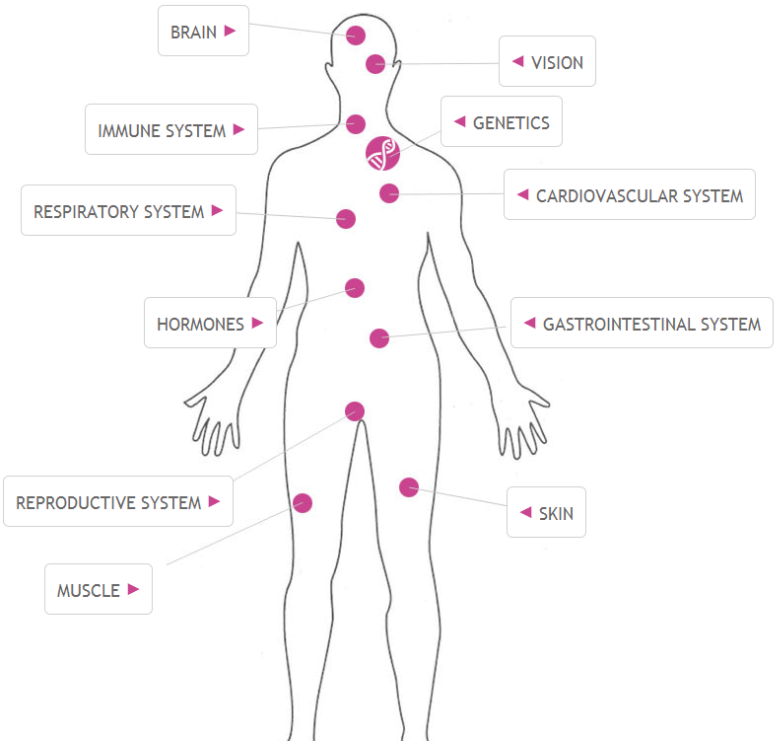

Figure 2: Myotonic dystrophy is a complex multisystemic disorder

Body system	Possible Effects
Skeletal muscles	• Muscle weakness (myopathy) • Muscle stiffness and trouble relaxing a muscle (myotonia) • Muscle wasting that gets worse over time (atrophy) • Severe muscle weakness and delayed development in newborns and infants
Cardiac system	• Heart rhythm problems (arrhythmias) • Enlarged heart muscle • Low blood pressure • Sudden death
Respiratory system	• Breathing problems in newborns • Frequent lung infections • Aspiration of food or fluids into airways • Inability to breathe in enough oxygen • Sleep apnea
Gastrointestinal (GI) system	• Difficulty swallowing • Pain and bloating after meals • Constipation, diarrhea, irritable bowel syndrome, gastrointestinal reflux • Gallstones • Enlarged colon
Brain and central nervous system (CNS)	• Difficulty with thinking and problem-solving • Emotional and behavior problems • Excessive daytime sleepiness • Nerve damage in feet and hands
Reproductive system	• Small testes, low sperm count, low testosterone • Higher risk of miscarriage and stillbirth; early menopause • Problems with pregnancy and delivery • Newborn complications
Hormones	• Insulin resistance • Premature frontal balding in men
Immune system	• Lower levels of antibodies in bloodstream
Tumors	• Higher risk of benign skin tumor (pilomatrixoma)
Vision	• Cataracts • Damage to the retina • Drooping eyelids (ptosis)

Table 2: Myotonic dystrophy is a complex multisystemic disorder

In this regard, a multidisciplinary approach and multidisciplinary teams are involved in the management of patients suffering the disease, as illustrated in **Table 3** (extracted from reference [43]).

Table 3: Symptoms and specialists involved in myotonic dystrophy management

Medical specialist	Complaint
Primary Care Physician	Exhaustion, inability to sleep well, excessive daytime sleepiness, feeling faint
Pediatrician	Hypotonia (also known as floppy baby syndrome) or child with learning and behavioral problems
Ophthalmologist	Blurry or dimmed vision (possible cataracts), eye muscle weakness, droopy eyelids (ptosis)
Cardiologist	Abnormal heartbeat, heart damage (cardiomyopathy), fainting spells
Pulmonary Specialist	Chronic respiratory problems, sleep apnea, frequent chest colds that do not go away, aspiration pneumonia caused by swallowing issues
Endocrinologist	Insulin resistance, benign thyroid mass
Dermatologist	Benign tumors associated with hair follicles (pilomatrixoma)
Gastroenterologist	Chronic diarrhea, constipation, unexplained stomach pain, gallstones, swallowing problems
Urologist and Reproductive Endocrinologist	Ectopic pregnancies, low testosterone, infertility, miscarriage, stillbirths
Psychiatrist	Depression, personality abnormalities such as excessive apathy, socialization issues, and attention deficit
Neurologist	Nerve and muscle complaints including weakness, stiffness, and chronic muscle pain, cognitive development delays, reduced executive function
Anesthesiologist	Respiratory failure before and after general anesthesia
Orthopedic Surgeon	Foot deformities, curvature of the spine
Podiatrist	Gait issues and muscle weakness
Plastic Surgeon/Oral Surgeon	Jaw and mouth bone deformities that disturb chewing and speech
Audiologist	Hearing loss
Speech Pathologist	Delayed or impaired speech, swallowing difficulties
Physical Therapist	Gait irregularities and muscle weakness

Dystrophia myotonica (DM) was first disclosed by the German doctor Hans Steinert in 1909.[44] Although he reported only six patient cases, they already exhibited the complexity in onset and inheritance pattern as it is currently known for the disease. In fact, Steinert described the classical form that is presently identified as myotonic dystrophy type 1 (DM1, OMIM 160900).

However, it was not until 1992 when the root cause of the disease was elucidated, as an unstable expansion of the cytosine-thymine-guanine (CTG) trinucleotide repeat in the 3' end untranslated region (UTR) of a transcript encoding a protein kinase family member, the *DM1 protein kinase* (DMPK) gene located in chromosome 19 locus 19q13.3, which codes for a myosin kinase expressed in skeletal muscle.[45,46,47] Moreover, that research led to the correlation between the number of repeat copies and the impact on patients, from unaffected individuals bearing less than 27 copies, to minimally affected patients with at least 50 repeats, and more severely affected patients from 50 to several thousand kilobase pairs.

In 1994, the identification of a new multisystemic disorder with similar characteristics to DM1 such as myotonia, proximal muscle weakness greater than distal weakness, and cataracts[48] but lacking the CTG repeat expansion[49] suggested the discovery of a new disease, which was referred to as 'Proximal Myotonic Myopathy' (PROMM). In 2001, genetics revealed that the origin of PROMM was associated to the unstable repeat expansion CCTG in intron 1 of the *CCHC-type zinc finger nucleic acid-binding protein* (CNBP) gene, previously known as zinc finger 9 gene, ZNF9, on chromosome 3 locus 3q21[50]. Currently, we refer to this disease as Myotonic dystrophy type 2 (DM2, OMIM 602668).

More than forty diseases following a non-Mendelian genetic inheritance pattern caused by expansions of simple oligonucleotide sequence repeats have been discovered as per 2022. Most of these currently known as 'repeat expansion diseases' impair the nervous system, both central and peripheral.[51] As a repeat expansion disease, myotonic dystrophy shows the characteristics of genotype-phenotype positive correlation between repeat length and disease severity, wherein phenotype may range from an asymptomatic form in short repeat sequences to very severe life-threatening impairments. Repeat length is associated with the onset of symptoms, being the longer the earlier it manifests. Anticipation, the tendency for disease severity to increase in successive generations of a family, is also a common feature, leading to the most severe disease congenital forms.[51] In myotonic dystrophy, the congenital form (CMD) which was identified in 1960[52] exists only in DM1.[38]

DM1 affects equal men and women, although the severity of the disability symptoms varies into genders,[53] as shown in **Table 4**:

Table 4: Comparison of clinical manifestations between genders (adapted from reference [53])

Most frequent clinical manifestations in males		**Most frequent clinical manifestations in females**	
	Severe muscular disability		Cataracts
	Marked myotonia		Dysphagia
	Muscle weakness		Digestive tract dysfunction
	Cardiac problems		Urinary incontinence
	Respiratory involvement		Thyroid disorder
	Developmental abnormalities		Obesity
	Facial dysmorphism		
	Cognitive impairment		
	More frequent hospitalizations		
	Higher mortality rate		

Table 5 compares basic genetic differences and clinical manifestations between the myotonic dystrophy forms DM1 and DM2. Roughly, DM2 is less severe than DM1 and does not have any congenital form already known.

Table 5: Comparison of clinical manifestations between DM1 and DM2 (adapted from reference [54])

Clinical features	DM1	DM2
Genetic features		
OMIM	160900	602668
Gene affected	*DMPK*	*CNBP*
Chromosome	19	3
Locus	19q13.3	3q21
Oligonucleotide repeat expansion	$[CTG]_n$	$[CCTG]_n$
Repeat count (n)		
Healthy	<37	10-26
Pre-mutation	38-49	27-74
Affected	50-4,000	75-11,000
Inheritance pattern	Autosomal dominant	Autosomal dominant
General features		
Estimated prevalence	4.76:10,000[55a] 9.27:100,000[55b]	2.29:100,000[55b]
Epidemiology	Widespread (heterogeneous)	European (Northern EU)
Age of onset (years)	0 to adult	8-60
Anticipation	Always present	Exceptional
Congenital form	Present	Absent
Life expectancy	Reduced	Normal range
Core features		
Clinical myotonia	Evident in adult-onset	Present in <50%
EMG myotonia	Always present	Absent or variable in many
Muscle weakness	Disabling at age 50	Onset after age 50-70
Cataracts	Always present	Present in minority
Muscle symptoms		
Facial and jaw weakness	Always present	Usually absent
Bulbar weakness-dysphagia	Always later	Absent
Respiratory muscles weakness	Always later	Exceptional
	Always prominent	Rare
Distal limb muscle weakness	May be absent	Main disability in most patients, late
	Always prominent	Prominent in few
Proximal limb muscle weakness	Absent or mild	Most disabling symptom in many
Sternocleidomastoid weakness	Face, temporal, distal hands and legs	Usually absent
Myalgic pain	Absent	Present in ≥50%
Systemic features		
Tremors	Absent	Prominent in many
Behavioural change	Early in most	Not apparent
Cognitive disorders	Prominent	Not apparent
Hypersomnia	Prominent	Infrequent
Cardiac arrhythmias	Always present	From absent to severe
Male hypogonadism	Manifest	Subclinical in most
Manifest diabetes	Frequent	Infrequent

Since the first Steinert's paper in 1909, more than 7,000 scientific papers reporting scientific findings on myotonic dystrophy (DM1, DM2 and CMD) have been published, as retrieved from PubMed database as per January 2022, pointing out at the interest and relevance of these diseases within the scientific community.

1.5. *Myotonic dystrophy type 1*

1.5.1. Epidemiology

Myotonic dystrophy type 1 is the most frequent inherited muscular dystrophy in adults. The disease seems to be spread worldwide, with a commonly reported estimated global prevalence of 1 in 8,000 individuals (*i.e.* 12.5 in 100,000) as a consensus value.[54] which is in fact an average prevalence from Western Europe and North American populations data only. Since clear ethnic differences have been reported,[56,57] a more cautious approach estimates the prevalence range between 1 in 3,000 and 8,000 individuals globally.[58] However, some recent studies point out to the possibility that these figures are in fact underestimated, and that the prevalence of individuals with CTG repeat expansions in *DMPK* could be up to 5 times higher (4.76 in 10,000) than the previously reported prevalence.[55] **Table 6** illustrates the prevalence variability of myotonic dystrophy type 1 along representative regions of the world.

Differences in geographic prevalence of the disease were associated with the North Eurasian origin of the mutation,[59] which was spread by the migrations in the 17th century from Europe to North America to the current US and Canada territories. Particularly, in the Saguenay-Lac-Saint-Jean region (Quebec, Canada), DM1's prevalence is 30 to 60 times the world's prevalence, due to a founder effect that could be traced back to the ancestors, from which the mutated gene passed on over 10 to 14 generations.[61] This is the region with the highest reported prevalence worldwide: carrier rate reaches 1/550, compared with 1/5,000 to 1/50,000 elsewhere.[61]

Table 6: Prevalence of DM1 per 100,000 inhabitants broken into regions and main countries

Continent / Country	DM1 prevalence per 100,000	Year	Comments	Reference
North America				
USA	2.0	1984	-	[60]
Canada	182.0	2005	Quebec region	[61]
Europe				
Albania	-	-	No studies found†	-
Austria	-	-	No studies found†	-
Belgium	3.7	2010	-	[62]
Bulgaria	-	-	No studies found†	-
Croatia	8.5-18.1	1997	Istria region	[63]
Cyprus	-	-	No studies found†	-
France	-	2019	Unknown	[64]
Finland	36.0	2011	55.0 for DM2	[65]
Germany	9.0	2018	DM2 ~ DM1	[66]
Greece	-	-	No studies found†	-
Iceland	27.0	2005	-	[67]
Ireland	11.95	1999	-	[68]
Italy	9.6	2016	-	[69]
The Netherlands	-	-	No studies found†	-
Norway	13.4	2021	Northern region	[70]
Poland	-	-	No studies found†	-
Portugal	-	-	No studies found†	-
Serbia	5.3	2002	-	[71]
Slovenia	-	-	No studies found†	-
Spain	35.9	2016	Northern region	[72]
Sweden	17.8-19.7	2017	Western region	[73]
Switzerland	7.8	1970	-	[74]
Turkey	-	2013	No studies found†	[75]
UK	10.6	2009	Northern region	[76]
Asia				
China‡	0.37	2001	Children <19	[77]
Israel	5.7-47.3	2003	Ethnicity related	[78]
Japan	0.2-9.1	2014	Several regions	[60]
Russia	>12.5	2005	Yakutia region	[79]
Taiwan	0.46	2003	-	[80]
Africa				
Egypt	-	-	No studies found†	-
South Africa	14.3	1985	Transvaal region	[81]
Sub-Saharan	Absent	1996	-	[82]
Oceania				
Australia	-	-	No studies found†	-
New Zealand	11.6	2006	European descent	[83]

Notes:

† No studies found in PubMed database as per January 2022

‡ DM1 is not well studied in the Chinese population[84]. According to the latest 11 September 2021 definition, myotonic dystrophy would not be considered a rare disease in China.[10]

1.5.2. Genetic basis and pathogenic mechanisms

DM1 is originated due to an unstable expansion of the cytosine-thymine-guanine $(CTG)_n$ trinucleotide repeat in the 3' end untranslated region (UTR) of a transcript encoding a protein kinase family member, the *myotonic dystrophy protein kinase* (DMPK) gene located in chromosome 19 locus 19q13.3. Myotonic dystrophy type 1 is transmitted by an autosomal dominant inheritance pattern $(CTG)_n$. Accordingly, the mutation is inherited even from one carrier parent, independently of the gender of the progenitor, in a 50% probability per descendent. Family history and genetic testing are fundamental for a proper genetic diagnosis.[85]

As a common characteristic in repeat expansion diseases, the $(CTG)_n$ expansion size somehow correlates with the severity and age of onset of the disease. In this regard, wild type individuals have polymorphic 5 to 37 repeats, pre-mutation range is associated to 38 to 50 repeats, asymptomatic proto mutation is recognized between 51 and 100 repeats, and full mutation for which symptoms tend to manifest is considered beginning in 100 repeats to several thousands.[85] Congenital form is associated to mutation length above 2,000 repeats.[85]

Mutant DMPK transcripts CUG^{exp} RNA expansions fold into stable double-stranded hairpin loop structures with U-U mismatches, which are found in skeletal muscle, heart, and brain tissue, are retained in the cell nucleus in microscopically visible ribonuclear foci, which are the most prominent histopathological hallmark of the disease.

Myotonic dystrophy type 1 is characterized by repeat instability, the tendency of the repeat expansion to continuously increase throughout the life of the affected individual. Repeat instability is associated with the progressive nature of the disease,[86] and it is expressed by somatic mosaicism, anticipation and variable penetrance:

- Somatic mosaicism (instability in clonal transmissions, from one cell to another): the number of genetic repeats is heterogeneous in different cells along different tissues in the same individual. Larger expansions are found in skeletal muscles and heart than in peripheral blood,[87,88] explaining why the skeletal and cardiovascular are the most severely affected systems in DM1.
- Anticipation (instability in intergenerational transmissions): the number of $(CTG)_n$ repeats in the affected genes tends to expand through consecutive affected generations.[89] This repeat gain elicits earlier and more severe manifestations of DM1 symptoms in each successive generation.
- Variable penetrance: the extension and severity of the symptoms varies widely among people with the disease, even among individuals from the same family, due to their different degree of mosaicism.

However, the presence of repeat expansions, their size and even the somatic mosaicism *per se* are not enough to explain the pathogenic mechanism and the multisystemic affectation in DM1.

In this regard, although the expanded CTG repeats disrupt normal cellular processes at the RNA when the mutation is transcribed, mutation is not translated at the protein level. Accordingly, DM1 is not caused by the haploinsufficiency of the DMPK mutant gene,[90] as the DMPK knock-out mice models later demonstrated.[91]

In fact, once mutant CTG repeat expansions present in DNA are transcribed into RNA, the corresponding CUG-containing *DMPK* transcripts fold in RNA alternative structures, such as a stable double-stranded stem-loop, that accumulate in the nucleus of DM1 cells as RNA foci.[92] RNA foci exhibit a strong affinity for RNA-binding proteins, which are misregulated and are unable to perform their normal function.[93] Particularly, the CUG-BP1 and Elav-like (CELF)[94] and muscleblind-like (MBNL)[93,95] protein families were found to be directly involved in this mechanism, although through opposite functions: whereas MBNL protein member MBNL1 is sequestered in foci and functionally depleted in the nucleoplasm,[96] CELF protein member CELF1 is upregulated,[97] resulting in gain of function. Accordingly, the initially discovered loss-of-function mechanism[98] was in fact a broader RNA gain of toxic function through antagonistic misregulation of the above-mentioned RNA-binding proteins.[99]

Particularly, MBNL is a family of key splicing regulators in mammals, composed of three paralogs (MBNL1, MBNL2 and MBNL3) with differential expression in various tissues along foetal to adult development: MBNL1 is predominant in adult skeletal and cardiac muscle, MBNL2 is predominant in brain and MBNL3 in liver and human foetal placenta.[100] MBNL1 plays a central role in DM1 pathogenesis. Typically, MBNL1 translocates from cytoplasm to the nucleus in the postnatal period to induce adult-type splicing, but in DM1 it is depleted from the nucleoplasm because it is recruited extensively into the ribonuclear foci.

The complexity in DM1 pathologic mechanism increases due to the fact that both CELF and MBNL family proteins are splicing regulators,[101,102] *i.e.,* proteins involved in the process by which i) introns are removed from the primary messenger RNA transcript, and ii) exons are joined together to form the mature messenger RNA, that ribosomes further translate into proteins, mainly in eukaryotes. Beyond the normal cut and paste single-mode, alternative splicing may take place, through several processes by which different combinations could generate different protein-coding isoforms. Therefore, different proteins from single genes are obtained by alternative splicing, wherein alternative splicing is a certain use of alternative exons in target transcripts, which changes in a coordinated way at different times of development. Alternative splicing contributes not only to protein diversity,[103] but also to some human diseases known as spliceopathies.[104] Spliceopathies are originated by the misregulation of alternative splicing by RNA-binding proteins that alter transcription, translation, cell signalling, transcription factors and regulated pathways of miRNA and double-stranded RNA processing.[105] It is well established in the state of the art that functional depletion of MBNLs in human prevents the foetal to adult pattern transition that results in DM1.[100] As a result, there is an abnormal persistence of foetal patterns of alternative splicing in adult tissues that finally impact in cell differentiation and lineage determination, tissue identity acquisition and maintenance and organ development in DM1.[106] Skeletal muscle is one of the tissues with the highest level and complexity of splicing programs, wherein the MBNL and CELF RNA-binding protein families are among the most important splicing regulators.[107]

Figure 3 illustrates step by step the relationship from the DNA mutant expansion to the DM1 symptoms. **Table 7** illustrates some of the main known genes affected, their corresponding RNA-binding splicing regulator and the correlation to the main symptomatic effects in DM1.

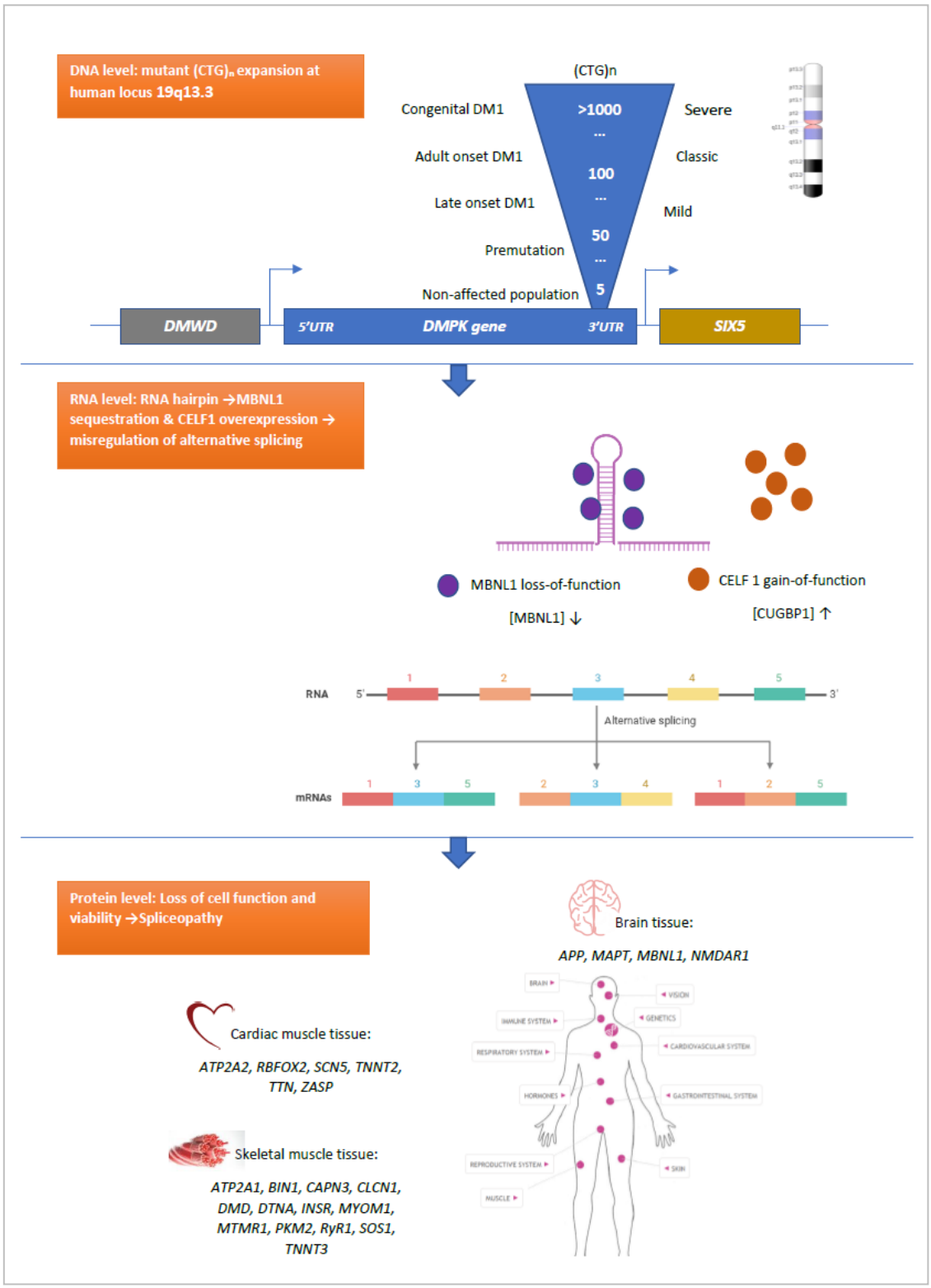

Figure 3: From genes to symptoms in myotonic dystrophy type 1

Table 7: Correlation between genes, splicing factors and multisystemic effects in skeletal muscle in DM1 (adapted from reference [107])

Gene	Gene name	Regulated Exon/Intron	Regulated by	Related to
Skeletal muscle tissue				
ATP2A1 (SERCA1)	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase 1	Exon 22	MBNL1	Muscle degeneration
BIN1	Bridging integrator 1	Exon 11	MBNL1	Muscle weakness
CACNA1S	Calcium channel CaV1.1	Exon 29	MBNL1 and CELF1	Muscle weakness
CAPN3	Calpain 3	Exon 16	MBNL1	Muscle weakness
CLCN1	Muscle-specific chloride channel	Intron 2 Exon 7a	MBNL1 and CELF1	Myotonia
DMD	Dystrophin	Exon 71 Exon 78	MBNL1	Impairs mobility and muscle architecture
DTNA	Dystrobrevin-α	Exon 11a Exon 12	MBNL1 and CELF1	Muscle weakness
INSR	Insulin receptor	Exon 11	MBNL1 and CELF1	Insulin resistance
MYOM1	Myomesin 1	Exon 17a	MBNL1-3	Muscle abnormalities
MTMR1	Myotubularin-related protein 1	Exon 2.1 Exon 2.2 Exon 2.3	MBNL1	Impairs myogenesis in DM1 mice
PKM2	Pyruvate kinase M2	Exon 9 Exon 10	CELF1 and MBNL1	Atrophy
RyR1	Ryanodine receptor	Exon 70	MBNL1	Reduced muscle contraction
SOS1	Son of sevenless homolog 1	Exon 25	MBNL1	Muscle hypertrophy
TNNT3	Fast troponin T3	Exon 23	CELF1	Alteration of the sarcomere structure

Table 7 (continuation): Correlation between genes, splicing factors and multisystemic effects in cardiac muscle and brain tissue in DM1 (adapted from reference [107])

Gene	Gene name	Regulated Exon/Intron	Regulated by	Related to
Cardiac muscle				
ATP2A2 (SERCA2)	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase 2	Intron 19	MBNL1	Cardiac conduction impairment
RBFOX2	RNA Binding Fox-1 Homolog 2	3 nt	CELF1	Cardiac conduction delay and arrhythmogenesis
SCN5A	α- subunit of the cardiac voltage channel NaV1.5	Exon 6a	MBNL1	Arrhythmia
TNNT2	Cardiac troponin T	Exon 5	MBNL1 and CELF1	Alteration contractile properties
TTN	Titin	Zr4 Zr5 Mex5	MBNL1	Myofibril elasticity and structural integrity
ZASP (LDB3)	LIM Domain Binding 3	Exon 5 Exon 11	CELF1	Morphological abnormalities cardiac fibre
Brain tissue				
APP	Amyloid beta precursor protein	Exon 7	CELF1	Unknown
MAPT	Microtubule-associated protein tau	Exon 2 Exon 3 Exon 10	MBNL2	Impair neuronal microtubules network
MBNL1	Muscleblind-like type 1	Exon 6 Exon 8	MBNL1	Splicing defects several tissues
MBNL2	Muscleblind-like type 2	Exon 7 Exon 8	MBNL2	Splicing defects in brain
NMDAR1	N-methyl-D-aspartate receptor	Exon 5	Unclear	Memory impairment

Limited availability of MBNL1/2 contributes the most to DM1 phenotypes. MBNL1 loss of function accounts for more than 80% of missplicing events and nearly 70% of expression defects in a murine model that expresses 250 CTG repeats in the context of human skeletal actin.[108,109,110] Furthermore, overexpression of MBNL1 rescues aberrant alternative splicing of muscle transcripts and myotonia of HSALR mice.[111] Overexpression of MBNL1 is well tolerated in skeletal muscle in mice and early and long-term overexpression prevents myotonia,

myopathy, and alternative splicing alterations typical of DM1 mice,[112] suggesting that MBNL1 overexpression may serve as a therapeutic strategy for DM1.[113]

Last but not least, beyond the misregulation of alternative splicing, some aspects of DM1 could be explained by several other actions of MBNL and CELF proteins, and even through other still unknown network mechanisms.[105] In this regard, further future research may still provide more insights to contribute to the understanding of such a polyhedral disease.

1.5.3. Clinical subtypes

The $(CTG)_n$ mutant expansion involved in DM1 disease can vary from 50 to more than 1,000 repetitions, contributing to a broad range of symptoms and different phenotypes.

From a clinical perspective DM1 may be classified into four different subtypes: mild; classical; juvenile and congenital types.[54] However, there is still no formal consensus or guidelines on this fragmentation since some phenotypes overlap due to the somatic mosaicism of the repeat alleles, the repeat instability for life, gender, the large variability in symptoms among individuals, and the diverse grades of severity that the same person may experience during life.[114,115]

Currently, scientists have tried to segment the complexity found in DM1 patients according to three main grades (mild, classic and severe) and four to five main clinical categories (congenital, infantile/juvenile, adult-onset or 'classic DM1' and late-onset or 'mild DM1') according to the age of onset in conjunction with the length of $(CTG)_n$ expansion repeats.

Some of the core characteristics of four DM1 phenotypes are disclosed below and summarized in **Table 8**:

- Late-onset or Mild DM1: individuals in the lower repeat range could be asymptomatic. Mild symptomatic patients may have premature cataracts and baldness as the sole clinical features, whereas myotonia, weakness and excessive daytime sleepiness are normally not present. Although individuals affected by the mild phenotype tend to have a normal life span, in some cases cardiac conduction abnormalities may arise, resulting in a shorter life span.[54]
- Classical or adult-onset DM1: The age of onset is typically in the second or third decade of life. The most frequent symptoms are weakness, myotonia and cataracts. Distal weakness manifests at the end of the limbs toward the feet and hands, leading to symptoms relating to the strength and fine use of the hands and foot drop, which increases the incidence of stumbling and the impression that DM1 individuals may seem clumsy. In addition, facial weakness may result in ptosis, a characteristic droopy eyelid due to muscle atrophy, and the typical myopathic 'hatchet aspect'. Since myotonia impairs several muscles, individuals experience difficulties in talking, chewing and swallowing, as well as cardiac involvement, whose impairment contributes significantly to the morbidity and mortality of the disease.

  Central nervous system dysfunction, fatigue, excessive daytime sleepiness, and gastrointestinal tract involvement, as well as endocrine abnormalities such testicular atrophy, are usual.

Apathy, lack of initiative, excessive daytime sleepiness and fatigue can be distressing for people living with DM1, themselves, their caregivers and the rest of family members. Beyond life span, these features have a significant impact on the quality of life.[54]

Pulmonary complications are the leading cause of death in DM1 patients, followed by cardiac complications often asymptomatic.[116] Due to the complexity of the disease, consensus-based care recommendations for adults with myotonic dystrophy type 1 have been published to help to standardize and improve care for this patient population.[116]

- Childhood/Juvenile DM1: this form resembles the classical form of myotonic dystrophy, but is less severe and less associated with the classical symptoms. In contrast, it is more clearly associated with cognitive deficits and behavioral abnormalities, for example, difficulties in learning and socialization at school. Muscle involvement may be minimal in the juvenile presentation, but cardiac conductions abnormalities should be routinely monitored.[114]

- Congenital DM1: Although it is the most severe form of DM1, it is not considered as an early form of classical DM1, since the age of onset occurs at birth itself. It is estimated that children with congenital form account for 10-30% of the DM1 population,[117] and its incidence has been disclosed to be 2.1/100,000 births in Canada.[118]

  Polyhydramnios and poor foetal movements precede the birth of an infant with congenital DM1. The affected parent is nearly always the mother and congenital DM1 occurs in a quarter of the offspring of affected DM1 mothers. The neonate suffers severe generalized weakness with the tented upper lip, mild ptosis, hypotonia and respiratory distress that require intubation and immediate ventilation support at birth. There is a high mortality rate in the perinatal period, mainly due to respiratory failure. Infants are floppy babies with failure to thrive and severe respiratory distress. Facial and jaw muscles are weak and experience difficulties in feeding and suckling. Myotonia is typically absent clinically and may be difficult to detect initially. On the other side, cerebral atrophy, and therefore, mental retardation and developmental delay are common. Life span is really reduced, in the range of 16 to 41% in the early childhood, and around 50% mortality by the mid-thirties, mainly to respiratory and cardio-respiratory complications.[119]

  In a similar effort done for adult patients, also for minors suffering myotonic dystrophy type 1 consensus-based care recommendations for congenital and childhood-onset myotonic dystrophy type 1 have been recently published to standardize and improve the care of children with myotonic dystrophy,[120] from neonatal care to cardio-respiratory, skeletal muscle, gastrointestinal, neurodevelopmental, physicological, endocrine management, and the like.

Table 8: Correlation of DM1 phenotypes, clinical findings and CTG repeat length (adapted from references [54] and [116])

Phenotype (severity)	Clinical findings	(CTG)n size	Age of onset	Life span
Congenital (CDM)	Polyhydramnios	>1,000	Birth	From perinatal to infant or shortened life span
	Reduced foetal movements			
	Severe weakness			
	Infantile hypotonia			
	Cerebral atrophy			
	Intellectual disability			
	Cardio-respiratory problems			
	Respiratory failure			
Childhood onset	Similar symptoms as congenital DM1, but less severe	50-1,000	1 month - 10 years	Shortened
	Facial weakness			
	Myotonia			
	Low intellectual quotient			
	Heart conduction defects			
Juvenile onset	Similar symptoms as adult DM1 but more severe	400-800	10 – 18 years	Shortened
Adult-onset "classic DM1"	Distal muscle weakness	50-1,000	1-30 years	Shortened to normal
	Facial weakness and ptosis			
	Myotonia			
	Cataracts			
	Cognitive impairment			
	Excessive daytime sleepiness			
	Heart conduction defects			
	Insulin resistance			
	Gastrointestinal distress			
	Respiratory failure			
Late onset / Asymptomatic /mild	Mild myotonia	50-100	20-70 years	Normal
	Cataracts			
Pre-mutation	None	38-49	NA	Normal

1.5.4. Management of DM1

Aligned with the multidimensional characteristics of the disease, a multidisciplinary approach through several medical disciplines is required to manage the challenging wide spectrum and diversity of symptoms that a DM1 patient may suffer, which tend to worsen through lifetime gradually, and progressively limit the quality of life and life span itself. DM1 patients need to attend multiple specialists that should be globally managed through coordinated clinical care, which is usually led by the neurology specialists. An illustrative list of related disciplines managing DM1 patients has been previously disclosed in **Table 3**. Management is focused on anticipatory guidance and monitoring of treatable complications.

Several general recommendations and guidelines are provided by Working Groups of clinical professionals.[43,116] Although limited, the main relevant current management to provide a certain quality of life through different approaches are disclosed herein (**Table 9**).

Table 9: Current recommendations for the management of myotonic dystrophy type 1 (adapted from references [43] and [116])

Treatment	Symptom
Medications	
Anti-myotonic drugs (ex. mexiletine)	To control myotonia that impairs normal activities. *Mexiletine is contraindicated in patients with cardiac involvement.[116]
Anti-diabetic drugs (ex. insulin)	To manage diabetes symptoms
Wakefulness-promoting drugs (ex. modafinil).	To control excessive daytime sleepiness when coexisting CNS alteration is suspected as the cause of excessive daytime sleepiness.[116]
Non-steroidal anti-inflammatory drugs	To manage muscle pain
Anti-diarrhea (ex. loperamide, antibiotics, mexiletine)	To manage diarrhea
Laxative drugs	To manage constipation
Rehabilitative therapy	
Physiotherapy	To manage muscle weakness, myotonia and contractures
Speech therapy	To help with swallowing and pronunciation issues
Psychiatric therapy	To address behavioural and physicological issues
Individualized support	To help with learning disabilities and cognitive delays
Devices	
Assistive devices (neck braces, arm and food braces, canes, walkers, scooters and wheelchairs)	To ensure safe mobility
Eye crutches	To support droopy eyelids (ptosis)
Pacemaker or implantable cardioverter defibrillator	To address irregular heartbeat issues
Continuous positive airway pressure device	To ensure respiratory sufficiency
Surgery	
Orthopedic surgery	To correct gait issues and contractures
Cataract removal	To improve vision
Eyelid surgery	To correct droopy eyelids

The survey 'The Christopher Project' performed within the myotonic dystrophy community in the United States and Canada and published in 2019, revealed that the top five symptom prevalence in DM1 were muscle weakness, daytime sleepiness, fatigue, myotonia and balance issues, followed by many others.[121] However, the same report disclosed that only a quarter of responders take any medication to manage myotonia (19%), daytime sleepiness (18%) or gastrointestinal issues (27%), with a moderate level of satisfaction with the use of these medications.[121]

In such a complex disease as myotonic dystrophy is, and beyond the traditional medical care model focused on the treatment of organic systems impairments (respiratory, cardiovascular, muscular, skeletal, endocrine and the like), the care of DM1 patients should be based on an integrative approach, including specific preventive treatments, guidance and anticipation of future care needs, nutrition, social evaluations to monitor patients for complications and the like.[122]

1.5.5. Therapeutic approaches to DM1

As per June 2022, there are no medications approved explicitly for myotonic dystrophy type 1 and still no cure exists, although some promising approaches are currently under development, associated with the fact that the past two decades have witnessed the generation of breakthrough knowledge regarding the molecular causes and conceptual approaches to treat neuromuscular disorders.

The main straightforward approach would consider targeting the root cause of the disease in order to short circuit the subsequent downstream related deleterious effects. In this regard, targeting mutant DNA is the focus of novel genome editing tools.

However, in the previous years the strategies have been mainly focused on the mutant RNA and protein levels in order to reverse the RNA gain of toxic function associated with DM1 and previously disclosed herein, either by: 1) degradation of the mutant transcripts containing expanded CUG repeats, 2) steric blockage to unfold the secondary structure of the CUGexp-RNA, 3) inhibition of the deleterious binding of MBNL proteins to the expanded CUG repeats, 4) MBNL1 overexpression, and the like.[123] Particularly, MBNL loss of function is well-recognized as significantly contributing to DM1 phenotypes.[108,109,110]

During the execution of the present research project, we reviewed the existing drug development programs in DM1, encompassing advanced candidates from the preclinical stage to human clinical trials.[124] Due to the complexity of DM1 pathogenesis, multiple approaches are being targeted, the genetic origin of the disease itself or attenuation of symptomatic burden. Therapies are further classified into three broad categories of small molecules, oligonucleotide-based therapies, and gene therapies, encompassing mechanisms of action targeting root cause disease events up to only specific clinical symptoms. A summary of the main findings is discussed herein.[124]

1.5.5.1. Small molecule drugs

Several small molecules are under different stages of research and development for DM1. Strategies cover from *de novo* drug discovery of new active substances to drug repurposing for a second medical use in DM1. Drug repurposing (also known as drug repositioning) is a process of identifying new therapeutic uses for existing drugs, for which safety in humans is already well established. The repurposing approach is based on previous target information, experimental data or virtual screening methodologies to explore known active pharmaceutical ingredients as second medical use candidates.

In this category, we mainly identified repurposed pre- and investigational new drugs (INDs), which were initially developed to treat a different condition, compared with only one new chemical entity (NCE) IND with first therapeutic use in DM1. Since drug repurposing candidates have already demonstrated safety in humans, they can be tested in patients much quicker and less costly and risky than for entirely new drugs, which explain that they have taken the lead as the closest to reaching market authorization. Mexiletine,[125,126] metformin,[127] tideglusib,[128] erythromycin,[129] flumazenil,[130] ranolazine,[131] cannabinoids[132,133] and pitolisant[134] are examples of small molecules under preclinical or clinical testing to manage several aspects of DM1.

Beyond the drug approaches, a complementary strategy based on the nutritional management of DM1 through the dosaged administration of the natural alkaloid methylxanthines caffeine and theobromine was additional included.[124]

1.5.5.2. Oligonucleotide-based therapeutics

Oligonucleotide-based therapies can potentially target any gene in the genome and are, thus, relevant candidates to address the diversity among rare genetic diseases. As per 2021, at least ten oligonucleotide-based therapies were already approved for clinical use.[135] In DM1, relevant approaches involving the design of antisense oligonucleotides (ASOs) that are able to degrade *DMPK* transcripts via the activation of RNase H machinery in the nucleus (*i.e.*, gapmers) or able to prevent MBNL1 sequestration by CUG repeats (or displace prebound MBNL1 proteins) by an occupancy-based mechanism (*i.e*., mixmers) have been recently reviewed.[136]

Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded oligodeoxynucleotides (less than fifty monomers) that can alter RNA and reduce, restore, or modify proteins.[137] Antisense drugs are intermediate in size between biologicals and small-molecules.[138] Chemically modified ASOs serve as highly selective sequence pairs to specific regions of mRNA and regulate the translation of genetic material into functional proteins. Depending on the type of chemistry introduced and the design of the antisense construct, ASOs can mitigate RNA toxicity by two possible mechanisms: (1) competing with MBNL1 for CUGexp binding and/or displacing prebound MBNL1 proteins (steric block mechanism); (2) inducing degradation of DMPK mRNA via the RNase H machinery. Both mechanisms would result in functional restoration of MBNL1 activity, and consequently, correction of missplicing.

ASOs bind complementary RNA by Watson-Crick base pairing and can suppress gene expression through a number of different antisense mechanisms. The chemical design of the ASO, the exact position binding position on the RNA and the exact binding position in the cell drive the effect of oligonucleotide. Extensive reviews covering mechanisms of actions,[138,139] toxicity,[140] medicinal chemistry to improve drug properties,[141] drug delivery[142] and therapeutic application in neurological[143] and muscular diseases[144] have been published. ASO candidates are designed to address the genetic basis of DM1 by reducing the levels of mutant *DMPK* RNA in the nucleus, releasing splicing proteins, allowing normal mRNA processing and translation of normal proteins and potentially stopping or reversing disease.

However, in contrast to their high specificity, oligonucleotide-based therapies suffer from poor delivery to the muscle tissues that, in the case of DM1, is further aggravated because cells display membrane integrity that do not facilitate cell uptake.[145]

1.5.5.3. Gene therapy approaches

The most straightforward approach to overcome the limited biodistribution of ASO is to promote its endogenous expression using gene therapy vectors. Adeno-associated viruses (AAVs) are being evaluated for drug development in several muscular dystrophies.[146] Benefits of this avenue in DM1 were initially disclosed with a proof-of-concept approach for an AAV-delivered RNAi in the HSA^{LR} mice model.[147]

Recently, the CRISPR/Cas9 genetic scissors have also emerged as innovative approaches to treat DM1 by targeting the removal of the expansions at the DNA level, preventing their transcription, or targeting degradation of the toxic RNA, therefore effectively targeting the underlying DM1 etiology.[148,149]

Although gene therapies may provide the definite cure for DM1 through the permanent correction of genetic defects, certain complex challenges also arise, and future studies in large animals will be needed to assess further the safety, dose levels, and immunosuppression regimens required for safe and long-term treatments.

In conclusion, over the past decade, the DM1 community has smoothly transitioned from basic to translational research with the common goal of improving the quality of life and life span of people suffering from this very complex disease. Considering the intense research and development activity worldwide, it seems feasible that the DM1 community will have at least one therapeutic option targeting the genetic cause of the disease and potentially several options to improve their quality of life within the next few years.

Further details for therapies under active clinical and preclinical stages are disclosed in **Table 10** and **Table 11**, respectively.

An even more extended drug development pipeline for myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2), including therapeutic approaches from the earlier discovery phase to clinical trials, promoted by Patient Associations and research centres in muscular dystrophies is continuously updated.[150]

Table 10: Myotonic dystrophy drug candidates currently in clinical trials

Phase	Clinical trial	Status	Clinical trial information / Sponsor	Results
Mexiletine (Repurposed small molecule) - Orphan Drug Designation by FDA and EMA				
3	NCT04700046	Not yet recruiting 2021-2023	Study to Investigate the Efficacy and Safety of Mexiletine in paediatric patients With Myotonic Dystrophy Type 1 and Type 2 (MIND). Sponsor: Lupin, Ltd.	Not posted
3	NCT04624750	Recruiting 2020-2024	Safety, efficacy and steady-state pharmacokinetics of mexiletine in paediatric patients with myotonic disorders. Lupin, Ltd.	NP
2	NCT01406873	Completed 2011-2018	Effects of mexiletine on ambulation, myotonia, and muscle function, strength, pain, gastrointestinal functioning, cardiac conduction, and quality of life in DM1. University of Rochester	[125]
Metformin (Repurposed small molecule)				
3	2018-000692-32	Ongoing 2019-2022	Efficacy of metformin on motility and strength in DM1. Tor Vergata	NP
2	2013-001732-21	Completed 2013-2017	A randomized, double-blind, placebo-controlled phase II study of metformin in myotonic dystrophy type 1 patients. Centre d'Etude des Cellules Souches /Istem	[127]
Tideglusib (Repurposed small molecule) - Orphan Drug Designation by FDA				
2/3	NCT05004129	Enrolling by invitation 2021-2023	Safety and Efficacy of Tideglusib in Congenital Myotonic Dystrophy for children and adolescents who participated in and completed the preceding AMO-02-MD-2-003 study. Sponsor: AMO Pharma Ltd	NP
2/3	NCT03692312	Recruiting 2020-2022	Randomized, multicenter, double-blind, placebo-controlled, Phase 2/3 study of patients (aged 6 to 16 years) diagnosed with Congenital DM1. AMO Pharma Ltd	NP
2	NCT02858908	Completed 2016-2018	Safety, efficacy and PK of Tideglusib in the treatment of adolescents and adults with congenital and juvenile-onset DM1. AMO Pharma Ltd	[128]
Pitolisant (Small molecule/repurposing)				
2	NCT04886518	Recruiting 2021-2022	Safety and Efficacy of Pitolisant on Excessive Daytime Sleepiness and Other Non-Muscular Symptoms in Patients With Myotonic Dystrophy Type 1. Sponsor: Harmony Biosciences, LLC	[134], NP

Phase	Clinical trial	Status	Clinical trial information / Sponsor	Results
Erythromycin (Repurposed small molecule)				
2	jRCT2051190069	Recruiting 2019 -ongoing	Blinded, Placebo-Controlled Study to Assess the Safety, Tolerability, and Efficacy of MYD-0124 to DM1 Adult Patients. Hospital Osaka University	NP
AO1001 (Oligonucleotide-based therapy)				
1/2	NCT05027269	Recruiting 2021-2023	Safety, tolerability, pharmacokinetics and pharmacodynamics of single and multiple-doses of AOC1001 Administered Intravenously to Adult DM1 patients. Sponsor: Avidity Biosciences, Inc	[151], NP
IONIS-DMPKRx (ISIS 598769) (ASO)				
1/2	NCT02312011	Completed 2014-2016	Safety, tolerability, and pharmacokinetics of multiple escalating doses of ISIS 598769 administered subcutaneously to adult patients with DM1. IONIS-Biogen	NP
Flumazenil, ERX-963 (Repurposed small molecule)				
1	NCT03959189	Completed 2019-2020	Safety, tolerability and potential reduction of excessive daytime sleepiness/hypersomnia and improvement of cognitive function in patients with DM1. Expansion Therapeutics, Inc.	[130]
Ranolazine *Ralexa™* (Repurposed small molecule)				
1	NCT02251457	Completed 2014-2017	Preliminary data to determine the safety and efficacy of ranolazine in the symptoms of DM1. Ohio State University / Gilead Sciences	[131]
Caffeine and Theobromine formulation *MYODM™* (Natural compounds)				
NA	NCT04634682	Completed 2020-2021	Effect of the food supplement MYODM™ in the treatment of excessive daytime sleepiness and quality of life in adults with DM1. Myogem Health Company, S.L.	[152]

NA: Non-applicable
NP: Not posted

Table 11: Myotonic Dystrophy drug candidates in preclinical stages

Preclinical status	Preclinical information / Sponsor	Results
Cannabinoids CBD/THC (Small molecule/repurposing)		
IND enabling phase	Management of chronic neuropathic pain, myotonia, and myalgia. A pilot survey in DM1, DM2, and congenital myotonia, with two patients per disease. Sponsor: Nexien Biopharma.	[153]
ISIS 486178 (Oligonucleotide-based therapy)		
Lead optimization	Systemic treatment resulting in myotonia and cardiac conduction improvement, correction of splicing defects, reduction in RNA foci, and redistribution of MBNL1. DMSXL and DM200 mice models. Sponsor: Ionis Pharmaceuticals	[154, 155, 156]
FORCE-DMPK candidate (Oligonucleotide-based therapy)		
IND enabling phase	*In vivo* dose-dependent correction of splicing and myotonia in the HSA^{LR} model after a single low dose. DMPK mRNA reduction in mouse and non-human primates. FORCE™ platform. Sponsor: Dyne Therapeutics.	[157]
Pip6a-PMO (Oligonucleotide-based therapy)		
Lead optimization	Intravenously injected in HSA^{LR}. Nuclear foci reduction, MBNL1 redistribution, splicing, and myotonia correction. Sponsor: Oxford University	[158]
NTC0200 (Oligonucleotide-based therapy)		
IND enabling phase	*In vitro* ability to target and open up aberrant DM1-linked secondary RNA structure in the mutant transcript, thereby displacing sequestered splice proteins. PATrOL™ platform. Sponsor: Neubase.	[159]
Arthex-01 (Oligonucleotide-based therapy)		
Lead optimization Start of Phase 1/2 by 2022	Subcutaneous injection in HSA^{LR}. Enhanced MBNL1/2 protein levels, recovered missplicing, muscle strength, and myotonia. Long-lasting activity of antagomiR-23b for up to 45 days. Sponsor: Arthex Biotech.	[160, 161]
AAV-PIN-dCas9 (Gene therapy)		
Lead optimization Start of IND-enabling studies by 2021	Intramuscular/systemic delivery in adult and neonatal HSA^{LR}. RNA-targeting Cas9 lasted for up to three months. Elimination of RNA foci, reversal of splicing biomarkers, and myotonia. Sponsor: Locanabio.	[162, 163]
AT-466 (Gene therapy)		
Lead optimization	AAV delivery to overcome the biodistribution limitations of ASO-based therapies. Sponsor: Audentes.	[164]
AAV-CRISPR-Cas9 (Gene therapy)		
Preclinical proof of concept	*In vivo* genome editing for DM1: deletion of the CTG repeat tract leading to a reduction of nuclear foci in muscle fibres after intramuscular injection of SaCas 9 and sgRNA rAAV9 vectors in DMSXL mice. Sponsor: Genethon-INSERM	[165]

Background research

The first evidences that certain natural products, such as caffeine, increased MBNL1 *in vitro* in myoblasts from DM1 patients, arose from the collaborative project titled 'Development of novel treatments for Myotonic Dystrophy: *In vivo* Drug Discovery',[166] from the public-private partnership consortium among three research Institutions: Universitat de València Estudis Generals (particularly, the Faculty of Biological Sciences) represented by the Coordinator of the full project, Prof. Rubén Artero; Institut Químic de Sarrià (IQS) from Universitat Ramon Llull, represented by Prof. José I. Borrell and Dr. Jordi Teixidó; and the R&D company IUCT, from Inkemia IUCT group, under the supervision of Dr. Josep Castells and Dr. David Miguel.

The collaborative project was funded by La Marató de TV3 Foundation,[167] a non-profit organization aimed at fostering and promoting biomedical research of excellence, as well as raising social awareness about the diseases. In the 2009 edition, the call was particularly focused on fundraising projects intended to promote the prevention, diagnosis, prognosis and treatment of Rare Diseases. Among the projects of excellence that were selected for funding, the collaborative project titled 'Development of novel treatments for Myotonic Dystrophy: *In vivo* Drug Discovery',[168] was the origin of the research presented herein.

The main objectives of the collaborative project were the identification, design and chemical synthesis of new molecules as future therapeutic agents in myotonic dystrophy type 1, since there was, and still currently there is, no effective therapy for this rare disease. Given the multidisciplinary nature of the consortium, the drug discovery collaborative project was based on three complementary approaches (**Figure 4**): i) Random screening of drug-like chemical libraries, ii) Computational design, synthesis and biological activity evaluation of potential inhibitors of MBNL1-CUG binding and iii) Computational design, synthesis and biological activity evaluation of potential inhibitors of CUG folding. Positive results were validated in a cell model of the disease developed from human myoblasts cultured from DM1 patients.

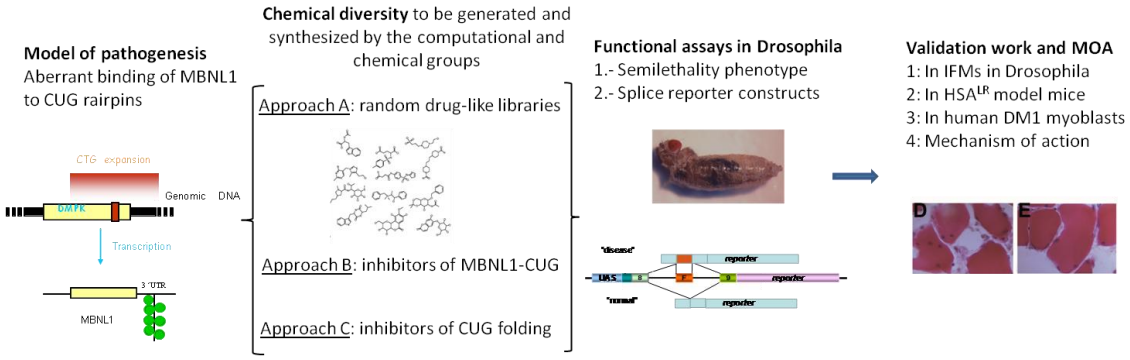

Figure 4: Outlines of the work plan of the collaborative project, which addressed the generation of chemical diversity according to three complementary approaches (A, B, C)

Chemical diversity generated by the computational group was next synthesized by the chemical group of the consortium and tested in vivo in Drosophila models. Primary hits were next validated in Drosophila indirect flight muscles (IFMs) and cell models. The mechanism of action of promising lead compounds was explored.

A sample set of 779 compounds were obtained through the chemical diversity based and/or generated from the three approaches. All the compounds were tested in the *Drosophila* DM1 models. As the main output of the collaborative project, six prototype compounds (or hits) were identified, from natural products to repositioned drugs and rationally designed new compounds.

Among the natural compounds tested, caffeine (1,3,7-trimethylxanthine), a compound found in multiple natural products, rescued missplicing and locomotor performance typically reduced in DM1 model flies. In addition, when tested in DM1 transdifferentiated fibroblasts, caffeine increased fusion index and increased MBNL1 disperse distribution in the cell's nuclei.

Due to the relevance of MBNL1 in DM1, the results obtained through the collaborative project were protected in the form of the patent applications 'Caffeine for the treatment of Myotonic Dystrophy type 1 and type 2'[169] and 'Compounds for the treatment of Myotonic Dystrophy type 1 and type 2'[170] before being publicly presented to the scientific community at the Fundació La Marató TV3 XVI Symposium on Rare Diseases, in 2015.[168]

Figure 5: Poster disclosing the goals, results and applications of collaborative project titled 'Development of novel treatments for Myotonic Dystrophy: *In vivo* Drug Discovery', prepared by Fundació La Marató de TV3 for the XVI Symposium on Rare Diseases

Afterwards, the consortium licensed both patent applications to Myogem Health Company, a start-up company that was a spin-off from the Inkemia IUCT group founded *ad hoc* with the commitment to drive those preliminary results into valuable products that could reach the people affected by DM1 by reaching the market.

Given the effect of caffeine on MBNL1,[169] our hypothesis was that other natural xanthines might also exhibit some anti-DM1 activity. Accordingly, the further extension of the research was focused on in-depth exploration of the behavior of these compounds in DM1 models. Moreover, combinations of xanthines in general, or methylxanthines, as well as combinations of caffeine with at least another xanthine were expected to exhibit some synergistic effects in *in vitro and/or in vivo* DM1 models. This rationale was the origin of the present research project.

Xanthines and methylxanthines

1.6. *Introduction*

In chemical terms, xanthines are purine bases, heterocyclic organic compounds comprising coupled pyrimidinedione and imidazole rings. Methylxanthines are xanthines wherein at least one of the nitrogen positions in the skeleton is covalently bonded to a methyl group, generating monomethylated, dimethylated and trimethylated derivatives. Theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) are examples of naturally occurring dimethylxanthines, whereas caffeine (1,3,7-trimethylxanthine) is the single naturally occurring trimethylxanthine (see **Figure 6** and **Table 12**).

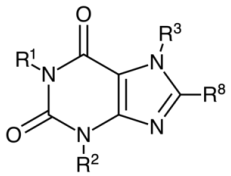

Figure 6: Chemical structure of xanthine scaffold

Table 12: Natural purine alkaloid structures based on xanthine skeleton

Compound	Trivial name	R^1	R^2	R^3	R^8	Naturally found in
Xanthine	-	H	H	H	H	Plants
1-Methylxanthine	-	CH_3	H	H	H	Plants
3-Methylxanthine	-	H	CH_3	H	H	Plants
7-Methylxanthine	-	H	H	CH_3	H	Plants
1,3-Dimethylxanthine	Theophylline	CH_3	CH_3	H	H	Plants
1,7-Dimethylxanthine	Paraxanthine	CH_3	H	CH_3	H	Animals, by-product
3,7-Dimethylxanthine	Theobromine	H	CH_3	CH_3	H	Plants
1,3,7-Trimethylxanthine	Caffeine	CH_3	CH_3	CH_3	H	Plants
2,6,8-Trihydroxypurine	Uric acid	H	H	H	OH	Animals, by-product

In biological terms, methylxanthines are secondary plant metabolites from purine nucleotides naturally found in the berries, seeds and leaves of nearly 100 species in 13 orders of the plant kingdom, although highly accumulated in certain parts of that species such as in *Coffea* seeds, *Theobroma* seeds and *Camellia* leaves,[171] where particularly caffeine is supposed to have a chemical defence crop protection role as well as acting as naturally occurring pesticide.[172]

In caffeine-containing plants, the main biosynthetic pathway to caffeine is composed by a four steps enzymatic route that transform the initial substrate xanthosine nucleoside to the intermediate 7-methylxanthosine (step I), which is enzymatically hydrolyzed to 7-methylxanthine (step II), that is enzymatically methylated into theobromine (step III), which is further methylated to caffeine in a second methylation step (step IV). Some other minor pathways may also occur due to substrate unspecificities of the enzymes.[171]

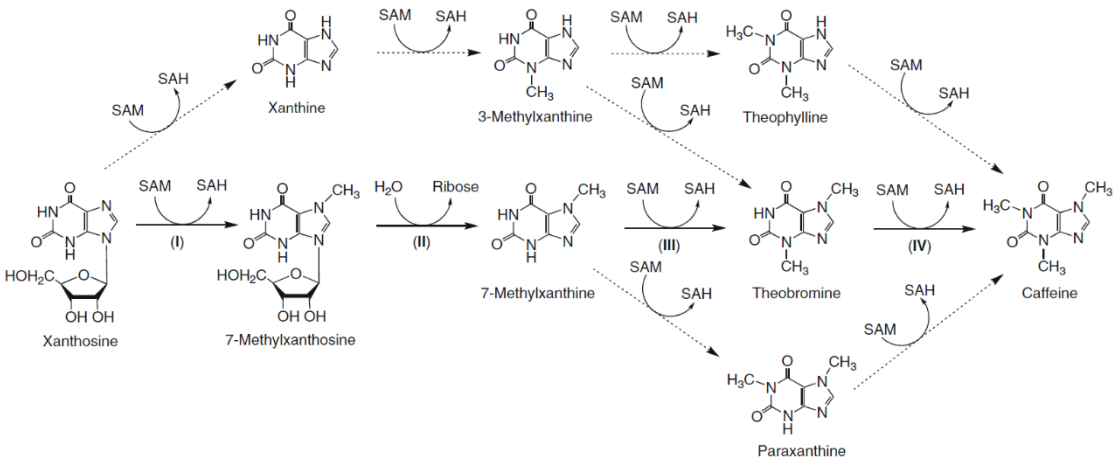

Figure 7: The main biosynthetic pathway (in solid arrows) and some minor pathways (in dotted arrows) to caffeine from xanthosine
Enzymes: 7-methylxanthosine synthase (EC 2.2.2.158, steps I and II), *N*-methylnucleosidase (EC 3.2.2.25, step II) theobromine synthase (EC 2.1.1.159, step III) and caffeine synthase (EC 2.1.1.160, step IV), as well as SAM (*S*-adenosyl-*L*-methionine) and SAH (*S*-adenosyl-*L*-homocysteine).

Once formed, caffeine is slowly degraded to uric acid which is further degraded to CO_2 and NH_3 via a first limiting step through theophylline and/or theobromine. Accordingly, caffeine is naturally accumulated in most plants containing methylxanthines, except for *Theobroma cacao* where theobromine is the most important methylxanthine, even more than caffeine itself.

Beyond the naturally occurring methylxanthines, synthetic methylxanthines bearing chemical substitutions different than methyl in their structure have been obtained in the laboratory due to the interest in certain therapeutic properties related to the diverse mechanism of actions of methylxanthines. **Table 13** shows some examples:

Table 13: Examples of synthetic purine compounds based on xanthine skeleton

Compound	Trivial name	R^1	R^2	R^3	R^8	Uses
8-Chlorotheophylline	-	CH_3	CH_3	H	Cl	Antiemetic as dimenhydrinate salt
8-Bromotheophylline	-	CH_3	CH_3	H	Br	Diuretic as pamabrom form
3-Propylxanthine	Enprofylline	H	C_3H_7	H	H	Phosphodiesterase inhibitor, bronchodilation
3-Isobutyl-1-methylxanthine	IBMX	CH_3	C_4H_9	H	H	Phosphodiesterase inhibitor, reduction of inflammation
Methylxanthine [1-(5'-oxohexyl)-3-methyl-7-propylxanthine]	Propentofylline	$C_6H_{11}O$	CH_3	C_3H_7	H	Phosphodiesterase inhibitor, neuroprotection

1.7. *Pharmacodynamics*

1.7.1. Mechanisms of action

Therapeutic uses of methylxanthines are mostly related to the treatment of certain respiratory diseases, such as asthma,[173] apnea of prematurity[174] and other paediatric respiratory tract diseases,[175] but also in cardiovascular diseases, obesity and diabetes, coadjuvant of analgesics, diuretics and even cancer.[176]

The behavioural stimulant effects of methylxanthines in the brain were first disclosed to involve the blockage of adenosine receptors.[177] Currently, at least four different cellular mechanisms of action have been described to explain the effects of methylxanthines *in vivo*:

- Antagonism of adenosine receptors
- Inhibition of phosphodiesterases (PDEs)
- Modulation of GABA receptors
- Regulation of intracellular calcium levels

Adenosine receptor antagonism is the main mechanism underlying methylxanthines at physiologic concentrations, as well as at plasma concentrations reached upon dietary intake.[178] On the other side, the mechanisms mediated by inhibition of phosphodiesterases, modulation of GABA receptors and regulation of intracellular calcium levels require higher pharmacological doses of methylxanthines.[179, 180, 181] Accordingly, they can only be modulated through medications administered *ad hoc* in respiratory disease, cardiovascular disease, cancer, obesity and diabetes, neurological and neurodegenerative diseases, human fertility, and the like,[176] which are beyond the goals and framework of the present research.

Table 14: Methylxanthine effectiveness in different pharmacological contexts[176]

Action	Ranking
CNS stimulation	Caffeine > Theophylline > Theobromine
Respiratory stimulation	Caffeine > Theophylline > Theobromine
Coronary dilatation	Theophylline > Theobromine > Caffeine
Cardiac stimulation	Theophylline > Theobromine > Caffeine
Smooth muscle relaxation (bronchodilation)	Theophylline > Caffeine; Theobromine
Diuresis	Theophylline > Caffeine > Theobromine
Skeletal muscle stimulation	Caffeine > Theophylline > Theobromine

Probably, CNS stimulation is the best-known effect of methylxanthines, through which they increase arousal and vigilance, decrease fatigue, increase work capacity, decrease motor reaction time for some tasks and elevate mood in the body systems.[182]

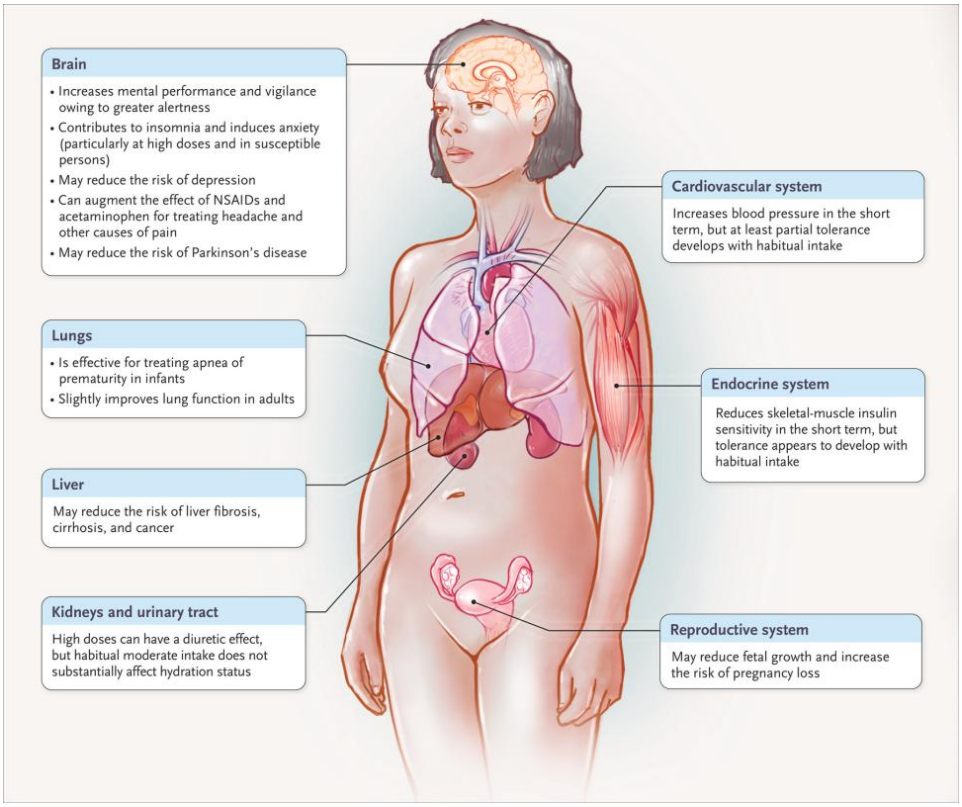

Figure 8: Effects of caffeine intake on health, according to the organ system
Reproduced from van Dam *et al.,*[183] with permission.

1.7.2. Antagonism of adenosine receptors

Adenosine receptors (AR) are a family of G-protein coupled receptors widely distributed in almost all human body tissues and organs where they participate in a large variety of physiopathological responses, including vasodilation, pain, and inflammation. In all those key processes, the endogenous purine nucleoside adenosine acts as a cellular regulator by modulation of the adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 which are expressed through the body, in the nervous, cardiovascular, respiratory, gastrointestinal, urogenital, and immune systems, as well as in bone, joints, eyes, and skin,[184] as shown in **Table 15**.

Table 15: Biological effects of adenosine

System	Effect	Receptor subtype
Central nervous system	Inhibition of neurotransmitter release	A_1
	Neuroprotection	A_1 / A_3
	Anxiolytic activity	A_1
	Anticonvulsant pain	A_1
	Reduction of pain	A_1 / A_3
	Excitatory activity	A_{2A}
	Stimulation of glutamate and acetylcholine release	A_{2A}
	Reduction of locomotor activity	A_{2A}
	Trophic effects	A_{2A} / A_{2B}
Cardiovascular system	Negative inotropic effect	A_1
	Negative chronotropic effect	A_1
	Negative dromotropic effect	A_1
	Ischemic preconditioning	A_1 / A_3
	Vasodilation	A_{2A} / A_{2B}
	Inhibition of platelet aggregation	A_{2A}
Immune system	Inhibition of reactive oxygen species	A_{2A} / A_3
	Neutrophils	A_1 / A_3
	Increase of chemotaxis	A_1
	Decrease of chemotaxis	A_3
Lymphocytes	Immunosuppression	A_{2A} / A_3 / A_{2B}
Monocytes/macrophages	Inhibition of proinflammatory cytokines release	A_{2A} / A_3 / A_{2B}
Mast cells	Stimulation of degranulation	A_3 / A_{2B}
Respiratory system	Bronchoconstriction	A_1 / A_3 / A_{2B}
Renal system	Vasoconstriction	A_1
	Vasodilation	A_{2A}
	Reduction of the glomerular filtration rate	A_1
	Inhibition of diuresis	A_1
	Inhibition of renin secretion	A_1
Gastrointestinal system	Inhibition of acid secretion	A_1
	Stimulation of intestinal chloride secretion	A_{2B} / A_3
Cellular metabolism	Inhibition of lipolysis	A_1
	Inhibition of insulin secretion	A_1
	Stimulant of gluconeogenesis	A_{2A}
	Production of glucose	A_{2B}

Certainly, due to their structural similarity between methylxanthines and the purine base adenine in adenosine (see **Figure 9** considering caffeine as a comparison example), methylxanthines can act as competitive inhibitors of all subtypes of adenosine receptors, although they mostly exert their action through inhibition of A_1 and A_{2A} receptor subtypes.[181]

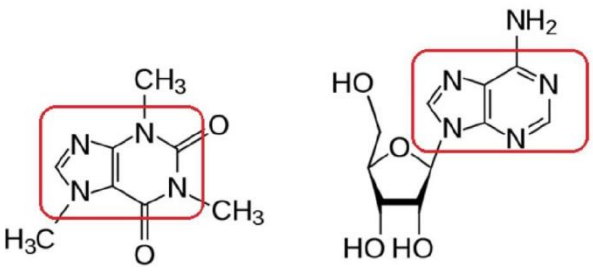

Figure 9: Comparison between the chemical structures of caffeine and adenosine

Therefore, methylxanthines antagonize the effect of adenosine and counterbalance the effects disclosed in **Table 15**.

1.8. *Pharmacokinetics*

1.8.1. Caffeine

Caffeine is rapidly and completely absorbed after oral intake in humans (t_{max} 30-120 minutes), 99% in just 45 minutes, mainly in the small intestine (80%) and in the stomach (20%), without any significant hepatic first-pass effect.[185]

Once absorbed, caffeine enters the intracellular tissue water and freely crosses all body barriers, reaching plasma, cerebrospinal fluid, saliva, bile, semen, breast milk, umbilical cord blood and all tissue organs. However, it is not long-term accumulated. The plasma half-life of caffeine is about 4 h, ranging from two to eight hours.

Caffeine's main metabolite in humans is paraxanthine (1,7-dimethylxanthine), by cytochrome CYP1A2 in the liver, accounting for 70-80% of metabolism, as well as theophylline and theobromine in minor quantities. Metabolites are further metabolized and excreted through urine, wherein about 70% of the administered oral dose of caffeine is eliminated in young to elderly, whereas the elimination is impaired in neonates and children due to their immature metabolizing hepatic enzyme systems.

It is widely recognized that the metabolism, clearance, and pharmacokinetics of caffeine is influenced by several different factors, including but not limited to age, gender, hormonal status, liver disease, obesity, smoking habits, medications affecting the activity of CYP1A2 isoform of cytochrome P450, and diet. Noteworthy, genetic variability among individuals, particularly polymorphisms in the enzymes metabolizing caffeine, such as the CYP1A2, which metabolizes 95% of the caffeine ingested, and *N*-acetyltransferase 2, as well as in the expression of the adenosine A2A receptor, the main brain target of caffeine, contribute to explain the large variability in caffeine effects observed on the human body by mechanisms that still are not fully understood.[186]

Gender is a controversial source of variation in caffeine pharmacokinetics. In this regard, whereas some studies disclose that gender has no effect on caffeine pharmacokinetics in men and women,[187] some other studies conclude that males and females differ in their responses to caffeine, being males more prone to respond to caffeine in lower doses than females.[188]

Oral lethal dose (LD_{50}) of caffeine in humans is 150-200 mg/kg body weight.[189]

1.8.2. Theophylline

Like caffeine, theophylline is rapid and completely absorbed after oral intake in humans, [185] with peak concentrations occurring from 0.5 to 2h.[190] Distribution is largely affected by plasma protein binding, which could reach 53% to 65%.

Once metabolized in the liver by several CYP isoenzymes including CYP1A2, CYP2E1 and CYP3A4, theophylline is eliminated by biotransformation in the liver into several metabolites, including 3-methylxanthine, that are finally excreted by urine. Elimination is affected by age, body weight, diet, smoking habits, other drugs and cardiorespiratory or hepatic disease.[191]

No data is available for the oral lethal dose (LD_{50}) of theophylline in humans.[189]

1.8.3. Theobromine

Theobromine pharmacokinetics has been less studied than caffeine and theophylline.[185] In contrast to caffeine, theobromine is fat-soluble, reaches peak blood concentrations in 2–3 hours after ingestion,[192] and has an estimated half-life from 2h to 6h, without accumulation.

In humans, theobromine is distributed throughout the total body water, wherein it can be found in plasma, saliva and breast milk, before being metabolized and excreted through urine.

Theobromine is toxic for a variety of mammals such as dogs.[193] The oral lethal dose (LD_{50}) in humans is 1,000 mg/kg.[189]

1.8.4. Paraxanthine

Paraxanthine (1,7-dimethylxanthine) is the main caffeine metabolite.[185] It is fully absorbed in the gastrointestinal tract, and distributed in the blood and tissues, but not in the brain, where it seems to be excluded from crossing the blood-brain-barrier.

In humans, approximately 60% of orally administered paraxanthine may be recovered as unchanged in the urine. Its main metabolite is 1-methylxanthine.

Final remarks

Myotonic dystrophy type 1 (DM1) has been disclosed as one of the more complex diseases already known.

DM1 could be described as a spliceopathy regulated by the RNA-binding MBNL and CELF protein families, more specifically by the MBNL1 and CELF1 isoforms. Loss of MBNLs activity in DM1 is an effect of the expression of pathological CUG repeats that attract and sequester in the nuclei much of the available cellular pool of MBNLs. The sequestration of MBNLs directly impairs splicing of several key regulatory pre-mRNAs in muscles and neural cells and prevents fetal to adult pattern transition. At least several tens of splicing events are misregulated in DM1 due to MBNL1 loss-of-function and CELF1 gain-of-function, such as muscle chloride channel (CLCN1), insulin receptor (INSR), bridging integrator 1 (BIN1) and calcium channel voltage-dependent L type alpha 1S subunit (CACNA1S), wherein most of these events directly correlate with several symptoms of the disease such as myotonia, insulin resistance and muscle weakness, and therefore, explain the basis of this multisystemic disease.

No efficacious treatments or cure still exist for this multisystemic and degenerative disease. Those families suffering DM1 have a very limited quality of life, in terms of vitality, pain, general health perception, physical, emotional and social role functioning and mental health.

Given the fact that previous background research suggested that caffeine could elicit some effects on MBNL1, the hypothesis beyond the present thesis was that xanthines, as a compound family, might exhibit some positive effects in DM1. Particularly, methylxanthines are natural products consumed worldwide in food and beverages, whose properties, and beneficial[194,195,196] as well as deleterious effects[197] in health have been extensively described.

In the light of the ´Leave No-one Behind´ commitment behind Sustainable Development Goals (SDG),[198] and particularly, SDG3 devoted to good health and well-being and SDG10 devoted to reducing inequality, paying attention to the needs of disadvantaged populations, the dietary management in rare diseases might provide an extraordinarily valuable option for improving the quality of life of people living and suffering from some of these minority diseases.

The present research project aims to evaluate the potential of methylxanthines and their combinations as key ingredients in the nutritional management of DM1 through a nutritional approach to the disease.

GOALS

The present project aims to investigate the potential of methylxanthines as key ingredients in food products that could contribute to improve the health-related quality of life in myotonic dystrophy type 1 (DM1), through a nutritional management of the disease.

Main goal:

To scientifically substantiate properly formulated methylxanthines-based products as a novel nutritional management approach in myotonic dystrophy type 1.

Specific goals:

1. To delve into the state of the art of nutritional needs in the DM1 community, as well as the natural sources of methylxanthines and their content in foodstuffs.

2. Define the food product formulated with methylxanthine according to the current regulatory framework to be complied with.

3. To evaluate the role of methylxanthines, individually or in combination, in DM1 by assessing their *in vitro* and *in vivo* effect in validated models of the disease.

4. To establish the composition and dosage of the selected methylxanthine-based formulation in a specifically developed food product.

5. To evaluate the relationship between dietary methylxanthines and the quality of life in DM1.

6. To evaluate the effect of the investigational food product developed herein in the health-related quality of life in DM1 patients.

METHODOLOGY

Technological surveillance

Beyond the initial bibliographic search performed to launch this project, continuous scientific and technological monitoring have been carried out along the project lifetime, by specific searches in scientific databases, intellectual property databases, dissemination publications generated through patient associations and/or food products associations, regulatory agencies, the competent administration, and the like.

Regulatory framework

The legal bases discussed along Chapter 2 have been retrieved from the corresponding competent authorities and official sources:

1. Regulation of the European Parliament and of the Council has been retrieved from the European Union web page, EUR-Lex (https://eur-lex.europa.eu/homepage.html), that provides the official and most comprehensive access to EU Law and EU legal documents.
2. Regulation of the Spanish Member State and Spanish Law has been retrieved from *AESAN, Agencia Española de Seguridad Alimentaria* (https://www.aesan.gob.es/), *ACSA, Agència Catalana de Seguretat Alimentària* (https://acsa.gencat.cat/) and the *Boletin Oficial del Estado* (BOE) (https://www.boe.es/) web pages.
3. Regulation of the Belgium Member State and Belgium Law has been retrieved from the Belgium Federal Public Service – Health, Food chain safety and Environment web page (https://www.health.belgium.be/).
4. Regulation of the French Government and French Law has been retrieved from Légifrance. *Le service public de la diffusion du droit de la République Française* web page (https://www.legifrance.gouv.fr/), that encloses the *Journal Officiel* de la République Française (JORF).
5. Regulation of the Italian Government and Italian Law has been retrieved from *Ministerio della Salute* web page (www.trovanorme.salute.gov.it) and *Gazzeta Ufficiale della Reppublica Italiana* web page (https://www.gazzettaufficiale.it/).

In vitro models for DM1 studies

The *in vitro* models for DM1 have been accessed through a kind collaboration with the Translational Genomics group at the Genomics Department, University of Valencia (*Universitat de València, INCLIVA*), led by Professor Ruben Artero. The main methodology used in the *in vitro* experiments providing the results discussed in Chapter 3 is further disclosed herein.

1.1. *Compound binding to CUG repeats. Polarization fluorescence assays:*

This was assessed in fluorescence polarization spectroscopy experiments. In these tests, when polarized light excites a fluorophore conjugated to a small molecule, it undergoes rotational diffusion faster than the time required for light emission occurs, resulting in a random arrangement of the molecule in the fluorescence emission time (depolarization). However, the rotation of the molecule becomes slower depending on the viscosity of the medium or the molecular volume, increasing the polarization of the emitted light. Thus, by measuring changes

in polarization of RNA with 23 CUG repeats conjugated to the fluorophore carboxyfluorescein, FAM (CUG) 23, one can evaluate whether a candidate molecule binds to the RNA. Pentamidine, a compound previously shown to be attached to the CUG repeats, was used as positive control,[199] whereas a pretesting compound known to be unable to bind CUGs served as negative control.

Fluorescent CUG RNA (carboxyfluorescein (6-FAM)-labelled) was incubated with increasing concentrations of tested compounds. Whereas 6-FAM-CUG RNA molecules do not fluoresce in any particular polarization axis, binding to a molecule slows down the rotational movement of the molecule and increases polarization values. By binding to CUG RNA, compounds have the ability to modify the toxicity of CUG repeats by a number of mechanisms including altering the conformation of the secondary structures that these RNAs have in solution, or competitively inhibiting the sequestration of MBNL proteins.

Binding to CUG repeats was assessed in fluorescence polarization spectroscopy experiments in which fluorescent CUG RNA (carboxyfluorescein (6-FAM)-labelled) was incubated with increasing concentrations of the tested compounds. For a given compound, polarization is sensitive to concentration.

Carboxyfluorescein-labelled CUG RNA (6-FAM-CUG23) at 6 nM was incubated with the compounds at different concentrations in binding buffer (50 mM Tris-HCl pH 7.0, 250 mM NaCl, 50 mM $ZnCl_2$, 10% glycerol, 1 mM DTT) on ice for 20 min in the dark. Polarization was measured in an EnVision® Multilabel Reader using an excitation filter FP480 and emission filter FP535**.**

1.2. *Toxicity study*

To assess the toxicity of compounds in culture cells, different concentrations of compounds (from 0.1 to 100 μM) were added to standard media in DM1 fibroblasts and survival was studied using the CellTiter 96 Aqueous Non-Radioactive cell proliferation assay protocol; a colorimetric assay that determines viable cells.

As the percentage of survival was always above 50% in comparison to non-treated cells and above 75% in comparison to DMSO treated cells, all the concentrations were continued being used in the following assays of activity, as their toxicity was very low.

1.3. *Reduction of the number of ribonuclear foci per cell*

The reduction of ribonuclear foci per cell is typically used as evidence of desired activity in myotonic dystrophy in cell culture models, because MBNL proteins are sequestered in ribonuclear foci. Since MBNL proteins are limited in myotonic dystrophies, an increase in free MBNLs is expected to improve DM1 pathological situation.

1.4. *Evidences in human myoblasts*

Cell culture conditions

Cell model of the disease (provided to Prof. Artero's lab by the D. Furling's laboratory, Institute of Myologie, Paris) consisted of normal and DM1 (1300 CTG repeats) immortalized (hTERT)

skin fibroblasts expressing conditional MyoD. Fibroblast cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L of glucose, 1 % of penicillin and streptomycin (P/S) and 10% foetal bovine serum (FBS) (Sigma). Fibroblasts were transdifferentiated in myoblasts by inducing expression of MyoD. Cells were plated in muscle differentiation medium (MDM) made of DMEM 4.5 g/L glucose with 1% P/S, 2% horse serum, 1% apo-transferrin (10 mg/ml), 0.1 % insulin (10 mg/ml) and 0.02 % doxycycline (10 mg/ml) for 48 h. For compound testing purposes, fibroblasts were aliquoted in a 24-well plate with 3.5 x 10^4 cells per well and were differentiated as previously mentioned. Compounds were added to a final concentration of 37.5 mM, 75 mM and 125 mM in Modified Dulbecco's Medium (MDM) and cells were incubated for 48 h.

Foci detection

In situ hybridization with CUG repeat RNA. Cells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature followed by several washes in PBS 1x. Fixed cells were incubated in pre-hybridization buffer (SSC 2x, 30% deionized formamide) for 10 min at room temperature, hybridized with Cy3-(CAG)7-Cy3 labelled probe diluted 1:100 in hybridization buffer (40% formamide, 2x SSC, 0.2% BSA, 10% dextran sulphate, 2 mM vanadyl complex, 10% tRNA (10mg/ml), 10% herring sperm) for 2 h at 37°C, washed twice in pre-hybridization buffer for 15 min at 45°C, washed in PBS 1x for 15 min at room temperature and mounted in Vectashield (Vector) with 2 mg/ml DAPI. Images were taken using a Leica DM2500 fluorescence microscope and foci were manually counted from at least 50 cells per compound.

1.5. *Quantification of Muscleblind protein (Mbl) expression or localization*

Immortalized transdifferentiated DM1 fibroblasts were seeded in a 94-well plate (8000 cell/per well) in standard media. To achieve the differentiation of the fibroblast to myoblasts, MyoD expression was induced by changing the standard media to differentiation media with Doxycycline and without serum. 24h after induction, the compounds were added to the cells at different concentrations, always maintaining the concentration of the solvent (DMSO) at 1% in the media. Every concentration of compound was tested in three different wells. 24h after adding the compounds the cells were fixed with paraformaldehyde 4% in PBS and Mbl detection using monoclonal anti-MBNL1 antibody was performed counterstaining with Hoechst to detect the nuclei. Incell 200 plate reader confocal was used for detection and quantification of the parameters selected and the data was analyzed using Graphpad software for statistical comparison of results.

1.6. *Foci quantification*

DM1 fibroblasts were seeded in a 94-well plate (8000 cell/per well) in standard media one day before adding the compounds to the media. Compounds were added to the cells at different concentrations, always maintaining the concentration of the solvent (DMSO) at 1% in the media. Each concentration of compound was tested in three different wells. 24h after adding the compounds the cells were fixed with paraformaldehyde 4% in PBS and FISH to detect CUG RNA was performed using a (CAG)23 probe labelled with Cy3. Cells were counterstained with Hoechst to detect the nuclei. Incell 200 plate reader confocal was used for detection and

quantification of the parameters selected and the data was analyzed using Graphpad software for statistical comparison of results.

In vivo models for DM1 studies

The fruit fly and vinegar *Drosophila melanogaster* and humans share many genes. It is estimated that two-thirds of the genes associated with human diseases have their homologous genes in *Drosophila* (666 of 991). In particular, metabolic pathways and signalling mechanisms are highly conserved at the cellular level: circadian rhythm, learning and memory, sleep, and CNS diseases such as Huntington's, Parkinson's and Alzheimer's diseases. The *Drosophila* model has been used successfully in screening compounds for the aforementioned diseases, as well as in cancer and anti-aging products.[200,201]

The *in vivo* models for DM1 have been accessed through a kind collaboration with the Translational Genomics group at the Genomics Department, University of Valencia (*Universitat de València, INCLIVA*), led by Professor Ruben Artero. The main methodology used in the *in vivo* experiments providing the results discussed in Chapter 3 is further disclosed herein. Animal welfare has been strictly considered in all the experiments.

1.7. *Functional assays in Drosophila melanogaster model*

Artero's group previously reported important functional deficits in flies expressing 480 interrupted CUG repeats under the muscle-specific driver Myosin-heavy chain (MHC)-Gal4.[202] These DM1 model flies displayed a 10% reduction in climbing velocity and a drastic 80% reduction of flying ability measured as landing distance. To test the effect of compounds on these phenotypes, MHC-Gal4 and UAS-(CUG)480 flies were crossed in bottles with nutritive media containing the compounds so the F1 (first offspring) was in contact with the compound throughout development. As control of disease non-treated flies, the same flies were crossed in standard nutritive media and as control of non-disease flies, MHC-Gal4 flies were crossed with the yellow-white (yw) genetic background in standard nutritive media.

Functional assays were performed according to the well established procedures disclosed herein:

Climbing assay

To assess climbing velocity, flies were transferred, after emerging, to tubes with compounds or the solvent DMSO 1% in standard nutritive media. Groups of twenty 7-day-old males were transferred into pipettes (1.5 cm in diameter and 25 cm in height) after a period of 24 hours without anaesthesia. The height reached from the bottom of the vial by each fly in a period of 5 s was recorded with a camera. For each genotype, approximately 30 flies were tested.[203] The results show the mean speed in mm/s. We used a t-test to assess the statistical difference between the two groups of flies, fed with or without compound.

Flying assay

To assess the effect of compounds on flying performance, the flies were transferred after emerging, to tubes with compound or the solvent DMSO 1% in standard nutritive media. Flying

assays were performed on day 5,[204] using 50 male flies per group. Landing distance was compared between the two groups using t-test and considered significantly different when $p<0.05$.

Drosophila lifespan analysis

A total of 100–150 newly hatched flies per genotype were collected, placed in tubes containing standard nutritive medium and kept at 25ºC. The number of deaths was scored on a daily basis, and flies were transferred into fresh medium every 3–4 days. Survival curves were obtained using the Kaplan–Meier method, and statistical curve comparisons were carried out according to the log-rank (Mantel–Cox) test ($\alpha= 0.05$).

Quality standards

All the work carried out along the present doctoral thesis has been performed within the framework of the in force quality standards of application in the corresponding thematic areas disclosed herein.

1.8. *International Organization for Standardization (ISO) quality standards*

In general terms, ISO 9001 quality management system standard certification has been the wider standard covering the quality aspects of this research. International Organization for Standardization (ISO)[205] standards are by far the most widely accepted set of quality standards that support innovation and provide solutions to global challenges in all types and sizes of organizations in the world. This quality management system is based in the Deming cycle or PDSA (Plan-Do-Study-Act)[206] as a continuous improvement of processes and product quality to meet the established set of requirements for a given goal.

By the time this research was performed, IUCT, the organization where the experimental part of this doctoral thesis was carried out, held the ISO 9001:2008 quality management system standard certification in several areas of its activity, including research and development.

1.9. *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) quality standards*

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)[207] is a unique organization in the world bringing together the regulatory authorities and pharmaceutical industry to discuss scientific and technical aspects of pharmaceuticals and develop technical documents recommendations, named the ICH guidelines. ICH guidelines[208] are core documents in the pharmaceutical industry standards in order to provide an harmonized framework to guide the drug development process in terms of quality, safety and efficacy, as well as to provide an harmonized structure for the Common Technical Document, aimed at drug registry purposes to achieve the marketing authorization licence.

Even though ICH guidelines are quality standards in the pharmaceutical industry, and as such, not applicable in the basic or common food industry, the fact that food for special medical purposes are food products intended for the dietary management (under medical supervision)

of individuals who suffer from certain diseases, disorders or medical conditions, and in this particular case, intended for a complex rare disease, encouraged the application of the highest quality standards beyond the strictly requested framework.

Moreover, according to current regulation on food for special medical purposes and the dossier's content as recommended by the European Food Safety Agency (EFSA) (see Chapter 2 for further details), a food for special medical purposes is expected to fulfil quality standards, regarding stability testing for instance, closer to pharmaceutical standard than to food standards.

In this regard, the corresponding current Good Practices (cGxP) quality guidelines have been applied to the different specific fields covered along the present work.

1.10. *Current Good Manufacturing Practices quality standards (cGMP)*

Current Good Manufacturing Practices (cGMP) have been the quality framework for the development, manufacture and analytical tasks performed on the food product disclosed herein.

As defined by the World Health Organization (WHO), 'Good manufacturing practice (GMP) is that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization'[209]. As defined by the European Medicines Agency (EMA), 'Good manufacturing practice (GMP) describes the minimum standard that a medicine manufacturer must meet in their production processes'.[210] Accordingly, GMPs apply to human and veterinary medicines, but not to food.

However, according to current regulation on food for special medical purposes and the dossier's content as recommended by the European Food Safety Agency (EFSA) (see Chapter 2 for further details), a food for special medical purposes is expected to be manufactured according to a GMP quality system.

By the time this research was performed, IUCT, the organization where the experimental part of this doctoral thesis was carried out, held the GMP certification for the Manufacture and Control of Medicines and Research Medicines by the Spanish Agency of Medicines and Medical Devices (AEMPS), under laboratory authorization number 4155-E. The Pharmaceutical Pilot Plant was led by Dr. David Miguel, co-Director of this thesis, as the Qualified Person.

The investigational food product for the clinical trial was manufactured by a contract manufacturer organization (CMO) which held cGMP accreditation for dietary supplements, in accordance with FDA quality standards for such products.

1.11. *Good Clinical Practices*

As defined by EMA, 'Good clinical practice (GCP) is an international ethical and scientific quality standard for designing, recording and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety and wellbeing of trial subjects are protected and that clinical-trial data are credible'.[211]

Commission Directive 2005/28/EC of 8 April 2005 laying down principles and detailed guidelines for good clinical practice as regards investigational medicinal products for human use, as well as the requirements for authorisation of the manufacturing or importation of such products[212] (also known as the Good Clinical Practice Directive) regulates, among others, 'the principles of good clinical practice and detailed guidelines in line with those principles, for the design, conduct and reporting of clinical trials on human subjects involving such products'.

Even though the food product is not a medicinal product as such, current Good Clinical Practices (cGCP) have been the quality framework for the clinical trial performed on human volunteers testing the investigational food product in the present research.

Ethical issues and in human research laws

The research with human samples and the pilot clinical trial have been carried out in accordance with the International, EU and Spanish requirements as expressed and/or regulated in:

- Nuremberg Code (1947)[213]
- Declaration of Helsinki (revision of Fortaleza, October 2013)[214]
- Universal Declaration about Human Genome and Human Rights of UNESCO (1997)[215]
- ETS No 164. Additional Protocol of European Committee for the protection of Human Rights and Human Dignity in Biology and Medicine Applications (Oviedo convention, 1997)[216]
- CTES 195. Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research (Strasbourg, 2005)[217]
- Recommendation CM/Rec(2016)6 of the Committee of Ministers to member States on research on biological materials of human origin (Strasbourg, 2016)[218]
- EMA Guideline for Good Clinical Practice E6(R2) (current effective version 2016)[219]
- Spanish Biomedical research Law (*Ley 14/2007, de 3 de julio, de Investigación biomédica*).[220] This law establishes the basic requirements for the authorization and functioning of biobanks with biomedical research purpose and for the processing of human samples
- Royal Decree related to the basic requirements for authorization and operation of biobanks for biomedical research purposes and the treatment of biological samples of human origin, and regulates the operation and organization of the National Registry of Biobanks for biomedical research (*Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica*)[221]
- Royal Decree related to observational studies in human (*Real Decreto 957/2020, de 3 de noviembre, por el que se regulan los estudios observacionales con medicamentos de uso humano*)[222]

The study was carried out in accordance with the protocol, which was submitted to the Ethics Committee for Clinical Research of the Health Area of Gipuzkoa (Study Protocol, Participant

Information Sheet and Consent Form) for its evaluation and approval, according to Spanish regulations. Moreover, the above-mentioned Ethics Committee has followed up the implementation of the study. All the interventions, including text blood sampling procedures, have been performed under the informed consent approved by the Ethics Board of the Institution Hospital Universitario Donostia. All samples are stored at the Donostia node of Basque Biobank of DNA and Tissues for Research, under the approval of the Biobank Ethics Committee. Written informed consent has been obtained from all patients (or legal guardians of patients) participating in the study.

Data protection

As general considerations, all the parties involved in the study, sponsor, researchers and participants accepted the national and international ethical standards on research. The project and treatment, communication and transfer of personal data of all participant subjects had strictly complied with the provisions in strict compliance with applicable international and EU law, particularly, Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data,[223] and repealing Directive 95/46/EC (General Data Protection Regulation)), as well as national law (Organic Law 3/2018 of December 5, on the Protection of Personal Data and Guarantee of Digital Rights).

Statistical analysis

The data from the observational and the interventional clinical trials was collected in the corresponding questionnaires in prospective form. Once both studies were finished after recording the data of the last participant included, the databases were closed and the statistical analysis was carried out. Accordingly, the content of each database was exported to the corresponding files under a suitable format used for the statistical analysis.

During the development of the study, deviations from the protocol may arise that have to be taken into account in the analysis phase. If for any reason a patient drops out of the study, they would not be replaced by another subject.

Descriptive and exploratory analysis

In the descriptive analysis, the distribution of each variable was analyzed, checking that all the data had values within the expected range, and that there were no logical inconsistencies between variables, or impossible data. If any of these circumstances occurred, the information registered in the database was corrected by comparing the information with that of the clinical history.

As a result of the descriptive analysis, the estimators of the variables were obtained using the most appropriate statistic for the nature and scale of measurement of each one.

The Shapiro-Wilk normality test (p>0.05) and Levene's homogeneity test (p>0.05) were used for each variable at each time point in order to assess the parametric or non-parametric distribution of each data set.

Descriptive analyzes (measures of central tendency and dispersion, expressed by the statistics mean, standard deviation, median, interquartile range -IQR) of the scores of the domains from the questionnaires were carried out, as well as the confidence intervals were calculated. The parametric or non-parametric behaviour is expressed in **bold** in the tables of Chapter 4 and Chapter 5.

The mean or median differences at two timepoints *between* arms were studied using parametric (unpaired Student's t-test) or non-parametric (independent samples Mann-Whitney's U tests), when appropriate. The mean or median differences between two timepoints *within* arms were studied using parametric (paired Student's t-test) or non-parametric (Wilcoxon signed-rank) tests, when appropriate.

Correlation between variables was explored using Pearson or Spearman's correlation test, according to the parametric or non-parametric distribution of the sample, respectively, as well.

Throughout the study, 95% confidence was considered when determining statistical significance (p<0.05).

RESULTS AND DISCUSSION

The **Results and Discussion** section of the present study is scheduled in five chapters according to the objectives they aim to achieve:

- **Chapter 1** introduces the concept of nutritional management, evaluating nutritional studies on DM1, research on natural sources of xanthines and methylxanthines, as well as research on dietary content and consumption of methylxanthines from natural sources. Accordingly, this Chapter addresses the specific goal nr 1.

- **Chapter 2** delves into the regulatory framework of food products through the legal basis that regulates the principles of food supplements and food for special medical purposes. The laws that regulate these categories are analyze herein, as well as requirements for launching both types of food products on the market. In this regard, Chapter 2 discloses the requirements of the regulatory framework to achieve the specific goal nr 2.

- **Chapter 3** discloses the development of the product, from the definition of its composition through *in vitro* and *in vivo* studies on DM1 models, to the definition of the dosage and the development of dosage form. Intellectual property issues, such as patent protection status and freedom to operate studies are also evaluated throughout this Chapter. Then, Chapter 3 addresses the specific goals nr 3 and nr 4.

- **Chapter 4** discusses the observational nutritional study code MD Project that measures the nutritional habits, dietary methylxanthine intake pattern and its relationship to quality of life in a cohort of adults with DM1. Chapter 4 fulfills specific goal nr 5.

- **Chapter 5** reveals the design, execution and results of the interventional study code NCT04634682 (Effect of MYODM on Quality of Life, Fatigue and Hypersomnia in Patients with Myotonic Dystrophy Type 1), whose objective is to evaluate whether the formulated composition containing *Theobroma cacao* supplemented with caffeine, improves the quality of life of DM1 patients, through the management of their excessive daytime sleepiness. Some insights on DM1 patients under COVID-19 pandemic, the time frame in which this study was carried out. Chapter 5 responds to the specific goal nr 6.

CHAPTER 1: NUTRITIONAL ASPECTS IN DM1

Chapter 1 introduces the concept of nutritional management, evaluating nutritional studies on DM1, research on natural sources of xanthines and methylxanthines, as well as the research on the dietary content and consumption of methylxanthines from natural sources. Accordingly, this Chapter addresses the specific goal nr 1.

1.1. Introduction

Although health was already defined in ancient times, the modern official definition of health was established in 1948 during the Constitution of the World Health Organization (WHO)[224] declaring that '*Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity*' and that '*The enjoyment of the highest attainable standard of health is one of the fundamental rights of every human being without any distinction of race, religion, political relief, economic and social condition*'. Accordingly, the main objective of WHO (Article 1)[224] is 'the attainment by all people of the highest possible level of health'.

In 1977, through WHO's strategy 'Health for All' and the Declaration of Alma-Ata[225], health definition was extended to include the social and economic concepts aimed at 'the attainment by all peoples of the world by the year 2000 of a level of health that will permit them to lead a socially and economically productive life'. Since then, health definitions have been extended and reviewed by several authors.[226,227]

Overall, the current health concept embraces a holistic approach since health is not merely the absence of disease or impairment, either physical or mental, but an equilibrium state in one's daily life demands, in a social environment.

WHO recognizes nutrition as a critical part of health and development.[228] However, malnutrition in all its forms, including undernutrition and overnutrition, is expanding all over the world. In this regard, whereas between 720 and 811 million people in the world faced hunger in 2020[229] and 462 million people are underweight,[230] 1.9 billion adults were overweight or obese in 2021.[230]

Overweight and obesity are defined by WHO as abnormal or excessive fat accumulation that may impair health.[231] Moreover, obesity is one of the main driving forces related to noncommunicable diseases (NCDs)[232], leading to cardiovascular diseases, strokes, cancers, and diabetes. WHO classifies adult underweight, overweight, and obesity according to body mass index (BMI), defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2).[233]

Table 16: WHO classification[234] of adult underweight, overweight, and obesity according to BMI

WHO Classification			**BMI (kg/m^2)**
Underweight	< 18.50	Severe thinness	< 16.00
		Moderate thinness	16.00 – 16.99
		Mild thinness	17.00 – 18.49
Normal range	18.50– 24.99	Normal range	18.50 – 24.99
Overweight	≥ 25.00	Pre-obese	25.00 – 29.99
Obese	≥ 30.00	Class I	30.00 – 34.99
		Class II	35.00 – 39.99
		Class II	≥ 40.00

Unhealthy diets and poor nutrition are among the top risk factors for many life threatening diseases. Therefore, even considering that different nutritional needs are required along the life, related to age, sex, existence of disease conditions, physical status and the like, a healthy diet helps to protect against malnutrition as well as noncommunicable diseases.[235]

A healthy diet might provide an appropriate caloric intake, preferably from plant-based origin foods, low amounts of animal source foods, unsaturated rather than saturated fats, and very limited amounts of refined grains, processed or ultra-processed foods, and added sugars.[236] Altogether, a healthy diet should optimize health.[236]

When referred to a healthy diet for adults, WHO include[235]:

- Fruit, vegetables, legumes, nuts and whole grains.
- At least 400 g (i.e. five portions) of fruit and vegetables per day, excluding potatoes and other starchy roots, to provide an adequate intake of dietary fiber.
- Ideally, less than 5% of total energy intake from free sugars (*i.e.* 50 g) for a person of healthy body weight consuming about 2000 calories per day.
- Less than 30% of total energy intake comes from fats, wherein unsaturated fats (less than 10% of total energy intake) are preferable to saturated fats and *trans*-fats of all kinds (to less than 1% of total energy intake). Moreover, industrially-produced *trans*-fats should be avoided.
- Less than 5 g of salt (preferably iodized) per day.

As defined by the Food and Agricultural Organization of the United Nations (FAO)[237] based on the current available scientific evidence, 'food-based dietary guidelines (FBDG) are intended to establish a basis for public food and nutrition, health and agricultural policies and nutrition education programmes to foster healthy eating habits and lifestyles. They provide advice on foods, food groups and dietary patterns to provide the required nutrients to the general public to promote overall health and prevent chronic diseases'.

In this regard, FBDG aims to guide food and diet patterns recommendations to promote health, while being adapted to the cultural dietary habits of the particular population they target. Several reviews related to distinct geographic areas have been reported, summarizing the global situation of FBDG,[238] a comparison of North and South American countries,[239] a comparison among European countries,[240] as well as at national level, such as FBDG for the Spanish population,[241] based on the Mediterranean diet, among many others. As per 2021, the current version of the Spanish food-based dietary guidelines developed by the Spanish Society of Community Nutrition (SENC) was visually summarized in the healthy eating and hydration pyramids[242] shown in **Figure 10** to **Figure 12**:

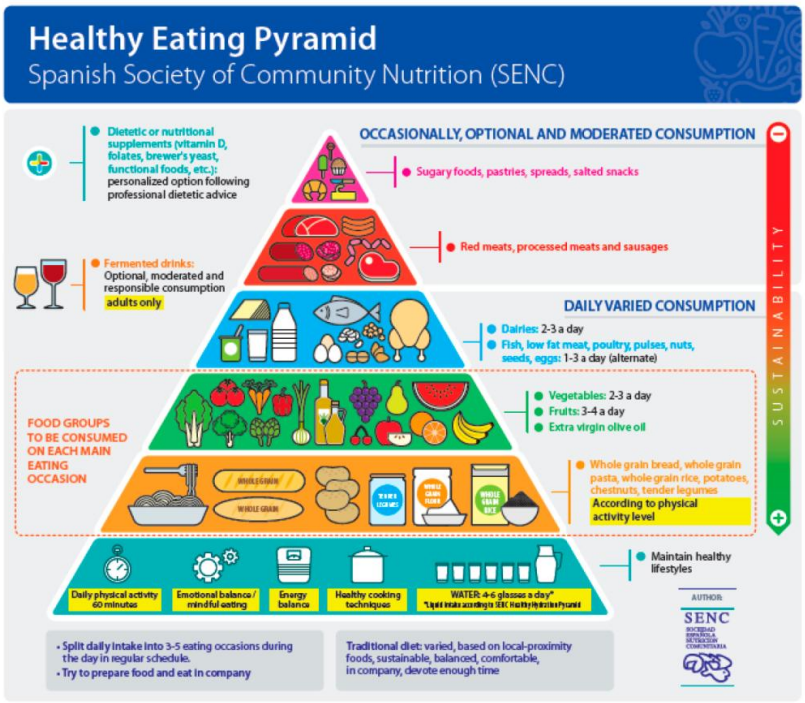

Figure 10: Healthy Eating Pyramid for adults

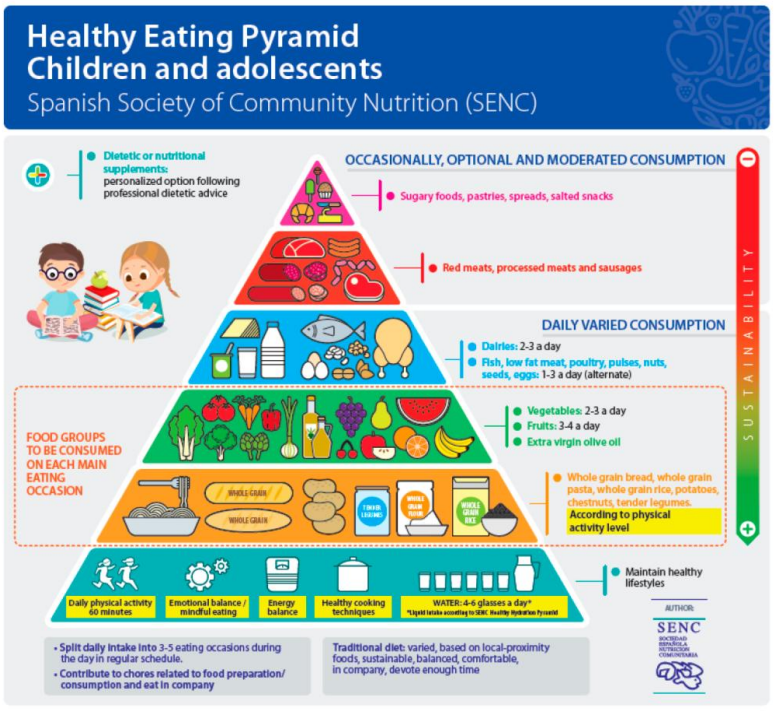

Figure 11: Healthy Eating Pyramid for children and adolescents

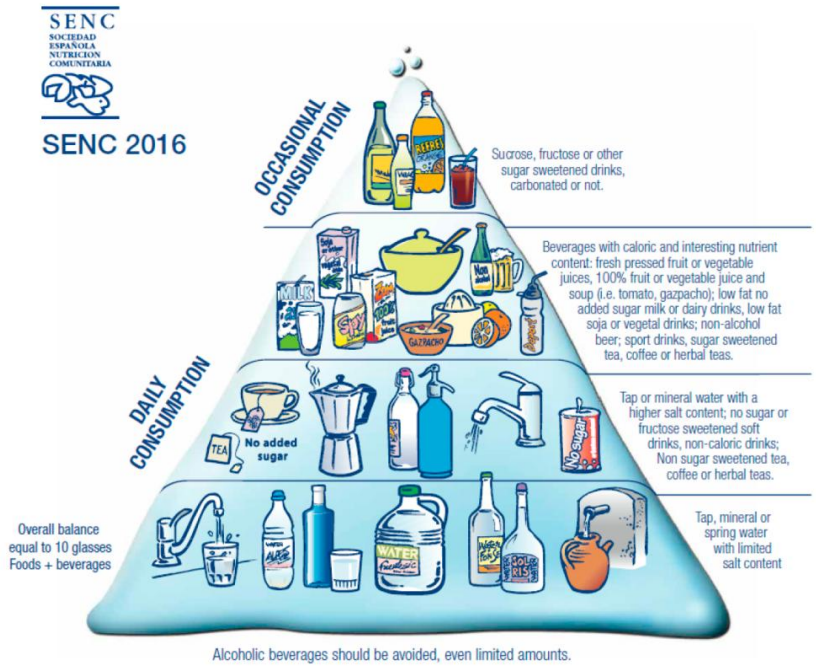

Figure 12: Healthy Hydration Pyramid for adults

Through the pyramid icon, the dietary guidelines are plenty of underlying information, wherein food is properly located from base to top according to its relevance and proportionality in the healthy diet. Moreover, the different colours for each level contain warning messages, which are inspired in the traffic lights in the middle to bottom part of the pyramid, from the green that invites to proceed, to the red one that somehow alerts about health dangers due to excessive consumption.

Regarding hydration, it is considered as a fundamental aspect of a healthy diet, wherein water is the basis for proper hydration, followed by several beverages without caloric content. The top of the pyramid is reserved for very occasional consumption of highly sweetened drinks. Alcoholic beverages have been fully omitted in the pyramid, with one single warning message to be avoided, even in limited amounts.

It is worth mentioning that the previous food suggestions are recommendations for the general population, which could request some adaptation in the case of specific population groups, according to their corresponding specificities, to meet the goal of optimizing health through the daily diet.

1.2. Nutrition and neuromuscular diseases

1.2.1. Introduction

Very often, the natural progression of the majority of neuromuscular diseases involves a decrease in mobility, particularly ambulation, a worsening of the pulmonary function and the deterioration of muscle strength, connected with an increase in intramuscular fat and adiposity. Accordingly, energy needs are highly related to the particular physical condition, age and daily habits, including the nutritional habits. An improper diet, either due to overnutrition or undernutrition, negatively impacts on the disequilibrium between energy needs and energy expenditures. In this regard, the prevention of both overweight and underweight situations in neuromuscular patients is extremely important to avoid further complications.

Some of the main factors that contribute to nutritional disturbances in patients with neuromuscular diseases are summarized below (adapted from Marinis[243]):

1. Gastrointestinal complications (dysphagia, gastroparesis, gastric distention, constipation, gastroesophageal reflux, and the like)
2. Swallow impairment due to muscle weakness
3. Fatigue (either for planning and preparing the meals, as well as for eating itself)
4. Increase work of breathing due to muscle weakness
5. Systemic inflammation
6. Factors contributing to obesity, such as:
    a. Diminished physical activity and caloric expenditure associated to muscular weakness
    b. Increase of intramuscular mass
    c. Poor diet choice (associated with frequent limited income levels)
    d. Steroid therapies

Moreover, since nutrition requirements evolve and change throughout life, neuromuscular patients' diet must be carefully monitored to provide the levels of macronutrients and micronutrients adapted to each particular evolving profile at each specific period of life, in accordance with the disease progression.

The maintenance of healthy nutritional habits is fundamental to counterbalance the previously mentioned limiting factors, since a proper diet and healthy lifestyles could help to:

1. Prevent or counterbalance gastrointestinal complications
2. Contribute to limit loss of muscular mass
3. Prevent obesity, hypertension, hypercholesterolemia, hypertriglyceridemia
4. Contribute to a correct growth in paediatric population

In general terms, nutritional recommendations are based on the healthy nutrition guidelines previously disclosed for the general population, but simply adapted to each particular situation in order to fulfil energy needs, maintain mass muscle or to deal with swallowing limitations and/or gastrointestinal issues. The main recommendations for these patients suggest to follow

a balanced diet comprising adequate macronutrients (carbohydrate, protein, fat and fluid), as well as certain micronutrient supplementation (calcium and vitamin D), in small portions and frequent meals.[243] Hence, nutritional recommendations for people living with neuromuscular diseases comprise a slightly enrichment in protein content (15-18% of the total caloric value), balanced in carbohydrates (50-60% of the total caloric value) and fats (30% of the total caloric value), limited to the recommended caloric intake for each individual. [244]

There is a lack of official guidelines on the management of (mal)nutrition in patients with DM1.[245] However, patient associations publish nutrition guides and healthy guidance for people living with these diseases. In this regard, our research was guided by the recommendations from neuromuscular patient associations[244] and myotonic dystrophy associations,[246] which had been properly assessed by the corresponding medical advisory committees.

1.2.2. Macronutrients

1.2.2.1. Caloric requirements

The determination of energy needs for a particular individual strongly depends on several key parameters, such as age, gender, weight and height, physical activity and even presence of certain illnesses. In this regard, nutrition specialists first determine the exact energy needs for that particular person, and then translate the needs into the most appropriate type of foods, serving sizes and number of meals, to provide the optimal nutritional schedule according to said particular needs.

Beyond the sedentary lifestyle and low activity levels, the energy expenditure in slowly progressive neuromuscular disease subjects has been reported to be 25% lower when compared to the 'normal' subjects taken as controls.[247] Moreover, an increased energy cost of physical activity has been disclosed for neuromuscular disease subjects.[248] Overall, energy needs should be carefully calculated when referring to people living with neuromuscular diseases.

1.2.2.2. Proteins

Progressive muscular weakness is a common feature in neuromuscular diseases. Accordingly, maintenance of muscular mass is a key target for diets in this special population.

Protein is the most fundamental component of tissues in animals and humans. Once hydrolyzed into amino acids and short chain oligopeptides (basically di- and tripeptides), dietary proteins provide the essential components nitrogen, sulphur and hydrocarbon skeletons, which cannot be obtained from any other nutrient.[249]

Whereas recommended values of daily protein intake ranges from 1.0 to 1.6 g protein per kg body weight per day, with a safe upper threshold of dietary protein intake up to 3.5 g protein per kg body weight per day, in healthy adults aged above 18 years old, the dietary requirement of protein for a healthy adult with minimal physical activity is currently established in 0.8 g protein per kg body weight per day.[249]

It is well established that a sedentary lifestyle quickly and deeply impacts skeletal muscle. As an example, young healthy males can decrease leg muscle mass by 3% due to a 7-day bed rest. On the contrary, moderate exercise improves skeletal muscle mass and health, suggesting that the combination of dietary protein and moderate activity provide synergistic effects that foster skeletal-muscle protein synthesis.[249]

Related to protein, a progressive loss of skeletal muscle contractile protein is a characteristic of patients with neuromuscular diseases and is ultimately due to an imbalance between muscle protein synthesis and protein degradation.

In this regard, an slightly increase in protein consumption is recommended for people living with neuromuscular diseases, but not exceeding 1.1 g protein per kg body weight per day, wherein the protein should come 50% from animal source and 50% from plant based source.[244]

1.2.2.3. Carbohydrates

The most common forms of carbohydrates are simple carbohydrates (monosaccharide and disaccharides) and complex carbohydrates (*i.e.* polysaccharides, which are divided into starches and fibers). They can be found in grains, fruits, dairy products, legumes, starchy vegetables, juices, but also in snack foods and sweets, as well as in sweetened drinks.

Carbohydrates are the main source of the caloric intake, overall contributing to 50-60% thereof. According to the different sources of carbohydrates, it is recommended to combine 45%-50% complex carbohydrates from bread, pasta, rice or legumes with daily 25-30 g dietary fiber to prevent constipation, while limiting the amounts of simple carbohydrates (such as glucose from sugar, honey or sweetened drinks) to less than 10% of the overall caloric intake.[244]

1.2.2.4. Fats

Fats are the third group of macronutrients needed in human diet, jointly with carbohydrates and proteins. However, among the three types of macronutrients, fats provide the major caloric density (1 g fat accounts for 9 Kcal) in comparison to protein and carbohydrate (1 g protein or carbohydrate provide 4 Kcal), according to the Atwater conversion factors.[250]

Chemically, fats are esters of fatty acids, usually triple esters of glycerol, also known as triglycerides, that could be found as liquids (or vegetable oils), as well as solid or semisolid structures at room temperature. Fats are insoluble in water.

Biologically, fats from food provide an important source of energy, as well as structural and metabolic functions, such as energy storage and thermal insulation. Moreover, fat facilitates the absorption of essential vitamins that are not water soluble, but liposoluble, such as vitamin A, vitamin D, vitamin E and vitamin K. However, fat that is not used for the body is stored as body fat and triggers obesity,[251] insulin resistance and inflammation[252] and cardiovascular diseases.[253]

Fats from food should provide 30-35% of the daily overall caloric intake[232]. However, it is not only quantity but also quality that must be well-balanced. Preferred fats are the mono and polyunsaturated fats (structurally containing double or triple bonds in their structures), over the saturated fats (formed by single bonds linkages) and trans fatty acids (unsaturated fats with at least one double bond in trans relative configuration).[254]

Mono and polyunsaturated fatty acids are present in vegetable oils, such as olive oil, fruits such as avocados, fish as salmon, or nuts and seeds, such as almonds. Saturated fats naturally exist from many animal sources, including meat and dairy products. Trans fats naturally exist in ruminant-based meat and dairy products, or could be synthetically produced due to certain food industry manufacturing processes.[255] WHO recommends limiting the total trans fat intake to less than 1% of total energy intake.[232]

In summary, fat consumption should be well balanced in the case of people living with neuromuscular diseases, especially in the case of sedentary lifestyle, to avoid overweight, obesity and derived related disorders.[256]

1.2.3. Micronutrients (vitamin D, Ca)

Vitamin D (calciferol) is a fat-soluble vitamin involved in calcium and phosphate absorption, bone mineralization and growth, muscle contraction, nerve conduction, general cellular function in all cells of the body, with immunomodulatory and cardioprotective properties,[257,258,259] among many other biological and physiological functions.[260]

It is naturally present as vitamin D_2 (ergocalciferol) or D_3 (calciferol) pre-formed precursors, in a few foods such as fatty fish (such as trout, salmon, tuna, and mackerel) or produced endogenously in the skin from 7-dehydrocholesterol by ultraviolet light during sun exposure.[257]

Vitamin D controls calcium absorption in the body under the endogenously form calcifediol. Calcium is an essential nutrient that plays a vital role in many different vital functions, structural and non-structural. In the form of divalent calcium salts, it is involved in neuromuscular function, provides rigidity to the skeleton, and is involved in the majority of metabolic processes.[257]

Calcium accounts for 1.9% of the body by weight, wherein 99% of total body calcium is located in the skeleton and the remaining 1% is equally distributed between the teeth and soft tissues, with only 0.1% in the extracellular fluid.[257] Calcium deficiencies, situations in which the intake, absorption and excretion are not properly balanced, dramatically affect body performance, and usually lead to falls and fractures, especially in the elderly.[261,262]

Overall, vitamin D and calcium are critical micronutrients along human life, but especially relevant for those people living with neuromuscular diseases.[263,264,265]

1.2.4. Fluids

Water accounts for about 50–60% of total human body mass.[266] Water plays a central role in key body functions, as metabolism, substrate transport across membranes, cellular homeostasis, temperature regulation, and circulatory function.[267]

It is estimated that 5-10% of the total body water content is renewed every day.[266] In this regard, body water content must be maintained in relatively narrow safe limits to maintain body activity and to prevent diseases. However, when water is not properly replaced by means of appropriate fluid intake, even for mass deficits as tiny as 1% dehydration, deleterious health effects easily arise in the form of loss of alertness and ability to concentrate, tiredness, headaches and loss of cognitive function.[266] Hypohydration may also have deleterious effects on cardiovascular health.[268] Under even more severe 10-15% body mass water losses, death is expected.

In neuromuscular diseases, which are characterized by muscular weakness, dehydration could provide additional loss of psychomotor movements and physical performance that contribute to limiting daily life.

The European Food Safety Agency (EFSA) provides scientific opinion on water intake recommendations. Mean daily intake was established at 2000 mL per adult women and 2500 mL per adult men,[267,269] ingested either from solid food or directly drunk from liquid sources. Recommendations for people living with neuromuscular diseases do not differ from these general guidelines, taking into account gender and age.[244]

1.2.5. Nutritional recommendations for people living with NMD

General recommendations according to macronutrients and key micronutrients have been disclosed in the Introduction section in the present chapter.

Some concrete nutritional recommendations have been published by several Neuromuscular Patient Associations, and translated into types of foods, number of servings and portions. For the purposes of this introductory chapter, the dietary recommendations for people living with neuromuscular diseases from the Spanish Federation of Neuromuscular Diseases (ASEM) and the Spanish Society in Endocrinology and Nutrition (SEEN) have been enclosed herein (**Table 17**). However, it should be understood that this nutritional guidance would need to be even more adjusted and adapted to each particular and usually complex personal situation, including overweight or underweight, obesity, anaemia, fatigue, swallowing difficulties, gastrointestinal issues, and the like.

Table 17: Nutritional recommendations for people living with neuromuscular diseases (adapted from reference [244])

Food group	Number of servings	Size of each servings
Potatoes, rice, bread, pasta	4-6 servings/day (whole grain preferred)	60-80 g pasta or rice 40-60 g bread 150-200 g potato
Vegetables and greens	≥ 2 servings/day	150-200 g fresh and clean
Fruit	≥ 3 servings/day	120-200 g
Olive oil	3-6 servings/day	10 mL
Milk and dairy products	2-4 servings/day	200-250 mL milk 200-250 g yogurt 40-60 g cured cheese 80-6125g fresh cheese
Fish	4-5 servings/week	125-150 g
Lean meats, poultry, and eggs	4-5 servings/week (alternate consumption)	100-125 g
Legumes	3-4 servings/week	60-80 g
Nuts	3-7 servings/week	20-30 g
Sausages and fatty meats	Occasional and moderately	-
Sweets, snacks, sweetened non-alcoholic beverages	Occasional and moderately	-
Butter, margarine and pastries	Occasional and moderately	-
Drinking water	4-8 servings/day	200 mL
Wine and beer	Optional and moderately (adults only)	100 mL wine 200 mL beer
Physical activity practice	Daily (30 minutes/day), adapted to the clinical situation	

1.3. Nutrition and DM1

Among the heterogeneous group of neuromuscular diseases, we focused our research in muscular dystrophies, particularly in myotonic dystrophies, and more particularly, in myotonic dystrophy type 1 for the reasons already disclosed in the introductory chapter.

In line with previous recommendations already discussed along this chapter, when referred to muscular dystrophies, the National Institute of Health (NIH) states that "proper nutrition is essential for overall health. Limited mobility or inactivity resulting from muscle weakness can contribute to obesity, dehydration, and constipation. A high-fiber, high-protein, low-calorie diet combined with recommended fluid intake may help".[270]

Beyond these general nutritional recommendations, the present research intended to delve deep into the nutritional habits that people living with DM1 could already have. In this regard, understanding the DM1 patients' nutrients intake was a first step in the present research to evaluate further potential nutritional approaches.

To the best of our knowledge, up to date, only a few studies and all of them in Canada, have evaluated the nutritional habits of patients suffering DM1:[271,272]

- Motlagh's study[271] prospectively evaluated nutritional intake, body composition and muscle strength in twenty-nine adult patients with DM1 (15 male and 14 female), and found that a substantial number of adults with muscular dystrophy did not meet current dietary reference intake (DRI) recommendations for the general population.

- Brien's study[272] evaluated the eating habits and dietary nutritional intakes of fifty-two adult patients with DM1 (20 male and 32 female). Deficient macronutrient and micronutrient intakes were found in comparison to recommendations for the general population, with no significant differences related to gender. Results from Brien's study suggested that many DM1 patients have a deficient diet.

In this regard, there is an important lack of scientific literature reviewing nutrition adequacy in DM1 in other countries around the world, and there is an even more important lack of scientific literature supporting nutritional recommendations properly evaluated through controlled clinical trials.

Both Motlagh's and Brien's studies report very similar findings regarding nutritional habits, and macronutrients and micronutrients consumption through diet. Lessons learned from these studies are further disclosed below.

1.3.1. Macronutrients

Related to energy and macronutrients group, Motlagh' study revealed that:[271]

- About 62% of the DM1 group did not meet the daily recommendations for energy.
- About 10% of the DM1 group did not meet the daily reference intake for protein.
- About 55% of DM1 patients had a daily fat intake above the acceptable macronutrient

distribution range.

Obesity within the DM1 community has been disclosed in several studies, ranging from 10%[271] to more than 50%.[273] Brien's study evidenced that almost 47% female and 60% male were overweight or obese, whereas in contrast, 13% female and 15% male were underweight.[272] Overweight has been related to impaired lung volume and impaired inspiratory muscle strength.[274]

In a survey performed in Canada, the majority of DM1 patients (76%) were found to be physically inactive as compared to the reference population (47.9%).[273]

Due to obesity and a sedentary lifestyle, people living with DM1 are at high risk of developing other chronic diseases, such as metabolic syndrome (MetS).[275,276] Metabolic syndrome is defined by the concomitant presence in an individual of several risk factors, such as central obesity, insulin resistance, impaired glucose tolerance, dyslipidemia and hypertension that increase the risk of type 2 diabetes mellitus, cardiovascular disease and many cancers.[277,278] The complex interactions between these risk factors contribute to chronic organ damage such as cardiovascular remodelling and an increased fat deposition in hepatocytes, leading to reduced liver function in patients with metabolic syndrome. MetS enhances the risk for various diseases such as diabetes, cardiovascular disease, asthma, ovarian cysts and some cancers.[279] Moreover, non alcoholic fatty liver disease (NAFLD) is also associated with metabolic syndrome.[280]

In this regard, the manifestations of metabolic syndrome, including anomalies of the lipid metabolism, insulin resistance, hyperglycemia, hypertriglyceridemia and a low level of HDL cholesterol, are found at varying levels in almost all individuals with DM1. In DM1 patients, the frequency of MetS is significantly higher than in the general population, which is attributed to the sedentary lifestyle related to muscle weakness, as well as an increased percentage of body fat and insulin resistance due to impaired alternative splicing of the insulin receptor and dyslipidemia with high levels of serum triglycerides and low density lipoprotein (LDL).[276] Accordingly, up to 20% of DM1 patients are at risk of suffering MetS.[276,281]

1.3.2. Micronutrients

Vitamin deficiency was reported in DM1 community.[271] In fact, a large proportion of DM1 patients did not meet the dietary reference intake for vitamins nor minerals. Of particular relevance to muscular dystrophy was the extremely low intake of vitamin D and vitamin E, even though vitamin K, folate, biotin, calcium and magnesium were insufficiently ingested by almost the majority of the study population,[271] as well.

Regarding vitamin D, its relevance in DM1 disease and reduced intake by DM1 patients have also been disclosed,[282] wherein highly prevalence of severe vitamin D deficiency subsequently affecting muscle strength in proximal muscles was found. Moreover, suboptimal vitamin D levels have been associated to age-associated sarcopenia,[283] and severe deficiency of vitamin D can result in myopathy and osteopenia.[271]

Although vitamin intake levels are critical for proper muscle function, people living with DM1 seem not to reach the optimal intake levels through a balanced diet. In this regard, vitamin supplementation could provide the optimum amounts to improve body performance.[271,282,284]

Regarding calcium, over 70% patients were found not to reach its dietary recommended intake.[271] Jointly with vitamin D deficiency, this situation could eventually lead to bone demineralization and osteopenia in these patients.

Last but not least, minerals as copper and zinc, which correlated with measurements of pulmonary function and strength, were additionally found at lower levels than the dietary recommended intake reference values.[271]

1.3.3. Socioeconomic and household issues

Diet quality is directly affected by income levels.[285] In this regard, higher-quality diets (rich in whole grains, lean meats, fish, fresh vegetables and fruit, with a high content of vitamins and minerals) are associated with greater socioeconomic status, whereas energy-dense diets that are nutrient-poor but cheaper (high in refined grains, added sugars, added fats with low contents of micronutrients) are preferentially consumed by people with more limited economic resources.[285] Considering that food price influences food choice[285,286] and that low-cost food is usually high in fats and sugars,[287] people at low income levels are more prone to consume unhealthy diets that contribute to worsening their quality of life.

Beyond the complex health situation, the socioeconomic status of people living with DM1 has been reported to be more challenging than that in the average population.[273] Moreover, socioeconomic deprivation was found to be correlated with CTG length.[288]

According to the Christopher project[289] survey performed among the myotonic dystrophy community in the USA and Canada in 2019:

- 52% of overall respondents aged 16 to 64 reported they were unable to work
- 30% of overall respondents reported total annual household income less than $25.000, compared to 17% nationally
- 86% of overall respondents reported challenges in handling objects or opening jars
- 59% of overall respondents reported challenges in preparing meals
- 57% of overall respondents reported challenges in using cutlery and kitchen utensils
- 59% of overall respondents reported challenges in swallowing, eating and drinking

Certainly, common and usually simple activities related to eat and drink, as planning meals, doing the shopping and cooking could be really challenging for people living with DM1,[290] apart from the social embarrassment to eat with others due to gastrointestinal problems,[291] difficulties in swallowing and speaking, fatigue, reduced initiative, and sometimes autonomy limitations and dependency from caregivers during meals.[290,292]

1.4. Food sources of xanthines and methylxanthines

1.4.1. Introduction

The main dietary xanthines include caffeine, theobromine and theophylline, and minor amounts of xanthine and hypoxanthine.[171]

Theobromine, theophylline, 3-methylxanthine and xanthine are transient intermediates in the plant biosynthetic as well as catabolic pathway that produces caffeine from xanthosine and degrades caffeine into CO_2 and NH_3, respectively. However, with the exception of theobromine in *Theobroma cacao*, very limited amounts of these compounds are found in plants.

Paraxanthine (1,7-dimethylxanthine) is the main caffeine metabolite found in animals, and particularly in humans, but it is almost absent in foods. The monomethylated xanthines 1-methylxanthine and 7-methylxanthine account for minor human metabolites, but are also minor intermediates in the catabolic and biosynthetic pathways, respectively, in caffeine-containing plants.

The methylxanthines caffeine, theobromine and theophylline are naturally occurring plant-produced natural products belonging to 28 genera and over 17 families, wherein the most traditional foods sources are coffee, tea and cocoa, although other natural sources such as *maté*, *guarana* and kola nuts contain different amounts of methylxanthines, as well.

Coffee is obtained from the fruit or a small tree of the genus *Coffea*, mainly from the species *arabica* and *robusta*, which together account for 99% of the world's coffee. Caffeine is the main methylxanthine found in coffee. However, the caffeine content in a cup of coffee varies considerably according to several factors, such as the caffeine concentration contained in the natural source from which it has been obtained (*robusta* has twice caffeine content than *arabica*, for instance), the brewing method and the size of serving, which are usually cultural aspects of coffee preparation and consumption.

Tea is a drink made from the leaves of *Camellia sinensis*, a large tree from which several quality leaves can be obtained. Further processing related to drying, crushing and optionally oxidizing the collected leaves render green tea, red tea or black tea, all of them with very different taste and methylxanthine content. Although caffeine is the main methylxanthine found in tea, its concentration is lower than that found in coffee, followed by theophylline and theobromine.

Chocolate is a drink made from cocoa extract, which is obtained from *cacao tree (Theobroma cacao)* seeds. The name *Theobroma* means 'food for the gods'. Once the pulp from the seedpod is removed by fermentation, the seeds are dried and further processed by roasting, pressing and grounding to enhance flavour, remove cocoa butter and finally produce cocoa powder. Even more processing by mixing with sugar, cocoa butter, milk or milk solids eventually renders a high variety of chocolate products with very different taste and smell, and a variety of methylxanthine content. Theobromine is the main methylxanthine found in chocolate, although caffeine in minor amounts is also present.

The tea-like drink *maté* is made from the leaves of the ilex plant, *Ilex paraguarinensis,* which contains between 1% and 2% caffeine.

Guarana is the paste made from the seeds of *Paullinia cupana*. Once dried in the sun, powdered and mixed with water it can be consumed as a drink that could be further sweetened with sugar for a better taste. *Guarana* contains up to 6% caffeine in the seeds.[293] For this reason, it is considered the most potent of the natural sources of caffeine. Other methylxanthines found in smaller amounts (below 0.3%) include theobromine and theophylline.[293]

Soft drinks such as the cola beverages Coca-Cola® and Pepsi-Cola® use nuts from trees of the genus *Cola (kola nuts)* to naturally flavour these beverages. The nuts contain caffeine and theobromine, although the majority of caffeine content in the cola soft drinks comes from synthetic sources, and are added later in the process. Caffeine has been included in Coca-Cola® composition since 1885, originally from kola nuts.

Energy drinks are defined as a variety of non-alcoholic beverages containing caffeine, taurine and vitamins (often in combination with other ingredients), marketed for their actual or perceived effects as stimulants, energizers and performance enhancers.[294] Energy drinks are caffeinated beverages that usually contain guarana, mate and kola nuts, sweeteners and other additives such as taurine, vitamins, ginkgo, as well as additional supplementation in caffeine. Currently, energy drinks are the beverages containing a higher content in methylxanthines, more than 150 mg/L.

1.4.2. Food content on xanthines and methylxanthines

The content of methylxanthines in the food consumed in diet is largely different than their content in the beans, seeds and leaves present in the natural source, which are largely different from the grounded, dried and powdered processed beans, seeds and leaves that are used to prepare the homemade hot beverages coffee, tea and chocolate.

Moreover, the content of methylxanthines in homemade and even that coffee, tea and chocolate beverages prepared in coffee shops, cafeterias and restaurants vary significantly depending on different factors, such as the origin of the crop, its processing and the extraction process, since the extraction itself depends on different factors such as temperature, pressure, volume of hot water used as extracting liquid, extraction time and contact time after extraction, among others.

However, the content of methylxanthines, especially caffeine, in commercially prepared food and beverages, such as cola and chocolate beverages, or energy drinks, is well established in the manufacturing process and normally available in the label, according to current legislation.[295] Caffeine and theobromine are listed as evaluated substances in the Union list of flavouring substances, and their content is limited from 70 mg/kg to 150 mg/kg, depending on their uses.[296]

1.4.2.1. Caffeine content in foods and beverages

The main sources of dietary caffeine are coffee, tea, caffeinated soft and/or energy drinks and chocolate. In Europe, beverages and foods containing caffeine at a concentration above 150 mg/L must be labelled with the statement: 'High caffeine content. Not recommended for children or pregnant or breast-feeding women'.[295]

Several authors have reported different contents of caffeine in food (as reviewed by Sanchez[297] and references cited therein). Around the world, several coffee, tea and even yerba mate brewing methods[298] explain the different caffeine levels in diet.

Due to this variability in literature, for the purposes of the present research, EFSA was preferably considered as the official source. Accordingly, the values disclosed herein were extracted from EFSA's publications or sources directly cited by EFSA. Even under this constriction, caffeine content in all these foods and beverages vary from different publications[294,299] (for a comparison, see **Table 18** and **Figure 13**).

Table 18: Caffeine content in foods and beverages

Food or beverage	**Caffeine content (mg/L or mg/kg)**	
	EFSA[300] based on **Fitt *et al***[301]	**Zucconi *et al***[294] based on **Zoumas *et al***[302]
Coffee		
Espresso	1340	1916
Instant, ground, ice coffee, …	445	400
Cappuccino	273	250
Decaffeinated	21	20
Tea		
Instant, tea bag, ice tea, …	165	100
Black tea	220	-
Green tea	151	-
Decaffeinated tea	25	-
Chocolate		
Hot chocolate	-	255
Cocoa beverages based on cocoa powder	168	457
Cocoa beverages based on cocoa-beverages preparation powder (chocolate milkshake)	42	-
Chocolate bars		
Dark chocolate	525	340
Milk chocolate	111	183
White chocolate	-	-
Chocolate snacks		
Dark chocolate	-	264
Milk chocolate	168	142
White chocolate	-	-
Cola and energy drinks		
Cola drinks	108	-
Energy drinks	320	-

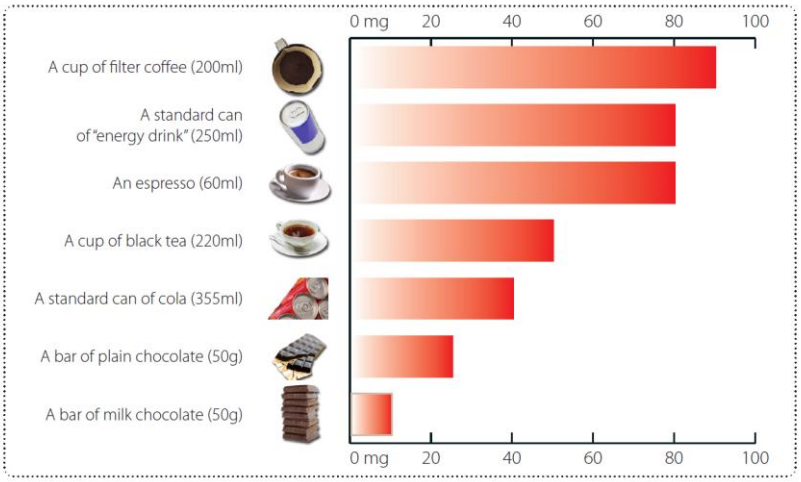

Figure 13: Caffeine amount in different foodstuffs
EFSA.[299]

Although coffee is probably the most popular beverage in the world, it contains virtually no food value.[303] According to data provided by the U.S. Department of Agriculture, on a 100 g portion basis, brewed coffee is composed of 99.5 g water and provides minor amounts of both macronutrients (carbohydrates, proteins and fats) as well as micronutrients.[304] Although fat-soluble and water-soluble vitamins are present in the beans, they are destroyed during the roasting process.[305] Some minerals such as potassium, magnesium, sodium, calcium, iron, manganese or copper are also present in the beans, at trace levels.[305]

However, other studies evaluated lipid content in coffee brews,[306] for which remarkable differences have been disclosed related to the preparation method, especially whether the brew is finally filtered (leading to 8-335 mg/L overall lipid content) or non-filtered (leading to 1000-2260 mg/L lipid content). In all cases, triglycerides account for more than 80% of the lipid content in coffee brews. In this regard, lipid content could reach 163 mg in boiled but not filtered coffee per 150 mL cup serving.

Carbohydrates account for more than 50% dry weight of coffee beans,[303] but are extracted after roasting and therefore they mainly do not reach the final brewed coffee.

Last but not least, coffee as well as tea and chocolate based beverages could include calories from sugars and saturated fat from added cream and whole milk that should be limited in a healthy diet.

1.4.2.2. Theobromine content in foods and beverages

Theobromine is the main methylxanthine in foods and beverages derived from cocoa.

Cocoa is the dry, roasted and powdered, non fat natural product obtained from *Theobroma cacao L.* tree seeds. After a multi-step manufacturing process that comprises seed cleaning and winnowing to obtain the nibs, nibs are subsequently fermented (oxidized), dried, roasted and

ground to paste to render the cocoa liquor. Finally, cocoa liquor could be pressed to remove the fatty part, named the cocoa butter fraction, from the non fatty part, known as cocoa powder.[307,308]

Cocoa liquor is the starting material from which all confectionery chocolate based products (either foods and beverages) are manufactured, along with some other functional ingredients such as sugar, milk or milk derivatives to provide the high variety of chocolate derived products.

In the European countries, Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption [309] harmonizes cocoa and chocolate products regarding composition and labelling, and defines cocoa and chocolate products as it is disclosed in **Table 19**:

Table 19: Names and characteristics of the cocoa and chocolate products intended for human consumption in EU[309]

Type of product	Cocoa butter[i]	Dry cocoa solids	Dry non-fat cocoa solids	Sugars	Other components
Cocoa powder (cocoa)	≥ 20%	-	-	-	Water ≤ 9%
Fat-reduced cocoa powder	< 20%	-	-	-	-
Powdered chocolate	≥ 32%	-	-	+	-
Sweetened cocoa powder (drinking chocolate)	≥ 25%	-	-	+	-
Fat-reduced sweetened cocoa powder	< 20%	-	-	+	-
Chocolate	≥ 18%	≥ 35%	≥ 14%	+	-
Milk chocolate	≥ 21.5%	≥ 25%	≥ 2.5%	+	Dry milk solids ≥ 14% Milk fat ≥ 3.5%
Cream chocolate	≥ 19.5%	≥ 25%	≥ 2.5%	+	Dry milk solids ≥ 14% Milk fat, ≥ 5.5%
Family milk chocolate	≥ 20%	≥ 20%	≥ 2.5%	+	Dry milk solids ≥ 20% Milk fat, ≥ 5%
White chocolate	≥ 20%	-	-	+	Dry milk solids ≥ 14% Milk fat, ≥ 3.5%
Chocolate a la taza	≥ 18%	≥ 35%	≥ 14%	+	Flour or starch ≤ 8%
Chocolate familiar a la taza	≥ 18%	≥ 30%	≥ 12%	+	Flour or starch ≤ 18%

[i] According to Directive 2000/36/EC of 23 June 2000, **cocoa butter** designates the fat obtained from cocoa beans or parts of cocoa beans with not more than 1.75% free fatty acid contents (expressed as oleic acid) and not more than 0.5% of unsaponifiable matter (except press cocoa butter, where it will be not more than 0.35%) determined using the technique disclosed therein.

Cocoa solids, cocoa butter, sugar, and lecithin are the basic ingredients in the formulation of chocolate, which are found in different content in dark, milk and white chocolates.

Therefore, chocolate's nutritional composition contains a high content in carbohydrates and fats. Carbohydrates, mainly sugars, account for up to 45%, whereas fat represents up to 30% total content.[310] Overall, energy content in chocolate and chocolate products is higher than 3000 kcal/kg of product,[310] which must be taken into account in a healthy diet.

Regarding micronutrients, cocoa products present high levels of some essential elements, such as calcium, iron, copper, potassium, manganese, magnesium, zinc and phosphorus.[305] However, some other metals with safety concerns, such as aluminium and cadmium are also naturally present and must be controlled.[305]

Methylxanthines are only present in the cocoa fraction, either isolated or non-isolated from the cocoa liquor, but not in the cocoa butter part.

In cocoa, theobromine ranges from 26.000 mg/kg (about 2.6% of dry weight), whereas minor amounts of caffeine are also present, about ten-fold lower (2.400 mg/kg, 0.24% of dry weight).[311]

The ratio theobromine to caffeine varies widely among different chocolate products. Some authors have disclosed theobromine to caffeine ranging in chocolates from 2.3:1 to 25.0:1, with an average 7.9:1.[302]

Due to this variability in literature, for the purposes of the present research, EFSA was preferably considered as the official data source. Accordingly, the values disclosed herein were extracted from EFSA's publications or sources directly cited by EFSA. Even under this constriction, theobromine content in all these foods and beverages vary from different publications and quite wide ranges are provided (see **Table 20**).

Table 20: Theobromine content in foods and beverages[302]

Type of product	Theobromine content (mg/L or mg/kg)		Caffeine content (mg/L or mg/kg)	
	Range	Mean	Range	Mean
Chocolate				
Hot chocolate	-	4580	-	280
Chocolate bars				
Dark chocolate	3590-6280	4630	170-1250	690
Milk chocolate	1350-1860	1530	50-540	220
White chocolate	-	-	-	-

1.4.2.3. Theophylline content in foods and beverages

Biologically, theophylline is the degradation product of caffeine in caffeine-producing plants such as tea, coffee and mate. However, theophylline is naturally present in very minor amounts in food and drinks, even in tea.[312] Overall, theophylline is even below the limit of detection of very sensitive analytical techniques in all the food and beverages tested.[313,314]

1.4.2.4. Xanthine content in foods and beverages

The content of purine bases (including adenine, guanine, hypoxanthine and xanthine) in common foodstuffs deserves special attention since the end product of their metabolism in humans is uric acid. Although uric acid is involved in blood vessels protection, elevated levels of uric acid are related to gout and hyperuricemia.[315] For this reason, daily intake of dietary purines should be restricted in healthy diets. **Table 21** illustrates the content of hypoxanthine[ii] and xanthine in some common foodstuffs.

Table 21: Hypoxanthine and xanthine content in foodstuffs, short selection from reference [316]

Foodstuffs	**Content (mg/100g)**	
	Hypoxanthine	**Xanthine**
Vegetables		
Asparagus (upper part)	3.8	0.3
Broccoli	5.7	5.3
Green pepper	7.0	10.7
Parsley	32.3	0.0
Spinach (leaf)	0.0	7.7
Meat		
Beef		
Heart	96.6	15.2
Liver	ND	50.2
Topside (heated)	87.2	13.3
Chicken		
Breast	98.4	1.0
White meat	110.2	0.0
Wing	92.5	0.0
Pork		
Liver	34.0	66.9
Shoulder	53.1	0.0
Processed meat		
Bacon	43.6	ND
Prosciutto	92.1	0.0
Salami	83.0	7.6
Vienna sausage	32.7	ND
Fish		
Fresh fish		
Bonito	170.1	0.0
Herring	103.4	ND
Salmon	91.2	0.0
Tuna	129.3	0.0
Dried fish		
Anchovy	381.8	4.0
Shellfish and mollusk		
Oriental shrimp	103.4	117.1
Oyster	12.2	82.1
Sakura shrimp (dried)	512.2	29.0
Seasonings		
Powdered Umami broth	657.0	1.5
Soy sauce (dark)	33.7	9.0

ND: Not detected

[ii] Formally, hypoxanthine is a purine derivative, but not a methylxanthine. However, its natural occurrence in food has also been evaluated herein.

1.4.3. Consumption of foods and beverages containing methylxanthines

Coffee is among the most consumed beverages worldwide. According to the International Coffee Organization, around 167 million of 60 kilogram bags of coffee were consumed worldwide in 2020-2021, almost 44% exported from Brazil, being the European Union and the United States of America the main importers.[317] The average coffee intake per capita per year in the EU was 4.8 kg in 2008, with the Nordic countries leading the figures (Finland 12.6 kg, Norway 9.0 kg, Sweden 8.3 kg), whereas in the United States, coffee consumption was reported to be around 3.3 kg per capita per year.[318]

Figure 14 graphically depicts the percentage of caffeine-containing beverages (coffee, tea, soft drinks and energy drinks) volume sales divided into continents.

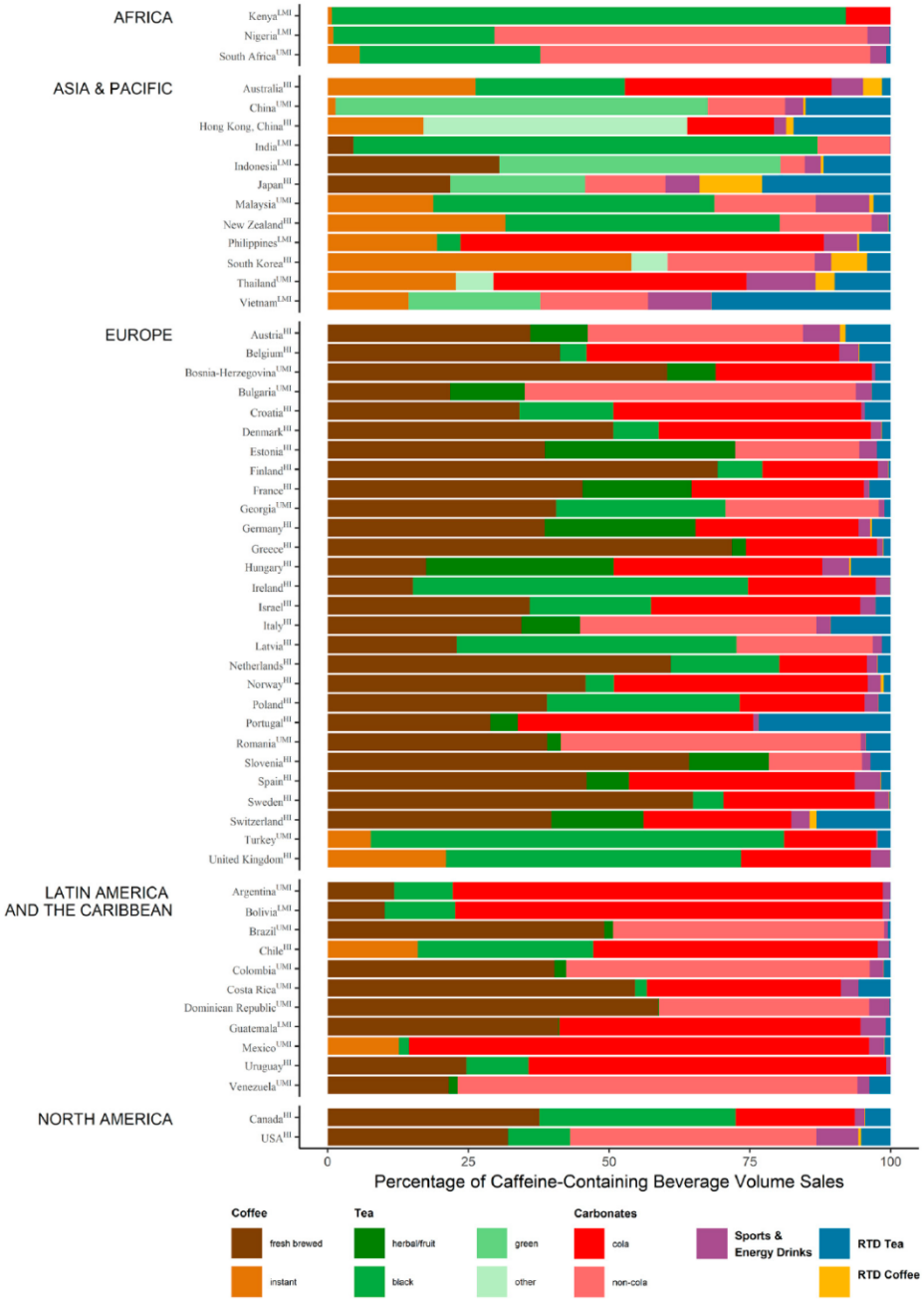

Figure 14: Percentage of caffeine-containing beverage volume sales per beverage (Data source Euromonitor 2017, copied with permission from reference [298]).

World tea production reached 5.7 million tonnes in 2016, mainly harvested in China (43%), India (22%), Kenya (18%) and Sri Lanka (11%), black tea accounting for two out of three parts of the overall production total.[319]

Unlike coffee and cocoa, main tea producers are also main consumers, and 75% tea is consumed locally where it is produced.[320] According to 2016 data, China consumed almost 39% of world tea consumption (1.5 kg per capita per year), followed by India (19%). Western countries consume much less tea than coffee. In Europe, only Ireland and UK are reported to consume 1.5 kg per capita per year,[320] whereas the United States tea consumption accounts for 0.4 kg per capita per year.[319] **Figure 15** illustrates the consumption growth rate (2007-2016) *vs* share of global consumption (2016), and the dynamism of tea market.

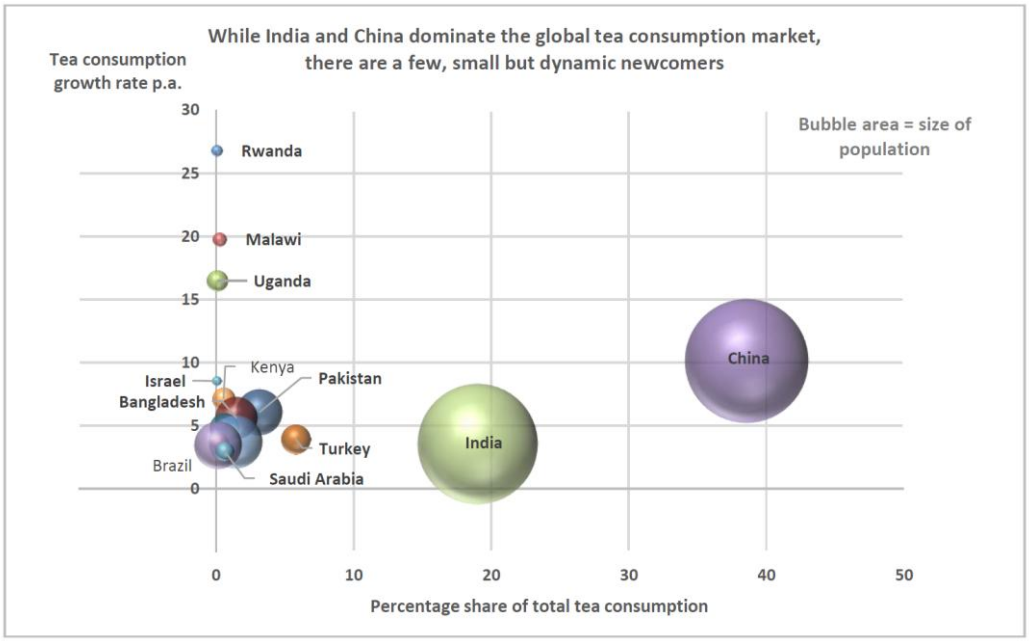

Figure 15: Consumption growth rate (2007-2016) vs share of global consumption (2016) (Data source FAO 2018, extracted from reference [320])

Regarding cocoa, 4.8 million tons of cocoa beans were reported to be produced worldwide in 2018/2019, mainly harvested in West Africa but mostly consumed in Europe (45%) and America (32%), followed by Asia and Oceania (12%).[321]

Chocolate consumption per capita in 2017 was led by European countries, mainly Switzerland (8.8 kg per capita per year), whereas Russia and US accounted for 4.8 kg and 4.4 kg per capita per year, respectively. Considerably less consumption was reported in South America, Japan, South Africa and China (**Figure 16**).

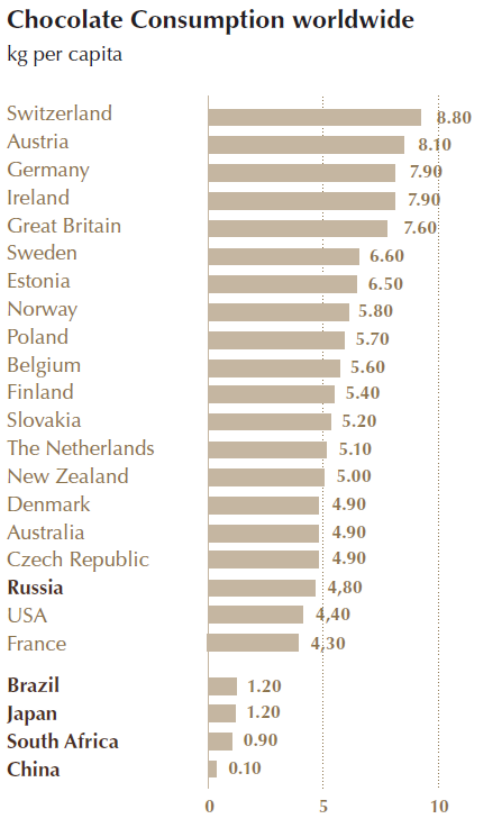

Figure 16: Chocolate consumption worldwide
(Data source Euromonitor 2017, extracted from reference [322]).

Due to the high diversity of methylxanthines containing foods and beverages, as well as their very different consumption patterns worldwide, translating food consumption into methylxanthines consumption could render quite variable data.

For the particular case of caffeine intake, a very detailed approach was reported by EFSA through the EFSA Comprehensive European Food Consumption Database, constructed from thirty-nine surveys conducted in twenty-two European countries for a total of 66.531 participants. The Database discloses food sources trends according to age (**Table 22**) and mean caffeine intake (**Table 23**) among Member States.[300]

Interestingly, coffee is the main source of caffeine in adults to the very elderly, whereas chocolate is the main source in toddlers to adolescents.

Table 22: Food sources contributing to daily caffeine intake[300]

Age class	%	Food sources contributing to daily caffeine intake (%)				
		Coffee	Tea	Chocolate	Cola drinks	Energy drinks
Toddlers (12 to <36 months)	Median	0	34	56	3	0
	Range	0 - 13	0 - 73	20 - 100	0 - 58	0
Children (3 to < 0 years)	Median	2	13	42	10	0
	Range	0 - 40	0 - 68	18 - 98	0 - 52	0 - 3
Adolescents (10 to < 18 years)	Median	19	22	29	17	0
	Range	3 - 53	1 - 65	8 - 92	0 - 42	0 - 13
Adults (18 to ≤ 65 years)	Median	78	12	2	3	0
	Range	33 - 94	1 - 59	1 - 17	0 - 18	0 - 4
Elderly (65 to ≤ 75 years)	Median	84	12	1	1	0
	Range	24 - 97	2 - 74	0 - 10	0 - 1	0
Very elderly (≥75 years)	Median	81	11	2	0	0
	Range	28 - 93	4 - 79	1 - 18	0 - 1	0 - 1

Table 23: Daily caffeine intakes by age class across different dietary surveys among Member States[300]

Age class	Mean caffeine intake			
	mg per day		mg/kg bw/day	
	Min	Max	Min	Max
Toddlers (12 to <36 months)	0.3	30.3	0.0	2.1
Children (3 to < 0 years)	3.5	47.1	0.2	2.0
Adolescents (10 to < 18 years)	17.6	69.5	0.4	1.4
Adults (18 to ≤ 65 years)	36.5	319.4	0.5	4.3
Elderly (65 to ≤ 75 years)	22.6	362.1	0.3	4.8
Very elderly (≥75 years)	21.8	416.8	0.3	6.0

Although data was extracted from European dietary surveys, similar pattern consumption was reported in the United States of America,[323] wherein it was estimated than 85% of the population consumes at least one caffeinated beverage per day,[324] 60% adults were coffee consumers,[325] but only 21% were declared to be tea consumers.[325] An average 4 mg/kg mean daily caffeine intake was disclosed for US adult consumers and a mean daily caffeine intake 1 mg/kg for children younger than 18 years old.[325]

Similar caffeine consumption patterns have been reported at national level in different countries worldwide.[326]

According to the scientific opinion disclosed by EFSA on the safety of caffeine, single doses of caffeine up to 200 mg (about 3 mg/kg body weight for a 70-kg adult) or up to 400 mg per day (about 5.7 mg/kg body weight for a 70-kg adult) do not give rise to safety concerns for the general healthy adult population.[300]

Based on data from **Table 18**, 150 mL expresso as a single coffee serving would provide 200 mg caffeine. However, in case that the natural source was chocolate, 380 g dark chocolate bar or 1800 g milk chocolate bar per serving would be required.

To the best of our knowledge, no limitation for theobromine in the human diet has been regulated nor published. The maximum dose of theobromine tested in controlled clinical trials on healthy volunteers was 1000 mg/day. Several side effects were reported at 1000 mg/day, whereas no side effects were reported at 500 mg/day.[327]

Based on data from **Table 20**, 42 g dark chocolate bar or 130 g milk chocolate bar per serving would provide a single consumption of 200 mg theobromine. Nutritional facts for illustrative examples of dark and milk chocolates at those serving sizes are provided in **Table 24**.

Table 24: Nutritional facts for illustrative examples of dark and milk chocolate servings providing *ca* 200 mg theobromine

Nutrition facts	Dark chocolate, 70% cocoa†, 100 g	Dark chocolate, 70% cocoa†, 42 g serving	Milk chocolate, 30% cocoa‡, 100 g	Milk chocolate, 30% cocoa ‡, 130 g serving
Energy (kcal)	566	238	545	708
Energy (kJ)	2350	987	2276	2959
Carbohydrate	34	14	56	73
of which sugars	29	4	55	72
Total fat	41	17	32	42
of which saturated fat	24	10	19	25
Trans fat	0	0	ND	ND
Proteins	9.5	4	7	9
Dietary fiber	12.2	5.1	ND	ND

†Lindt Excellence Dark Chocolate 70% Cocoa - 100 g tablet
‡Lindt Milk Chocolate Classic Recipe 30% Cocoa minimum - 125 g tablet
ND: not disclosed

The data shown along this section reveals that due to the high energy, carbohydrate and fat content, a properly balanced diet is not compatible with daily hundreds gram amounts of chocolate products as the main source of theobromine in their commercially available foodstuffs form.

1.5. Concluding remarks

Although scarce, nutritional studies suggest that people living with DM1 suffer from nutritional inadequacy, even higher than expected. According to literature, the majority of DM1 patients would be overweight, and up to 10-15% would be obese.

Some of the nutritional deficiencies found among individuals with neuromuscular disease include excess fat intake, low vitamin D and vitamin E consumption, as well as calcium deficiencies.

Studies refer nutritional deviations of DM1 patients' nutrient intake from the accepted recommendations based on Dietary Reference Values (DRVs)[328] or dietary reference intake (DRI)[329] for the general healthy population. Nevertheless, it is still unknown if people living with DM1 request the same level of nutrients as the general population.

Due to muscle weakness, excessive daytime sleepiness, potentially lower metabolic rate[330] and sedentary lifestyle, recommended nutrient intake levels for general population may overestimate the real dietary needs of people living with DM1.[271,272] In this regard, further studies are required to determine the specific needs of this challenging population.

The main dietary xanthines found in coffee, tea, cocoa, *maté*, meats and fishes include caffeine, theobromine and theophylline, and minor amounts of xanthine and hypoxanthine. Their amounts in food and beverages are highly variable depending on many different factors, which are related to their natural origin, manufacture and method of preparation.

The top methylxanthine containing food and beverages worldwide consumers are Finland for coffee, with 12.6 kg per capita per year, Turkey for tea, with 4.0 kg per capita per year and Switzerland for chocolate, accounting for 8.8 kg per capita per year.

According to EFSA, single doses of caffeine up to 200 mg (about 3 mg/kg body weight for a 70-kg adult) or up to 400 mg per day (about 5.7 mg/kg body weight for a 70-kg adult) do not give rise to safety concerns for the general healthy adult population. No limits have been disclosed for theobromine.

For the methylxanthine theobromine, carbohydrate and fats nutrients naturally present in chocolate products could negatively alter nutritional healthy recommendations, and further elicit the energy imbalance usually found in people living with neuromuscular diseases, since the natural progression of muscular dystrophy leads to a decrease in mobility and activity, as well as a decrease in lean body tissue and an increase in intramuscular fat and adiposity, that lead to a decline in energy expenditure. Overfeeding and underfeeding are separate but serious concerns in the DM1 population.

In summary, nutritional values of food and beverages containing methylxanthines, specially their content in carbohydrates and fats either naturally present or added for providing a better taste, deserve special attention in the particular case of people living with neuromuscular diseases, for which energy imbalances could promote overfeeding and serious concerns, and must be limited in the context of a healthy diet.

CHAPTER 2: REGULATORY FRAMEWORK

Chapter 2 delves into the regulatory framework of food products through the legal basis that regulates the principles of food supplements and food for special medical purposes. The laws that regulate these categories are analyzed herein, as well as requirements for launching both types of food products on the market. In this regard, Chapter 2 discloses the requirements of the regulatory framework to achieve the specific goal nr 2.

2.1. Introduction

2.1.1. Background

Food in its broader scope, and particularly, food safety and quality, are extremely regulated by a complex network of laws at national and supranational level, regulating the composition, production, control, handling, labelling, transport, conservation, trade, storage and residues of food products, in order to demonstrate that the quality requirements are subjected to food law, as well as to protect consumers' health. Since this is a very dynamic field, the food regulatory landscape is under constant revision and frequent amendments.

At the European Union level, and according to EU treaties, regulations scope may cover from binding laws at European level and applied to all EU Member States, to just at national level and applied to a few or even a concrete Member State. As disclosed by the European Commission in terms of EU Law,[331,332] the following definitions apply:

Legislative (or Legal) Act

European legal instrument that is available to the European institutions to carry out their tasks. Legislative acts are adopted following one of the legislative procedures set out in the EU treaties.

EU Treaty

The treaties lay down the objectives of the European Union, the rules for EU institutions, how decisions are made and the relationship between the EU and its Member States. The EU treaties have from time to time been amended to reform the EU institutions and to allow new EU countries to join the EU.

The treaties are negotiated and agreed by all the EU countries and then ratified by their parliaments, sometimes following a referendum.

Regulations

Regulations are EU legal acts that apply automatically and uniformly to all EU Member States as soon as they enter into force, without needing to be transposed into national law. They are binding in their entirety on all EU countries. National authorities must ensure they are correctly applied and their compliance.

Directives

Directives are legislative acts that require EU Member States to achieve a certain result, but leave them free to choose how to do so in order to reach the goal. Following the Directive, each EU Member State must adopt measures to incorporate it into its corresponding national law (in a process known as 'transposition') in order to achieve the objectives set by the directive. National authorities must communicate these measures to the European Commission. Each directive contains a deadline by which EU countries must incorporate its provisions into their national legislation (*i.e.* law transposition) and inform the Commission.

Decisions

A decision is an EU legal act that is binding in its entirety. A decision which specifies those to whom it is addressed shall be binding only on them, for instance, a particular EU country or an individual company.

Recommendations

A recommendation is an EU legal act that allows the institutions to make their views known and to suggest a line of action without imposing any legal obligation on those to whom it is addressed. They have no binding force.

Opinions

An 'opinion' is an instrument that allows the institutions to make a statement in a non-binding fashion, without imposing any legal obligation on those to whom it is addressed. It can be issued by the main EU institutions (Commission, Council, Parliament), the Committee of the Regions and the European Economic and Social Committee. While laws are being made, the committees give opinions from their specific regional or economic and social viewpoint.

A complete list of the vast EU Laws regulating food may be accessed through the Eur-Lex (Access to the European Union Law) website,[333] where the following main contents are structured into a detailed network of Regulations, Directives, Decisions, Recommendations and Opinions grouped under various topics:

Food safety – Food regulation in EU (as per May 2022):[333]

- General rules
- Food safety bodies – European Food Safety Agency (EFSA)
- Labelling and nutrition
- Food labelling legislation
- Food supplements
- Natural mineral waters
- Food for specific groups
- Nutrition and health claims
- Food improvement agents
- New foodstuff types
- New (novel) foods
- Biological safety
- Antimicrobial resistance
- Food hygiene
- Food irradiation
- Crisis preparedness and management
- Chemical safety
- General rules
- Contaminants
- Food packaging and containers
- Hormones in meat

The legal bases discussed along this Chapter have been retrieved from the corresponding competent authorities and official sources already disclosed in the 'Methodology' section.

2.1.2. Food regulation

REGULATION (EC) No 178/2002 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 28 January 2002[334] laying down the general principles and requirements of Food Law, establishing the European Food Safety Authority and laying down procedures in matters of food safety "*provides the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, taking into account in particular the diversity in the supply of food including traditional products, whilst ensuring the effective functioning of the internal market*" (Article 1). It establishes the general food law (Chapter 2), and the European Food Safety Authority, named European Food Safety Agency (EFSA),[335] along with its mission, tasks, organization, operation and values.

EFSA (thereafter, the Authority) provides independent scientific advice, scientific opinions and technical support for the Community's legislation and policies in all fields which have a direct or indirect impact on food and feed safety, as well as risk communications (Article 22).

The rationale behind the need of the Regulation (EC) No 178/2002 and the Authority is to assure food and feed safety among the Community and their free movement within the Community on a common basis at Community level.

According to the above-mentioned Regulation, 'food' (or 'foodstuff') means any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. Remarkably, "food shall not include (…) medicinal products within the meaning of Council Directives 65/65/EEC[336] and 92/73/EEC[337]" (Article 2).

For the purposes of the present research, relevant food categories that will be further discussed herein are 'food supplements' and 'food for specific groups', in particular, food for special medical purposes, which in the light of food definition, are considered foods, and therefore the following definitions and legal basis apply:

1. **Food supplement**: DIRECTIVE 2002/46/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 10 June 2002[338] on the approximation of the laws of the Member States relating to food supplements defines 'food supplements' as "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*".[339]
2. **Food for special medical purposes**: REGULATION (EU) No 609/2013 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 June 2013[340] on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, defines '*food for special medical purposes' as "food specially*

processed or formulated and intended for the dietary management of patients, including infants, to be used under medical supervision; it is intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary food or certain nutrients contained therein, or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved by modification of the normal diet alone".

REGULATION (EU) No 1169/2011 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 October 2011 on the provision of food information to consumers,[341] amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 "*establishes the general principles, requirements and responsibilities governing food information, and in particular food labelling*" (Article 1) in order to provide "*a high level of protection of consumers' health and interests by providing a basis for final consumers to make informed choices and to make safe use of food, with particular regard to health, economic, environmental, social and ethical considerations*" (Article 3).

The Regulation keeps vigil on food information, which must fulfil fair information practices, and must not be misleading (…) "*by attributing to the food effects or properties which it does not possess*" (Article 7). Moreover, the Regulation provides mandatory food information (Chapter IV) in terms of names, list of ingredients and quantities, durability, nutrition declaration, allergens, among others, as well as the inclusion of special information for foods for which the labelling must include one or more additional particulars, as in the case of caffeine (Annex III).

REGULATION (EC) No 1924/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 20 December 2006 on nutrition and health claims made on foods[342] "*harmonises the provisions laid down by law, regulation or administrative action in Member States which relate to nutrition and health claims in order to ensure the effective functioning of the internal market whilst providing a high level of consumer protection*" (Article 1), wherein 'claim' means any message or representation (…) which states, suggests or implies that a food has particular characteristics" (Article 2).

In the light of this Regulation, the following definitions apply (Article 2):

- **'Claim'** means any message or representation, which is not mandatory under Community or national legislation, including pictorial, graphic or symbolic representation, in any form, which states, suggests or implies that a food has particular characteristics.
- **'Nutrition claim'** means any claim which states, suggests or implies that a food has particular beneficial nutritional properties due to the energy (calorific value) it (i) provides, (ii) provides at a reduced or increase rate, or (iii) does not provide; and/or the nutrients or other substances it (i) contains, (ii) contains in reduced or increased

proportions, or (iii) does not contain. Nutrition claims are fully disclosed in Chapter III and Annex of Regulation (EC) No 1924/2006.

- **'Health claim'** means any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health. Health claims are fully disclosed in Chapter IV of Regulation (EC) No 1924/2006.

Both nutrition and health claims may be used in the labelling, presentation and advertising of foods placed on the market in the Community only if they comply with the provisions of the above mentioned Regulation, "avoiding being false, ambiguous or *(containing)* misleading information" (Article 3). Moreover, any of these claims must be scientifically substantiated by generally accepted scientific data (Article 6).

Nutrition claims are detailed disclosed in Annex to Regulation (EC) No 1924/2006. Current permitted nutrition claims include: low energy, energy-reduced, energy-free, low-fat, fat-free, saturated fat-free, low sugar, sugar-free, with no added sugar, low sodium/salt, very low sodium/salt, sodium-free or salt-free, source of fibre, high fibre, source of protein, high protein, source of (name of vitamin/s) and/or (name of mineral/s), high (name of vitamin/s) and/or (name of mineral/s), contains (name of the nutrient or other substance), increased (name of the nutrient), reduced (name of the nutrient), light/lite, and naturally/natural; providing that any of them are in accordance with the conditions disclosed in the cited Regulation.

Health claims are extensively disclosed in Chapter IV of Regulation (EC) No 1924/2006. As stated in Article 10, "*health claims made on food shall be prohibited* (…)" unless they strictly comply with the specific requirements, are authorised in accordance with the present Regulation and included in the list of permitted claims published by the Commission. The list of permitted claims includes the wording of the claims, specific conditions of use of the claims, and where applicable, conditions or restrictions of use and/or an additional statement or warnings. COMMISSION REGULATION (EU) No 432/2012 of 16 May 2012 established a list of permitted health claims made on foods,[343] other than those referring to the reduction of disease risk and to children's development and health. Since then, the list is continuously updating in electronic format.

Application for authorisation of a health claim by a food business operator is disclosed in Article 15 of Regulation (EC) No 1924/2006. The applicant must submit to the competent authority of the Member State the administrative information and scientific data, including, where available, independent, peer-reviewed studies to demonstrate that the health claim complies with the criteria provided under Regulation EC No 1924/2006. Once evaluated by the Authority and considering its public opinion, the Commission shall submit its decision on granting or non-granting authorisation in accordance with the opinion of the Authority and the Community law.

The Commission establishes and maintains a Community Register of nutrition and health claims made on food, named 'EU Register on nutrition and health claims',[344] which includes the nutrition claims and the conditions applying thereof, the authorised health claims and the conditions applying thereof, as well as the rejected health claims and the reasons for their rejection (Article 19).

Granted health claims benefit from protection of proprietary data, in the sense that the claim can only be used by the applicant for a period of five years after the entry into force of the relevant legal act. After the five years period of exclusivity, the health claim may be used by any food business operator in light of Regulation EC No 1924/2006 articles. The list of health claims for which the right to use the claim is restricted to the benefit of the applicant is published by the 'EU Register of the European Commission.[344]

As per May 2022, the Register contains 2.343 overall entries, from which 266 are authorised claims, whereas 2.077 are rejected claims. On the other side, the list of granted health claims with protection of proprietary data is limited to six entries.

Figure 17 summarizes the regulatory framework previously introduced, and further discussed in the next sections.

Figure 17: Overview of the regulatory framework

For the purposes of the present research, authorized health claims related to the substances caffeine and theobromine have been investigated.

Regarding the substance '**theobromine**', it is not listed in the EU Register as per May 2022. The food 'cocoa', ***Theobroma cacao***, is involved in two non-authorized applications, related to the maintenance or achievement of a normal body weight and enhancement of mood. Finally, the food **'chocolate'** is involved in two claim applications related to protection of lipids from oxidative damage, not authorized based on lack of scientific substantiation.

Regarding the substance **'caffeine'** by the same date, it is listed in the EU Register in eleven applications related to reduction of body fat mass, increase of physical performance, contribution to the maintenance or achievement of a normal body weight or helping to increase alertness (*intakes of caffeine between 40-75 mg per serving).* None of them have been authorised to the present time.

No other methylxanthines or xanthines are listed in the EU Register.

2.2. Food supplements

2.2.1. Introduction and harmonized regulation

An adequate and varied diet should, under normal circumstances, provide the necessary nutrients for a healthy life in quantities established and recommended by generally acceptable scientific data. However, several studies demonstrate that this ideal situation is not achieved for all nutrients and by all groups of population in all countries.[345,346] In this regard, concentrated sources of nutrients properly formulated in the form of food supplements may help to complement the common diet.

Food supplements are partially harmonized along European countries.[347] For the purpose of DIRECTIVE 2002/46/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements,[338] "***food supplements*** *means foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*"; wherein **'nutrients'** mean vitamins and minerals (Article 2), and more particularly, vitamins and minerals listed in the positive list disclosed in Annex I and Annex II of this Directive:

> Vitamins include Vitamin A, C, D, E, K, B1, B2, B6, B12, niacin, pantothenic acid, folic acid, and biotin, whereas minerals include Ca, Mg, Fe, Cu, I, Zn, Mn, Na, K, Se, Cr, Mo, F, Cl, and P (Annex I) in the structural forms permitted according to Annex II of Directive 2002/46/EC.

In 2009, the Annexes of Directive 2002/46/EC were updated and replaced, respectively, by Annexes I and II of REGULATION (EC) NO. 1170/2009, OF THE COMMISSION, of November 30, 2009, in regarding the lists of vitamins and minerals and their forms that can be added to food, including food supplements:[348]

> Vitamins include Vitamin A, C, D, E, K, B1, B2, B6, B12, niacin, pantothenic acid, folic acid, and biotin, whereas minerals include Ca, Mg, Fe, Cu, I, Zn, Mn, Na, K, Se, Cr, Mo, F, Cl, P, B, Si (Annex I) in the structural forms permitted according to Annex II, extended from the former list.

Further amendments to Directive 2002/46/EC were incorporated by COMMISSION REGULATION (EU) No 1161/2011 of 14 November 2011,[349] COMMISSION REGULATION (EU) No 119/2014 of 7 February 2014,[350] COMMISSION REGULATION (EU) 2015/414 of 12 March 2015,[351] COMMISSION REGULATION (EU) 2017/1203 of 5 July 2017,[352] and COMMISSION REGULATION (EU) 2021/418 of 9 March 2021.[353] However, in spite of the abovementioned amendments, the list is still limited to vitamins and minerals.

Remarkably, Article 6 in Directive 2002/46/EC, establishes that "*the labelling, presentation and advertising must not attribute to food supplements the property of preventing, treating or*

curing a human disease, or refer to such properties", since the present Directive does not apply to medicinal products as defined by Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use.

According to the same Article, the information which is allowed to be added in the label is limited to:

> (a) The names of the categories of nutrients or substances that characterise the product or an indication of the nature of those nutrients or substances;
>
> (b) The portion of the product recommended for daily consumption;
>
> (c) A warning not to exceed the stated recommended daily dose;
>
> (d) A statement to the effect that food supplements should not be used as a substitute for a varied diet;
>
> (e) A statement to the effect that the products should be stored out of the reach of young children.

Directive 2002/46/EC partially harmonizes food supplements along the European Union, regarding labelling and uses of vitamins and minerals. However, it is important to highlight that current European Directives regulating food supplements do not apply to any other form of nutrients different from vitamins and minerals.

In this regard, reference is made to the report on the use of substances other than vitamins and minerals in food supplements by the Commission of the European Communities,[354] wherein regarding regulatory framework in force in the EU, the Commission concludes that it "constitutes a sufficient legislative framework for regulating this area and does not consider it opportune to lay down specific rules for substances other than vitamins or minerals for use in foodstuffs", even though according to report from the same Commission,[355] in 2008 the number of substances other than vitamins and minerals used in food supplements on the European market was estimated to be over 400, and the share of vitamins and minerals in those figures accounted for 50%, according to the same source.

Therefore, the use of nutrients different from vitamins and minerals still lays down under the national laws in force in each Member State, without any harmonization for the present time.

2.2.2. Non harmonized regulation and the Mutual Recognition principle in food supplements

As defined by the European Commission when referring to the internal or single market, *"(it) refers to the EU as one territory without any internal borders or other regulatory obstacles to the free movement of goods and services. A functioning single market stimulates competition and trade, improves efficiency, raises quality, and helps cut prices*".[356] Therefore, the Union Treaties ensure the free movement of goods in the internal market, and restrictions on imports or measures having similar effect are prohibited among Member States.
However, some goods are not fully harmonized by the Union laws. In these cases, particular national regulation applies, wherein regulation may differ among Member States on a case-by-case basis.

In order to avoid obstacles in the free movement of goods in such cases, the European Parliament and the Council of the European Union regulated the **Mutual Recognition principle**, in the REGULATION (EC) No 764/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 9 July 2008 laying down procedures relating to the application of certain national technical rules to products lawfully marketed in another Member State,[357] later repealed by REGULATION (EU) 2019/515 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 19 March 2019 on the mutual recognition of goods lawfully marketed in another Member State.[358]
As stated in Regulation (EC) No 724/2008, "*The principle of mutual recognition applies to products which are not subject to Community harmonisation legislation, or to aspects of products falling outside the scope of such legislation. According to that principle, a Member State may not prohibit the sale on its territory of products which are lawfully marketed in another Member State, even where those products were manufactured in accordance with technical rules different from those to which domestic products are subject. The only exceptions to that principle are restrictions which are justified on the grounds set out in Article 30 of the Treaty, or on the basis of other overriding reasons of public interest and which are proportional to the aim pursued*".

Therefore, the principle of mutual recognition exclusively applies to products which are not subject to EU harmonisation legislation or to aspects falling outside the scope of such legislation, but not to products where EU legislation is harmonised.
For the purposes of the present research, in the particular field of foods as goods, the mutual recognition principle applies to food supplements, since only certain requirements such as labelling are harmonized under current regulations,[338,348] but not important aspects such as their composition.

The European Commission Enterprise and Industry Directorate-General published a Guidance document. The application of the Mutual Recognition Regulation to food supplements[359] to clarify how Regulation (EC) No 764/2008 (the 'Mutual Recognition Regulation') applies to the marketing of food supplements within the EU. In this regard, the application of the mutual recognition procedure only applies when all the following conditions are met:

1. The food supplement is already lawfully marketed in one Member State.
   "*A Member State cannot forbid the sale on its territory of products which are lawfully marketed in another Member State, even if they were manufactured according to different technical rules*".

2. The food supplement is not subject to harmonised EU law.
   In this regard, composition other than vitamins and minerals in the list in force is non-harmonized, whereas names, labelling, presentation, advertising, and nutrition and health claim are harmonized

3. The procedure only applies to a food business operator, and not to any legal person.

4. The decision (when referred to the mutual recognition at the concerned Member State) is based on a technical rule.
   Wherein, a technical rule is referred to any non-harmonized characteristics required of the product (levels of quality, performance or safety, or dimensions, the name under which it is sold, terminology, symbols, testing and test methods, packaging, marking or labelling), or of protecting consumers or the environment.

Having considered the above-mentioned conditions, the administrative decision on the mutual recognition procedure applied to the food supplement which is intended to be placed in a further Member State market, may be either:

1. The permission to lawfully enter into the market of the second Member State
2. The prohibition of the placing on such market of that food supplement
3. The requirement for modification or additional testing of that food supplement before it can be placed or kept on such market
4. The withdrawal of that food supplement from such market

Figure 18 summarizes the Food supplements' regulatory framework in the light of the Mutual Recognition Principle:

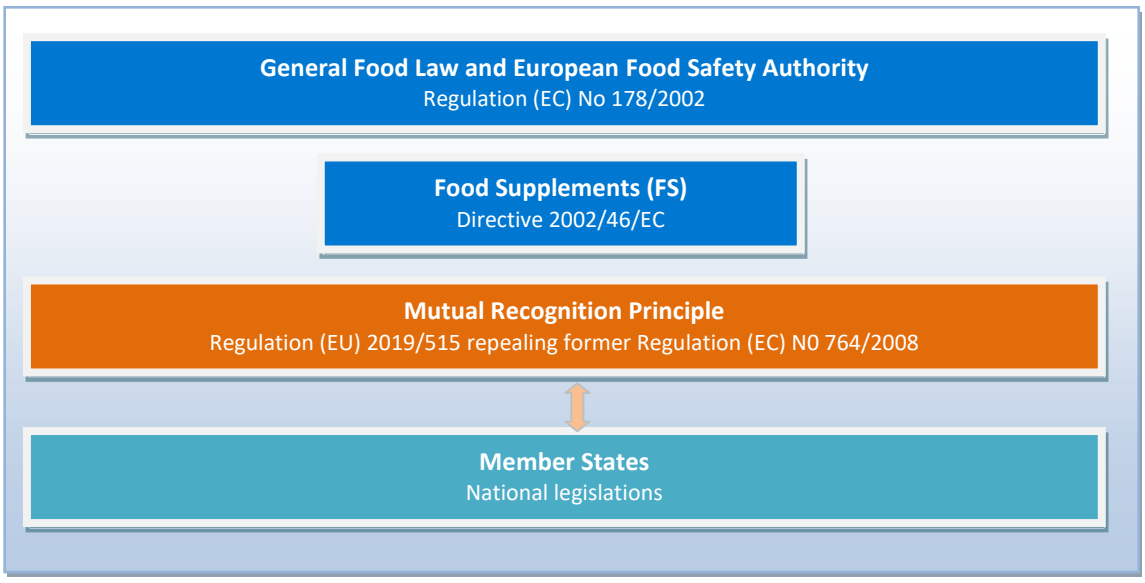

Figure 18: Overview of the regulatory framework, including the Mutual Recognition Principle

2.2.3. Comparative situation among Member States regarding components different than vitamins and minerals in food supplements' composition

As previously introduced in this Chapter, Directive 2002/46/EC partially harmonizes food supplements along the European Union, particularly regarding labelling and uses of vitamins and minerals, but still excludes from harmonization any other form of nutrients different from vitamins and minerals. In this regard, Directive is transposed into each Member State law, and complemented (or not) with parallel national regulation that could be considerably different along the EU territory.

The European Commission Health & Food Safety Directorate - General publishes and updates the List of competent authorities of the Member States within the meaning of Article 4(6) of Directive 2002/46 on food supplements.[360] Whereas in some Member States the competency corresponds to the Ministries of Health, some other Member States regulate food supplements under more specific food and nutrition divisions, as detailed in **Table 25**. This situation somehow illustrates the different consideration that this food category holds along different jurisprudences.

In this regard, considering their corresponding specific national regulations, some Member States components have extended food supplements' ingredients beyond vitamins and minerals to **botanicals** (including whole, fragmented, cut plants or parts of plants, algae, fungi, lichens) **and other substances**.

As an example of the vast differences along the EU territories in terms permitted and non-permitted substances in food supplements' composition, **Table 26** illustrates the situation among the Member States regarding the existence of positive and/or negative lists of plants (*i.e.* permitted or not permitted as nutrients different than vitamins and minerals) under their corresponding national regulations.[361]

Table 25: List of competent authorities of the Member States within the meaning of Article 4(6) of Directive 2002/46 on food supplements[360]

Member State	Competent Authority (Article 4(6) of Directive 2002/46/EC)
AUSTRIA	Bundesministerium für Gesundheit Federal Ministry for Health
BELGIUM	SPF Santé publique, Sécurité de la Chaîne alimentaire et Environnement Service Denrées alimentaires, Aliments pour Animaux et Autres Produits de Consommation
BULGARIA	Food Control and Border Control Directorate Bulgarian Food Safety Agency
CROATIA	Ministry of Health
CYPRUS	Medical and Public Health Services, Ministry of Health
CZECH REPUBLIC	Ministry of Health
DENMARK	Danish Veterinary and Food Administration Chemistry and Food Quality Division
ESTONIA	Office for Retail, Organic Farming and Food of Non-animal Origin, Veterinary and Food Board
FINLAND	Elintarviketurvallisuusvirasto
FRANCE	Direction Générale de la Concurrence, Consommation et Répression des Fraudes (DGCCRF) Bureau D3 - Secteur Nutrition
GERMANY	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
GREECE	Particular Nutrition Food, Food Supplement and Biocides Assessment Section, National Organization of Medicines
HUNGARY	Országos Élelmezés- és Táplálkozástudományi Intézet (OETI) (National Institute for Food and Nutrition Science)
IRELAND	Food Safety Authority of Ireland
ITALY	Ministero della Salute DSVET DGSAN
LATVIA	Ministry of Agriculture Food Center of the Food and Veterinary Service
LITHUANIA	National Nutrition Centre at the Ministry of Health
LUXEMBOURG	Ministère de la Santé
MALTA	Malta Standards Authority Regulatory Affairs Directorate Foodstuffs, Chemicals, Pesticides and Cosmetics Unit
NETHERLANDS	Ministerie van Volksgezondheid, Welzijn en Sport Directie Voeding, Gezondheidsbescherming en Preventie
POLAND	Department of Health Promoting Foods
PORTUGAL	DGAV – Direção Geral de Alimentação e Veterinária
ROMANIA	Ministry of Health (MH) Institute of Public Health Timisoara
SLOVAK REPUBLIC	Department of Food Safety and Improvement of Nutrition Public Health Authority SR
SLOVENIA	Ministry of Health of Republic of Slovenia Sector for Food Safety
SPAIN	Ministerio de Consumo, Agencia Española de Seguridad Alimentaria y Nutrición (AESAN)
SWEDEN	National Food Administration
ICELAND	Environmental and Food Agency of Iceland
NORWAY	Norwegian Food Safety Authority

Table 26: Positive and/or negative lists of plants allowed in food supplements in EU

Positive and/or negative lists of plants allowed in food supplements available			
Member State	**Positive list**	**Negative list**	**Status**
	Y/N	**Y/N**	**Legally binding / official guideline/ industry guideline/ …**
AUSTRIA	Y	Y	Official guideline
BELGIUM	Y	Y	Legally binding
BULGARIA	N	Y	Legally binding
CROATIA	Y	Y	Legally binding
CZECHIA	Y	Y	Legally binding
DENMARK	N	Y	Official guideline
ESTONIA	N	Y	Official guideline
FINLAND	N	Y	Official guideline
FRANCE	Y	N	Legally binding (BELFRIT list is applied for mutual recognition)
GERMANY	Y	Y	Official guideline
GREECE	N	N	---
HUNGARY	N	Y	Official guideline
IRELAND	N	Y	Official guideline
ITALY	Y	N	Legally binding
LATVIA	N	N	---
LITHUANIA	N	Y	Legally binding
NETHERLANDS	N	Y	Legally binding
POLAND	Y	N	Unofficial internal guideline
PORTUGAL	N	N	---
ROMANIA	Y	Y	Negative list - legally binding. Positive list - unofficial guideline
SLOVAKIA	N	N	---
SLOVENIA	Y	Y	Official guideline
SPAIN	N	N	---
SWEDEN	N	Y	Official guideline

Along the EU, the market size of other sources of ingredients other than vitamins and minerals is considerably profitable in Italy, Germany, UK, France and Belgium compared to the other Member States,[354] mainly driven by their broader scope in the allowed substances as food supplements' ingredients.

For the purposes of the present research, aimed at a better knowledge on the permitted or non permitted inclusion of methylxanthines as ingredients in the composition of the future food product, a thorough research into national regulation of ingredients different from vitamins and minerals was performed. The Member State Spain was selected based on the proper jurisprudence to be met. Additionally, Belgium, France and Italy were alternative jurisdictions known for their dynamism in the food supplements' field. Particularly, Belgium, France and Italy harmonize certain aspects of food supplements' compositions through the common BELFRIT list,[362] which will be further disclosed. Accordingly, beyond vitamins and

minerals, the research was focused on how these EU territories had regulated the categories 'botanicals' or 'plants' and 'other substances'.

Regarding **botanicals**, a common project among Belgium, France and Italy known as the BELFRIT list tried to harmonize and to facilitate mutual recognition in the case of botanicals, due to their heterogeneity, biological and chemical complexity. The BELFRIT list integrates the three national lists of plants into one common single list, considering the safety assessments already performed on national level. Plants in the list were previously considered safe on the basis of the traditional use (*i.e.* they are not novel foods,[363] which are explicitly excluded), and of the whole complex of available scientific evidence, which can be used because of their physiological effects. The list includes the scientific name of the plant as it appears in the recognized references. It also specifies the parts of plants traditionally used as well as the chemical classes and substances of interest. The list was adopted into each Member State's legislation, as discussed herein.

The category **other substances** encompasses substances, other than nutrients or plant substances, having a nutritional or physiological effect. As in the case of plants, its legal situation as ingredients in food supplements should be revised and compared along different Member States.

2.2.3.1. Regulatory situation in Spain and its territories

The *Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios*,[364] transposed the Directive 2002/46/CE into this Member State. Subsequently, this Royal Decree was modified by the *Real Decreto 130/2018, de 16 de marzo por el que se modifica el Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios*,[365] by providing a positive list of substances different from vitamins and minerals that are allowed in the manufacture of food supplements along the Spanish territory, as well as their chemical form, purity and maximum daily amounts, according to Annexes I and II in the above-mentioned Royal Decree.

The positive list includes the following categories:

Category 1: Fatty acids
Category 2: Amino acids and some other nitrogen-containing compounds
Category 3: Dipeptides and peptides
Category 4: Coenzymes
Category 5: Flavonoids and carotenoids
Category 6: Nucleotides
Category 7: Polysaccharides and oligosaccharides
Category 8: Other substances

Neither botanicals nor plants are permitted in Spain as food supplements ingredients. Regarding the term 'other substances', although it is not defined in the Royal Decree as such, it should be understood as other substances (different from minerals, vitamins and substances falling under Categories 1 to 7) with nutritional or physiological effects that can be used in the manufacture of food supplements. This category comprises: choline, chondroitin sulphate, creatine monohydrate, glucosamine, inositol, wheat germ, pollen, royal jelly, beer yeast, soy

lecithin and propolis. For food supplements' compositions different from the former, the Mutual Recognition principle may apply.

Beyond the reflection to Spanish law of the Directive 2002/46/CE into this Member State, the *Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios*, details the way for food business operators to place the product on this Member State market, through notification to the national competent authority of that placing on the market, by forwarding it a model of the label before or simultaneously to the first entry in such market. In compliance to Royal Decree 191/2011, of February 18 *Real Decreto 191/2011, de 18 de febrero, sobre Registro General Sanitario de Empresas Alimentarias y Alimentos*,[366] food business operators in Spain must be registered on the *Registro General Sanitario de Empresas Alimentarias y Alimentos* (General Sanitary Registry of Food and Food Companies). Particularly, for the purpose to place either a food supplement or a food for special medical purposes on the market, food business operators will be registered under key code nr 26.[367]

In this Member State, the competent authority may be either the Autonomous Communities' food safety authorities (*Agència Catalana de Seguretat Alimentària,*[368] ACSA, in Catalonia) or the national competent authority *Agencia Española de Seguridad Alimentaria y Nutrición*[369] (AESAN, Spanish Agency for Food Safety and Nutrition), depending on the place where the business operator has registered its office (in the Member State itself, in another Member State or in a Third Country).

2.3.2.2. Regulatory situation in Belgium

Belgium transposed Directive 2002/46/CE into this Member State through *ARRÊTÉ ROYAL du 15 MAI 2003 - Arrêté royal modifiant l'arrêté royal du 3 mars 1992 concernant la mise dans le commerce de nutriments et de denrées alimentaires auxquelles des nutriments ont été ajoutés*,[370] which is a modification of the Royal Decree of 3 March 1992, currently in force.

Food supplements have a long tradition in Belgium where they are regulated by three specific Royal Decrees, according to the nature of the ingredients in the composition thereof:

- **Nutrients**: Food supplements and ordinary foodstuffs enriched with nutrients are regulated by Royal Decree of 3 March 1992 (*ARRÊTÉ ROYAL du 3 MARS 1992 concernant la mise dans le commerce de nutriments et de denrées alimentaires auxquelles des nutriments ont été ajoutés*).[371] As defined therein, nutrients are nutritional substances that the human body needs and cannot normally produce itself and at a sufficient quantity of which must be supplied to the organism through food.
- **Plants**: Food supplements containing plants (botanicals) are regulated by Royal Decree of 29 August 1997 relating to the manufacture and trade in foods composed of or containing plants or plant preparations (*ARRÊTÉ ROYAL du 29 AOÛT 1997 relatif à la fabrication et au commerce de denrées alimentaires composées ou contenant des plantes ou préparations de plantes*).[372] Annex in the decree is divided into several lists, aimed at differentiating molecules exerting the desired physiological activity on the body, but also the molecules capable of exerting adverse effects, as well as specific molecules or markers for the chemical characterization of the plant:

List 1: Dangerous plants which may not be used as or in foodstuffs
List 2: Edible mushrooms
List 3: Plants to be notified if in pre-dosed form

- **Other substances**: Food supplements containing other substances are regulated by the Royal Decree of 12 February 2009 (*ARRÊTÉ ROYAL du 12 FÉVRIER 2009 relatif à la fabrication et au commerce de compléments alimentaires contenant d'autres substances que des nutriments et des plantes ou des préparations de plantes*).[373]

Particularly, the exact identification of other substances for which their use in food supplements is linked to conditions allowed by the Belgium law are those included in the short list in appendix of Ministerial Decree of 22 December 2021 relating to the manufacture and trade of food supplements containing other substances than nutrients and plants or plant preparations (*ARRÊTÉ MINISTÉRIEL du 22 DÉCEMBRE 2021 relatif à la fabrication et au commerce de compléments alimentaires contenant d'autres substances que des nutriments et des plantes ou des préparations de plantes*),[374] repealing Ministerial Decree of 19 February 2009 relating to the manufacture and trade of food supplements containing other substances than nutrients and plants or plant preparations (*ARRÊTÉ MINISTÉRIEL du 19 FÉVRIER 2009 relatif à la fabrication et au commerce de compléments alimentaires contenant d'autres substances que des nutriments et des plantes ou des préparations de plantes*).[375] However, according to Belgium law, food supplements containing other substances may only be placed on the market if they satisfy the conditions related to the minimum or maximum daily portion as regulated therein.

2.3.2.3. Regulatory situation in France

France transposed Directive 2002/46/CE into this Member State through Decree n° 2006-352 of March 20, 2006 relating to food supplements (*Décret n°2006-352 du 20 mars 2006 relatif aux compléments alimentaires*).[376]

As in the case of Belgium, this mother Decree is complemented with further implementing orders devoted to the particular ingredients they intend to regulate:

- **Nutrients**: Nutrients are regulated in France under Order of 9 May 2006 relating to nutrients that can be used in the manufacture of food supplements (*Arrêté du 9 mai 2006 relatif aux nutriments pouvant être employés dans la fabrication des compléments alimentaires*),[377] wherein Annex I lists in detail nutrients that are considered vitamins and minerals, Annex II discloses the vitamins and mineral substances that can be used for the above mentioned purpose, Annex III discloses maximum daily doses thereof, and Annex IV discloses vitamins and minerals that could be used for the manufacture of dietary supplements until 31 December 2009.
- **Plants**: Botanicals are regulated under Decree n° 2006-352 of March 20, 2006 relating to food supplements (*Décret n°2006-352 du 20 mars 2006 relatif aux compléments alimentaires*).[376] The BELFRIT list in France was adopted through the Order of June 24, 2014 establishing the list of plants, other than fungi, authorized in food supplements and the conditions for their use (*Arrêté du 24 juin 2014 établissant la liste des plantes, autres que les champignons, autorisées dans les compléments alimentaires et les*

conditions de leur emploi).[378] The list is incorporated in the Order through Annex 1: Plants authorized for use in dietary supplements and their conditions of use.

- **Other substances**: As defined under Article 2 in Decree n ° 2006-352 of March 20, 2006 relating to food supplement *(Décret n°2006-352 du 20 mars 2006 relatif aux compléments alimentaires*),[376] the term other substances refers to "substances for nutritional or physiological purposes (are) chemically defined substances having nutritional or physiological properties, with the exception of the nutrients defined in 2 *(i.e.* vitamins and minerals) and substances having exclusively pharmacological properties. More particularly, Order of September 26, 2016 establishing the list of substances for nutritional or physiological purposes authorized in food supplements and the conditions for their use *(Arrêté du 26 septembre 2016 établissant la liste des substances à but nutritionnel ou physiologique autorisées dans les compléments alimentaires et les conditions de leur emploi*)[379] regulates the exact list of substances for nutritional or physiological purposes, mentioned in the aforementioned decree of March 20, 2006. These substances are permitted in the manufacture of food supplements alone or as components in a mixture, provided that they do not present a danger to the health nor mislead the consumer for whom the product is intended, at the proposed doses, according to available scientific evidence.
  As opposed to Regulation (EC) n° 1925/2006 which defines 'other substances' as anything other than vitamins and minerals and *de facto* includes plants, micro-organisms or any other complex ingredient, the concept of substances for nutritional or physiological purposes are understood in French regulation in a restrictive manner, covering only chemical pure compounds, that is, pure chemicals made up of atoms linked together by chemical bonds, and excluding mixtures of different substances. Moreover, explicit exclusion is made to new substances within the scope of Regulation (EU) 2015/2283 relating to novel foods and new ingredients.[363]

2.3.2.4. Regulatory situation in Italy

Italy is one of the most dynamic jurisprudences in EU in terms of food supplements, with a long history of use, and therefore an extended body of laws are in force in this Member State, wherein the competent authority, *Ministero de la Sallute*, regulates vitamins, minerals, other substances with a nutritional or physiological effect, herbal substances and preparations, probiotics and prebiotics, adjuvants of low-calorie diets, and the like.[380]

Italy transposed Directive 2002/46/CE into this Member State through Decree n° 2004-169 of May 21, relating to food supplements (*Decreto legislativo 21 maggio 2004, n. 169: Attuazione della direttiva 2002/46/CE relativa agli integratori alimentari*).[381]

Regarding composition, in a very similar structure as previously disclosed for Belgium and France, Italian regulation encloses and distinguishes among:

- **Nutrients**: Nutrients are regulated in Italy under Decree n 2004-169 of May 21, relating to food supplements (*DECRETO LEGISLATIVO 21 maggio 2004, n. 169 Attuazione della direttiva 2002/46/CE relativa agli integratori alimentari*),[381] wherein Nutrients are considered vitamins and minerals, and wherein Annex I list in detail said vitamins and

minerals, whereas Annex II discloses the vitamins and mineral substances that can be used for the above mentioned purpose, as well as the admitted daily amount thereof.

- **Plants**: Botanicals were regulated under the Ministerial Decree of 9 July 2012 on the "Discipline of the use of plant substances and preparations in food supplements", which supplements the Legislative Decree of 21 May 2004, no. 169 (*Decreto 9 luglio 2012 Disciplina dell'impiego negli integratori alimentari di sostanze e preparati vegetali*).[382] Later, the BELFRIT list was incorporated by Decree of 27 March 2014 (*Decreto dirigenziale 27 marzo 2014. Aggiornamento del DM 9 luglio 2012 sulla Disciplina dell''impiego negli integratori alimentari di sostanze e preparati vegetali*)[383] in the form of Annex 1.bis[384] to adopt a new single list of admitted plants used in supplements as a source of substances and preparations plants.

  As per May 2022, the regulation in force in Italy regarding botanicals in food supplements is the Executive Decree of 9 January 2019 (*Ministero della Salute Decreto dirigenziale 9 gennaio 2019 ed il relativo allegato contente l'elenco aggiornato delle sostanze e i preparati vegetali utilizzabili negli integratori alimenti*)[385] which contains the list of plant substances and preparations that can be used in food supplements, an update of the list as it was previously regulated by the Annex 1 to Ministerial Decree 10 August 2018 - Discipline of the use of vegetable substances and preparations in food supplements (*Decreto 10 agosto 2018. Disciplina dell'impiego negli integratori alimentari di sostanze e preparati vegetali*),[386] which repeals the decree of the Minister of Health 9th July 2012. Decree 26 July 2019[387] updates Decree 9 January 2018 regarding curcuma.
- **Other substances**: When referred to 'Other substances with a nutritional or physiological effect', Italian regulation discloses the list of substances with a nutritional or physiological effect that includes some of the substances that can be used in supplements (*Ministero della Salute Altri nutrienti e altre sostanze ad effetto nutritivo o fisiologico, Revisione settembre 2019*).[388] The list includes nutrients other than vitamins and minerals and other substances with a nutritional or physiological effect permitted for use in food supplements, although it is not exhaustive. For substances not included in the list, use is permitted under Italian regulation if they have a history of significant consumption, such as to prove in favour of safety. Otherwise it is novel food pursuant to Regulation (EU) 2015/2283 on New Foods.

Figure 19 summarizes the Food supplements' regulatory framework previously introduced, as discussed in the preceding sections along this Chapter.

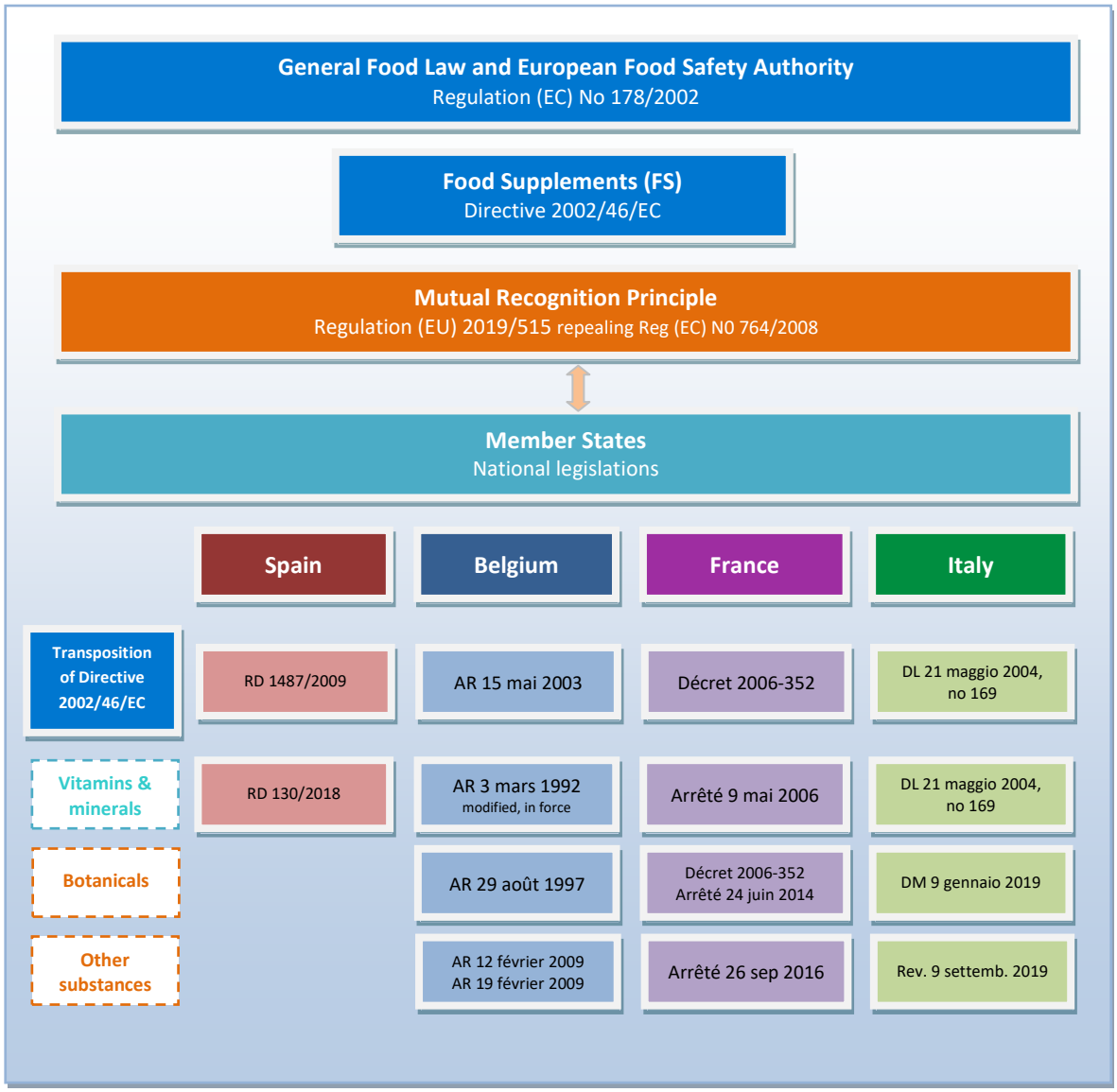

Figure 19: Overview of the regulatory framework to be met for food supplements in Spain, Belgium, France and Italy

2.2.4. Comparative regulatory situation of methylxanthines as authorized ingredients in food supplements in Spain, Belgium, France and Italy

For the purposes of the present research, none of the methylxanthines including caffeine, theobromine, theophylline, paraxanthine and the like (chemical compounds *per se*, falling under the category of 'other substances'), nor *Coffea arabica, Camelia sinensis or Theobroma cacao as '*botanicals' (providing that these plants are the main natural sources of natural methylxanthines), are allowed in the food supplements' composition in the Member State Spain nor its territories. In spite of this limitation, in light of Regulation (EC) No 724/2008, the Mutual Recognition principle among EU Common market "(…) a Member State may not prohibit the sale on its territory of products which are lawfully marketed in another Member State, even where those products were manufactured in accordance with technical rules different from those to which domestic products are subject".

In this regard, the plant *Theobroma cacao,* from the *Malvaceae* family, is included in the BELFRIT regulated list of plants considered safe on the basis of their traditional use. More specifically, the part of the plant traditionally used and particularly cited in the list is seeds (reference is made to the corresponding Annexes in all the above mentioned regulations in Belgium, France and Italy). However, falling down the 'other substance' classification, theobromine is not allowed in any of the four Member States as such.

Certainly, the most regulated methylxanthine is caffeine, which is included in the Belgian, French and Italian regulated list of other substances, but not allowed in Spain. Caffeine content is limited to a different maximum amount under different legislations:

- Belgium: limited to maximum 80 mg/day (total content)[374]
- France: limited to maximum 200 mg daily portion[379]
- Italy: limited to maximum 200 mg daily portion[388]

2.3. Food for special medical purposes

2.3.1. Introduction and regulation

REGULATION (EU) No 609/2013 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009,[389] (also known as the Regulation on 'Food for Specific Groups of the population' or 'FSG Regulation'), defines "***food for special medical purposes*** *means food specially processed or formulated and intended for the dietary management of patients, including infants, under medical supervision; it is intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary food or certain nutrients contained therein, or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved by modification of the normal diet alone*" (Article 2).

As in the case of food supplements, this Regulation provides a Union list (Chapter III, Article 15 and Annex), limiting their content to:

> (a) Vitamins: A, D, E, K, C, thiamin, riboflavin, niacin, B6, B12, folate, biotin, pantothenic acid;
> (b) Minerals: K, Ca, Mg, Fe, Zn, Cu, Mn, F, Se, Cr, Mo, I, Na, B;
> (c) Amino acids;
> (d) Carnitine and taurine;
> (e) Nucleotides;
> (f) Choline and inositol.

Food for special medical purposes is a wider and more diverse category than food supplements, since each specific disease and patient ages may need different compositions according to product's intended use. In this regard, Regulation (EU) No. 609/2013 extremely tightened the composition, and it was earlier realized that Regulation may seriously limit the evolution of scientific knowledge of such an innovative food category.

Accordingly, a further more flexible regulation was required to meet these needs, in the form of COMMISSION DELEGATED REGULATION (EU) 2016/128 of 25 September 2015, which supplemented Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for food for special medical purposes.[390]

The Delegated Regulation (EU) 2016/128 states that "*Food for special medical purposes is developed in close cooperation with health care professionals to feed patients affected by or malnourished because of a specific diagnosed disease, disorder or medical condition that makes it impossible or very difficult for those patients to satisfy their nutritional needs through the consumption of other foods. For that reason, food for special medical purposes must be*

used under medical supervision, which may be applied with the assistance of other competent health professionals".

In the light of this Regulation, Food for special medical purposes is classified in the following three categories (Article 2):

> (a) **Nutritionally complete food with a standard nutrient formulation** which, used in accordance with the manufacturer's instructions, may constitute the sole source of nourishment for the persons for whom it is intended;
>
> (b) **Nutritionally complete food with a nutrient-adapted formulation specific for a disease**, disorder or medical condition which, used in accordance with the manufacturer's instructions, may constitute the sole source of nourishment for the persons for whom it is intended;
>
> (c) **Nutritionally incomplete food with a standard formulation or a nutrient-adapted formulation specific for a disease**, disorder or medical condition which is not suitable to be used as the sole source of nourishment.

The basis for food for special medical purposes is the fact that the dietary management of the associated disease/disorder/medical condition "*cannot be achieved by modification of the normal diet alone*". Accordingly, for a product to be accepted as food for special medical purposes the nutritional needs of that group of patients cannot be met by consuming exclusively common food in the framework of the normal diet.

Regarding specific information to the consumer, this category must include (Article 5):

> (a) A statement that the product must be used under medical supervision
>
> (b) A statement whether the product is suitable for use as the sole source of nourishment
>
> (c) A statement that the product is intended for a specific age group, as appropriate
>
> (d) Where appropriate, a statement that the product poses a health hazard when consumed by persons who do not have the disease, disorder or medical condition for which the product is intended
>
> (e) The statement 'For the dietary management of …' where the blank shall be filled in with the disease, disorder or medical condition for which the product is intended
>
> (f) Where appropriate, a statement concerning adequate precautions and contra-indications
>
> (g) A description of the properties and/or characteristics that make the product useful in relation to the disease, disorder or medical condition for the dietary management of which the product is intended, in particular, as the case may be, relating to the special processing and formulation, the nutrients which have been increased, reduced, eliminated or otherwise modified and the rationale of the use of the product

(h) Where appropriate, a warning that the product is not for parenteral use

(i) Instructions for appropriate preparation, use and storage of the product after the opening of the container, as appropriate

Nutrition declaration must also be included in the labelling, in particular (Article 6):

(a) The amount of each mineral substance and of each vitamin listed in Annex I to this Regulation and present in the product

(b) The amount of components of protein, carbohydrate, fat and/or of other nutrients and their components, the declaration of which would be necessary for the appropriate intended use of the product

(c) Information on the osmolality or the osmolarity of the product where appropriate;

(d) Information on the source and the nature of the protein and/or protein hydrolysates contained in the product

Remarkably, Article 7 states that "*Nutrition and health claims shall not be made on food for special medical purposes*".

Figure 20 summarizes the Food for special medical purposes' regulatory framework. It is relevant to highlight that, the combination of Regulation (EU) No 609/2013, and Commission Delegated Regulation (EU) No 2016/128, fully harmonize FSMP composition along the Member States. Accordingly, on a completely different situation to the case of food supplements, the Mutual Recognition Principle is not applicable in the food for special medical purposes category:

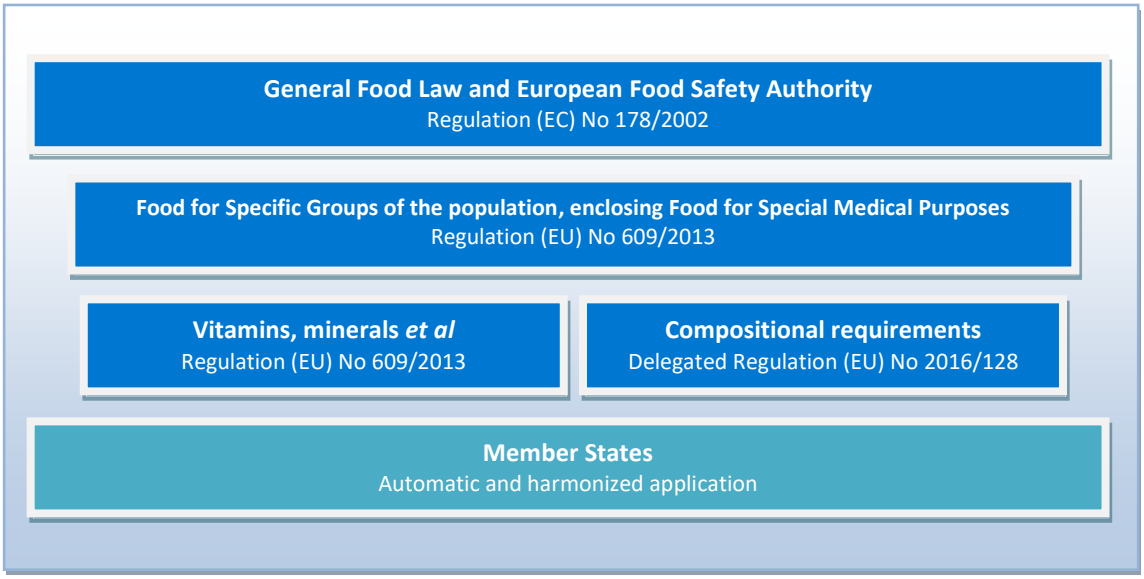

Figure 20: Overview of the harmonized regulatory framework for FSMP

The Official Journal of the European Union published in 2017 the "Commission Notice on the classification of Food for Special Medical Purposes (C/2017/7716)"[391] aimed to provide guidelines to assist both national competent authorities and stakeholders in classifying and marketing their products under the appropriate EU legal framework.

Moreover, this Commission Notice clarifies the basic concepts laying down the legal definition of food for special medical purposes, among others. In the light of this publication, the definition of food for special medical purposes is broadly discussed for the proper understanding of this food category, as well as differences between food for special medical purposes and other types of products different from foods (for instance, medicinal products).

2.3.2. On the differences between FSMP and medicinal products

The concept is broadly discussed in the 'Commission Notice on the classification of Food for Special Medical Purposes (C/2017/7716)'.[391] According to Regulation (EU) No 609/2013 of the European Parliament and of the Council, food for special medical purposes is defined as 'food', in Article 2, even though it is intended for the dietary management of patients, including infants, under medical supervision.

Moreover, Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, previously disclosed in this Chapter, defines 'food' (or 'foodstuff') in Article 2 as *"(…) any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. (…) 'Food' shall not include: (…) (d) medicinal products within the meaning of Council Directives 65/65/EEC and 92/73/EEC*".

Council Directives 65/65/EEC and 92/73/EEC are no longer in force, currently repealed by Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use[336] which defines a **medicinal product** as: "*(a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or (b) Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis*", in Article 1.

Therefore, the definitions and regulations on food and medicinal products are mutually exclusive. However, wherein reasonable doubts regarding product classification as food for special medical purpose or medicinal product, Directive 2001/83/EC of the European Parliament and of the Council regulates that "*In cases of doubt, where, taking into account all its characteristics, a product may fall within the definition of a 'medicinal product' and within the definition of a product covered by other Community legislation the provisions of this Directive shall apply*" (Article 2). Therefore, in case of uncertainty on the proper classification of borderline products, when referring to food for special medical purposes and their effect on the disease they are intended to dietary manage, the product must be considered under the scope of a medicinal product, rather than under the scope of a foodstuff.

Last but not least, it is noteworthy that possible differences may exist among Member States in the classification of products, since national authorities decide whether a product is to be classified as a medicinal product on a case-by-case basis. However, even under these circumstances, any product that is intended to prevent or to treat a disease should be considered as a medicinal product, and not a food.

2.3.3. Classification and place on the market of FSMP

EU law allows food business operators to place the product on their Member State market by means of notification to the national competent authority of that placing on the market, without any previous authorisation, on the basis of the food business operators' own assessment that the product falls within the scope of this category. Notification is accomplished by forwarding a model of the label to the competent authority of the corresponding Member State before or simultaneously to the first entry in such a market. In spite of not being centralized, the notification system allows an efficient monitoring of this food category among Member States.

Even though only the label is required for the notification, it is reasonable to provide complementary information supporting the relationship with the disease, disorder or medical condition for which the product is intended to use, since in accordance with Regulation (EC) No 178/2002 of the European Parliament and of the Council, national competent authorities are responsible to enforce the relevant legislation and verify the correct classification and the accomplishment of legal requirements for any product placed on its market as food for special medical purposes.

However, Member States may have reasonable doubts whether a product meets the regulatory definition of food for special medical purpose, to properly classify the product as such. The improper classification may lead to differences in EU law among different Member States affecting consumers' rights and protection, free circulation of goods in the common market and unfair competition in different Member States among food business operators.

Therefore, when discrepancies regarding classification of a food for special medical purpose arise, the European Commission may apply the European Food Safety Authority (EFSA) for a scientific evaluation, opinion and judgement on the information provided by the food business operator, in which the FSMP classification has been built. Particularly, EFSA assessment must focus on the evaluation whether the specific group of patients suffering from the medical condition for which the product is intended to use has nutritional needs that are impossible, impractical, unsafe or nutritionally/clinically disadvantageous to satisfy through the exclusive consumption of food other than the FSMP product for which data is provided.

The information must be provided to EFSA in the form of a well-structured product dossier in a common format, according to the scientific and technical guidance template "Scientific and technical guidance on foods for special medical purposes in the context of Article 3 of Regulation (EU) No 609/2013"[392], adopted in 2015.

Upon EFSA's opinion, the Commission can adopt decisions stating whether the specific product placed on the market as FSMP is appropriately classified as such or not, in the light of Article 3

of Regulation (EU) No 609/2013 ("In order to ensure the uniform implementation of this Regulation, the Commission may decide, by means of implementing acts: (a) whether a given food falls within the scope of this Regulation; (b) to which specific category of food referred to in Article 1(1) a given food belongs").

2.3.4. Guidance on the assessment of FSMP

Scientific and technical guidance on foods for special medical purposes in the context of Article 3 of Regulation (EU) No 609/2013[392] is aimed at providing structured information on the following key issues, closely related to the food for special medical purposes definition:

1. The extent to which the specific food product is sufficiently characterised
2. The extent to which the disease/disorder/medical condition for which the specific product is intended is sufficiently characterised
3. The extent to which patients suffering from the specific disease for the dietary management of which the product is intended; a) are in the impossibility or difficulty to take, digest, absorb, metabolise or excrete ordinary foodstuffs, or certain nutrients contained therein or metabolites, or b) have specific medically-determined nutrient requirements, typical to the disease, that cannot be reasonably or realistically satisfied by modifying the normal diet
4. The specific role of the product in the dietary management of the disease or medical condition for which it is intended, in particular the extent to which the specific product is different from or more suitable than foods that are not FSMPs
5. Any potential restrictions of use, referred to safety to the consumers

The above-mentioned information and supporting data is scheduled into six sections:

1. **Part 1**: contains administrative and technical data, related to the product and food business operator.
2. **Part 2**: contains more detailed information on the specific food product: name and characteristics, list of ingredients, energy and nutrient content, information on the special formulation or processing, a description of the manufacturing process, and stability information.
3. **Part 3**: contains information relative to the proposed use: the target patient population, the disease/disorder or the medical condition, the conditions of use and the restrictions of use, if any.
4. **Part 4**: contains information relative to the characterisation of the disease/disorder or the medical condition, and of the target patients.
5. **Part 5**: contains information on the specific role of the food product in the dietary management of patients under each proposed use, and benefits over it is more convenient than the exclusive use of foodstuffs that are not FSMPs.
6. **Part 6**: contains information on the conditions and restrictions of use, referred to quantity and pattern of consumption, the route of administration, and the reasons for medical supervision.
7. All the information enclosed in the dossier, either provided, non applicable or absent, must be accompanied by the proper reasons, justification and data. Scientific data,

either published and unpublished, in favour and not in favour should be provided. Remarkably, the entire dossier may not be claimed as confidential. Therefore, specific parts considered as confidential by the party responsible for the dossier should be kept at minimum and must be verifiable by the Authority.

8. Each dossier should be exclusively prepared for each specific food for special medical purposes' product.

The full table of contents to provide for the FSMP evaluation according to EFSA guidance is provided in **Figure 21**.

Moreover, due to the relevance of the dossier content for the purposes of the present research project, the expected detailed information to enclose in the scientific dossier for the FSMP evaluation according to EFSA guidance is transcribed herein and further discussed in the following pages.

efsa European Food Safety Authority

Guidance for the assessment of FSMP

TABLE OF CONTENTS

Figure 21: General contents on the guidance for the assessment of an FSMP[392]

2.3.4.1. Part 1: Administrative and technical data

1.1. Comprehensive table of contents of the dossier

1.2. Identification form

1.3. Party responsible for the dossier

1.3.1. Company/organisation

1.3.2. Contact person

1.4. Specifications

1.4.1. Specific food product

1.4.2. Specify whether the dossier refers to:

- A ***nutritionally complete*** food product with a *standard nutrient* formulation which may constitute the *sole source of nourishment* for the persons for whom it is intended.
- A ***nutritionally complete*** food product with a *nutrient-adapted* formulation specific for a disease, disorder or medical condition which may constitute the *sole source of nourishment* for the persons for whom it is intended.
- A ***nutritionally incomplete*** food product with a standard formulation or a nutrient-adapted formulation specific for a disease, disorder or medical condition which is *not suitable* to be used *as the sole source of nourishment*.

1.4.3. Describe the proposed use(s) for the specific food product

For each proposed use, the target patient population, the disease/disorder or the medical condition, the standard conditions of use and, where applicable, the restrictions of use, should be identified and disclosed.

1.5. Confidential data

State whether the dossier includes confidential data. If it does, the related Part in the dossier with confidential data must be specified, stating section and page number, and verifiable justification/declaration for the confidentiality must be provided.

2.3.4.2. Part 2: Characterisation of the specific food product

In Part 2, the party responsible for the dossier discloses to EFSA:

2.1. Name and characteristics

Information to provide:

- The source and specifications of the food product (e.g. physical and chemical properties, composition, and where applicable microbiological constituents)
- The list of ingredients and their sources
- The energy and nutrient content of the product and the methodology used to assess these values
- Analytical methods to provide quantitative analysis of the energy and nutrient content
- Laboratory where the measurements have been performed to certificate data
- The particular quality system for control/documentation for data (e.g. GLP and ISO17025)
- Any relevant information on the special formulation of the product

2.2. Manufacturing process

Information to provide:

- The description of the manufacturing process
- The particular quality system for manufacturing the product (e.g. GMP). If confidentiality restrictions arise, at least a non-confidential summary of the manufacturing process should also be provided.
- Any relevant information on the special processing of the product

2.3. Stability information

Where applicable:

- A brief summary of the studies undertaken (e.g. conditions, batches and analytical procedures)
- The results of the stability studies
- Conclusions with respect to storage conditions and shelf-life

2.4. References

References and supporting documentation quoted under Part 2, copies/reprints of published data and/or full reports of unpublished data must be included and attached herein.

2.3.4.3. Part 3: Proposed use(s)

In Part 3, the party responsible for the dossier discloses to EFSA:

> The proposed use(s) for the specific food product is described in this section. In case of several proposed uses for the same food product, for each proposed use, the target patient population, the disease/disorder or the medical condition, the conditions of use and, where applicable, the restrictions of use should be described in this section.
>
> **Proposed use No.1:**
>
> **Proposed use No.2:**
>
> (…)
>
> *If more than one use is proposed for the specific food product, parts 4, 5 and 6 should be filled out for each proposed use.*

2.3.4.4. Part 4: Characterisation of the disease/disorder or the medical condition, and of the patients for whom the specific food product is intended

In Part 4, the party responsible for the dossier discloses to EFSA:

4.1. Diagnosis of the disease/disorder – description of the medical condition

For the disease/disorder or the medical condition for which the specific food product is intended, it should be specified whether it is a disease/disorder or a medical condition (by replying yes or not to the classification):

a) It is a **disease/disorder**, wherein for the purpose of the dossier and according to EFSA guidance document, "disease/disorder means a pathological process, acute or chronic, inherited or acquired, of known or unknown origin, having a characteristic set of signs and symptoms which are used for its diagnosis and the management of which requires nutritional intervention under medical supervision. In this guidance document, the terms disease and disorder are considered as synonymous and have the same meaning".

b) It is a **medical condition**, wherein for the purpose of the dossier and according to EFSA guidance document, "medical condition denotes any structural or functional alteration, either acute or chronic, which may result from one or more disease or disorders, the management of which requires nutritional intervention under medical supervision". Non limiting illustrative examples may include kidney failure due to viral hepatitis, dysphagia from cancer of the upper gastrointestinal tract, and the like.

In case the selected option is 'disease/disorder', additional scientific information on the well-defined, objective criteria widely accepted by the medical community and verifiable by a physician, should be provided

In case the selected option is 'medical condition', medical condition itself should be fully disclosed.

4.2. Characterisation of the patients for whom the specific food product is intended

This section should contain information on:

- The patients suffering from the disease/disorder or the medical condition. Specifically, it should be disclosed if the specific food product is intended for all patients or only for a particular subgroup of patients suffering from the disease/disorder or the medical

condition. In the former case, the characteristics of such patients (e.g. age, sex, stage of the disease, clinical condition) need to be identified.

- The reason(s) why it is impossible, impractical or unsafe for patients for whom the specific food product is intended to consume exclusively foodstuffs that are not FSMPs, and/or the reason(s) why such patients would have a nutritional or clinical disadvantage from consuming exclusively foodstuffs that are not FSMPs.

  Following this description, the dossier contains some boxes to select a "yes/no response", and to provide reasoned response, on the reason for these impossibilities: whether they are related to inability or reduced ability to chew and/or swallow, to digest, to absorb nutrients, to metabolise and/or utilise, to excrete nutrients, and whether the disease/disorder or medical condition is aggravated by the consumption of foodstuffs that are not FMSPs or leads to specific medically-determined nutrient requirements, to select from: energy, carbohydrates, fat/fatty acids, protein/aminoacids, vitamins, minerals, water/electrolytes, other substances. In case that other reason applies, the exact reason needs to be identified.

4.3. Impact of the disease/disorder or the medical condition on the nutritional status of the patients for whom the specific food product is intended

This section is intended to check (yes/no) and to further disclose if the disease/disorder or the medical condition may have an impact on the nutritional status of the patients for whom the specific food product is intended. In the case of positive responses, then complementary information should be provided, whether the disease/disorder or the medical condition leads to energy/protein malnutrition, excess/deficiency of essential amino acids, excess/deficiency of essential fatty acids, vitamin deficiency, excess/deficiency of minerals, or other impacts on nutritional status.

4.4. References

References, supporting documentation, published data, unpublished data, full reports and the like, quoted Part 3 should be provided here.

2.3.4.5. Part 5: Specific role of the food product in the dietary management of patients under the proposed use

Part 5 contains outstanding information for substantiating the dossier, which specifically relates to the role of the food product in the disease/disorder or medical conditions it is intended to be used. Particularly, in Part 5 the party responsible for the dossier discloses to EFSA:

a) The **rationale for the specific composition and/or formulation** of the food product in relation to the proposed use.

b) The **rationale on the use** of the specific food product in the dietary management of patients for whom the product is intended is necessary or why it is more practical or safer than the exclusive use of foodstuffs that are not FSMPs, and/or why it has a **nutritional or clinical advantage** for the patient.

c) Any available **human data** documenting the use of the specific food product for the dietary management of patients for whom it is intended.

d) **Guidelines/consensus papers** published by **scientific (medical) societies** addressing the dietary management of patients for whom the specific food product is intended.

e) Any other pertinent information supporting the role of the product in regards its use.

5.1. References

References, supporting documentation, published data, unpublished data, full reports and the like, quoted **Part 5** should be provided here.

Part 5 encloses the concept "*any available **human data** documenting the use of the specific food product for the dietary management of patients for whom it is intended*". In this regard, the specific data that is needed to properly classify and place a product on the market under the Food for special medical purposes' category should be considered on a case-by-case analysis, as disclosed in the "Commission Notice on the classification of Food for Special Medical Purposes (C/2017/7716)"[391] Data must demonstrate the fulfilment of the product in regards the whole definition of FSMP, i.*e*., that the specific target patients suffering from a disease/disorder/medical condition have nutritional needs that are impossible, impractical, unsafe or nutritionally/clinically disadvantageous to satisfy through the exclusive consumption of food other than FSMP.

Reference is made to the term 'human data', which clearly emphasizes the origin of the expected data coming from human subjects in the dossier, by differentiating this data from any other data acquired through *in vitro* or *in vivo* sources, such as any other experimental model of the target disease.

2.3.4.6. Part 6: Conditions and restrictions of use

In Part 6, the party responsible for the dossier discloses to EFSA:

6.1. Conditions of use

a) Standard quantity and pattern of consumption

b) Specify whether the food product is intended to be used as the sole source of nourishment, a partial replacement of other dietary sources, a supplement/integration to the patient's diet

c) Route of administration: oral nutrition, tube feeding: in the stomach or in the jejunum

d) Directions for preparation and/or use.

e) The reasons why the use of the specific food product requires medical supervision

6.2. Restrictions of use

Where appropriate, a statement addressed to those individuals who should **avoid** using the food product proposed as FSMP, as well as the rationale.

2.4. Concluding remarks

For the purposes of the present research, and in the light of the above-mentioned regulatory information, the formulated product containing methylxanthines as main ingredients should fall under the food for special medical purposes classification, since according to its regulatory definition,[389] 'food for special medical purposes' means food specially processed or formulated and intended for the dietary management of patients, including infants, under medical supervision; it is intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary food or certain nutrients contained therein, or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved by modification of the normal diet alone".

In this regard, the food product should be considered as a '*nutritionally incomplete* food *product with a standard formulation or a nutrient-adapted formulation specific for a disease, disorder or medical condition which is not suitable to be used as the sole source of nourishment'.*

Last but not least, in terms of scientific outstanding information for substantiating the EFSA dossier, and more specifically, in terms of the role of the food product in the disease it is intended to be used (*i.e.* myotonic dystrophy type 1, DM1), human data documenting the use of the food product in the nutritional management of patients for whom it is intended should be provided. There are several FSMP to manage prevalent diseases such as oncology, stroke, critical care, frailty, epilepsy, and the like.[393] However, to the best of our knowledge, only very scarce examples of food for special medical purposes have been developed and marketed to manage certain aspects of rare diseases, such as in Alzheimer's disease,[394] whereas a decaffeinated green tea extract from *Camellia sinensis* is being marketed as a food supplement used in Down syndrome.[395,396]

Therefore, our group attempted this human data collection by means of an observational clinical study evaluating dietary methylxanthines intake habits and their potential relationship with quality of life (reference is made to Chapter 4), as well as a pilot interventional clinical trial evaluating the effect of the properly formulated composition containing methylxanthines on the quality of life, fatigue, and hypersomnia in adult patients with DM1, as a novel nutritional management strategy for DM1 (reference is made to Chapter 5), once the formulated product would had been properly developed (reference is made to Chapter 3).

CHAPTER 3: PRODUCT DEVELOPMENT

Chapter 3 discloses the development of the product, from the definition of its composition through *in vitro* and *in vivo* studies on DM1 models, to the definition of the dosage and the development of dosage form. Intellectual property issues, such as patent protection status and freedom to operate studies are also evaluated throughout this Chapter. The present Chapter addresses the specific goals nr 3 and 4.

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This project received funding by FEDER grant IDI-20151100 (CDTI-PID).

3.1. Composition

3.1.1. *Screening* of individual xanthines

In order to accomplish the first goal of the present research project, which was aimed at deepening in the effect of xanthines and methylxanthines (either individually or in combination) in DM1, the work was initiated by selecting the test items, as well as preparing suitable DMSO stock dissolutions for the *in vitro* and *in vivo* testing.

Accordingly, natural xanthines as well as certain synthetic xanthines and dimeric xanthines previously synthesized in our group, were selected as starting points in the screening cascade disclosed herein:

Table 27: Methylxanthines selection

Compound code	Compound name	CAS nr
0 or caff	Caffeine	[58-08-2]
1	Theophylline	[58-55-9]
2	Theobromine	[83-67-0]
3	Aminophylline	[317-34-0]
4	Xanthine	[69-89-6]
5	Hypoxanthine	[68-94-0]
6	Paraxanthine (1,7-Dimethylxanthine)	[611-59-6]
7	3-Isobutyl-1-methylxanthine (IBMX)	[28822-58-4]
8	3-Methylxanthine	[1076-22-8]
9	3-Ethyl-1-propylxanthine	[135462-23-6]
10	3-Allyl-1-ethyl-8-hydroxyxanthine	[194802-32-9]
11	3,8-Dimethyl-2-thioxanthine	[91725-06-3]
12	1-Ethyl-3-isobutylxanthine	[96654-24-9]
17	7,7'-(Heptane-1,7-diyl)bis(1,3-dimethyl-1H-purine-2,6(3H,7H)-dione)[iii]	[156234-12-7]
18	7,7'-(Nonane-1,9-diyl)bis(1,3-dimethyl-1H-purine-2,6(3H,7H)-dione)[iv]	[156234-13-8]
19	7,7'-((Propane-1,3-diylbis(oxy))bis(ethane-2,1-diyl))bis(1,3-dimethyl-1H-purine-2,6(3H,7H)-dione)[v]	[1927611-32-2]
20	7,7'-((Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))bis(1,3-dimethyl-1H-purine-2,6(3H,7H)-dione)[vi]	[1927611-33-3]
21	*N,N'*-(propane-1,3-diyl)bis(2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)acetamide)[vii]	[1927611-36-6]

Pentamidine, an antiparasitic agent used in DM1 research due to its disrupting properties on MBNL1-CUG repeats, was used as positive control.

[iii] Example 1 in WO 2016075285 A1 (reference [170])
[iv] Example 2 in WO 2016075285 A1 (reference [170])
[v] Example 4 in WO 2016075285 A1 (reference [170])
[vi] Example 5 in WO 2016075285 A1 (reference [170])
[vii] Example 6 in WO 2016075285 A1 (reference [170])

The missing codes for compounds 13, 14, 15 and 16, correspond to four additional compounds, chemically different than the xanthines scaffold, evaluated in different research projects running in parallel at that time in our lab. They were used herein for comparison purposes. Due to confidentiality reasons, neither the exact identity of these compounds nor experimental data on the compounds is disclosed herein.

The screening cascade was designed as a sequential evaluation of *in vitro* and *in vivo* events to assess the effect of the selected xanthines *per se* or properly formulated in validated models of the disease, accessed through a kind collaboration with Professor R. Artero, leader of the Translational Genomics Group, at the University of Valencia.

The main assays and workflow of the screening cascade are shown in **Figure 22** and further discussed in the following sections of the present Chapter.

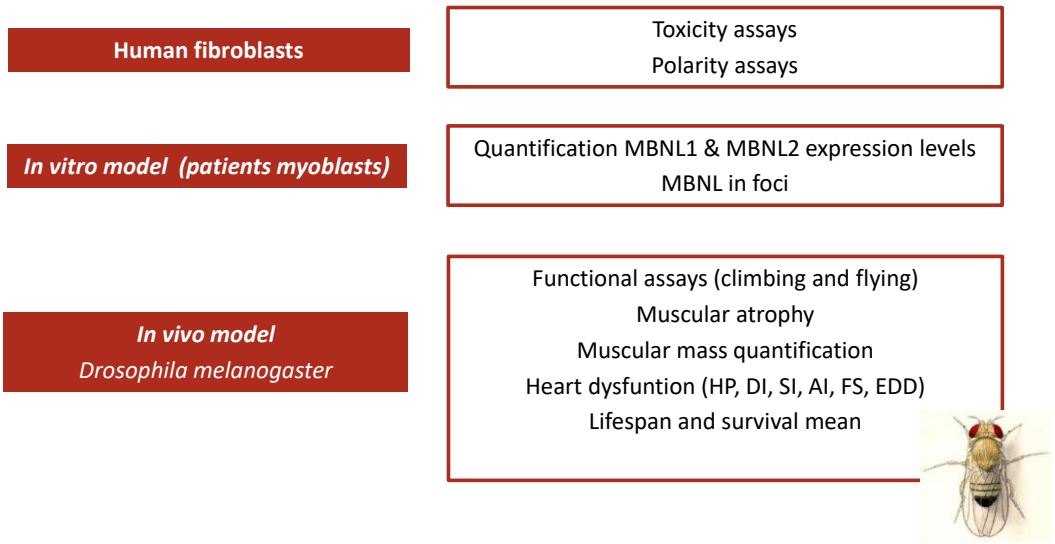


Figure 22: Workflow of the screening cascade in the DM1 models

3.1.1.1. In vitro models of DM1

3.1.1.1.1. Toxicity assays

To assess the toxicity of compounds in culture cells, several concentrations of compounds (from 0.1 to 100 µM) were added to standard media in DM1 fibroblasts. Survival was studied using the CellTiter 96 Aqueous Non-Radioactive cell proliferation assay protocol, a colorimetric assay that determines viable cells. The results obtained for compounds from **Table 27** are shown in **Figure 23**.

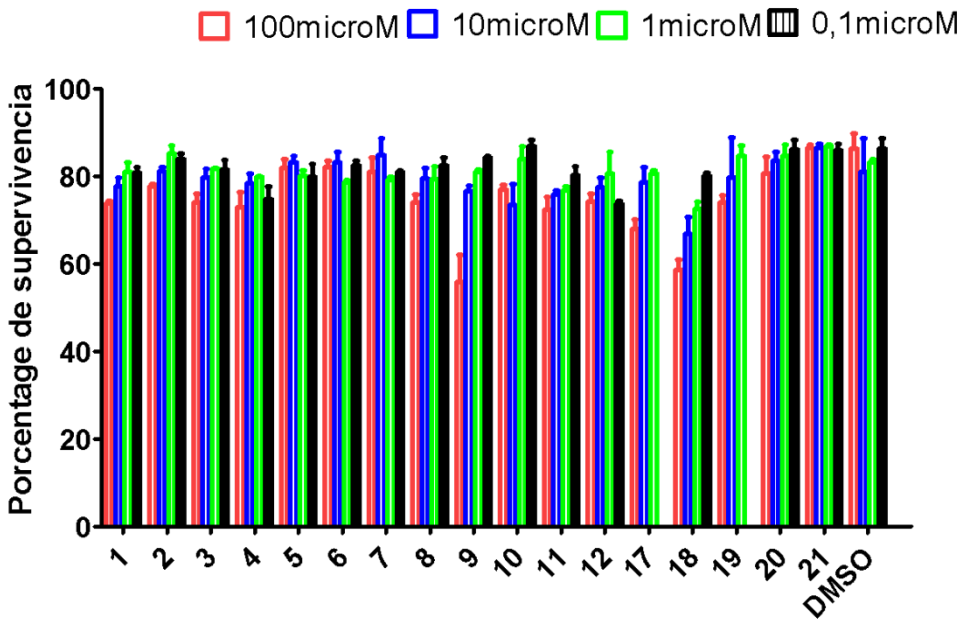


Figure 23: Percentage of survival of DM1 fibroblasts after administration of different concentrations of compounds in the media
The values of survival are normalized to non-treated DM1 fibroblasts which have 100% of survival. However, in all cases, the compounds were dissolved in DMSO. Then, the cells treated only with DMSO are considered the most appropriate controls for comparison. Bar graphs show mean ± SEM from three independent experiments. Missing values for compounds 17 and 19 at 0.1 µM due to an experimental error. DMSO was prepared at 100 µM.

All the compounds screened rendered a percentage of survival above 50% in comparison to non-treated cells, and above 75% in comparison to DMSO treated cells. Accordingly, as their toxicity was very low, the screening cascade continued using all the concentrations in the next assays.

3.1.1.1.2. Polarity assay: binding to CUG repeats (measurements of relative polarity)

In order to define whether the selected compounds had the ability to bind to CUG repeats, fluorescence polarization assays were performed. In these tests, when polarized light excites a fluorophore conjugated to a small molecule, it undergoes rotational diffusion faster than the time required for light emission occurs, resulting in a random arrangement of the molecule in the fluorescence emission time (depolarization). However, the rotation of the molecule becomes slower depending on the viscosity of the medium or the molecular volume, increasing the polarization of the emitted light. Thus, by measuring changes in polarization of RNA bearing 23 CUG repeats and conjugated to the fluorophore carboxyfluorescein, FAM (CUG) 23, it can be assessed whether a candidate molecule binds to the RNA. In this test pentamidine, a compound previously known to attach to the CUG repeats, was used as a positive control, and the solvent DMSO was used as negative control.

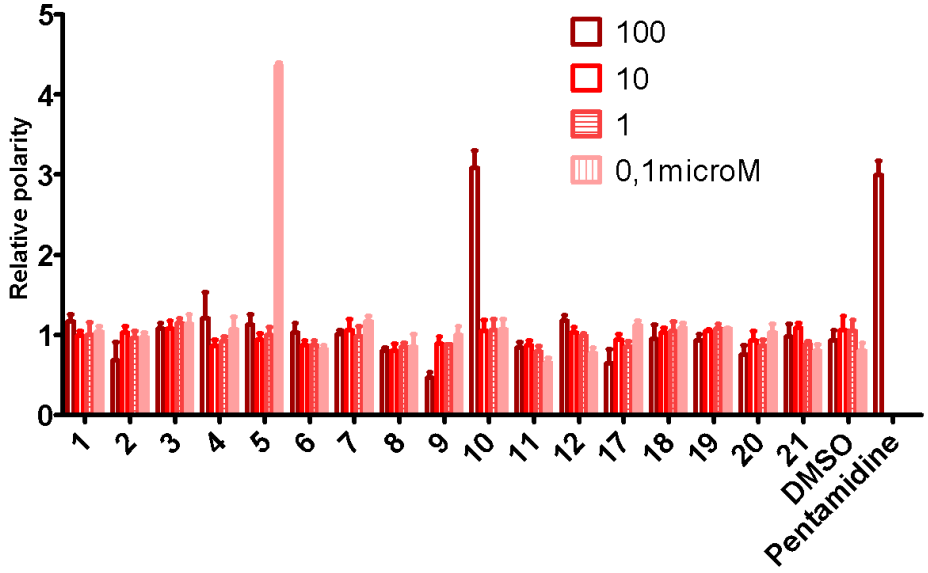


Figure 24: Polarization measurements relative to the values of polarization of the FAM (CUG) 23 probe without compound
Each compound shown in the x-axis was tested at increasing concentrations (from 0.1 to 100 µM). Higher polarization values indicate greater binding capacity to the CUG repeats. Bar graphs show mean ± SEM from three independent experiments, each one with three technical replicates. DMSO was prepared at different concentrations diluted in water.

As illustrated in **Figure 24**, in general terms, the selected xanthines didn't exhibit the capacity to bind to the CUG repeats, with the exception of hypoxanthine (compound 5) and 3-allyl-1-ethyl-8-hydroxyxanthine (compound 10), which seemed to have some affinity for FAM (CUG) 23. In order to confirm the obtained results, experiments were repeated using a FAM (CUG) 10 probe, a shorter RNA which was easier to polarize due to its smaller size (**Figure 25**). However, none of the compounds showed any significant affinity for the RNA repeats, suggesting that the tested xanthines did not promote their effect in DM1 through binding to the CUG repetitions.

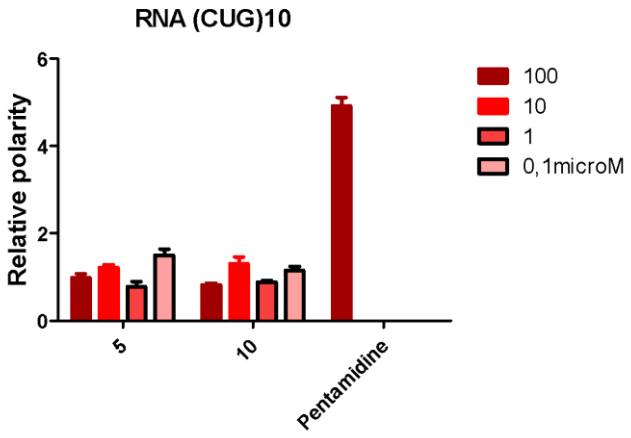


Figure 25: Polarization measurements relative to the values of polarization of the FAM (CUG) 10 probe without compound
Each compound shown in the x-axis was tested at increasing concentrations (from 0.1 to 100 µM). Higher polarization values indicate greater binding capacity to the CUG repeats. Bar graphs show mean ± SEM from three independent experiments, each one with three technical replicates.

3.1.1.1.3. Foci quantification in DM1 fibroblasts

The quantification of the number of foci in fibroblasts was performed in order to check if the tested compounds had the capacity to reduce these protein aggregates, since the state of the art suggests that the dissolution of these aggregates allows the release of MBNL and the recovery of its natural functions, reversing the symptoms associated with the disease.

DM1 fibroblasts were seeded in a 94-well plate (8000 cell/per well) in standard media one day before adding the compounds to the media. Compounds were added to the cells at different concentrations, always maintaining the concentration of the solvent (DMSO) at 1% in the media. Each concentration was tested in three different wells. Then, 24h after adding the compounds the cells were fixed with paraformaldehyde 4% in PBS and FISH. The detection of CUG RNA was performed using a (CAG)23 probe labelled with Cy3. Cells were counterstained with Hoechst to detect the nuclei. Incell 200 plate reader confocal was used for detection and quantification of the parameters selected and the data was analyzed using Graphpad software for statistical comparison of results, as shown in **Figure 26**.

The main conclusion from this experiment was that the tested compounds had a small effect on the number of foci. In general, most of the compounds induce a decrease in the number of foci, although some compounds (as compounds 11, 12, 20 and 21) tend to increase it.

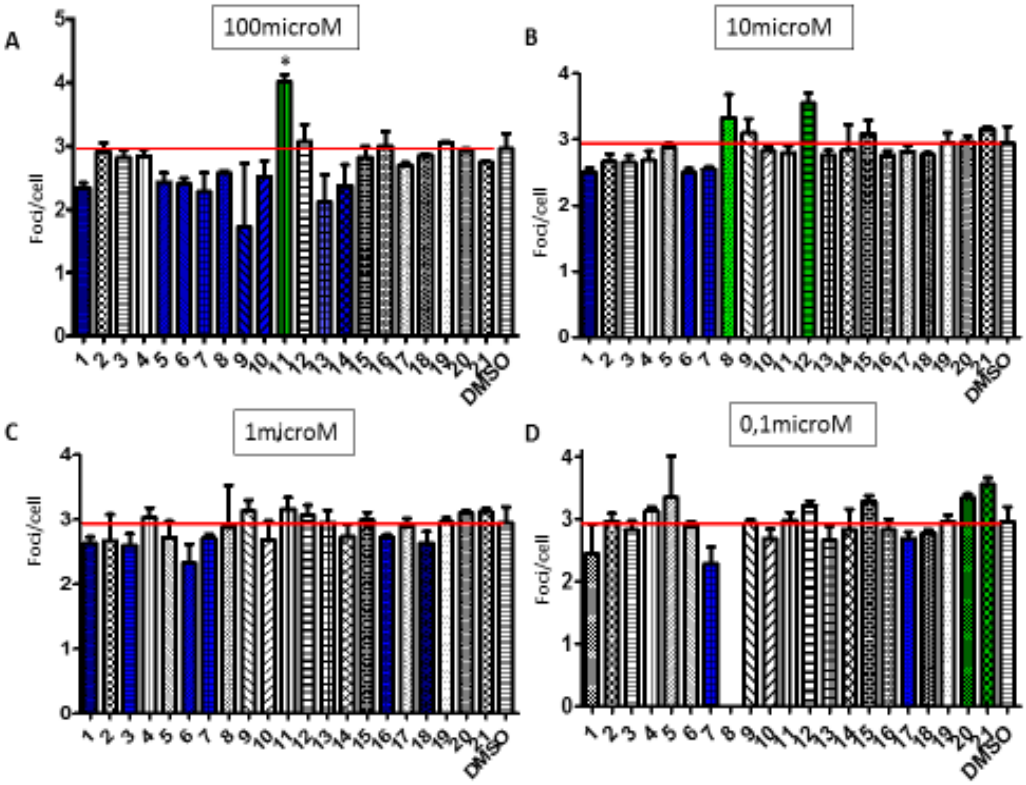


Figure 26: Effect of compounds on foci number per cell in DM1 fibroblasts
Bar graphs show the mean ± SEM foci number per nuclei in DM1 fibroblasts that had been treated with different concentrations of compounds (from 0.1 µM to 100 µM) or solvent (DMSO) during one day. Horizontal red line establishes the reference value of control cells treated only with DMSO. In green, compounds with a tendency to increase the number of foci per cell are remarked, and in blue, compounds with tendency to reduce foci number. Although some compounds such as compound 7 showed a reduction of foci even at lowest concentration, this reduction was not statistically significant. The only remarkable effect was the increase of foci achieved by compound 11 at the maximum concentration which is statistically significant. *p<0.05 (t-test). Missing values for compound 8 at 0.1 µM due to an experimental error.

3.1.1.1.4. Quantification of MBNL1 expression and localization

Current therapeutic strategies are based on candidate drugs that bind to CUG or CCUG repeats, thereby releasing MBNL proteins to regulate splicing, or other processes, of its pre-mRNA targets. An increase in free MBNL1 protein has been shown to reduce the severity of symptoms in animal models of DM1, while in contrast, a decrease of free MBNL1 protein is observed with longer expansions, which are correlated with more severe disease. The pathogenic role of the *MBNL1* gene is further substantiated by the fact that MBNL1 gene variants modify DM1 severity.[397] Similarly, MBNL proteins have been shown to get sequestered in DM2, thus, strategies aimed at increasing steady state levels of these proteins have the potential to become treatments for DM2. Then, the consensus strategy to reverse splicing abnormalities observed in DM1 and DM2, among other molecular alterations, is based in a reduction in nuclear foci and the concomitant reduction in nuclear sequestration of MBNL proteins.[199,398]

MBNL1 measures were performed to quantify the total protein amount, either in its free form or sequestered in MBNL foci, since the state of the art suggests that the increase in MBNL, through its release from foci or from an induced overexpression of the protein, has therapeutic properties in animal models of DM1. Therefore, the quantification of total MBNL1 protein in the nucleus was considered the best key performance indicator for the screening and ranking of the candidate compounds.

In order to facilitate the comparison, results were normalized in front of their control, the solvent DMSO, whose value was set at zero. **Figure 27** illustrates these results.

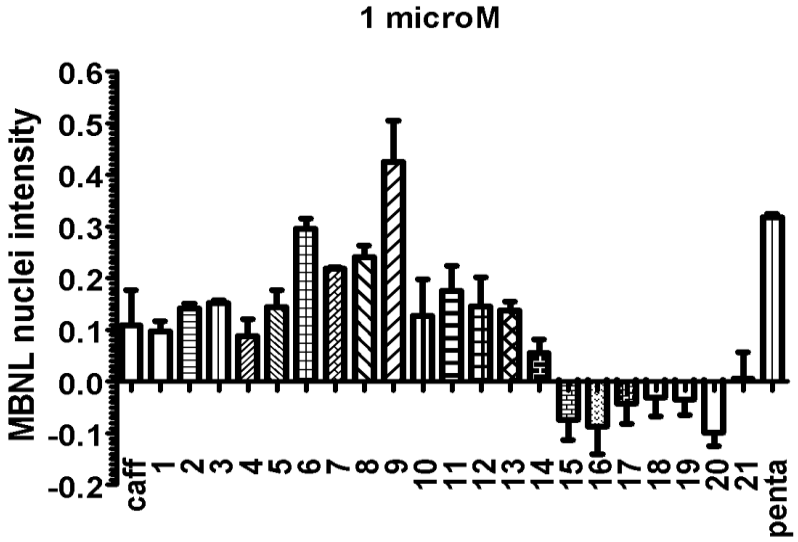


Figure 27: MBNL nuclei intensity normalized to DMSO of all compounds tested
Bar graphs show the mean ± SEM of MBNL intensity in nuclei of DM1 myoblasts treated with 1 µM concentration of different compounds. Pentamidine is also shown as positive control.

Compound 9 (synthetic xanthine) rendered the most intense response, followed by compound 6 (natural xanthine), at similar levels than the positive control pentamidine. It is worth mentioning that the dimeric methylxanthines (compounds 17 to 21) exhibited a completely different behaviour under this assay than the monomeric xanthines.

3.1.1.2. In vivo models of DM1. Functional assays

Model flies expressing 480 interrupted CUG repeats under the muscle-specific driver Myosin-heavy chain (MHC)-Gal4 exhibit important functional deficits. Particularly, these DM1 model flies displayed a 10% reduction in climbing velocity and a drastic 80% reduction of flying ability measured as landing distance. Therefore, they are reliable models for measuring the locomotor function and atrophy in DM1.

To test the effect of xanthines on the rescue of these phenotypes, MHC-Gal4 and UAS-(CUG)480 flies were crossed in bottles with nutritive media containing the compounds, so the F1 (first offspring) was in contact with the compound throughout development. As control of disease non-treated flies, the same flies were crossed in standard nutritive media and as

control of non-disease flies, MHC-Gal4 flies were crossed with the yellow white flies (yw), genetic background, in standard nutritive media.

3.1.1.2.1. DM1 functional phenotypes: climbing assay. Test of caffeine effect.

To assess climbing velocity, the flies were transferred, after emerging, to tubes with 1.7, 1 and 0.1 µM caffeine or the solvent DMSO 1% in standard nutritive media. Groups of ten 5-day-old males were transferred into vials of 1.5 cm in diameter and 25 cm in height, after a period of 24 hours without anaesthesia. The height reached from the bottom of the vial by each fly in a period of 10 s was recorded with a camera. For each genotype, approximately 30 flies were tested.[399] The results show the mean speed in mm/s. Student's t-test was used to assess the statistical difference between the two groups of flies, fed with or without compound (**Figure 28**).

According to the results obtained through this experiment, the flies expressing 250 CUG repeats in muscle have decreased climbing ability in comparison to control flies. Treatment with caffeine at concentrations as low as 0.1 µM dissolved in standard food significantly improved climbing performance. Importantly, the climbing velocity of control flies without CUG repeats (CONT, control) was not significantly altered by caffeine.

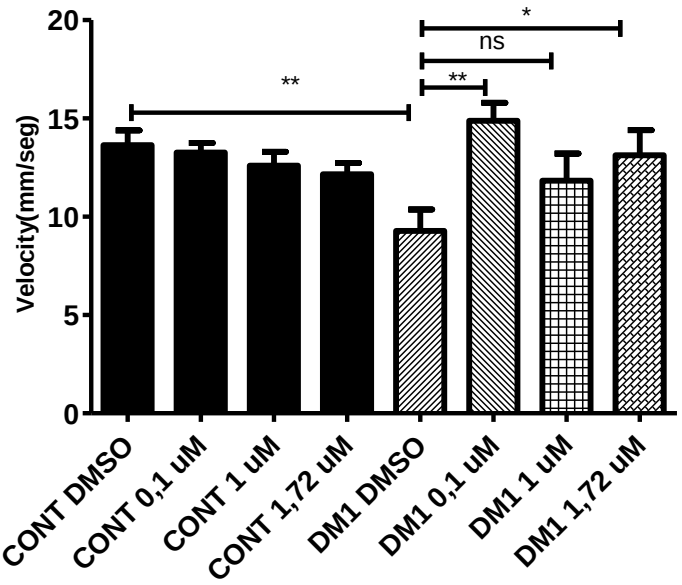


Figure 28: Functional climbing assay to assess the effect of caffeine on locomotor performance in DM1 model flies
Bars show mean ± SEM of flies fed with standard nutritive media with DMSO or caffeine to a final concentration of 1.72 µM. Two different genotypes are represented; CONT are control flies without repeats and DM1 are flies expressing 250 CUG repeats under the muscle specific driver MHC-Gal4. ns, not significant, * p<0.05, ** p<0.001 (Student's t test).

3.1.1.2.2. DM1 functional phenotypes: flying assay. Test of caffeine effect.

To assess the effect of caffeine on flying performance, after emerging, the flies were transferred to tubes with 1.7, 1 and 0.1 µM caffeine or the solvent DMSO 1% in standard nutritive media. Flying assays were performed on day 5, using 50 male flies per group. Landing distance was compared between the two groups using Student's t-test and considered significantly different if $p<0.05$, as previously discussed in the Methodology section (**Figure 29**).

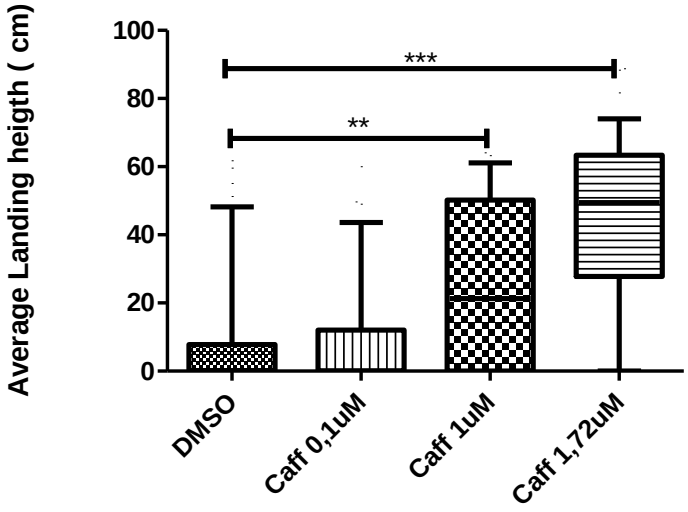


Figure 29: Notched box plot showing the median and the distribution of average landing height data obtained in the flight assay with the relevant genotypes
Flying disability observed in DM1 model flies is rescued by caffeine treatment at 1 and 1.72 µM. The horizontal lines inside the boxes represent median values, whereas bottom and top edges of the boxes represent the 25th and 75th percentiles and bottom and top whiskers reach the 10th and 90th percentiles, respectively. * p<0.05, ** p<0.001 (Student's t-test).

The functional tests in the fly model allow the assessment of muscle atrophy rescue achieved by a compound. In order to improve results in these functional tests, an increase of muscle mass, strength and coordination are required. The flight movement demands more complex coordination among different muscle types. In this regard, the rescue of climbing required lower doses of compounds. Accordingly, flight ability of DM1 flies was rescued by caffeine at 1 µM and 1.72 µM in a dose dependent manner. However, low doses of caffeine (0.1 µM), which were able to rescue climbing, did not show an effect on the flight capability improvement.

3.1.1.3. Selection of individual xanthines

Results from the MBNL1 normalized quantification were evaluated by considering two different sets of compounds: caffeine *per se* and the other xanthines, independently.

Briefly, the conclusions drawn from the previous *in vitro* data shown herein were that caffeine exhibited an increase in the intensity of MBNL in the nucleus and also in foci, which is consistent with a general increase in MBNL beyond the merely release from foci. This increase

in MBNL was enough to induce a functional improvement at the flight and climbing level in DM1 animal models (according to *in vivo* functional assays previously disclosed).

The analysis of results from the other xanthines confirmed that none of them showed binding to repeats. Although some of them showed some *in vitro* toxicity for cells, even at the highest concentration tested, survival was greater than 50%.

The most active compounds were deemed to be paraxanthine (compound 6), 3-isobutyl-1-methylxanthine (compound 7), 3-methylxanthine (compound 8), and 3-ethyl-1-propylxanthine (compound 9). However, according to the in force regulatory information that was simultaneously being reviewed at that time (reference is made to Chapter 2 data), none of these compounds *per se* (neither compound 6, 7, 8 or 9) were allowed as ingredients in the forthcoming food product composition. However, on the contrary than compounds 7 and 9, compounds 6 and 8 were caffeine metabolites that were worth exploring.

Finally, based on the experimental results filtered according to the natural origin of compounds, the six most active selected individual compounds to conduct further experiments were: caffeine (caff), theobromine (compound 2), xanthine (compound 4), hypoxanthine (compound 5), paraxanthine (compound 6) and 3-methylxanthine (compound 8). These were the individual products progressing to the next phase.

3.1.2. *Screening* of a combination of xanthines. Part 1

3.1.2.1. Design and formulation of combination of xanthines

Aimed at the identification of further synergistic effects, we proceeded to design and prepare binary mixtures containing the six selected xanthines in three molar stoichiometry ratios. The considered combined preparations are schematically represented in **Table 28**:

Table 28: Design of binary mixtures from the individually selected xanthines

Compound	Stoichiometry	Theobromine	Paraxanthine	3-MX	Hypoxanthine	Xanthine
Caffeine	1:1	✓	✓	✓	✓	✓
Theobromine	1:1	-	✓	✓	✓	✓
Paraxanthine	1:1	-	-	✓	✓	✓
3-MX	1:1	-	-	-	✓	✓
Hypoxanthine	1:1	-	-	-	-	✓

Compound	Stoichiometry	Theobromine	Paraxanthine	3-MX	Hypoxanthine	Xanthine
Caffeine	2:1	✓	✓	✓	✓	✓
Theobromine	2:1	-	✓	✓	✓	✓
Paraxanthine	2:1	-	-	✓	✓	✓
3-MX	2:1	-	-	-	✓	✓
Hypoxanthine	2:1	-	-	-	-	✓

Compound	Stoichiometry	Theobromine	Paraxanthine	3-MX	Hypoxanthine	Xanthine
Caffeine	1:2	✓	✓	✓	✓	✓
Theobromine	1:2	-	✓	✓	✓	✓
Paraxanthine	1:2	-	-	✓	✓	✓
3-MX	1:2	-	-	-	✓	✓
Hypoxanthine	1:2	-	-	-	-	✓

Regarding ternary mixtures, the fact that potentially there were hundreds of combinations to explore, drove us to impose certain restrictions. Accordingly, in a first step, preferred mixtures containing at least caffeine were defined.

A set of forty-five binary mixtures (samples codified as B) and ten ternary mixtures (samples codified as T) were prepared at the corresponding stoichiometry among components, in 1 µM concentration. For confidentiality reasons, the exact composition of the binary and ternary mixtures is left blinded.

The more relevant experimental results are shown in the forthcoming pages.

3.1.2.2. In vitro models of DM1

3.1.2.2.1. Quantification of MBNL expression intensity in combination mixtures of xanthines

Immortalized transdifferentiated DM1 fibroblasts were seeded in a 94-well plate (8000 cell/per well) in standard media. To achieve the differentiation of the fibroblast to myoblasts, MyoD expression was induced by changing the standard media to differentiation media with Doxocycline and without serum. 24h after induction, the compounds were added to the cells at an approximate concentration of 1 µM in 10% DMSO. Additions of 10% DMSO and pentamidine 100 µM in 10% DMSO were used as negative and positive controls, respectively. Every compound was tested in three different wells. 48h after adding the compounds the cells were fixed with paraformaldehyde 4% in PBS and MBNL detection using monoclonal anti-MBNL1 antibody was performed counterstaining with Hoechst to detect the nuclei. Incell 200 plate reader confocal was used for detection and quantification of MBNL intensity in nuclei and the data was analyzed using Graphpad software for statistical comparison of results. **Figure 30** and **Figure 31** (normalized) illustrate the obtained results.

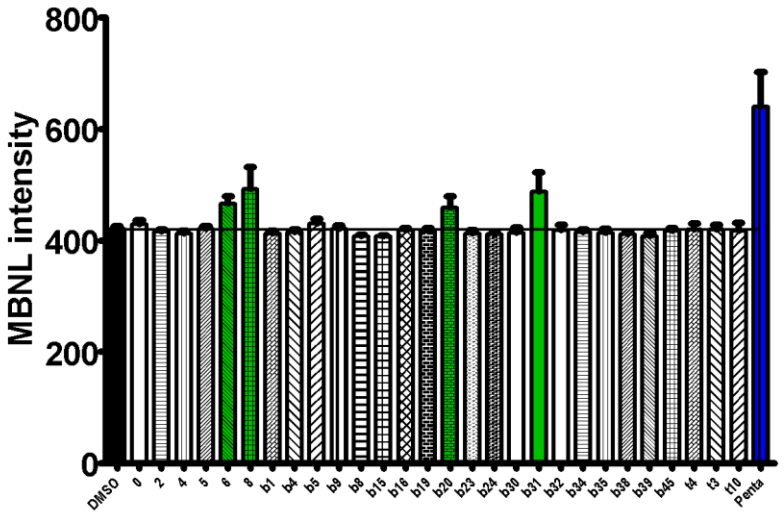


Figure 30: Quantification of MBNL expression intensity in nuclei
Bar graphs show the mean ± SEM of MBNL staining intensity in nuclei in DM1 myoblasts that had been treated with different compounds or solvent (DMSO) during two days. Horizontal line shows the reference value of control cells treated only with DMSO. In

green, compounds with a tendency to increase MBNL intensity in nuclei, in blue pentamidine and in black DMSO. *p<0.05, **p<0.01 (Student´s t –test to assess the significant differences between cells treated with a compound and cells treated with DMSO).

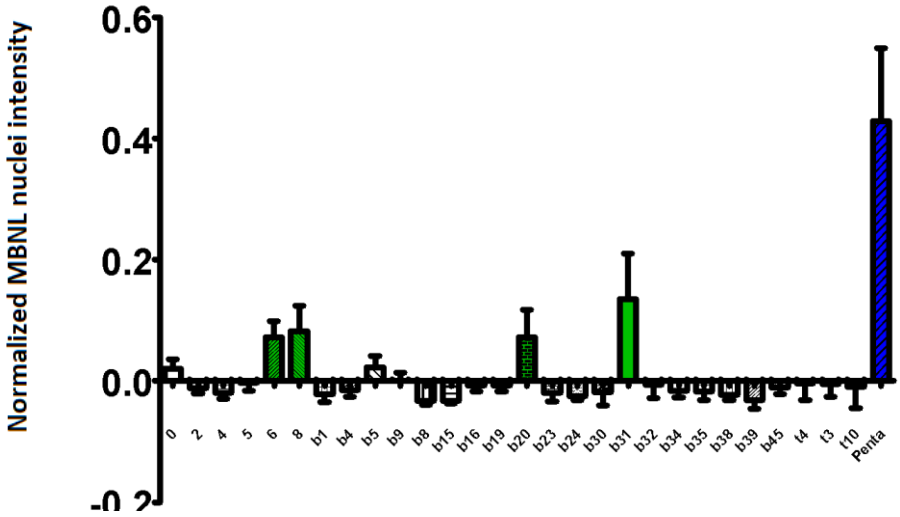


Figure 31: MBNL Intensity normalized to DMSO
DMSO values were taken as background and subtracted from the compound's data to allow easier visualization of differences. Compounds in green in this graph are the same as the compound in green in the previous figure.

According to the normalized data shown in **Figure 31**, individual xanthines (compound 6) and (compound 8), as well as binary sample B20, but especially binary sample B31 furnished the highest MBNL intensity in nuclei. In the case of compounds 6, 8 and binary sample B31 the difference with the levels of MBNL intensity measured in cells treated with DMSO were statistically significant.

It should be remarked that pentamidine was tested at 100 µM, according to the standard methodology used in the Translational Genomics Group, whereas xanthines were tested at 1 µM. Therefore, in this experiment, pentamidine should be understood as a mere positive control, since a quantitative comparison between the values obtained from pentamidine and xanthines values are not possible due to the differences in two orders of magnitude in their concentrations.

Regarding the most prominent result, binary sample B31, it was prepared as a composition comprising caffeine and theobromine in a 1:2 molar stoichiometry.

In order to confirm this result, the same composition was prepared and retested in a new experiment, in front of caffeine and theobromine (compound 2). In this experiment, paraxanthine (compound 6) instead of pentamidine was tested at the same concentration as samples and used as positive control, for the reasons previously disclosed.

As shown in **Figure 32**, combination of caffeine and theobromine in 1:2 molar stoichiometry promoted an increase in the MBNL1 levels compared to the baseline levels obtained from individual compounds, showing thereby a clear synergic effect, *i.e.* certain compositions

containing caffeine and theobromine significantly increased the rescue of MBNL in comparison to the individual compounds.

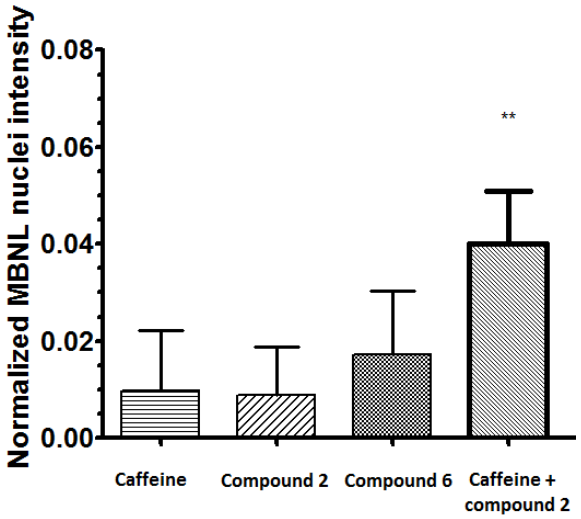


Figure 32: Quantification of MBNL expression intensity in nuclei, normalized to DMSO
DMSO value was taken as background and subtracted from the compounds. Data bar graphs correspond to the mean ± SEM of MBNL staining intensity in nuclei in DM1 myoblasts treated with the compounds shown or its combination. Individual samples were tested at 1 µM whereas the combination was tested at 1 µM caffeine: 2 µM theobromine, during two days. * Indicates p-value<0.05, ** indicates p-value<0.01, *** indicates p-value<0.001 (Student's t –test to assess the significant differences between cells treated with a compound and cells treated with DMSO).

Several combinations of caffeine and theobromine at different concentrations and proportions between both compounds were evaluated (data not shown). Surprisingly, different effects were obtained for the different compositions. However, the composition containing caffeine and theobromine in 1:2 molar stoichiometries was identified as the optimum formulation between caffeine and theobromine for maximizing the amount of MBNL1 (**Figure 32**).

3.1.2.2.2. MBNL1 detection in DM1 myoblasts by immunocytochemistry

To assess the effect on MBNL levels and distribution, immunocytochemistry was used to detect MBNL1 in DM1 myoblasts and MBNL1 nuclear intensity, by means of the images taken with Incell, including caffeine as a positive control.

As shown in **Figure 33**, MBNL1 (in green) was found dispersed in nuclei and cytoplasm in control myoblasts and retained in ribonuclear foci in DM1 myoblasts. Treatment of DM1 cells with caffeine (labelled as 'caff'), but specially, the formulated composition comprising caffeine and theobromine in 1:2 molar stoichiometry ratios (labelled as MYO-DM) significantly increased the presence of dispersed MBNL in cytoplasm and nuclei in DM1 myoblasts.

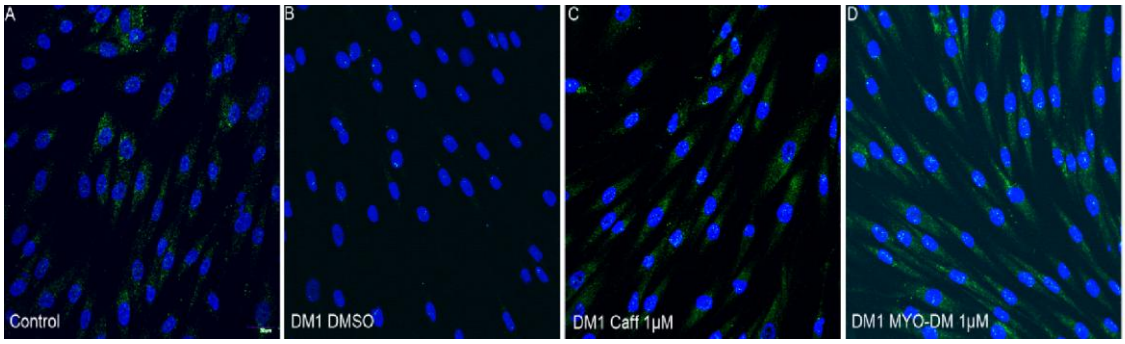


Figure 33: The formulated composition comprising caffeine and theobromine in 1:2 molar stoichiometry ratio significantly increased free MBNL1 in DM1 myoblasts
Representative immunofluorescent images of control (A) and DM1 myoblasts grown in DMSO (B), caffeine 1 µM (C, caff) and caffeine: theobromine 1:2 molar ratio, 1 µM caffeine: 2 µM theobromine (D).

3.1.2.3. Selection of combination of methylxanthines

Therefore, in the light of the confirmation of the synergistic effect between caffeine and theobromine, as well as the regulatory information from Chapter 2, the present research moved forward with the composition containing caffeine and theobromine in 1:2 molar stoichiometry ratios, which was equivalent to 1:1.85 weight/weight relationships. Along with the text and figures, the above-mentioned composition is named MYODM or MYO-DM as an internal code.

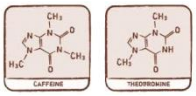
3.2. Dosage

3.2.1. Introduction and goals

A key issue in the progress of the present research was to translate the results obtained from *in vitro* models (wherein individual samples had been prepared at 1 µM concentration, and the most active combination contained caffeine and theobromine at 1 µM and 2 µM concentration, respectively), to the dose units required in the *in vivo* functional assays that used *Drosophila melanogaster* as DM1 model. Accordingly, the proper metrics should be defined.

Moreover, in a further step forward, even a more relevant and strategic decision to face was the dose interspecies translation, from the *Drosophila* model to the human species, while strictly fulfilling the stoichiometry of the active samples, the regulatory constraints on permitted daily doses per each different ingredient, their permitted regulated form as part of plants or other substances, and last but not least, considering the pharmaceutical dosage form in which the ingredients would be formulated, preferably as oral dosage forms.

In this regard, the main challenge to face at this point of the research was how to join all the pieces of the puzzle, with at least the following incoming data illustrated in **Figure 34**.



Sample B31 composition:
Molar stoichiometry: caffeine:theobromine 1 µM:2 µM
Weight/weight ratio: caffeine:theobromine 1:1.85 (w/w)
Source: Chapter 3

Caffeine: limited to maximum 80 mg/day (total content)
Equivalence to a maximum 1.33 mg/kg/day (human, 60 Kg)
Source: Chapter 2, Chapter 3, FDA guideline on MRSD in FIH

Theobroma cacao from cocoa bean powder:
Theobromine content: 7-12% (w/w)
Caffeine content: 0.18-0.20% (w/w)
Source: Chapter 3

Body reference weight (median): 0.27 mg
Flying assay: statistically significant at 1 µM
Feed amount: 20 mL (twice, from larvae to adult)
Almost 65% feed not eaten by adult flies
Cohort in functional assays: 30-50 flies
Source: Translational Genomics Group, UVEG

Body reference weight (median): 60 kg
Source: FDA guideline on MRSD in FIH[400]

Safe daily caffeine intakes up to 400 mg per day (5.7 mg/kg/day for a 70-kg healthy adult)
Source: EFSA's Scientific Opinion on the safety of caffeine[300]

Soft capsules as oral dosage forms:
Size # 0 (expected maximum capacity 340-400 mg)
Size # 00 and 000 rejected due to potential swallowing difficulties
Size # 1 and 2 rejected due to low charge capacity
Source: Chapter 3

Figure 34: Incoming data and regulatory constraints to consider along the product development stage

3.2.2. From animal models to humans: Human Equivalent Dose (HED)

3.2.2.1. Algorithm for the animal to human data conversion

The current effective version of the FDA Guideline for estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers,[400] discloses an algorithm for deriving the maximum recommended starting dose (MRSD) for the first-in-human clinical trials (FIH) of new molecular entities in adult healthy volunteers, and among much other information, the guideline provides common conversion factors for deriving a human equivalent dose (HED) from different animal species.

The concept HED refers to the dose in humans that provides the same level of effect as that observed in animals, at the administered dose, by application of the conversion factor recommended by the FDA. HED is expressed in mg/kg, considering an adult of average weight 60 kg.[400]

According the algorithm disclosed in the FDA guideline, HED can be calculated from preclinical animal data, based on the different body surface area of several species, including the most common rodents, mini-pigs, rabbits, dogs and primates, providing that animal weights range from 20 g to 40 Kg. For any other preclinical animal species not included in the list, or for animal weights outside the above-mentioned working weight range, the guideline suggests the use of the formula (Eq. 1):

Eq. 1.

$$\text{HED} = \text{Animal dose (mg/kg)} \times [\text{animal weight (kg)} \div \text{human weight (kg)}]^{0.33}$$

wherein, the human body reference weight is established as 60 Kg for human adults, and 20 Kg for a human child.

The FDA algorithm shown in Equation 1 has been the base of the calculations disclosed herein.

3.2.2.2. Concentration to dose translation

3.2.2.2.1. Approach 1: HED from in vitro data (concentrations used in fibroblasts assays)

An initial approach to extrapolate the concentration value *in vitro* (expressed in µM units) to the dose value *in vivo* (expressed in µg/kg or mg/kg) was to multiply by a factor of 10, according to common practice in some preclinical labs:[viii]

$$[\text{sample}]_{\text{in vivo}} = 10 \times [\text{sample}]_{\textit{in vitro}}$$

However, taking into consideration the upper limit constraints, especially for caffeine, before directly jumping to this very simple approach, some previous calculations were performed to translate those values to HED to confirm these values before launching the experimental phase.

[viii] Strategy commonly used by the Genomics Translational group (personal communication).

Step 1: Dose translation to Drosophila

Since sample B31 (caffeine:theobromine 1:2 ratio, at 1 µM:2 µM concentrations, respectively) rendered the best *in vitro* results, our first approach was to reproduce the same conditions in the 20 mL[ix] with which the fly population would be fed in the *in vivo* experiments. Equations 2 to 4 and **Table 29** illustrate the calculations behind this approach.

Eq. 2: Caffeine 1 µM in 20 mL feed (equivalent to the total caffeine content):

$$20\ mL \cdot \frac{1\ \mu mol\ CF}{10^3\ mL} \cdot \frac{1\ mol\ CF}{10^6\ \mu mol\ CF} \cdot \frac{194.19\ g\ CF}{1\ mol\ CF} \cdot \frac{10^6\ \mu g}{1\ g} = 3.9\ \mu g\ CF\ in\ 20\ mL\ feed$$

Eq. 3: Theobromine 2 µM in 20 mL feed (from Theobroma cacao powder, containing theobromine at approximately 9% w/w, according to its corresponding certificate of analysis):

$$20\ mL \cdot \frac{2\ \mu mol\ TB}{10^3\ mL} \cdot \frac{1\ mol\ TB}{10^6\ \mu mol\ TB} \cdot \frac{180.14\ g\ TB}{1\ mol\ TB} \cdot \frac{100\ g\ extract}{9\ g\ TB} \cdot \frac{10^6\ \mu g}{1\ g} = 80\ \mu g\ powder\ in\ 20\ mL\ feed$$

Eq. 4: Thebromine contained in the 80 µg Theobroma cacao powder:

$$80\ \mu g\ powder \cdot \frac{9\ \mu g\ TB}{100\ \mu g\ Theobroma\ cacao\ powder} = 7.20\ \mu g\ TB\ from\ Theobroma\ cacao\ powder$$

Since caffeine is also present in the *Theobroma cacao* powder in a *ca* 0.2% w/w amount (according to its corresponding certificate of analysis), the caffeine amount provided by the natural source extract should also be considered as part of the total caffeine content:

Eq. 5:

$$80\ \mu g\ powder \cdot \frac{0.2\ \mu g\ CF}{100\ \mu g\ Theobroma\ cacao\ powder} = 0.16\ \mu g\ CF\ from\ Theobroma\ cacao\ powder$$

Therefore, in order to reproduce the same concentrations in 20 mL feed as those administered to cells in the binary sample B31 (caffeine:theobromine 1:2, at 1 µM:2 µM concentrations, respectively), the following amounts of caffeine, theobromine and *Theobroma cacao* powder as the natural source of theobromine would be required (**Table 29**):

[ix] The complete 20 mL feed was considered in the algorithms, even though feed consumption varies along the evolution growth from larvae to adult stages and especially at the end of the experiments, not consumed food remained not eaten (Genomics Translational group personal communication).

Table 29: Calculated amounts of caffeine, theobromine and cocoa powder as per Approach 1

Parameter (µg)	Caffeine (CF)	Theobromine (TB)	*Theobroma cacao* powder
Total amount - Eq. 2 & 3 & 4	3.90	7.20	80.00
From the natural source powder - Eq. 5	0.16	7.20	80.00
To be supplemented‡	3.74	0.00	0.00

‡ Total amount minus the amount provided from the natural source powder

Providing that the *in vivo* experiments included 30 subject flies per assay, the sample concentration per fly in the 20 mL feed would be (**Table 30**):

Table 30: Calculated amounts of caffeine and theobromine to deliver to DM1 flies, as per Approach 1

Parameter	Caffeine (CF)	Theobromine (TB)
In vitro concentration (µM)	1	2
In vitro concentration/subject (µM/fly)	0.034	0.067
Total content (µg)	3.900	7.200
Total content/subject (µg/fly)	0.130	0.240

Step 2: HED determination

Next step consisted in the determination of the corresponding HED for caffeine and theobromine, using Eq.1. and previous data from Step 1. Animal dose could be determined providing that the fly animal weight ranged from 0.24 to 0.30 mg,[x] as illustrated in Equations 6 and 7:

Eq. 6

$$HED(CF) = animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \sqrt[3]{\frac{animal\ weight\ (kg)}{human\ weight\ (kg)}} = \frac{1.3 \cdot 10^{-4}\ mg\ CF}{2.7 \cdot 10^{-7}\ kg} \cdot \sqrt[3]{\frac{2.7 \cdot 10^{-7}\ kg}{60\ kg}} = 0.85\ mg\ CF/kg$$

Eq. 7

$$HED(TB) = animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \sqrt[3]{\frac{animal\ weight\ (kg)}{human\ weight\ (kg)}} = \frac{2.4 \cdot 10^{-4}\ mg\ TB}{2.7 \cdot 10^{-7}\ kg} \cdot \sqrt[3]{\frac{2.7 \cdot 10^{-7}\ kg}{60\ kg}} = 1.56\ mg\ TB/kg$$

Hence, the direct translation from the concentration value *in vitro* (expressed in µM units) to the dose value *in vivo* (expressed as µg/kg or mg/kg), simply multiplying by a factor of 10, furnished caffeine and theobromine human equivalent doses above the maximum daily intake recommended by EFSA, and therefore we would be overdosing the amounts of both methylxanthines. **Table 31** illustrates these data:

[x] Source: Genomics Translational group (personal communication). An average 0.27 mg animal weight was taken.

Table 31: Human equivalent dose obtained from direct translation according to Approach 1

HED (mg/kg) referred to 60 kg human weight	Caffeine (CF)	Theobromine (TB)
Direct translation from µM - Eq. 6 & 7	0.85	1.56
Direct translation from µM, extrapolation x 10	8.50	15.64
Maximum daily intake (mg/kg)	6.70[300]	8.30[327]
Dosage due to direct translation (%)	127	188

Based on all this rationale, the initial *Approach 1* was rejected.

3.2.2.2.2. Approach 2: HED from in vivo data (concentrations used in caffeine assays)

An additional data source to approach the concentration to dose translation issue were the flying results obtained for caffeine on DM1 functional phenotypes in the DM1 *Drosophila* model, previously disclosed in this Chapter. Briefly, in the flying assay a statistically significant effect was observed at least at the concentration corresponding to the addition of 30 µL 1 µM caffeine stock solution diluted into 3 mL feed, which was later administered to a group of 50 flies.

Now, considering the experimental values, the following results were obtained for caffeine (**Table 32**).

Table 32: Calculated amounts of caffeine to deliver to DM1 flies, as per Approach 2

Parameter	Caffeine $(CF)_{exp}$	Theobromine $(TB)_{exp}$
Total content in flying feed (mg) - Eq. 8	$5.83 \cdot 10^{-6}$	0
Total content in flying feed/50 flies (mg/fly)	$1.17 \cdot 10^{-7}$	0
HED (mg/kg) - Eq. 9	0.035	0

wherein

Eq. 8

$$30\ \mu L \cdot \frac{1\ \mu mol\ CF}{10^6 \mu L} \cdot \frac{1\ mol\ CF}{10^6 \mu mol} \cdot \frac{194.19\ g\ CF}{1\ mol\ CF} \cdot \frac{10^3 mg\ CF}{1\ g\ CF} = 5.83 \cdot 10^{-6}\ \ mg\ CF\ in\ 3\ mL\ feed$$

Eq. 9

$$HED(CF) = animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \sqrt[3]{\frac{animal\ weight\ (kg)}{human\ weight\ (kg)}} = \frac{5.8 \cdot 10^{-6}\ mg\ CF}{2.7 \cdot 10^{-7}\ kg} \cdot \sqrt[3]{\frac{2.7 \cdot 10^{-7}\ kg}{60\ kg}} = 0.038\ mg\ CF/kg$$

Although only caffeine was administered in the flying experiment disclosed herein (**Table 32**), the corresponding values for theobromine could be calculated from the caffeine:theobromine 1:1.85 w/w ratio, as determined in **Table 33**:

Table 33: Human equivalent dose obtained from direct translation according to Approach 2

Parameter	Caffeine $(CF)_{exp}$	Theobromine $(TB)_{calc}$
Total content in flying feed (mg)	$5.83 \cdot 10^{-6}$	$1.08 \cdot 10^{-5}$
Total content in flying feed/50 subjects (mg/fly)	$1.17 \cdot 10^{-7}$	$2.16 \cdot 10^{-7}$
HED (CF) (mg/kg)	0.038	-
HED (TB) (mg/kg)- Eq. 10	-	$1.4 \cdot 10^{-3}$
Maximum daily intake (mg/kg) in a 60-kg human	6.70^{300}	8.30^{327}

wherein

Eq. 10

$$HED(TB) = animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \sqrt[3]{\frac{animal\ weight\ (kg)}{human\ weight\ (kg)}} = \frac{2.16 \cdot 10^{-7}\ mg\ TB}{2.7 \cdot 10^{-7}\ kg} \cdot \sqrt[3]{\frac{2.7 \cdot 10^{-7}\ kg}{60\ kg}} = 0.0014\ mg\ TB/kg$$

Accordingly, the direct extrapolation from *in vivo* functional tests in *Drosophila* as determined from the flying experiment furnished HED values two to three orders of magnitude below the threshold of the maximum recommended daily dose. Apart from being unexpected, the underdosing values were somehow difficult to translate into the most common oral dosage forms (capsules, tablets or sachets). Therefore, based on these data, *Approach 2* for dose translation was also rejected.

3.2.2.2.3. Approach 3: HED from maximum regulated daily dose

Since neither *Approach 1* nor *Approach 2* were suitable to solve the *in vitro* concentration to the *in vivo* dose translation issue, a third option based on translating the maximum permitted daily dose from humans to the animal model, *Drosophila melanogaster,* was considered.

Briefly, as previously disclosed in Chapter 2, caffeine was limited as an ingredient in food supplements. The more restrictive value limiting its daily intake was 80 mg for an adult person. Regarding theobromine, although it was formally not limited, previous *in vitro* results showed that caffeine and theobromine should better maintain a molar ratio 1:2, respectively, or a weight ratio 1:1.85, respectively. Regarding the correlation between human and animal dose, the same algorithm disclosed in Equation 1 could be applied. Finally, a last input to consider was the galenic form as capsules and the most suitable frequency of daily dosing. All these inputs were considered in this approach for the calculations. **Table 34** shows the obtained results.

Table 34: Comparison of developmental parameters in front of daily caffeine intake

Parameter	Daily caffeine intake (mg)		
	80[xi]	**60**	**40**
Percentage of the maximum permitted daily amount (%)	100	75	50
Caffeine in 60 kg adult (mg/kg/day)	1.3	1.0	0.7
Theobromine[xii] (mg/day)	148.0	111.0	74.0
Theobroma cacao extract[xiii] (mg/day)	1644.4	1233.3	822.2
Caffeine naturally provided from the extract[xiv] (mg/day)	3.3	2.5	1.6
Caffeine to supplement to reach expected daily intake (mg/day)	76.7	57.5	38.4
Composition containing methylxanthines, total weight (mg/day)	1721.1	1290.8	860.6
Frequency of daily dosing[xv] (capsules/day, approximately)	4	3	2

Based on these data, it was possible to reach the maximum daily amount of 80 mg caffeine permitted in food supplements in certain EU regulations (see Chapter 2) providing that an adult person would ingest four capsules of the formulated product per day. Alternatively, the daily intake of three capsules of the formulated product would provide approximately 60 mg/day caffeine (75% of the 80 mg/day taken as reference) whereas the intake of two daily capsules of the formulated product would provide approximately 40 mg/day caffeine (50% of 80 mg/day taken as reference). All those values were deemed suitable for the purposes of the present research, being the three capsules per day slightly preferred over the four or the two frequencies of daily dosing, since three capsules could be easily scheduled in accordance to meals time.

Taken all together, *Approach 3* rendered a solid and coherent starting point, fulfilling regulatory and development constraints. However, translation from *in vitro* data in DM1 models to *in vivo* data in DM1 models were requested before moving from the *in vivo* models to humans. Accordingly, in a further step forward, the values shown in **Table 34** were translated into animal doses to correlate the biological response of the human doses and the *Drosophila* DM1 model (see **Table 35**).

[xi] This value was in accordance with the Italian and Belgium legislations. See Chapter 2 for further details.
[xii] Theobromine amount in accordance to the CF:TB 1:1.85 weight ratio.
[xiii] This value was obtained taking into account the theobromine content *ca* 8-9% of a representative sample of *Theobroma cacao* extract provided by a commercial supplier.
[xiv] This value was obtained taking into account the caffeine content *ca* 0.2% of a representative sample of *Theobroma cacao* extract provided by a commercial supplier.
[xv] Provided that mean capacity in capsules nr 00 was *circa* to 340-400 mg, according to the rheological properties of the solid mixture.

Table 35: Interspecies comparison of daily caffeine intake

Parameter	Daily caffeine intake (mg)		
	80[xvi]	**60**	**40**
Percentage of the maximum permitted daily amount (%)	100	75	50
Caffeine in 60 kg adult (mg/kg/day)	1.3	1.0	0.7
Caffeine dose in *Drosophila*[xvii] (mg/kg/day)	758	568	379
Caffeine dose in *Drosophila*[xviii] (mg/fly/day)	$2.0 \cdot 10^{-4}$	$1.5 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$
Caffeine dose already tested in *Drosophila* in the flying experiment[xix] (mg/fly/day)	$1.17 \cdot 10^{-7}$	$1.17 \cdot 10^{-7}$	$1.17 \cdot 10^{-7}$
Dose relationship between functional assays[xx]	$1.7 \cdot 10^{3}$	$1.3 \cdot 10^{3}$	$0.9 \cdot 10^{3}$

Based on the dose relationship between functional assays data shown herein, the translation to the animal model of a caffeine daily intake ranging from 40 mg to 80 mg in humans represented three orders of magnitude above the caffeine amount administered in the previous flying experiment. It was unknown whether these doses could be toxic to *Drosophila*. Since theoretical calculations should be substantiated by experimental data, a set of slightly different compositions fulfilling the above-mentioned constraints was prepared and tested in the models, as disclosed in the next section.

3.2.3. Screening of a combination of xanthines. Part 2

3.2.3.1. Selection of combinations

Aimed at defining a coherent correlation between experimental models and human doses, the previous section disclosed the rationale behind the translation of the previous data for caffeine and theobromine combination to the *in vivo* experiments in the DM1 fly model.

However, as previously stated, the translation of a caffeine daily intake ranging between 40 mg to 80 mg from human to the animal model represented three order of magnitud the caffeine amount administered in the previous flying experiment. Therefore, beyond their effect on *Drosophila*, it was uncertain whether these concentrations could be toxic to the experimental model.

To confirm the effect of the selected composition on the *in vivo* functional behaviour of dystrophic flies, six compositions were designed (sample set M1 to M6, see **Table 36**), to explore the equivalent effect of the 60 mg caffeine per day in humans to the animal caffeine dose at which *in vivo* functional effect was obtained in the flying test, while exploring intermediate situations and caffeine combination with the selected *Theobroma cacao* extract.

[xvi] Value in accordance with the Belgian legislation. See Chapter 2 for further details.
[xvii] Animal dose calculated from the HED caffeine values in the Table and Equation 1.
[xviii] Provided that fly's body reference weight is 0.27 mg (median value).
[xix] Provided that in the flying experiment, 1 µM caffeine solution was diluted 1/1.000 and administered to 50 flies. For correction purposes, it was assumed that only 50% of the feed was ingested by the animals.
[xx] Obtained from the quotient between caffeine dose in *Drosophila* (mg/fly/day) divided into caffeine dose already tested in the previous flying experiment.

Table 36: Rationale for the design of samples M1 to M6

Sample	HED_{CF} (mg/Kg)	Stoichiometry CF:TB	Comments	Caffeine source
M1	1	1:0	Equivalent to 60 mg CF daily dosage in humans. The potential toxic effect of this level of caffeine in flies was unknown.	Pure from synthesis
M2	1	1:2	Equivalent to the effect of 60 mg CF and 111 mg TB daily dosage in humans, that would be administered by means of three daily capsules of the formulated product.	From natural source + supplemented
M3	$1 \cdot 10^{-2}$	1:2	Equivalent to a 100-fold reduction of the dosage in sample M2, in order to prevent some potential toxicity in flies during the experiment.	From natural source + supplemented
M4	$2 \cdot 10^{-4}$	1:0	It was approximately 5000-fold lower than M1, trying to reproduce the flying experiment for CF administered alone. Therefore, sample M4 could be considered as an internal control.	Pure from synthesis
M5	$2 \cdot 10^{-4}$	1:2	Equivalent to sample M4, but including the synergistic effect of CF and TB at the 1:2 molar ratio.	From natural source + supplemented
M6	$1 \cdot 10^{-1}$	1:2	Equivalent to a 10-fold reduction of sample M2 dosage, in order to prevent some potential toxicity in flies during the experiment.	From natural source + supplemented

Samples were prepared at the proper caffeine and theobromine concentrations, considering the fly population in each experiment, feed schedule, how they were to be diluted in the feed, remaining unconsumed feed, and the like.

Once prepared, samples were sent to Translational Genomics Group's laboratory for their *in vivo* testing in the *Drosophila* models. The obtained results are disclosed in the next sections.

3.2.3.2. In vivo models of DM1

3.2.3.2.1. DM1 functional phenotypes: test of the effect of combinations of caffeine and theobromine in climbing assay

The experiment was performed as previously discussed in the Methodology section. Briefly, to assess climbing velocity, groups of twenty 7-day-old males were transferred into disposable pipettes (1.5 cm in diameter and 25 cm height) after a period of 24 h without anaesthesia. The height reached from the bottom of the vial by each fly in a period of 5 seconds was recorded with a camera. For each genotype, two groups of 20 flies were tested. Two-tailed t-test (p = 0.05) was used for comparisons of pairs of samples applying Welch's correction whenever necessary. Results are illustrated in **Figure 35**.

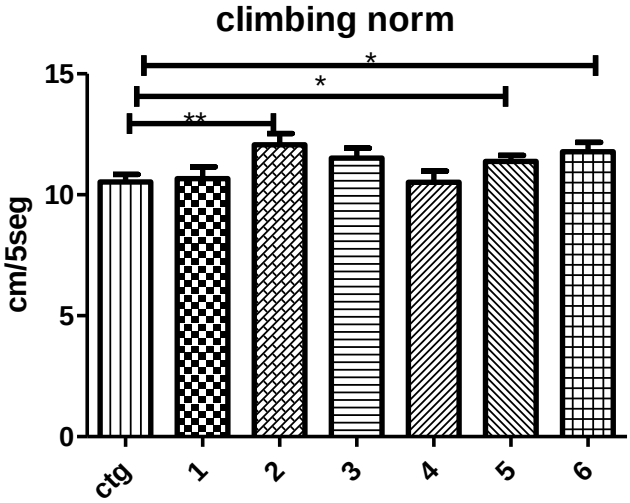


Figure 35: Climbing speed for samples M1 to M6
Bar Graphs showing the climbing speed as the mean speed ± SEM of 7-days-old disease flies fed with standard food (ctg) or food with compounds (samples M1 to M6, in the graph as 1 to 6, respectively). Climbing normalized to ctg. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test).

Interestingly, significant improvements in climbing velocity were observed after feeding the disease flies with samples M2, M5 and M6. Specifically, sample M2 (the most equivalent dose in flies corresponding to the effect of 60 mg caffeine and 111 mg theobromine daily dosage in humans), showed the most significant difference. On the contrary, samples M1 and M4 containing exclusively caffeine but not theobromine, showed the lowest effect.

3.2.3.2.2. DM1 functional phenotypes: test of the effect of combinations of caffeine and theobromine in flying assay

Since flying is considered a more exigent test than climbing, this test was performed in order to refine the differences among the effects of the sample set M1 to M6. Certainly, only the sample M2 rendered a significant improvement in fly movement, measured as landing distance (see **Figure 36**).

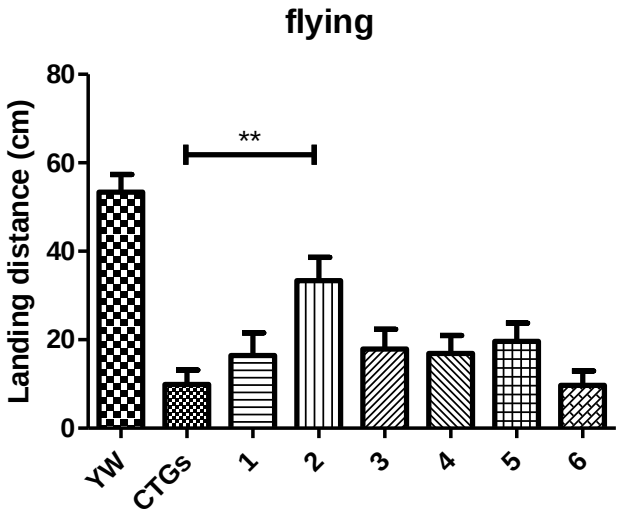


Figure 36: Landing distance for samples M1 to M6
Histogram showing the average landing height data ± SEM of 7-days-old control (YW) or disease control flies (CTGs), and the effect of samples M1 to M6 in diseased flies. Flight disability observed in the diseased flies (CTGs) was significantly rescued by feeding the flies with sample M2. Small improvement was observed with other samples, but they were not significant. $^{**}p < 0.01$ (Student's t-test).

In this experiment, the percentage of flight rescue in disease flies fed with the caffeine:theobromine combination sample M2 almost three-fold higher than the results obtained for disease flies fed with standard food. When the comparison was done with healthy flies (the yellow white type, YW), diseased flies fed with sample M2 rescued more than 50% of the original flight capacity.

As it was observed in the flying experiment shown herein, caffeine:theobromine combination at the particularly dose in flies corresponding to 60 mg caffeine and 111 mg theobromine in human (sample M2) was clearly superior to single caffeine dosed at the equivalent 60 mg (sample M1) and clearly superior to the rest of the samples corresponding to lower doses of caffeine and theobromine, even fulfilling the caffeine: theobromine 1:2 molar ratio. Caffeine *per se* rescued only half the landing distance that the equivalent caffeine dosed in sample M2, confirming the synergistic effect of both methylxanthines in the formulated product.

In summary, the caffeine:theobromine combination in 1:2 stoichiometry dosed in sample M2 demonstrated a clear improvement in the functional assays based on climbing and flying capacities in *in vivo* models of DM1. Particularly, flying capacity was almost three-fold increased due to the improvement in indirect muscles of flight, which could be translated as the possibility to achieve more than half of the flying capacity that the healthy control flies rendered.

3.2.3.2.3. DM1 functional phenotypes: quantification of the muscle area.

Previous data from several studies of muscle area quantification and functional flight analysis obtained from the Translational Genomics Group's laboratory allowed us to establish a direct relationship between muscle atrophy and flying capacity, according to the graph shown in **Figure 37**.

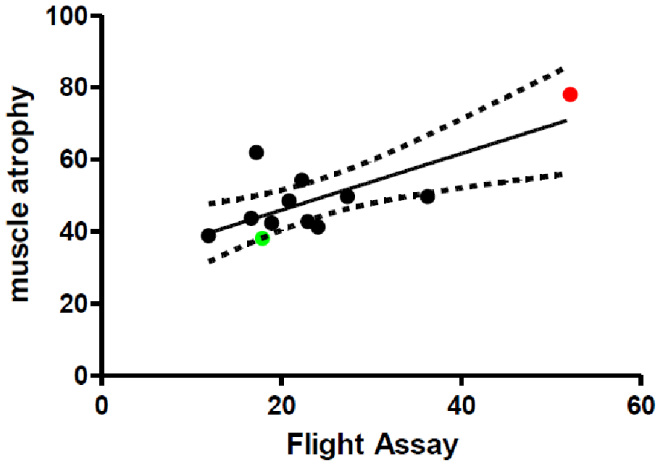


Figure 37: Pattern for Interpolation

The line was obtained by representing the results obtained in tests of quantification of muscle area (in the Y axis, as a percentage of muscle area) and in flight tests (in the X axis, as landing distance expressed in cm). The region between the dotted lines represents the 95% confidence interval. The red dot represents the value obtained in the case of a control fly. The green dot represents the value obtained by a diseased fly that expresses the repeats in the skeletal muscle. Black spots correspond to values measured in flies treated with different compounds.

Then, when the data obtained from the flight test performed in samples M1 to M6 was interpolated in the muscle atrophy pattern shown in **Figure 37**, we obtained the percentage of muscle area shown in **Figure 38**.

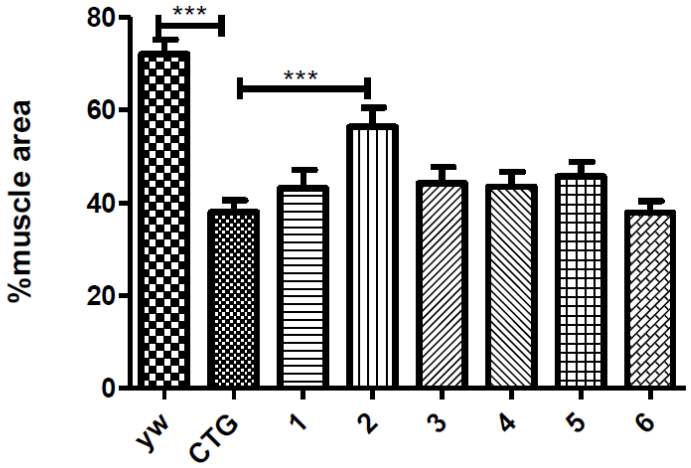


Figure 38: Muscle area for samples M1 to M6
Histogram showing the average percentage of muscle area ± SEM of 7- days-old control (YW) or disease flies (CTGs, samples M1 to M6). Reduction of muscle area observed in the diseased flies (CTGs) was rescued by feeding the flies with sample M2. Small improvements were observed with the rest of the samples, but they were not statistically significant. ***$p < 0.001$ (Student's t-test).

Reduction of muscle area obtained in the diseased flies (CTGs) was significantly rescued by feeding the diseased flies with sample M2, which rescued approximately 40% of muscle area of the disease model flies. Moreover, DM1 flies fed with the formulation M2 recovered almost 85% of muscle area of control healthy flies. Interestingly, sample M2 was the only one that achieved statistically significant improvement in diseased DM1 model flies.

Taken all together, none of the samples M1 to M6 showed any sign of toxicity in the DM1 *Drosophila* model. On the contrary, a clear correlation between compositions and results was observed, confirming the synergistic effect of caffeine and theobromine combination over caffeine administered alone, as previously observed in the *in vitro* experiments.

Moreover, in all these experiments, the sample M2 statistically rendered the more relevant improvements in climbing rescue, flying rescue and increment of muscular area. Therefore, the composition and dose labelled as sample M2 was definitively selected for the purposes of the present research. Therefore, the final experiments in the present section were exclusively performed using the sample M2 as the best candidate for product composition.

3.2.3.2.4. DM1 functional phenotypes: lifespan and median survival

Survival is reduced in DM1 patients and animal models. Model flies expressing 250 CUG repeats show a reduction in mean survival. To study the effect on fly survival of the formulated composition M2 (caffeine:theobromine in 1:2 molar ratio, dosed in flies to the corresponding HED 60 mg/day caffeine and 111 mg/day theobromine), the survival curves of a minimum of 50 control and DM1 model flies, fed either with DMSO or MYODM, at 29ºC were studied.
Since in previous experiments, the mean survival in the DM1 model flies was not altered when flies were fed with DMSO, DMSO was taken as control in the survival experiment. Results obtained when comparing control flies with DM1 model flies fed with DMSO and DM1 model flies fed with sample M2 composition are illustrated in **Figure 39**.

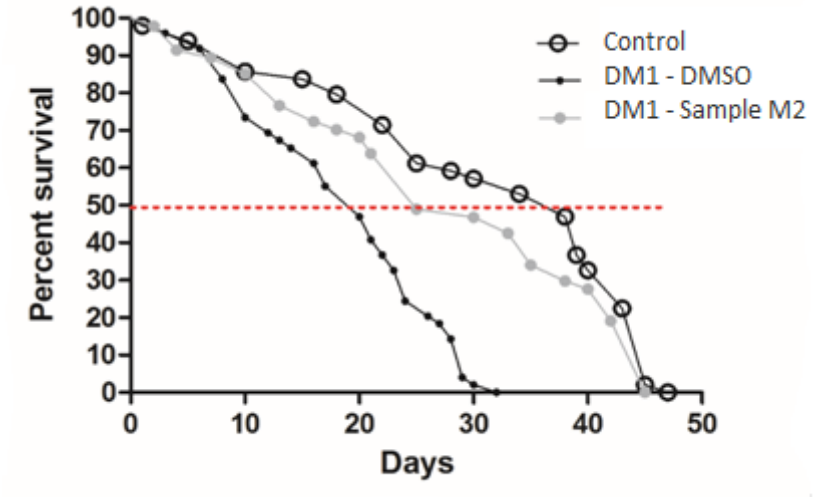


Figure 39: Survival curves of flies
Average percentage of alive flies versus age (in days). The GMH5-Gal4 driver was used to induce the expression of the innocuous reporter GFP (control) or the 250 CUG repeats (DM1) to *Drosophila* cardiomyocytes. The horizontal dotted line marks the median survival. The survival curves were highly significant ($p < 0.0001$, log-rank test).

Certainly, 50% survival is reduced from 38 days in control flies to only 20 days in DM1 DMSO flies. Lifespan was also greatly reduced from 48 to 32 days.
Feed supplementation with sample M2 in the model flies achieved an increase of both lifespan (46 days) and survival means (25 days). Taking the control flies data as reference, after the administration of caffeine and theobromine formulation in M2, lifespan was almost fully rescued, whereas mean survival was rescued *circa* 65% in DM1 flies compared to control healthy flies.

3.2.3.2.5. DM1 functional phenotypes: cardiac dysfunction

Cardiac involvement is a common complication of the skeletal muscle disorder in myotonic dystrophy type 1, occurring in 80% of cases.[401,402] Heart dysfunction is the second most common cause of fatality associated with the disease, after respiratory distress. Alterations of systolic and diastolic function, as well as decreased ventricular ejection fraction and increased arrhythmicity, have previously been reported in DM1 patients.[403,404]

The Translational Genomics Group had developed a heart dysfunction model of DM1 in flies, which was used in the present research.[402] Cardiac dysfunction in DM1 model flies is characterized by increased arrhythmicity measured as arrhythmicity index (AI), and a prolongation of the heart period (HP), including extension of the relaxation (diastolic interval, DI) and contraction periods (systolic interval, SI). Image analysis of heart contractions also provided cardiac chamber parameters, including end diastolic and systolic diameters, (EDD and ESD, respectively) and the proportional decrease in heart wall diameter during contraction, referred to as fractional shortening (FS), which provides an indication of the cardiac output. In DM1 model flies, a significant decrease in end diastolic diameter (EDD), and also a reduced fraction shortening (FS) is observed.

To study heart function, adult fly hearts dissected in artificial hemolymph were recorded with a digital video camera. Because previous studies had reported that heart function changes with age, one-week-old flies were selected for this study. Cardiac contractions were analyzed using a semi-automatic optical heartbeat analysis (SOHA) method to quantify the fly heart functional parameters. M-mode traces of movie clips provided details of the heart wall edge positions (*y*-axis) over time (*x*-axis), illustrating the rhythmicity and the dynamics of the heart contractions.[405]
For the characterization of the cardiac phenotype of 250 CUG-expressing flies, we compared their dynamic parameters with F1 flies from crossing the GMH5-Gal4 to UAS-GFP flies (abbreviated GFP) accounting for the dose of the UAS transgenes. To test the effect of the composition containing caffeine and theobromine in the M2 sample, we compared cardiac parameters of seven-day-old long-CUG expressing flies which had been fed either with standard food or with caffeine and theobromine formulation in M2 sample (codified herein as MYO-DM) mixed at 0.75% in standard food during the seven days of their adult life.

DM1 model flies treated with the composition containing caffeine and theobromine in the M2 sample experienced a significant rescue in the prolongation of the heart period, due to a decreased duration of the diastolic interval, although the systolic interval was not significantly modified. The percentage fractional shortening and end diastolic diameter were completely repaired by the caffeine and theobromine combination, suggesting a rescue of contractility

properties and heart tube dimensions. Finally, although sample M2 achieved a slight decrease of arrhythmicity index, the difference with DM1 flies that had not been fed with the composition was not statistically significant (**Figure 40**).

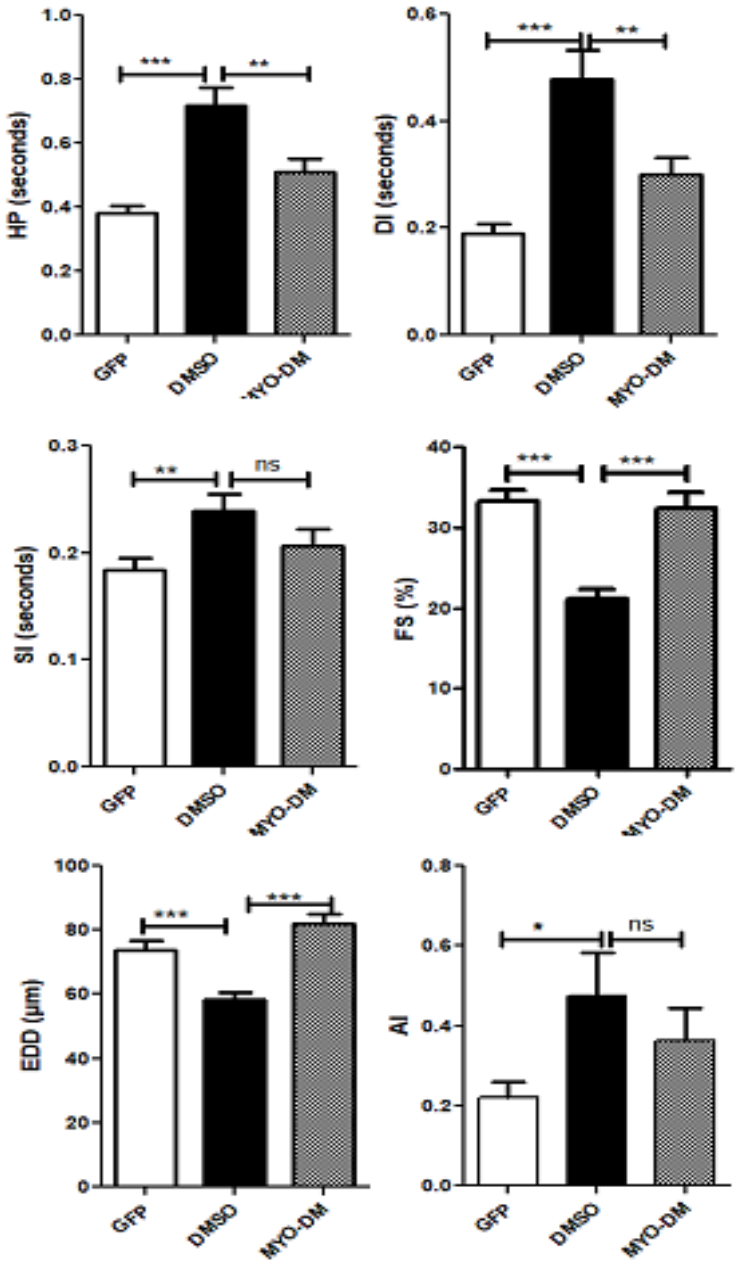


Figure 40: Treatment with caffeine and theobromine formulation according to sample M2 (identified herein as MYO-DM) rescued diastolic dysfunction and fractional shortening in DM1 model flies

Characteristic cardiac dysfunction of flies expressing long CUG repeats, compared to control flies expressing GFP reporter (GFP), include increased Heart period mean (HP), due to increase of Diastolic and Systolic Intervals (DI and SI, respectively), reduced percentage of Fractional Shortening (FS), due to a reduction of End Diastolic Diameter (EDD), and increased Arrhythmicity Index (AI). Treatment of adult flies with 0.75% sample M2 dissolved in DMSO in the food during 7 days (labelled herein as MYO-DM) rescued DI, %FS and EDD compared to DM1 model flies fed with the solvent DMSO (labelled herein as DMSO). The bars on the graph show mean values and their standard errors. *p < 0.05, **p<0.01, ***p < 0.001.

In a further step forward, in order to compare the results obtained with the formulation containing caffeine and theobromine in the previous experiment and the results obtained with pentamidine administration,[402] the percentage of improvement in all the parameters was calculated. Both experiments had been performed in separate time points and using different control and disease flies. However, when the results obtained with the compounds to the data of the dystrophic untreated flies used in the corresponding experiments were normalized, both compounds displayed similar improvement of all the phenotypes with the exception of the systolic interval (SI), which was only repaired by the composition containing caffeine and theobromine (**Figure 41**).

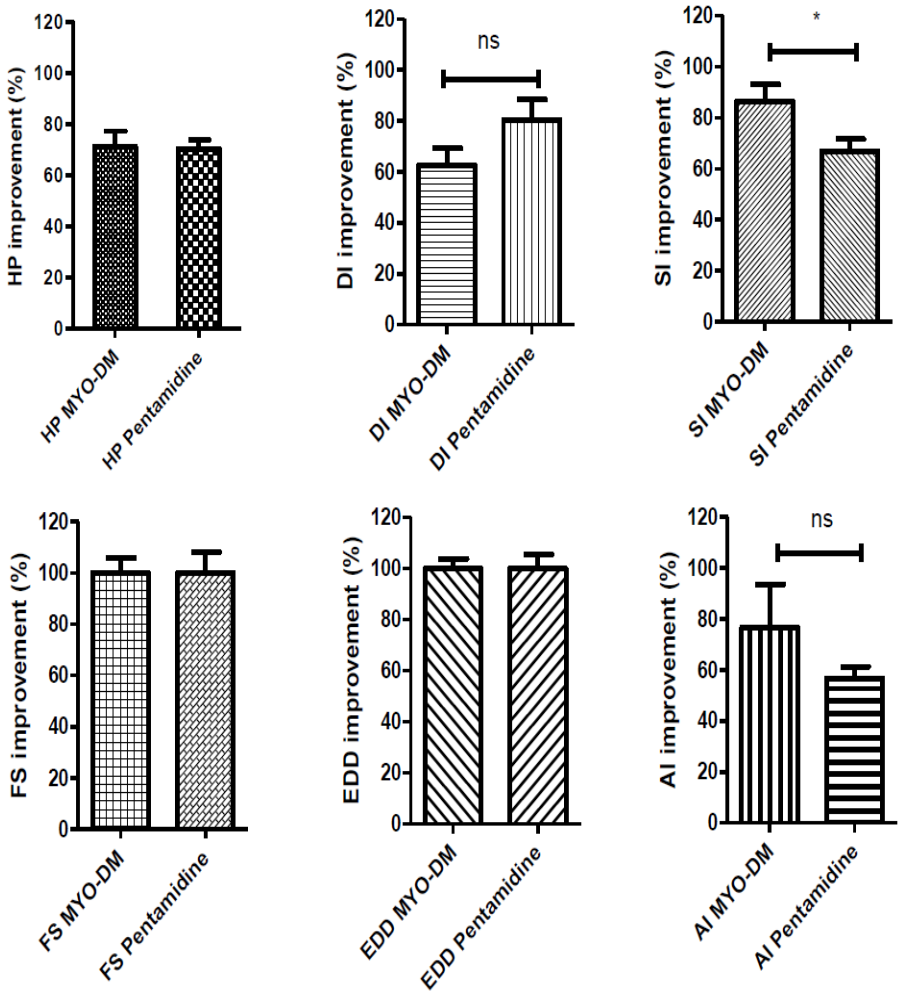


Figure 41: Comparative between the composition containing caffeine and theobromine sample M2 (labelled as MYO-DM) and pentamidine (both normalized from their corresponding controls), shown individually per each parameter
Both compounds have similar activities, but the activity of composition containing caffeine and theobromine sample M2 on the systolic interval was more pronounced. The bars on the graph show mean values and their standard errors. *$p < 0.05$, ns non-significant.

Taken together, data shown herein demonstrated that the formulation developed along this research project exhibited even better results than pentamidine in the rescue of heart dysfunction in dystrophic flies.

3.2.4. Summary of results

According to the data from the *in vitro* and *in vivo* experiments performed on the caffeine and theobromine composition reported along this Chapter:

- It increases MBNL1 and MBNL2 protein levels. It is worth mentioning that MBNL1 is involved in 80% of mechanisms regulating the multisystemic effects of DM1.
- It promotes a significant rescue in the functional climbing and flying capacity in the DM1 fly model. Particularly, flying capacity is three-fold increased due to the improvement in indirect muscles of flight, which is translated into achieving *circa* 60-70% a rescue in flying capacity of the healthy control flies.
- It promotes a significant improvement in the muscular area quantification of dystrophic flies, closer to the healthy fly strain situation. In particular, *circa* 40% improvement in muscle area of the disease model flies and almost 85% of muscle area of non-DM1 model control is reached when model flies are fed with the composition.
- It promotes a very important rescue in almost all the parameters measured in the heart dysfunction *in vivo* model on *Drosophila melanogaster*. Particularly, a rescue in the prolongation of the heart period, due to a decreased duration of the diastolic interval, a completely repair of the percentage fractional shortening and end diastolic diameter, suggesting a rescue of contractility properties and heart tube dimensions, and a slightly decrease of arrhythmicity index, which however was not statistically significant. When compared to pentamidine, the composition containing caffeine and theobromine displayed similar improvement of all the phenotypes with the exception of the systolic interval (SI), which was only repaired by the composition containing caffeine and theobromine, but not by pentamidine.
- The study on the survival effect in the *in vivo* DM1 model confirms that supplementing the diet of dystrophic flies with the composition containing caffeine and theobromine is able to significantly improve their life expectancy. In particular, a 96% increase in life expectancy and a 65% average survival of dystrophic flies compared to healthy flies were obtained.

3.3. Raw materials

3.3.1. Introduction

As previously disclosed in Chapter 2 in light of Regulation (EU) No 609/2013,[340] the formulated product containing caffeine and theobromine as main ingredients should fall under the food for special medical purposes classification, wherein "food for special medical purposes means food specially processed or formulated and intended for the dietary management of patients, including infants, under medical supervision; it is intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary food or certain nutrients contained therein, or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved by modification of the normal diet alone". Regulation (EU) No 609/2013 does not limit the nutrients or ingredients that the food product may contain, since each specific disease and patient ages may need different compositions according to product's intended use.

Nevertheless, when defining the caffeine and theobromine sources, we considered more appropriate to adhere to the selection of the *Theobroma cacao* beans as the main natural source of theobromine from the BELFRIT list, since it is considered safe on the basis of their traditional use. Beyond its content in theobromine, the seeds may naturally contain certain levels of caffeine as well.

Several suppliers as well as representative samples of *Theobroma cacao* extracts were evaluated for the proper selection of this key raw material. Considering the certificates of analysis and their content on theobromine, Naturex (a leader company producing plant extracts and natural ingredients for food, beverages, nutrition and health, now part of Givaudan) was finally selected as the supplier for the natural ingredient.

The *Theobroma cacao* (cocoa) beans dry extract evaluated was a chocolate brown colour powder exhibiting a characteristic cocoa flavour. The powder had an average particle size between 45-65 µm, without the presence of aggregated particles. Regarding its composition, a preliminary evaluation of the certificates of analysis for a set of three different batches (see **Table 37**) evidenced significant variability inter-batches, especially in the theobromine content, that could affect the standard formula along its batch to batch future manufacture.

Table 37: Methylxanthine content in *Theobroma cacao* raw material

Item	*Theobroma cacao* dry extract Naturex batch	Theobromine content (% w/w)	Caffeine content (% w/w)
1	A197/033/A14	8.9	0.21
2	A321/039/A15	9.7	0.16
3	A096/067/A16	12.1	0.18

Plant extracts may suffer from certain inter-batch variations that could affect their quantitative composition, which definitely would impact the already established caffeine:theobromine optimum ratio in the present project.

Therefore, during the product development phase, the annual trends for several batches of *Theobroma cacao* (cocoa) beans were evaluated in order to define safe margins and thresholds in the product development, as illustrated in **Table 38**.

Table 38: Theobromine and caffeine annual trends in *Theobroma cacao* bean raw material

Batch	Theobromine content (% w/w)	Caffeine content (% w/w)	Humidity (% w/w)
A102/009/A15	9.70	0.20	4.0
A204/064/A15	7.86	0.14	4.0
A208/035/A15	9.13	0.14	4.3
A168/011/A15	9.70	0.16	4.0
A293/075/A15	9.70	0.16	4.0
A321/039/A15	9.70	0.16	4.0
A096/067/A16	12.08	0.18	1.7
A111/006/A16	9.70	0.20	4.0
A173/045/A16	9.70	0.20	4.0
Mean	9.70	0.17	3.78
SD	1.08	0.02	0.78
CV (%)	11.2	14.4	20.7

According to these data, the trends in the cocoa bean batches manufactured along one year period rendered 9.70±1.08 % mean theobromine content and 0.17±0.02 % mean caffeine content, wherein caffeine:theobromine ratio was 1:57 (w/w), significantly exceeding the 1:1.85 (w/w) ratio that was deemed to induce the synergistic effect in the DM1 experimental models.

Therefore, there was a need to supplement the endogenous caffeine in the natural extract with exogenous caffeine to fulfil the optimum stoichiometry ratio between both methylxanthines in the formulated product. Accordingly, caffeine compliant with the European Pharmacopoeia monograph quality specifications was selected as the second source of this methylxanthine in the composition.

The following sections disclose the specifications that were established for the selected raw materials *Theobroma cacao* dry extract and caffeine, as well as the nutritional values for the plant extract.

3.3.2. Theobroma cacao specifications

Table 39: Cocoa bean dry extract EA848227 specifications

Parameter	**Specifications**
Organoleptic quality	
Appearance	Powder
Colour	Light brown to brown
Flavour	Characteristic
Solubility	>75 % in water
Analytical quality	
Theobromine content	9 %-10 %
Caffeine content	0.17 %-0.20 %
Loss on drying	< 4 %
Particle size	>95 % through 40 mesh (or 420 µm)
Total heavy metals	< 10 ppm
Microbiological quality	
Total plate count	< 10.000 cfu/g
Yeast and molds	< 100 cfu/g
Coliforms	< 10 cfu/g
E. coli	Negative/g
Salmonella spp.	Negative/25 g
Bile tolerant Gram negative bacteria	< 100 cfu/g

3.3.3. Caffeine specifications

Table 40: Caffeine anhydrous specifications

Parameter	Specifications
Product	
IUPAC name	1,3,7-Trimethyl-3,7-dihydro-1*H*-purine-2,6-dione
Other names	1,3,7-Trimethylxanthine
CAS nr	[58-08-2]
Molecular formula	$C_8H_{10}N_4O_2$
Molecular weight	194.18 g/mol
Organoleptic quality	
Appearance	Odourless white crystalline fine powder
Physicochemical analysis	
Assay	98.5%-101.0%
Water content	< 0.5%
Loss on drying	< 0.5%
Residue on ignition	< 0.10%
Sulphuric ashes	< 0.10%
Total heavy metals	<10 ppm
Related substances	< 0.10%
Residual solvents	As per ICH Q3C[406]
Microbiological quality	
Total aerobic count	< 1.000 cfu/g
Yeast and molds	< 100 cfu/g
E. coli	Negative/g
Salmonella spp.	Negative/25 g
Pseudomonas aeruginosa	Negative/g
Staphylococcus aureus	Negative/g
Storage	
Conditions	Keep in closed container. Keep protected from light and humidity

3.3.4. Theobroma cacao nutritional values

Table 41: Cocoa bean dry EA848227 nutritional breakdown

Nutrients	**Amount per 100 g**
1. Caloric value (Kcal)	329
2. Caloric value (KJ)	1377
3. Fat (g)	< 7
Of which saturated fatty acids (g)	< 4
4. Carbohydrates (g)	> 54
Of which sugars (g)	< 3
5. Protein (g)	< 10
6. Water (g)	< 8
7. Fiber (g)	< 5
8. Sodium (mg)	< 15
9. Salt (equivalent)	< 0.038

3.3.5. Nutritional values for the product unit dosage

Considering that the daily 60 mg caffeine would be provided in three product unit dosages distributed along a day, one product unit dosage would contain 20 mg caffeine. Based on the caffeine:theobromine 1:1.85 (w/w) ratio and the 9.7% mean theobromine content in the *Theobroma cacao* beans, the product unit dosage would contain 37 mg theobromine in 380 mg cocoa extract. Therefore, a single product unit dosage would correspond to 400 mg of the composition fulfilling the criteria already disclosed in the previous sections.

Moreover, three daily product unit dosages would provide 60 mg caffeine and 111 mg theobromine in 1140 mg cocoa extract, rendering a total product weight corresponding to 1200 mg in the three daily services.

For these calculations, the presence of additional excipients was provisionally left aside.

Table 42: Expected nutritional facts for the product composition under development

		NUTRITION FACTS				
		Typical values per 100 g			Typical values per unit dosage	
		Theobroma cacao	Caffeine	TOTAL (per 100 g)	One unit dosage	Three unit dosages
Amount	g	95.0	5.0	100.0	0.4	1.2
Calories	KJ	1377.0	0.0	1308.0	5.2	15.6
	Kcal	329.0	0.0	312.0	1.2	3.6
Fat	g	7.0	0.0	6.6	0.0	0.1
of which saturated	g	4.0	0.0	3.8	0.0	0.0
of which monounsaturated	g	0.0	0.0	0.0	0.0	0.0
of which polyunsaturated	g	0.0	0.0	0.0	0.0	0.0
Carbohydrates	g	54.0	0.0	51.3	0.2	0.6
of which sugars	g	3.0	0.0	2.8	0.0	0.0
Fibre	g	5.0	0.0	4.5	0.0	0.1
Proteins	g	10.0	0.0	9.5	0.0	0.1
Salt	mg	38.0	0.0	0.0	0.0	0.0
Vitamins and minerals	mg	0.0	0.0	0.0	0.0	0.0

3.4. Intellectual property rights. Freedom to operate analysis

3.4.1. Introduction and goals

Having protected the invention and having defined the methylxanthines composition and dose already disclosed in the previous sections, one further verification before launching the galenic formulation development and subsequent manufacture of the product consisted on a Freedom to operate (FTO) analysis, an assessment through which it is intended to verify whether the manufacture, use or commercialization of a property product can be carried out without infringing the existing industrial property rights (IPRs) of a third party.

The freedom to operate analysis should be carried out as early as possible in the development, even in the idea or prototype stage of the product or process, to avoid wrong allocation of resources and useless costs, as well as to reduce the risk of patent infringement, future litigation and/or product recall in further stages.

Moreover, given that the IPRs are territorial, the FTO analysis should be oriented to specific countries or regions where the company focuses its markets, since the results of the FTO analysis make it possible to assess the risk of entering that market.

Usually, an FTO analysis includes the following sequential steps:

1. Search for keywords in specialized patent databases
2. Review of the identified documents, including the description but especially the claims
3. Verification of the global patent dossier or status of application/granting of each document considered relevant within the territorial scope of interest
4. For granted patents, review of their validity (*i.e.*, the holder is up to date with the payment of fees), as well as priority date and expiration date. It is worth mentioning that the analysis must clearly determine whether a potentially conflictive patent document is in force in the target market territory
5. Conclusion of the analysis and / or recommendations

Accordingly, the objective of this section is to disclose the research done in the verification that the production, commercialization and / or distribution of a product manufactured in the form of capsules, tablets or sachets, containing as main ingredients a mixture of methylxanthines, among which at least and preferably caffeine and theobromine, could infringe any previous in force intellectual property right of a competitor, or if on the contrary, there were no risk for the launch of said product to the market.

3.4.2. Methodology

The search was done in the professional patent database Questel[407], global intellectual property business intelligence software dedicated to patent research and analysis, based on relevant keyword combinations, restricted to certain fields, including but not limited to countries and patent/patent application status.

Keywords search included ‘caffeine AND theobromine AND +xanthine+’ or ‘caffeine AND

theobromine AND +xanthine+ AND food', wherein '+xanthine+' was intended to include any word containing 'xanthine', encompassing but not limited to 'methylxanthine', in the claims *per se* or in the claims AND description, in patent applications already in process or granted worldwide, in Europe, USA or Spain.

Overall, almost two hundred patent references were retrieved from this search, each result accompanied by its corresponding file including document title, holder, inventors, priority date, and associated patent family, abstract and main claims related to the searched keywords. Moreover, each file contained links to the files of the related documents in pdf format to facilitate access, reading and review.

3.4.3. Patent analysis

In a first stage, key information was obtained from the abstracts and the extract of the claims on the files, although the whole content of all the files was fully reviewed to debug those documents not related to the type of product, or the composition, or the uses fulfilling the goals of the present project. Finally, non granted patents or patents not in force in the European countries that were the primary target markets of the product developed herein were deleted from the list, as well.

This pre-selection furnished a patent set that deserved a deeper evaluation, in order to confirm whether their scope, in terms of claims of composition or food use, could limit the launch of the product developed along the present project. Claims and legal status of the patents, as well as their territorial validity were verified according to Espacenet[408] patent search engine provided by the European Patent Office.

Following this more exhaustive analysis, some documents were again deleted from the pre-selected list. Finally, the set of the selected patents for full evaluation was reduced to five files, as disclosed in **Table 43**:

Table 43: Selected patents for a detailed evaluation as potentially closest prior

Granted patent	Title	Applicant	Expiry date	Reference
ES2261685T3 US7048941B2	Novel chocolate composition as delivery system for nutrients and medication	NEW WORLD ENTERPRIZES	29/03/2022	[409]
EP1693057 B1 ES2294584T3	Confectionery products containing caffeine	PROCTER & GAMBLE	18/02/2025	[410]
EP1964477 B1 ES2347082T3	Confectionery composition comprising a xanthine derivative and low fructose	PROCTER & GAMBLE	01/03/2027	[411]
EP2258337 B1	Composition for hair	KAO	03/06/2029	[412]
EP2369939 B1 ES2537021T3	Food products enriched with methylxanthines	UNILEVER PLC	03/12/2029	[413]

The main comments and author's opinion on their impact on freedom-to-operate in Europe, as well as any warning related to potential patent infringement are disclosed in this section.

ES2261685T3 - US7048941B2: Novel chocolate composition as delivery system for nutrients and medications

Abstract:[409]

"A novel chocolate product for use in delivering medicaments and/or nutrients to animals, particularly humans, specially formulated so that the craving for such product by animals, particularly humans, is significantly greater than the craving for chocolate conventionally used in pharmaceutical compositions and the concentration, optimization, and the addition of endogenous and exogenous ingredients to increase such craving as well as to treat specific indications. The chocolate product contains: from about 0.5 to about 200 milligrams, more preferably from (A) about 5 to about 20 milligrams, of one or more biogenic amines per 1 gram of the chocolate product; (B) from about 10 to about 500 milligrams, more preferably from about 20 to about 200 milligrams, of one or more amino acids per 1 gram of the chocolate product; (C) from about 1 microgram to about 20 milligrams, more preferably from about 10 micrograms to about 10 milligrams, of one or more of: methyl tetrahydroisoquinoline, N-acylethanolamines, and/or anandamide and/or salsolinol per 1 gram of the chocolate product; (D) from about 0.2 to about 30 milligrams of at least one trace mineral per 1 gram of the chocolate product; and (E) from 0.6 to about 500 milligrams, more preferably from about 35 to about 100 milligrams, of one or more methylxanthine alkaloids per 1 gram of the chocolate product. The chocolate product used in this invention also preferably contains effective amounts of at least one chocolate aroma and at least one vanilla aroma".

Analysis:

The invention relates to a chocolate-based product and composition as a carrier or drug delivery system or vehicle for administering medication-containing compositions or nutrients, wherein said composition is formulated to increase the desire to consume the nutrients or drugs dispensed by said composition, in order to increase patient-adherence to oral treatments.
The product is chocolate-based, achieved by an unusual manufacturing process which is different from the natural one, details described therein, and supplemented with various ingredients, including methylxanthines such as caffeine, theobromine or theophylline, or a mixture thereof.

Opinion:

Provided that the product developed in the present project was not intended to be formulated in the form of a chocolate-matrix, which is a food form completely different than the pharmaceutical form as capsules or sachets expected in the present project, this document did not affect the project's operational freedom.

ES2294584T3 - EP1693057 B1: Confectionery products containing caffeine

Abstract:[410]

"Confectionery compositions comprising a xanthine derivative, a cooling composition and a warming composition are provided. The cooling and warming compositions are located in distinct and discrete regions within the confectionery composition and are adapted to provide sequential release profiles. The compositions herein provide xanthine derivatives as stimulants without negative aspects of xanthine derivative flavour perception".

Analysis:

The invention relates to confectionery compositions that effectively mask the bitter taste of xanthine derivatives, such as caffeine.
The confectionery products seem to be the same as in patent EP1964477 B1[411] from the same company, although the shell technology in the present patent seems to be superior to provide sequential release of the key components in the oral cavity. The confectionery composition comprises a xanthine derivative and some technological additional components as a cooling composition comprising a physiological cooling agent and a warming composition comprising a physiological warming agent, wherein said cooling composition and said warming composition are located in distinct and discrete regions within said confectionery composition and said cooling and warming compositions being adapted.
Regarding the confectionery form in which the confectionery composition is formulated, it includes hard boiled sweets, soft boiled sweets, chewing gums, gummy-based sweets, centre-fill confectionery, or lollies.

Opinion:

Provided that the product developed in the present project was not intended to be formulated in the form of any confectionery product, nor formulated as a confectionery composition, which are completely different matrices and compositions than those expected in the pharmaceutical form as capsules or sachets intended to be developed in the present project, it was considered that this document did not affect the project's operational freedom.

EP1964477 B1: Confectionery composition comprising a xanthine derivative and low fructose

Abstract:[411]

"Confectionery compositions comprising a candy base having improved stability are provided. Said candy base comprises one or more sugar bases, at least one edible organic acid, one or more xanthine derivatives and less than 1.9% of fructose by weight of said candy base. The confectionery compositions herein provide the stimulant effect of xanthine derivative and the energy of sugar bases without the negative aspects of opaqueness, stickiness and flavour modification".

Analysis:

According to the inventors, xanthine derivatives, in particular caffeine, seem to enhance

moisture absorption and to alter, distort or mask the taste of other flavours within the sugar based candy. Thus, a need exists for a stable sugar base candy comprising xanthine derivatives and edible organic acids.
The invention relates to confectionery products that comprise a caramel base, where said caramel comprises one or more sugars, at least one edible organic acid, one or more xanthine derivatives and more than 0.1 and less than 1.9% fructose by weight of said caramel base. Preferred xanthine derivatives are: xanthine, caffeine, theobromine and theophylline, their salts or mixtures thereof; and more preferably caffeine. The invention also discloses the manufacture method of said confectionery products.
The confectionery composition of the present invention preferably takes the form of a centre-filled candy comprising from 60% to 95%, by weight of the centre-fined candy.

Opinion:

Provided that the product developed in the present project was not intended to be formulated in the form of any confectionery product, nor formulated as a confectionery composition, which are completely different matrices and compositions than those expected in the galenic form as capsules or sachets intended to be developed in the present project, it was considered that the second document from Procter & Gamble did not affect the project's operational freedom to operate.

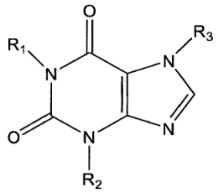
EP2258337 B1: Composition for hair

Abstract:[412]

"Present invention relates to a composition for hair comprising at least one xanthine or its derivative and at least one carboxylic acid according to the chemical structure given in the description below. Compositions of the present invention gives hair improved rigidity, grip, less elasticity and volume, but more body. Composition of the present invention can be in any form suitable for application onto hair such as in the form of a shampoo, cleansing - conditioning composition, or in the form of a conditioner used after washing hair with a cleansing composition. The latter is used either as rinse off or as leave-in conditioner".

Analysis:

Claim 1 refers to "Composition for hair characterized in that, it comprises at least one xanthine or its derivative according to the general formula



wherein R1, R2 and R3 are independent from each other H, or substituted or unsubstituted C1 to C5 alkyl at least one carboxylic acid selected from malonic acid and glyoxylic acid and at least one conditioning compound selected from oily substances, non-ionic substances, cationic amphiphilic ingredients, cationic polymers or their mixture".

According to the patent holder, the preferred xanthines are "xanthine, caffeine, theophylline and theobromine, and more preferred are caffeine, theophylline and theobromine", wherein “Particularly preferred is caffeine”.
The invention, particularly its independent claim 1, explicitly refers to the fact that said composition is used for hair. In the description section, KAO discloses that said compositions are preferably aqueous, and comprise at least 10% w/w of water. Moreover, the same section discloses that the form of application of the product is preferably in solution, dispersion, gel, emulsion, spray or foam, not mentioning any solid form thereof.

Opinion:

In light of the previous information, it was considered that KAO’s invention EP2258337 B1 did not affect the operational freedom of the present project for the development and commercialization of the products based on combinations of methylxanthines for oral administration as a food supplement or food for special medical purposes.

EP2369939 B1: Food products enriched with methylxanthines

Abstract:[413]

“A food product enriched with one or more methylxanthines is provided. The food product comprises a total amount of from 100 to 3000 milligram of methylxanthines per unit amount of the food product, and further comprises a polymeric polyphenol compound that has a molecular weight equal to or above 500 gram per mole and which is complexed with the one or more methylxanthines, wherein the weight ratio of the polymeric polyphenol compound to the one or more methylxanthines is from 10:1 to 1 :10. A method for the production of such food products is also provided”.

Analysis:

The invention relates to food products enriched with one or more methylxanthines such as theobromine, caffeine, theophylline, paraxanthine and isocaffeine, either from natural or synthetic source, the food products being in the form of cereal bar, chocolate bar, cookie, condiment, confectionery, desert, snack, spread, ice cream, dressing, mayonnaise, sauce, bakery product, shortening or cheese. The goal of fortification is to achieve the positive effects of methylxanthines and to improve the consumer's mood during and after consumption. Moreover, applicants disclose that the enrichment of food products with methylxanthines provides beneficial effects thereof when consumed as an ingredient in a food product. A final aspect of the invention is the use of a food product according to the invention to improve the mood of the person who consumes it.
In order to diminish the bitter taste profile in the food product due to the bitterness of methylxanthines, it further comprises a polymeric polyphenol compound with a molecular weight equal to or greater than 500 g/mol that forms a complex with one or more xanthines, in a ratio from 10:1 to 1:10. The origin of the polyphenol can be natural or synthetic, preferably being tannic acid.
The invention defines "food product" as a substance that can be eaten or drunk for nutritional or pleasure purposes. Pharmaceutical compositions are explicitly excluded.

Opinion:

For the purposes of this FTO analysis, Unilever's patent EP2369939 B1[413] was considered the closest document to the present project based on the development of a formulated methylxanthine-based product.
However, having considering the previous statements and the patent claims, it was concluded that the document did not affect the operational freedom for the development and commercialization of the product based on combinations of methylxanthines for oral use as a food supplement or food for special medical purpose, provided that the product was formulated in a pharmaceutical form, such as capsules, tablets, or sachets, excluding any of the matrices claimed by Unilever.
Nevertheless, to avoid any infringement of Unilever's patent, the most common food-product forms, such as a cereal bar, chocolate bar, biscuit, condiment, sweets, dessert, appetizer, spread, ice cream, dressing, mayonnaise, sauce, bakery products, butter or cheese, explicitly included in the granted claims in our patent[170] must be definitively excluded.
An additional aspect extracted from Unilever's patent was related to excipients' formulation. The technology core of Unilever's invention was the use of polymeric polyphenols with a molecular weight equal to or greater than 500 g/mol in a ratio of 10: 1 to 1:10, which form a complex with one or more xanthines in order to decrease their bitterness. Therefore, polymeric polyphenols or even different components for the same purpose must be avoided to eliminate the risk of literal infringement or infringement by equivalence of Unilever's patent, respectively, based on the exact nature of the polymeric polyphenol selected.

3.4.4. Final opinion

1. The main conclusion of the present FTO analysis was that, at least in the US, European and Spanish markets, there were no risk of infringement of any previous intellectual property rights of a third party in the production, marketing and / or distribution of a food product manufactured in the form of capsules, tablets or sachets, containing as main ingredients a mixture of methylxanthines, among which at least and preferably caffeine and theobromine.

2. Notwithstanding, there would exist the risk of certain infringement if the food product developed in the present project would be manufactured according to certain conditions and compositions, in the form of a food matrix, like chocolate matrix (as claimed in New World Entreprizes' patent[409]), confectionery product (as claimed in by Procter & Gamble's patent[411]) or cereal bar, chocolate bar, biscuit, condiment, sweet, dessert, snack, spread, ice cream, dressing, mayonnaise, sauce, bakery products, butter or cheese (as claimed in Unilever's patent[413]).

3. Unilever's patent in force EP2369939 B1[413] was considered the closest document to the present project based on the development of a formulated methylxanthine-based product, and the most relevant to take into account when designing the product formulation.

4. In this regard, it was specially recommended to avoid the use of any polymeric polyphenol compound with a molecular weight equal to or greater than 500 g/mol, and particularly, to avoid formulations with tannic acid, in the formulation of the product developed in the present project.

3.5. Selection of the dosaged product form

As previously disclosed in Chapter 2 in light of Regulation (EU) No 609/2013,[340] the formulated product containing caffeine and theobromine as main ingredients should fall under the food for special medical purposes classification, wherein "food for special medical purposes means food specially processed or formulated and intended for the dietary management of patients, including infants, under medical supervision; it is intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary food or certain nutrients contained therein, or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved by modification of the normal diet alone". However, Regulation (EU) No 609/2013 refers not only to food for special medical purposes, but also to infant formula and follow-on formula, processed cereal-based food and baby food and total diet replacement for weight control. In this regard, the single reference to the form in which these types of products can be placed in the market is "in the form of prepacked food" (Article 4.2).

On the other side, Directive 2002/46/EC,[338] defines food supplements as "foodstuffs (…) marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities".

As protected under property patents WO2016075288 A1[169] and WO2016075285 A1[170]:

- *"(…) the composition for use according to the present invention is formulated as a pharmaceutical composition, food, food ingredient or supplement, nutraceutical composition, additive for a natural product or is present in the extract of a natural product. Preferably, said composition is the form of a solid or liquid. More preferably, said composition is present in a dairy product, a beverage or cereals".*
- *(…) A composition of a "food" item (…) may in principle take any form suited for consumption by man or animal. In one embodiment the composition is in the form of a dry powder that can be suspended, dispersed, emulsified or dissolved in an aqueous liquid such as water, coffee, tea, milk, yogurt, stock or fruit juice and alcoholic drinks. To this end, the powder may be provided in unit-dosage form. In an alternative preferred embodiment the composition in the form of a dry powder is tablet. To that end, a composition for a food supplement according to the invention may very suitably be provided with fillers, such as microcrystalline cellulose (MCC) and mannitol, binders such as hydroxypropylcellulose (HPC), and lubricants such as stearic acid or other excipients. A composition of a food item or food supplement as described above may also be provided in the form of a liquid preparation wherein the solids are suspended, dispersed or emulsified in an aqueous liquid. Such a composition may be admixed directly through a food item or may e.g. be extruded and processed to grains or other shapes. In an alternative embodiment a food item or food supplement may take the shape of a solid, semi-solid or liquid food item, such as cereals, a bread, a bar, a cookie, a sandwich or a beverage, or as a spread, sauce, butter, margarine, dairy product, and the like. Preferably, the composition is included in a dairy product, such as for instance butter or margarine, custard, yogurt, cheese, spread, drink, or pudding or other dessert".*

Consequently, there were potentially different types of forms from which to select the product marketable form. Nevertheless, the freedom to operate analysis and evaluation of potential patent infringement of third parties' intellectual property rights and marketed products containing methylxanthines (see FTO section for more details) discouraged the development of the most common food-product forms, such as chocolate matrix, confectionery products, cereal bars, chocolate bars, biscuits, condiments, sweets, desserts, appetizers, spread, ice creams, dressing, mayonnaise, sauces, bakery products, butter or cheese.
Nevertheless, manufactured in the form of unit dosage form, such as the common pharmaceutical solid forms for oral administration (tablets, capsules or powders in sachets), there would be no objections for the development, since fully freedom to operate in the market could be anticipated (see next section for more details).

The European Pharmacopoeia[414] (current effective version) defines:

- Capsules:

As defined in the 0016 Monograph, "*Capsules are solid preparations with hard or soft shells of various shapes and capacities, containing a single dose of active substance(s). They are usually intended for oral administration*", wherein the capsule shells are normally made of gelatine and authorized excipients. Capsules are intended to contain solid, semi-solid or liquids, including the active substance(s) and proper excipient(s) that do not cause deterioration of the shell. Once ingested, the shell is attacked by digested fluids and the content is released.

According to the shell resistance once in the gastrointestinal tract, capsules may be hard, soft, gastro-resistant, modified-release or cachets.

- Tablets:

As defined in the 0478 Monograph, referred to oral administration, "*Tablets are solid preparations each containing a single dose of one or more active substances. They are obtained by compressing uniform volumes of particles or by another suitable manufacturing technique, such as extrusion, moulding or freeze-drying (lyophilisation). Tablets are intended for oral administration. Some are swallowed whole, some after being chewed, some are dissolved or dispersed in the water before being administered and some are retained in the mouth where the active substance is liberated*". Tablet composition includes the active substance(s) normally with excipients such as diluents, binders, disintegrating agents, glidants, lubricants, colorants, and the like.

Tablets may be uncoated, coated, gastro-resistant, modified-release, effervescent, soluble, dispersible, orodispersible, chewable or oral lyophilisates, according to the technology used for its manufacture. However, their common core technology is the compression of particle or particle aggregates produced by granulation methods.

- Oral powders:

As defined in the 1165 Monograph, "*Oral powders are preparations consisting of solid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients, and, if necessary, colouring matter authorised by*

the competent authority and flavouring substances. They are generally administered in or with water or another suitable liquid". (…) "*Each dose of a single-dose powder is enclosed in an individual container, for example a sachet or a vial*".

When considering the above oral dosage forms in manufacturing terms, capsules provided more advantages and less disadvantages than oral powders and tablets, mainly because they could more easily and less costly mask the bitter taste of methylxanthines, requiring fewer excipients and less development complexity. On the other side, dysphagia problems in some DM1 patients could somehow limit their acceptance, depending on the capsule size. This was certainly a risk that was assumed at the current stage of the project.

Therefore, the capsule was finally selected as the product unit dosage form to continue with its development.

3.6. Development of the dosaged product

This section discusses the development stages that provided the first pilot batches of the dosaged product, as prototypes of the marketable product form. The work disclosed herein was in accordance with Good Manufacturing Practices (GMPs) compliance.

Important note: In the light of the previous introduction, and in spite of being part of the technical team in charge of the entire research and development project, the GMP works disclosed in this section were not directly performed by the present author. However, for the sake of clarity within the overall project, a brief summary of the studies undertaken to manufacture the pilot batches, as well as the results obtained, are disclosed herein.

Galenic development, analytical development, method validation, preparation of pilot batches for stability and stability testing were performed at IUCT, S.A. which at that time was a Pharmaceutical Laboratory authorized by the Spanish Agency of Medicines and Medical Devices (AEMPS) for the manufacture of medicinal products and investigational medicinal products (Registry number 4155E), that held GMP certification and authorization for the compatibility of manufacturing food supplements and cosmetics in the same manufacturing area than medicinal products. Authorized activities in the GMP compliant laboratories and pilot plant comprised: preformulation and formulation studies, early development, development of the manufacturing process, validation of analytical methods according to ICH guidelines, stability testing according to ICH guidelines, manufacture of batches for clinical trials and/or for stability testing, physicochemical and microbiological analysis, preparation of the active substance master file (ASMF) dossier, preparation of the Investigational Medicinal Product Dossier (IMPD) and the like.

Led by Dr. David Miguel as the Qualified Person, the summary of procedures ran under the GMP quality guidelines, as well as the results and the conclusions achieved therein are provided below.

3.6.1. Analytical method development

The analytical methods were developed in order to assist product formulation, to support the quality control of the manufactured product as well as to monitor the stability testing.

Accordingly, an exhaustive bibliographic search was carried out for the determination of xanthines in general, and caffeine and theobromine in particular from several sources.

1. An HPLC method for the quantification of caffeine and theobromine was established using pure standard samples. Extractive methods were settled up to extract the ingredients from the natural source of *Theobroma cacao* beans dry powder. The reliability of the chromatographic method was verified by determination of linearity, detection limit and quantification limit for each methylxanthine.

2. Regarding microbiological control, the objective was to guarantee the safety of the product at the microbiological level. The European Pharmacopoeia[414] (current edition) established the corresponding specifications in chapter 5.1.8. 'Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation', criteria B for herbal medicinal products, relative to TAMC parameters (total aerobes), TYMC (moulds and yeasts), gram-negative bacteria bile -Tolerants, *Escherichia coli* and *Salmonella*.

3. Regarding the 'Uniformity of dosage units', according to the 'Dosage forms' chapter, in the Capsules section, the European Pharmacopoeia[414] (current edition) states that the determination of this parameter was deemed not necessary.

4. Regarding the 'Uniformity of content units', according to the 'Dosage forms' chapter, in the Capsules section, the European Pharmacopoeia[414] (current edition) states that this parameter must be determined only if the active substance is in amounts less than 2 mg or less to 2% of the total mass of the contents of the capsule. Since the methylxanthine capsules contained higher quantities than these levels, the determination of this parameter was deemed not requested.

3.6.2. Validation of the analytical method for methylxanthines

The HPLC analytical method to measure methylxanthine content was validated in order to ensure their reliability. Accordingly, the corresponding validation protocol was prepared, followed by experimental execution and launch of the audited report.

The main parameters under evaluation were:

1. Stability of the standard solutions and problem
2. Selectivity
3. Repeatability
4. Intermediate Precision
5. Accuracy
6. Linearity and Range

According to the results obtained therein, the method met all the validation criteria, and therefore it was considered suitable for the quantitative determination of caffeine and theobromine in the dosage product.

3.6.3. Preparation of the pilot batch for preliminary stability testing

A single batch sized 2100 units of the product in the dosage form of hard capsules was manufactured according to GMP guidelines at IUCT's Pilot Plant. The capsules were packaged into 40 jars ('Duma' high density polyethylene HDPE opaque jars) containing 50 capsules per jar.

3.6.4. Preparation of pilot batches for ICH stability testing

Three batches of the dosage product, each one containing 3600 capsules, were manufactured, each one packaged into sixty-nine jars ('Duma' HDPE jars) containing fifty capsules per jar.

During the preparation of the batches, the in-process controls corresponding to the manufactured capsules, such as the weight uniformity, were carried out. Once the process was completed, a physical-chemical and microbiological control of the finished products was performed for batch release, in order to get the stability study initiated as soon as possible. These were the time-zero analyses.

3.6.5. Stability testing

As defined in the ICH Q1A (R2) Guideline,[415] "the purpose of stability testing is to provide evidence on how the quality of the product changes along the time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the product, and recommended storage conditions".

Therefore, the stability studies on the product dosaged as capsules and packaged into HDPE jars, each jar containing fifty capsules, were performed as per the conditions disclosed in the former guideline, based on the stability protocol that included preliminary and long term conditions in the four climatic zones, as it is disclosed below.

3.6.5.1. Preliminary stability testing

For the particular case of preliminary stability testing, samples from one single batch were stored in the corresponding climatic chambers at the following temperature and humidity conditions during three months time:

- 25ºC ± 2ºC / 60% HR ± 5% HR
- 30ºC ± 2ºC / 65% HR ± 5% HR
- 30ºC ± 2ºC / 75% HR ± 5% HR
- 40ºC ± 2ºC / 75% HR ± 5% HR

At each condition, samples were analyzed at time points zero, month 1 and month 3. As a result, all the parameters analyzed met specifications up to the three months of the study.

3.6.5.2. Formal stability testing (long term and accelerated conditions)

The stability testing according to ICH conditions for climatic zones I, II, III and IV was carried out on three batches of the formulated and conditioned product, under the following conditions. For each condition, samples were analyzed at time zero, 3, 6, 9 and 12 months for long term and six month under accelerated conditions:

- 25ºC ± 2ºC / 60% HR ± 5% HR
- 30ºC ± 2ºC / 65% HR ± 5% HR
- 30ºC ± 2ºC / 75% HR ± 5% HR
- 40ºC ± 2ºC / 75% HR ± 5% HR

The physical-chemical controls were carried out according to the methods described in the internal standard operating procedures (SOPs) and in the European Pharmacopoeia.[414] The microbiological controls were carried out following the procedures described in the European Pharmacopoeia,[414] as well as it was established in the internal validation protocol and validation report prepared *ad hoc*.

Analytical data concluded that, under long term storage conditions, the analyzed parameters met the specifications for the three batches of the formulated product up to 12 months of study under the three ICH stability conditions.

Regarding the accelerated condition, the parameters analyzed met specifications for the three batches up to 6 months of study, as well.

3.6.5.3. Conclusions regarding to storage conditions and shelf-life

ICH Q1E Guideline (Evaluation for stability data) intends "to provide recommendations on how to use stability data generated in accordance with the principles detailed in the ICH guideline "Q1A(R) Stability Testing of New Drug Substances and Products" to propose a retest period or shelf life in a registration application".

In the light of ICH Q1E Guideline, when data in both the long term and also under the accelerated conditions in the stability studies render no significant changes in the tested parameters, it is possible to extend the retest period or shelf life beyond the period covered by long-term data, by extrapolation. Based on the ICH Q1E's Appendix A, Decision Tree for Data Evaluation for Retest Period or Shelf Life Estimation, and the twelve month period covered by the long term stability testing, twenty-four months retest date was proposed for the product under the climatic conditions:

Climatic zone II: subtropical and Mediterranean zone (25ºC ± 2ºC/60%HR ± 5%HR)
Climatic zone IVa: hot and humid climate (30ºC ± 2ºC/65%HR ± 5%HR)
Climatic zone IVb: hot and very humid climate (30ºC ± 2ºC/75%HR ± 5%HR)

Once the pilot batches had been developed and the stability testing results already achieved, the manufacturing process was transferred to a GMP compliant CMO (contract manufacturing organization) that scaled the process up, manufactured, quality controlled and released the first batch of the marketable product (M01), whose main quality attributes are provided in **Table 44**.

Table 44: Quality attributes of MYODM batch M01 manufactured as hard gelatine capsules

Parameter	Specifications	Result
Organoleptic characteristics	Maroon capsules nr 0 with brownish powder inside	Conforms
Mean weight entire capsule	496 mg ± 7.5%	491.8 mg
Disintegration	< 30 min	21 min
Humidity of the content	< 10%	3.93%
Caffeine content	19.2 mg/capsule ± 20%	18.9 mg/capsule
Theobromine content	33.9 mg/capsule ± 20%	36.3 mg/capsule
Total plate count (TAMC)	≤ 10.000 cfu/g	< 10.000 cfu/g
Total plate yeast and molds (TYMC)	≤ 100 cfu/g	< 100 cfu/g
Total coliforms	≤ 100 cfu/g	< 100 cfu/g
E. coli	Absence in 1 g	Absence in 1 g
Staphylococcus aureus	Absence in 1 g	Absence in 1 g
Salmonella spp	Absence in 25 g	Absence in 25 g

Subsequently, part of this batch was later tested in the interventional pilot clinical trial disclosed in Chapter 5 in a DM1 patients' cohort.

3.7. Concluding remarks

Composition was determined from a sequential screening cascade firstly on selected individual xanthines, followed by a certain designed combination of xanthines afterwards, using *in vitro* and *in vivo* models of DM1. Taking into account the regulatory constraints that the food product composition must meet, especially in the form of food supplements, and based on experimental data, caffeine and theobromine binary compositions were selected and more deeply evaluated.

The composition containing caffeine and theobromine in 1:1.85 (w/w), respectively, was selected from the proper combination of relevant inputs including: *in vitro* and *in vivo* data in DM1 models, European regulatory constraints in terms of permitted methylxanthines and permitted caffeine daily doses in humans, guidelines on the dose translation from animal model to human, as well as preliminary insights on product's unit dosage formulation.

Particularly, our results demonstrated that the selected composition promoted an increase in MBNL1 and MBNL2 protein levels in *in vitro* models, a three-fold flying capacity increase in DM1 model flies, rescuing *circa* 60-70% that of the healthy control flies, c*irca* 40% improvement in muscle area of the disease model flies and almost 85% of muscle area of non-DM1 model control, a very important rescue in almost all the parameters measured in the heart dysfunction in model flies (prolongation of the heart period, a completely repair of the percentage fractional shortening and end diastolic diameter, and a slightly decrease of arrhythmicity index, a repair in the systolic interval, which was not reached by the positive control pentamidine), and finally a 96% increase in life expectancy and a 65% average survival of dystrophic flies compared to healthy flies.

Animal to human translation approaches fulfilling regulatory and development constraints were considered to define dose, which was limited up to 80 mg caffeine/daily, the maximum daily amount permitted in humans in the most restrictive EU regulation for food supplements.

The freedom to operate analysis and evaluation of potential patent infringement of third parties' intellectual property rights and marketed products containing methylxanthines discouraged the development of the formulated food product in the form of chocolate matrix, confectionery products, cereal bars, chocolate bars, biscuits, condiments, sweets, desserts, appetizers, spread, ice creams, dressing, mayonnaise, sauces, bakery products, butter or cheese. Nevertheless, manufactured in the form of unit dosage form (tablets, capsules or powders in sachets), full freedom to operate in the market was anticipated. Once balanced pros and cons, hard gelatine capsule was selected as the product unit dosage form in the present development.

The administration schedule for dosage in humans consisted of three daily capsules of the formulated product containing *Theobroma cacao* supplemented with caffeine, equivalent to the effect of 60 mg daily caffeine and 111 mg daily theobromine.

Galenic development, analytical development, method validation, preparation of the pilot batches for stability, as well as the stability testing, were performed in house at the GMP-compliant pilot plant facilities, whereas manufacturing, quality control, analysis and release of the product launched to the market was performed by an external GMP-compliant CMO.

CHAPTER 4: OBSERVATIONAL STUDY

Chapter 4 discusses the observational nutritional study code MD Project that measures the nutritional habits, the dietary methylxanthine intake pattern and its relationship with the quality of life in a cohort of adults with DM1.

Chapter 4 fulfills specific goal nr 5.

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4.1. Introduction and purposes

Aimed at the fact that the present research study could serve as the basis for future studies or potential uses of methylxanthines in the DM1 community, it was deemed necessary to deepen in the potential relationship between the pattern of consumption of certain foods containing dietary methylxanthines and the quality of life of people living with DM1.

Accordingly, the research project "Observational study on the quality of life and eating habits in patients with Myotonic Dystrophy type 1 (DM1)", MD Project, was conceived as an observational single cohort study in which each patient would be evaluated based on the results of the SF-36 quality of life health questionnaire and a survey on eating habits and frequency of consumption.

The main objective of the present study was to evaluate the quality of life of participants affected by DM1 and to collect information about their eating habits, physical activity, medications and general health conditions, in order to establish and evaluate relationships between diet and quality of life. Particularly, the present research was devoted to establish and evaluate relationships and/or correlations between the consumption of dietary methylxanthines found in coffee, tea, chocolate and/or soft drinks and the quality of life of the participants in the study.

The Short Form-36 Health Survey (SF-36) was considered as an ideal instrument for assessing the Health-Related Quality of Life (HRQoL) in DM1, since it is a quick instrument, easy to fill in, with validation studies in Spain, and instructions written in Spanish.

The SF-36 Health Survey aims to measure eight generic concepts about health, both positive and negative physical health and emotional states, that is, concepts that are not specifically related to a concrete pathology, treatment group or age.

The SF-36 Health Survey is based on 36 questions that address different aspects related to the daily life of the person completing the questionnaire. The questions are grouped and measured in eight sections that are independently valued, rendering the eight dimensions (or health concepts): physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. In addition to the eight health concepts, the SF-36 includes the general concept of changes in the perception of the current state of health and in that of the previous year. Two summary scores related to mental and physical components were additionally included to facilitate data processing. By allowing numerical assessment of different aspects of the individual health, the SF-36 Health Survey is an excellent tool for any research related to health in general, and quality of life in particular.

4.2. Study goals and Protocol

The main goals of the observational research project 'Observational study on the quality of life and eating habits in patients with Myotonic Dystrophy type 1 (DM1)', hereinafter MD Project, were to assess the health outcomes quality of life and eating habits in participants with myotonic dystrophy type 1 and to deepen in the consumption pattern of dietary methylxanthines in this group of population.

The final objective of the present observational study was to evaluate the pattern of consumption, potential relationship and impact of dietary methylxanthines in the quality of life of the subjects affected by DM1.

There was neither control group nor follow-up of the participants once they responded to the MD Project survey.

The Patient Informed Consent form and the MD project survey are provided in Annexes 1 and 2, respectively.

Study title

Observational study on the quality of life and eating habits in patients with Myotonic Dystrophy type 1 (DM1)

Protocol code: MD 001

Acronym: MD Project

Study population

The study population was made up of patients older than 18 years of age (both men and women) diagnosed with myotonic dystrophy type 1 (DM1), who met the inclusion and exclusion criteria of this research project.

Design

It was a single cohort study in which each participant responded to the SF-36 quality of life test and a food consumption survey. There were no follow-up visits.

Inclusion and Exclusion criteria

Inclusion criteria:

1) Adult patients, men and women older than 18 years of age diagnosed with DM1
2) Patients who have given their informed consent to voluntarily participate in the study
3) Patients who do not present significant cognitive impairment

Exclusion criteria:

1) Subjects not being diagnosed with DM1
2) Patients who do not sign the informed consent to participate in the study
3) Under-age, men and women less than 18 years

Selection of participants and recruitment

The recruitment process was done on a voluntary basis among adult patients affected by DM1 who demonstrated the willingness to participate in the study, who met all the inclusion requirements, and who were part of the patient associations or direct patients of the specialists or any other patient who were interested in participating in the study.

All participants were widely informed on the benefits of this research survey, as well as the disadvantages of participating thereof. No patient was recruited without understanding the study and signing the informed consent.

Data collection and management

Once the volunteer accepted to participate in the study, the interviewer directly conducted the interview with the participant. Eventually, the participant's caregiver could respond on behalf of the participant. Eventually, the interviewer could travel to the participant's home.

All the data of each patient was collected. In order to maintain anonymity, the data was collected and anonymized using a code that was only available to the study manager.

Data analysis

The primary endpoint was health-related change in quality of life, as measured by the SF-36 scale.

Independent variables of the study were:

1) Weight (kg)
2) Age (years)
3) Consumption pattern of certain food and beverages containing methylxanthines

Statistical analysis

The data was collected in the corresponding questionnaires and stored in a database to carry out the statistical analysis using appropriate statistical packages.

The proposed statistical analysis methods shown below is a synthesis of the methods to be used on the data collected, in order to respond to the objectives of the study.

In the descriptive analysis, the distribution of each variable was analyzed, checking that all the data had values within the expected range, and that there were no logical inconsistencies between variables, or impossible data. If any of these exist, the information registered in the database was corrected by comparing the information with that of the original filled-in questionnaires.

As a result of the descriptive analysis, the estimators of the variables were obtained using the most appropriate statistic for the nature and scale of measurement of each one. The absolute and relative frequency distributions of the qualitative or categorical variables were presented, as well as the measures of central tendency and dispersion (mean, standard deviation, median, interquartile range) of the quantitative variables. The fulfilment of normality and homogeneity

were confirmed per each variable entering the analysis. Accordingly, the statistics mean ± standard deviation summarizes results for parametric variables, whereas median ± interquartile range summarizes results for non-parametric variables. The appropriate statistics are expressed in **bold** in the tables of results along this chapter.

The results obtained in the descriptive study were then used as part of an exploratory study wherein correlations among measured variables were analyzed.

Finally, the subjects could be grouped based on the independent variables in order to analyze whether the independent variables could have a significant effect on the results of the study.

Throughout the study, 95% confidence was considered when determining statistical significance (p=0.05).

For the analysis of the main goal, descriptive analysis (measures of central tendency and dispersion) of the scores of the questionnaire domains and the global score was carried out, as well as the 95% confidence intervals were calculated. The existence of statistically significant differences between comparison groups were studied using parametric (unpaired t-test) or non-parametric (Mann-Whitney's U test) analysis, depending on the distribution of the sample. Correlation between variables was determined using parametric (Pearson) or non-parametric (Spearman) correlation tests.

Sample size

Given the nature of the observational study, the minimum sample size of the study was defined in 30 patients, for statistically significance of the results.

Ethical issues

The study was carried out in accordance with the requirements expressed in the Declaration of Helsinki (revision of Fortaleza, October 2013) as well as the legislation in force in Spain relating to carrying out observational studies and strict compliance with Organic Law 3/2018 of December 5, on the Protection of Personal Data and Guarantee of Digital Rights. Further details had been previously disclosed in the Methodology section.

The study was carried out in accordance with the protocol, which was submitted to the Ethics Committee of the *Fundació Catalana d'Hospitals* for its evaluation and approval. The treatment, communication and transfer of personal data of all participating subjects had complied with the provisions in strict compliance with Organic Law 3/2018 of December 5, Protection of Personal Data and Guarantee of Digital Rights. As general considerations, all the parties involved in this study, sponsor and researchers accepted the national and international ethical standards on research.

Confidentiality

The treatment of personal data required in this study was governed by Organic Law 3/2018 of December 5, on the Protection of Personal Data and Guarantee of Digital Rights.

The study data was entered and processed in a file owned by the sponsor that was treated in accordance with that mentioned in Organic Law 3/2018 of December 5, on Protection of Personal Data and Guarantee of Digital Rights, exclusively for the development and good end of the study.

The data collected during the course of the study was identified by means of a numerical code to guarantee the confidentiality of the patient's identity.

4.3. Patient-reported outcome measures

The primary outcome in a clinical trial is the most important issue for evaluating the effect of an observation, intervention or treatment. However, additional secondary outcome measures may be additionally included for evaluating further aspects of the study that could render interesting information. Both primary and secondary outcomes may be a single or several measures.

The selected outcome measure in the MD Project observational study was the health related quality of life SF-36, as disclosed below.

Primary Outcome Measure: Individualized Short Form-36 (SF-36) Health Questionnaire

Scores from the self-administered SF-36 questionnaire were measured per each participant. Mean scores range from 0 (minimum) - 100 (maximum) with higher mean scores reflecting better outcomes.

The SF-36 Health Survey[416,417] is a generic HRQoL survey designed by the Health Institute, New England Medical Center, Boston (Massachusetts), which, based on 36 questions, aims to measure eight generic concepts about health, that is, concepts that are not specific to health, a pathology, treatment group or age, detecting both positive and negative states of physical and emotional health. The Short Form-36 (SF-36) is acknowledged as the gold standard generic health status measure, with an ‘energy and vitality’ dimension.

These 36 questions address different aspects related to the daily life of the person completing the questionnaire. They are grouped and measured in eight sections that are independently valued and give place to eight dimensions (health concepts), divided into two health domains: physical and emotional, wherein each domain is scored on a 0 to 100 scale, higher scores indicating a better perception of health/functioning:

Physical health concepts domain:

PF: Limitations in physical activities because of health problems
RP: Limitations in usual role activities because of physical health problems
BP: Bodily pain
GH: General health perceptions

Mental health concepts domain:

VT: Vitality (energy and fatigue)
SF: Limitations in social activities because of physical or emotional problems (SF)
RE: Limitations in usual role activities because of emotional problems
MH: General mental health (psychological distress and well-being)

In addition to the eight health concepts, the SF-36 includes the general concept of changes in the perception of the current state of health and in that of the previous year.[417] **Table 45** schedules the structure of items, scales and measures of the SF-36 questionnaire. Concrete items are aggregated into eight scales, wherein concrete scales aggregate into two distinct higher-ordered clusters related to the physical and mental health components.
The Spanish version of the questionnaire has been properly validated[418] and reviewed.[419] Accordingly, this version was deemed more convenient for the study participants in order to facilitate its comprehension.

Table 45: Structure of measurement model in SF-36 (adapted from Ware and Kosinski[420])

Items		Scales	Summary measures
3a	Vigorous activities	Physical functioning (PF)	Physical Health
3b	Moderate activities		
3c	Lift, carry groceries		
3d	Climb several flights of stairs		
3e	Climb one flight of stairs		
3f	Bend, Kneel		
3g	Walk mile		
3h	Walk several blocks		
3i	Walk one block		
3j	Bathe, Dress		
4a	Cut Down Time	Role-Physical (RP)	
4b	Accomplished Less		
4c	Limited in Kind		
4d	Had difficulty		
7	Pain-Magnitude	Bodily Pain (BP)	
8	Pain-Interfere		
1	EVGFP Rating	General Health (GH)	
11a	Sick Easier		
11b	As Healthy		
11c	Health to Get Worse		
11d	Health Excellent		
9a	Vitality	Vitality (VT) or Energy/fatigue (E/F)	Mental Health
9e	Energy		
9g	Worn Out		
9i	Tired		
6	Social-Extent	Social Functioning (SF)	
10	Social-Time		
5a	Cut Down Time	Role-Emotional (RE)	
5b	Accomplished Less		
5c	Not Careful		
9b	Nervous	Mental Health (MH)	
9c	Low morale		
9d	Peaceful		
9f	Blue/Sad		
9h	Happy		

4.4. Ethics Committee Approval

The official communication from the Ethics Committee in Clinical Research from the *Fundació Unió Catalana d'Hospitals (Fundació La Unió)* on the approval of the Protocol code MD 001, and title '*Estudio observacional de calidad de vida y hábitos alimentarios en pacientes con Distrofia Miotónica tipo 1 (DM1)',* Observational study on the quality of life and eating habits in patients with Myotonic Dystrophy Type 1 (DM1), was received in July 2017 under approval code CEI 17/50.

4.5. Participants enrolment and study execution

The study population consisted in 38 patients, both men and women, adults aged above 18 years and diagnosed with DM1, that were recruited between 2017 and 2018 mainly from the Neuromuscular Patient Associations BENE (http://www.asociacionbene.com/), Arene (http://arene.es/es/) and ASEM (https://asemcatalunya.com/). All the patients met the inclusion and exclusion criteria of this research project, as previously disclosed.

Each participant was uniquely identified by a Patient Identification (ID) and anonymized from ID01 to ID38. No further follow-up was performed after data collection.

4.6. Study results

4.6.1. General data

A total of 38 subjects aged from 18 to 69 years old (mean age, 45±14 years) voluntarily participated in this study. Female represented 66% whereas male represented 32%. One participant didn't respond to the gender question, and therefore his/her responses are referred from now as those from the 'not disclosed gender'.

All participants had a previous diagnosis of DM1, and a mean 405±261 $(CTG)_n$ repeat count. Congenital myotonic dystrophy was the phenotype in 13 out of 38 participants (34%), childhood/juvenile onset in 4 out of 38 participants (11%), adult onset in 18 out 38 participants (47%), whereas non-respondents account for 3 out of 38 participants (8%). Confirmation by genetic test was present in 34 out of 38 participants (90%). General data for all participants in the study is disclosed in **Table 46**.

Table 46: Summary of cases: General data for all patients, divided according to gender

				ID	Age (years)	Weight (kg)	Height (m)	BMI (kg/m^2)	BMI Class	CTGn (n)
Gender	**Male**	1		01	.	69	1.70	23.88	NW	.
		2		08	36	66	1.74	21.80	NW	.
		3		10	69	82	1.72	27.72	OW	75
		4		11	68	67	1.71	22.91	NW	154
		5		16	42	42	1.70	14.53	UW	.
		6		17	42	89	1.79	27.78	OW	.
		7		18	37	96	1.77	30.64	Obese class I	.
		8		22	39	72	1.65	26.45	OW	667
		9		25	42	92	1.83	27.50	OW	.
		10		26	42	85	1.75	27.80	OW	.
		11		32	42	73	1.86	21.10	NW	.
		12		36	26	71	1.72	24.00	NW	.
		Total	N	12	11	12	12	12	12	3
			Mean	-	**44**	**75**	**1.74**	**24.67**	-	**298**
			SD	-	**13**	**15**	**0.06**	**4.30**	-	**321**
	Female	1		02	.	76	1.50	33.78	Obese class I	.
		2		03	.	55	1.52	23.81	NW	.
		3		04	.	62	1.50	27.56	OW	.
		4		06	.	71	1.60	27.73	OW	.
		5		07	40	70	1.64	26.03	OW	.
		6		09	.	78	1.69	27.31	OW	500
		7		12	51	80	1.65	29.38	OW	.
		8		13	52	52	1.56	21.37	NW	.
		9		14	44	72	1.53	30.76	Obese class I	.
		10		15	37	59	1.62	22.48	NW	.
		11		19	69	70	1.56	28.76	OW	.
		12		20	48	59	1.72	19.94	OW	333
		13		21	64	70	1.52	30.30	Obese class I	.
		14		23	53	93	1.79	29.03	OW	.
		15		24	21	55	1.70	19.03	NW	700
		16		27	59	.	1.58	.	.	.
		17		28	31	42	1.55	17.50	UW	.
		18		29	64	.	1.52	.	.	.
		19		30	18	40	1.53	17.10	UW	.
		20		31	62	72	1.58	28.80	OW	.
		21		33	45	62	1.60	24.20	NW	.
		22		34	38	61	1.67	21.90	NW	.
		23		35	29	47	1.58	18.80	NW	.
		24		37	47	62	1.55	25.80	OW	.
		25		38	50	42	1.42	20.80	NW	.
		Total	N	25	20	23	25	23	23	3
			Mean	-	**46**	**63**	**1.59**	**24.88**	.	**511**
			SD	-	**14**	**13**	**0.08**	**4.72**	.	**183**
	Not disclosed	1		05	.	58	1.56	23.83	NW	.
		Total	N	1	-	1	1	1	1	-
			Mean	-	-	58	1.56	23.83	-	-
			SD	-	-	-	-	-	-	-
	Total	N		38	31	36	38	36	36	6
		Mean		-	**45**	**67**	**1.63**	**24.78**	.	**405**
		SD		-	**14**	**15**	**0.10**	**4.45**	.	**261**

Notes: UW: underweight, NW: normal weight, OW: overweight

Body mass index

Overweight was the most frequent body mass index classification in the majority of the participants, accounting for 15 out of 38 participants (40%), closely followed by normal weight, present in 14 out of 38 participants (37%). Underweight (8% participants) and obesity-type I (10% participants) were also present in the participant set. BMI distribution according to gender is shown in **Figure 42**.

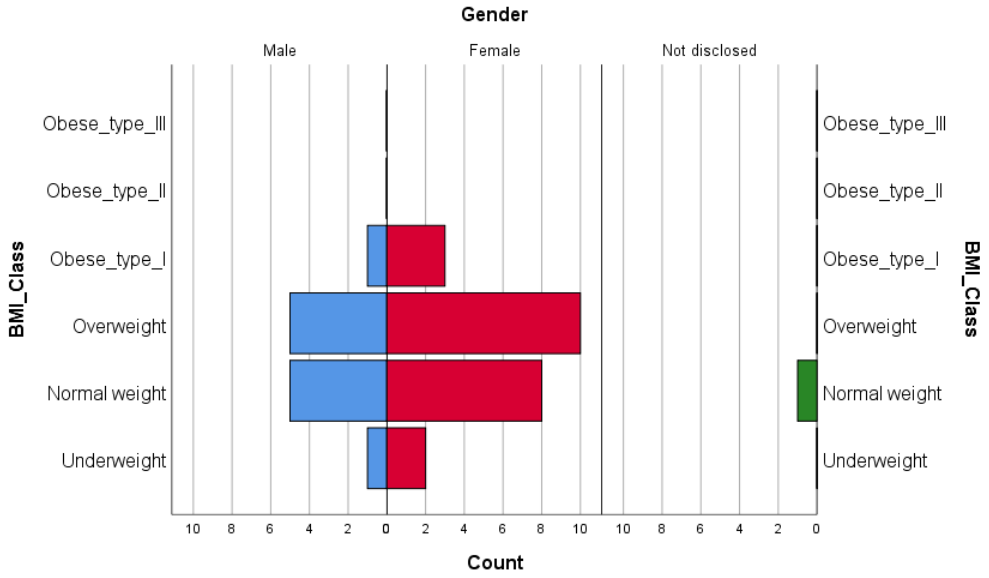


Figure 42: BMI distribution among study participants, according to gender.

These results are aligned with the studies already disclosed in Chapter 1, wherein overweight accounted for 47-60%, obesity from 10-50% and underweight from 13-15% in the DM1 population, as discussed.

Smoking habits and drugs

When asked about smoking habits either cigarette, cigars or pipe, 5 out of 38 participants (13%) reported smoking, 19 out of 38 participants (50%) reported not smoking, and 14 out of 38 participants (37%) declined to respond. Among smokers, daily mean units were 3.3±1.9.

No participant declared to take drugs, 24 out of 38 participants (63%) declared not to take drugs, and 14 out of 38 participants (37%) didn't respond to this question.

Pharmacological treatments and complementary treatments

Participants were asked about the pharmacological treatments they were using to treat myotonia, excessive daytime sleepiness and fatigue, gastrointestinal distress and cardiovascular control. In all cases, the majority of participants reported not to be under active therapeutic treatment for the above-mentioned DM1 symptoms. Summary of results is disclosed in **Table 47**.

Table 47: Pharmacological treatments to manage DM1 symptoms

Symptom	Use of pharmacological treatment		
	Yes	No	NR
Myotonia	6 (16%)	29 (76%)	3 (8%)
Excessive daytime sleepiness and fatigue	8 (21%)	27 (71%)	3 (8%)
Gastrointestinal distress	2 (5%)	32 (84%)	4 (11%)
Cardiorespiratory control	10 (26%)	26 (68%)	2 (6%)

NR: No response

Based on these results, the majority of patients did not use any pharmacological treatment for these DM1 symptoms, even though they are disclosed as the main factors that affect the quality of life of those living with DM1.

Moreover, participants were asked about complementary (non pharmacological) treatments. Again, the majority of participants reported not to be under complementary treatments to manage DM1 symptoms. **Table 48** summarizes these results.

Table 48: Complementary treatments to manage DM1 symptoms

Complementary treatments	Use of complementary treatment		
	Yes	No	NR
Vitamins and minerals	6 (16%)	28 (74%)	4 (10%)
Nutraceuticals	7 (18%)	16 (42%)	15 (40%)
Homeopathy	5 (13%)	27 (71%)	6 (16%)
Physiotherapy	13 (34%)	20 (53%)	5 (13%)

4.6.2. Nutritional habits

Most of the participants (87%) revealed not to follow any special diet, in front of 8% that did follow some special diet. Among food diet followers, 1 out of 3 participants reported to follow a low triglyceride diet, whereas no information was available for the rest of food diet followers.

The survey contained a special section in which participants were invited to disclose their particular pattern of consumption of several types of food and beverages, which were divided into the following categories:

- C.2. Cereals and derivatives
- C.3. Milk and derivatives
- C.4. Sugar and derivatives
- C.5. Oils and fats
- C.6. Vegetables and greens
- C.7. Legumes
- C.8. Fruits
- C.9. Eggs
- C.10. Meats and meat products

- C.11. Fish
- C.12. Mollusks
- C.13. Others (including chocolate products)
- C.14. Juices
- C.15. Coffee and derivatives
- C.16. Tea and derivatives
- C.17. Energy drinks
- C.18. Soft drinks
- C.19. Alcoholic beverages

The frequency of consumption per each category was divided into:

- Servings per day
- Servings per week
- Servings per month
- Serving size (small, medium, large)
- Number of units

Once the information per participant and category was compiled, the frequency of consumption expressed as servings per unit of time was determined from the information provided per participant at each category. For those categories in which recommended frequency of consumption should be expressed as 'servings per day', the information for participants was extracted either directly from 'servings per day' responses, and/or 'servings per week', divided into seven days and/or 'servings per month', divided into thirty days. In the cases where numeric discrepancies in the conversion from different frequencies arose, the minor frequency was taken.

Similarly, for those categories in which recommended frequency of consumption should be expressed as 'servings per week', the information for participants was extracted either directly from 'servings per week' responses, and/or 'servings per day', multiplied per seven days and/or 'servings per month', divided into four weeks. In the cases where numeric discrepancies in the conversion from different frequencies arose, the minor frequency was taken.

These conversions explain why in many cases, 'servings per day' and 'servings per week' are expressed as decimals numbers, and not as integer numbers.

The detailed intakes extracted from the participants' own responses in the food survey enclosed in the MD project protocol are detailed in the tables below, divided into genders. Servings per day's categories are expressed first, followed by servings per week's categories.

Table 49 to **Table 52** disclose results from those categories in which concrete serving frequency recommendations have been published. For comparison purposes, tables include the reference values for each category, as extracted from the nutritional guidelines for people living with neuromuscular diseases published in 2018 by the Spanish Federation of Neuromuscular Diseases (ASEM), with the scientific endorsement of the Spanish Society of Endocrinology and Nutrition (SEEN),[244] already disclosed in Chapter 1 (**Table 17**).

Table 49: Summary of cases. Pattern of consumption of selected groups of food and beverages. Males responses.

			ID	Rice, bread, pasta servings/day	Vegetables servings/day	Fruit servings/day	Olive oil servings/day	Milk, dairy servings/day	Fish servings/week	Lean meats, eggs servings/week	Legumes servings/week	Nuts servings/week
Gender Male	1		01	2.9	0.4	3.7	1.0	1.0	2.3	5.0	4.0	6.0
	2		08	6.0	0.0	0.2	2.0	4.0	3.0	4.0	0.0	0.0
	3		10	0.4	1.3	1.2	1.0	3.0	17.5	6.0	6.0	3.0
	4		11	.	.	.	.	.	.	.	.	.
	5		16	4.3	1.1	2.3	1.0	2.0	5.3	1.0	3.0	0.3
	6		17	3.3	2.0	0.0	2.0	0.0	0.0	0.0	1.0	0.0
	7		18	2.6	0.9	0.2	1.0	3.2	4.0	4.0	2.0	1.0
	8		22	0.0	2.0	5.0	3.0	1.0	6.0	7.0	9.0	7.0
	9		25	4.5	1.8	2.6	2.0	3.0	6.3	4.5	5.5	6.0
	10		26	4.3	1.9	2.4	0.7	1.0	0.5	2.3	5.3	0.0
	11		32	6.9	4.9	3.3	1.0	2.0	8.8	10.5	6.8	8.0
	12		36	5.6	1.0	0.6	2.0	2.3	3.0	10.0	0.0	2.5
	Total		12	11	11	11	11	11	11	11	11	11
		Mean	-	**3.7**	1.6	**1.9**	1.5	**2.0**	5.1	**4.9**	**3.9**	3.1
		SD	-	**2.2**	1.3	**1.6**	0.7	**1.2**	4.8	**3.3**	**2.9**	3.1
		Median	-	4.3	**1.3**	2.3	**1.0**	2.0	**4.0**	4.5	4.0	**2.5**
		IQR	-	3.0	**1.0**	3.1	**1.0**	2.0	**4.0**	4.8	5.0	**6.0**
ASEM/SEEN recommendations[244]			-	**4 - 6**	**≥ 2**	**≥ 3**	**3 - 6**	**2 - 4**	**4 - 5**	**4- 5**	**3- 4**	**3- 7**

Missing values for participant ID11

Table 50: Summary of cases. Pattern of consumption of selected groups of food and beverages. Females responses

			ID	Rice, bread, pasta servings/day	Vegetables servings/day	Fruit servings/day	Olive oil servings/day	Milk, dairy servings/day	Fish servings/week	Lean meats, eggs servings/week	Legumes servings/week	Nuts servings/week
Gender	**Female**	1	02	1.0	5.1	0.7	1.0	5.7	3.8	4.0	2.3	3.8
		2	03	5.1	4.3	0.1	4.0	0.3	0.0	6.0	1.0	0.0
		3	04	3.6	1.8	0.3	1.0	3.0	3.0	3.0	5.0	0.0
		4	06	3.0	0.3	2.1	1.0	3.0	5.0	6.0	4.0	5.0
		5	07	4.0	.0	0.9	3.0	2.0	9.0	5.0	0.0	0.0
		6	09	1.0	0.7	0.7	0.4	1.6	3.0	3.0	1.3	0.0
		7	12	0.3	0.4	1.0	2.0	2.0	1.0	2.0	0.8	7.0
		8	13	2.7	1.8	1.4	1.0	2.3	2.5	5.8	3.3	3.0
		9	14	2.1	1.6	5.5	1.0	1.0	7.5	9.0	0.5	0.0
		10	15	2.3	0.8	0.7	2.0	0.4	2.5	3.0	0.0	0.0
		11	19	.	.	.	.	.	.	.	.	.
		12	20	.	.	.	.	.	.	.	.	.
		13	21	0.3	3.3	5.0	1.0	2.0	4.5	3.0	1.5	0.0
		14	23	0.6	0.1	0.0	1.0	0.0	0.0	0.0	0.0	0.0
		15	24	0.0	4.5	3.8	2.0	3.0	3.0	2.0	3.0	14.0
		16	27	4.7	2.8	3.1	2.0	3.3	5.0	7.5	3.0	2.5
		17	28	5.3	0.6	1.1	1.0	2.3	3.0	9.5	3.0	2.0
		18	29	2.3	2.5	5.3	2.0	3.0	8.5	6.3	2.0	0.0
		19	30	1.4	0.8	0.6	3.0	0.0	5.8	6.0	2.0	0.0
		20	31	1.1	1.2	2.3	1.0	1.0	5.0	3.5	1.8	0.0
		21	33	6.3	3.3	4.4	2.0	2.4	4.5	10.0	3.0	2.0
		22	34	3.3	2.9	2.9	1.0	1.7	4.8	8.0	2.0	0.0
		23	35	3.3	0.5	2.7	1.0	4.3	3.5	6.0	2.0	0.0
		24	37	4.6	7.1	3.2	3.0	1.0	5.0	6.5	5.0	0.8
		25	38	7.3	2.0	2.8	2.0	2.4	6.5	4.5	1.0	8.0
	Total	N	25	23	23	23	23	23	23	23	23	23
		Mean	-	**2.9**	2.1	**2.2**	1.7	**2.1**	**4.2**	**5.2**	**2.1**	2.1
		SD	-	**2.0**	1.8	**1.7**	0.9	**1.4**	**2.3**	**2.6**	**1.4**	3.5
		Median	-	2.7	**1.8**	2.1	**1.0**	2.0	4.5	5.8	2.0	**0.0**
		IQR	-	3.6	**2.7**	2.5	**1.0**	2.0	2.0	3.5	2.0	**3.0**
ASEM/SEEN recommendations[244]			-	**4 - 6**	**≥ 2**	**≥ 3**	**3 - 6**	**2 - 4**	**4 - 5**	**4- 5**	**3- 4**	**3- 7**

Missing values for participants ID19 and ID20

Table 51: Summary of cases. Pattern of consumption of selected groups of food and beverages. Not disclosed gender's responses

			ID	Rice, bread, pasta servings/day	Vegetables servings/day	Fruit servings/day	Olive oil servings/day	Milk, dairy servings/day	Fish servings/week	Lean meats, eggs servings/week	Legumes servings/week	Nuts servings/week
Gender	**Not disclosed**	1	05	1.3	2.0	3.1	1.0	5.0	4.0	3.0	5.0	0.0
	Total	N	1	1	1	1	1	1	1	1	1	1
		Mean	-	1.3	2.0	3.1	1.0	5.0	4.0	3.0	5.0	0.0
		SD	-	-	-	-	-	-	-	-	-	-
		Median	-	1.3	2.0	3.1	1.0	5.0	4.0	3.0	5.0	0.0
		IQR	-	-	-	-	-	-	-	-	-	-
ASEM/SEEN recommendations[244]			-	**4 - 6**	**≥ 2**	**≥ 3**	**3 - 6**	**2 - 4**	**4 - 5**	**4 - 5**	**3 - 4**	**3 - 7**

Table 52: Summary of cases. Pattern of consumption of selected groups of food and beverages. All participants' responses

			N	Rice, bread, pasta servings/day	Vegetables servings/day	Fruit servings/day	Olive oil Servings/day	Milk, dairy servings/day	Fish servings/week	Lean meats, eggs servings/week	Legumes servings/week	Nuts servings/week
Gender	**All participants**	N	38	35	35	35	35	35	35	35	35	35
		Mean	-	**3.1**	1.9	2.1	1.6	**2.1**	4.5	**5.1**	2.7	2.3
		SD	-	**2.1**	1.6	1.7	0.8	**1.4**	3.2	**2.8**	2.2	3.4
		Median	-	3.0	**1.8**	**2.3**	**1.0**	2.0	**4.0**	5.0	**2.0**	**0.3**
		IQR	-	3.5	**2.2**	**2.5**	**1.0**	2.0	**2.8**	3.5	**3.0**	**3.8**
ASEM/SEEN recommendations[244]			-	**4 - 6**	**≥ 2**	**≥ 3**	**3 - 6**	**2 - 4**	**4 - 5**	**4 - 5**	**3 - 4**	**3 - 7**

Missing values for participants ID11, ID19 and ID20

The participants in this study did not reach the recommended four to six daily servings of the main carbohydrate sources (mean consumption 3.1±2.1 servings/day), being males mean consumption (3.7±2.2 servings/day) slightly higher than females mean consumption (2.9±2.0 servings/day).

Regarding the more than two daily servings recommendation for vegetable consumption, females revealed to consume a similar quantity (1.8±2.7 servings/day) than males (1.3±1.0 servings/day), with a general consumption pattern (1.8±2.2 servings/day) below the recommendation level.

In a similar way, participants' fruit consumption did not meet the more than three daily serving's recommendations as well, neither the entire cohort (2.3±2.5 servings/day), nor males individually (1.9±1.6 servings/day) or females (2.2±1.7 servings/day).

Taking all together, vegetables and fruit daily consumption in the MD Project study population (4.1±4.7 total servings/day) did not reach either the five portions recommended daily by WHO, as previously introduced in Chapter 1. Since fruit and vegetables are valuable sources of vitamins, the low intake levels determined herein might contribute in this study cohort to the vitamin deficiency that has been reported in the DM1 community.[271]

Recommendations for daily olive oil intake are three to six daily servings for people living with neuromuscular diseases.[244] In spite of the relevance of olive oil in the Mediterranean diet, MD Project participants did not meet the daily recommendations for this fat source. In fact, olive oil consumption among the entire cohort accounted for one serving per day (1.0±1.0 servings/day), and exactly the same values when considering males and females individually.

Notwithstanding, consumption of daily milk and dairy products, fish and lean meats and eggs did properly fulfil the guidelines from ASEM/SEEN[244] for each corresponding category.

In this regard, mean milk and dairy accounted for 2.1±1.4 servings/day in the entire cohort, divided into 2.0±1.2 servings/day for males and 2.1±1.4 servings/day for males, which even in the lower part of the range met the two to four daily servings' guidelines. As good sources of the calcium micronutrient, which is a critical micronutrient for those people living with neuromuscular diseases,[263] the daily intake of milk and dairy products should be even more encouraged within the DM1 community.

Due to its relevance in the maintenance of muscular mass, which is a key target for diets in neuromuscular diseases,[244] the pattern of consumption of the main protein sources in the present study, either from animal or plant-based categories, deserves special attention.

Fish intake in the entire cohort met the four to five weekly recommended servings (4.0±2.8 servings/week), with similar results for males (4.0±4.0 servings/week) and females (4.2±2.3 servings/week). Beyond the mean values, 19 out of 35 responders (54%), consumed more than four fish servings weekly, although only 9 out of 35 responders (26%) properly met the four to five weekly fish servings' recommendation, whereas 5 out of 35 responders (14%) revealed to consume one or less than one fish weekly servings.

Regarding lean meats and eggs, their relevance in this special population diet was also observed in the participants' results. The entire cohort was revealed to meet the four to five weekly recommended servings (5.1±2.8 servings/week), led by females (5.2±2.6 servings/week), and closely followed by males (4.9±3.3 servings/week). Up to 23 out of 35 participants (66%) revealed to consume more than four lean meats and eggs weekly servings. Particularly, 16 out of 35 participants' intake (46%) was even above the five weekly recommended servings, whereas 11 out of 35 participants (31%) definitively did not reach the recommendations. Only 3 out of 35 responders (8%) revealed to consume one or less than one lean meat or eggs weekly servings.

As plant-based protein sources, four to five weekly servings are recommended for legumes' intake. The entire cohort revealed not to meet the lowest recommended threshold (2.0±3.0 servings/week), mainly as a consequence of the females consumption habits (2.1±1.4 servings/week), whereas males values showed a higher trend (3.9±2.9 servings/week). In this regard, 7 out of 11 males (64%) revealed to consume more than four legumes' weekly servings in front of 8 out of 23 females (35%). On the other side, 11 out of 35 participants (31%) revealed to consume one or less than one weekly serving.

Accordingly, the participants in the MD Project seemed to have in good consideration the nutritional recommendations on the consumption of protein and its relevance for the maintenance of muscular mass in those affected by neuromuscular diseases. However, animal origin over plant-based sources was clearly preferred.

Last but not least, regarding nuts' consumption, three to seven weekly servings are recommended according to the mentioned guidelines. However, nuts seemed to be scarcely consumed in the entire study cohort (0.3±3.8 servings/week). These results were mainly conditioned due to the females group results (0.0±3.0 servings/week), whereas males revealed to be more habitual in their consumption (2.5±6.0 servings/week). Nuts' intake may be limited due to dysphagia and swallowing difficulties in DM1 participants.

Taken all together, the analysis on the intake habits of the entire cohort in the observational study MD Project revealed that rice, pasta, bread, vegetables, fruits, legumes, olive oil and nuts did not meet the recommended frequency of consumption on these food groups in people affected by neuromuscular diseases, as published by the Spanish Federation of Neuromuscular Diseases with the scientific endorsement of the Spanish Society for Endocrinology and Nutrition.[244] On the other side, the study revealed that for the food group's milk and dairy products, fish and lean meats and eggs, the frequency of consumption did properly fulfilled and even exceed the above-mentioned guidelines, suggesting that, at least in this cohort population, proper protein intake was considered a relevant asset in their nutritional habits.

Beyond the positively recommended food groups and results already discussed, the observational study compiled information on food categories for which only occasional and moderately consumption is recommended, mainly due to the fat, sugars and alcoholic content. **Table 53** to **Table 56** detail these results.

Table 53: Summary of cases. Pattern of consumption of selected groups of food and beverages with moderate to occasional recommended consumption. Males responses

			ID	Sausages[1]	Sweets, ice cream, pizza	Juices[2]	Soft drinks[3]	Alcoholic drinks
				servings/week				
Gender Male		1	01	2.0	6.0	1.3	0.0	0.5
		2	08	2.0	8.0	0.5	0.5	0.0
		3	10	2.6	0.5	5.0	5.0	7.5
		4	11	.	.	.	.	.
		5	16	1.0	0.3	0.0	2.0	0.0
		6	17	0.0	0.0	0.0	0.0	0.0
		7	18	0.0	0.5	7.0	0.0	0.0
		8	22	0.0	17.0	0.0	7.0	0.0
		9	25	4.1	2.0	0.0	6.3	3.8
		10	26	7.0	2.3	7.0	8.0	0.0
		11	32	3.0	3.0	2.5	5.5	10.5
		12	36	5.0	16.0	1.0	13.8	2.0
	Total	N	12	11	11	11	11	11
		Mean	-	**2.4**	5.0	2.2	**4.4**	2.2
		SD	-	**2.3**	6.2	2.8	**4.4**	3.6
		Median	-	2.0	**2.3**	**1.0**	5.0	**0.0**
		IQR	-	4.1	**7.5**	**5.0**	7.0	**3.8**

Missing values for participant ID11

Notes:

1. Sausages include sausages, tripe and visceras.
2. 'Juices' category includes only commercially packaged juices, whereas freshly squeezed juices from natural fruits have been included in the fruit category, wherein orange juice accounts for two orange servings.
3. 'Soft drinks' category encloses all commercial sweetened beverages, including caffeinated and non-caffeinated cola soft drinks.

Table 54: Summary of cases. Pattern of consumption of selected groups of food and beverages with moderate to occasional recommended consumption. Females responses

			ID	Sausages[1]	Sweets, ice cream, pizza	Juices[2]	Soft drinks[3]	Alcoholic drinks
				servings/week				
Gender Female		1	02	0.8	0.0	0.0	0.0	1.8
		2	03	14.0	1.3	0.0	0.0	0.0
		3	04	7.0	0.3	0.0	4.0	2.3
		4	06	6.0	6.0	0.0	0.0	1.0
		5	07	5.0	1.8	0.0	0.0	0.0
		6	09	3.0	1.0	8.0	7.0	0.0
		7	12	0.0	0.3	7.0	0.0	0.0
		8	13	4.0	11.0	2.0	4.0	0.0
		9	14	0.0	0.0	0.0	1.0	0.0
		10	15	5.0	1.0	0.0	0.0	2.0
		11	19	.	.	.	.	.
		12	20	.	.	.	.	.
		13	21	0.0	0.0	0.0	0.0	1.0
		14	23	0.0	0.0	0.0	0.0	0.0
		15	24	2.0	0.0	0.0	0.0	0.0
		16	27	2.0	3.5	0.5	0.5	0.0
		17	28	4.0	15.0	0.0	3.0	0.0
		18	29	0.0	0.0	0.0	0.0	1.0
		19	30	7.0	1.8	0.0	0.0	0.0
		20	31	0.0	0.0	7.0	0.0	0.0
		21	33	0.0	3.5	3.0	2.0	0.0
		22	34	1.0	1.5	0.0	3.0	0.0
		23	35	2.0	17.0	0.0	14.0	0.0
		24	37	4.0	3.5	0.0	2.5	0.0
		25	38	4.0	1.5	0.0	0.5	2.0
	Total	N	25	23	23	23	23	23
		Mean	-	3.1	3.0	1.2	1.8	0.5
		SD	-	3.4	4.8	2.5	3.2	0.8
		Median	-	**2.0**	**1.2**	**0.0**	**0.0**	**0.0**
		IQR	-	**5.0**	**3.5**	**0.5**	**3.0**	**1.0**

Missing values for participants ID19 and ID20

Notes:

1. Sausages include sausages, tripe and visceras.
2. 'Juices' category includes only commercially packaged juices, whereas freshly squeezed juices from natural fruits have been included in the fruit category, wherein orange juice accounts for two orange servings.
3. 'Soft drinks' category encloses all commercial sweetened beverages, including caffeinated and non-caffeinated cola soft drinks.

Table 55: Summary of cases. Pattern of consumption of selected groups of food and beverages with moderate to occasional recommended consumption. Not disclosed gender's responses.

			ID	Sausages[1]	Sweets, ice cream, pizza	Juices[2]	Soft drinks[3]	Alcoholic drinks
				servings/week				
Gender Not disclosed		1	05	3.0	7.0	0.0	0.0	0.0
	Total	N	1	1	1	1	1	1
		Mean	5	3.0	7.0	0.0	0.0	0.0
		SD	-	-	-	-	-	-
		Median	5	3.0	7.0	0.0	0.0	0.0
		IQR	-	-	-	-	-	-

Table 56: Summary of cases. Pattern of consumption of selected groups of food and beverages with moderate to occasional recommended consumption. All participants' responses.

		N	Sausages[1]	Sweets, ice cream, pizza	Juices[2]	Soft drinks[3]	Alcoholic drinks
			servings/week				
All Participants	N	38	35	35	35	35	35
	Mean	-	2.9	3.8	1.5	2.6	1.0
	SD	-	3.0	5.2	2.6	3.7	2.2
	Median	-	**2.0**	**1.5**	**0.0**	**0.5**	**0.0**
	IQR	-	**4.1**	**5.8**	**2.0**	**4.0**	**1.0**

Notes:

1. Sausages include sausages, tripe and visceras.
2. 'Juices' category includes only commercially packaged juices, whereas freshly squeezed juices from natural fruits have been included in the fruit category, wherein orange juice accounts for two orange servings.
3. 'Soft drinks' category encloses all commercial sweetened beverages, including caffeinated and non-caffeinated cola soft drinks.

The frequency of consumption of the food groups with moderate to occasional recommended intake in the entire cohort revealed that participants consumed sausages, sweets, ice cream and pizza approximately twice weekly (2.0±4.1 sausage servings/week and 1.5±5.8 sweets, ice cream and pizza servings/week), whereas commercially sweetened juices and soft drinks accounted for median 0.0±2.0 servings/week and 0.5±4.0 servings/week, respectively. Alcoholic drinks resulted in a 0.0±1.0 servings/week median consumption. Accordingly, the general trend in the entire cohort was in accordance with moderate to occasional consumption recommendations.

However, median values hid some individual frequency patterns that were clearly neither occasional nor moderately. In this regard, sweetened beverages, either juices, soft drinks, or both, were consumed almost daily (at least five servings per week) in 14 out of 35 responders (40%). Some consumers reported drinking sweetened drinks twice daily, as revealed in 4 out of 35 responders (11%). Caffeinated cola beverages were the most common soft drinks consumed, according to participants' responses. Notwithstanding, cola beverages contain approximately 35 g sugar per 330 mL serving, according to the values declared by the market leader in the label.

4.6.3. Consumption of dietary methylxanthines

Regarding the consumption of dietary methylxanthines from food and beverages in the form of coffee, tea, chocolate, caffeinated soft drinks (cola drinks) and energy drinks, 30 out of 35 responders (86%) revealed to consume different products in minor to moderate usual intensity, whereas 5 out of 35 participants (14%) revealed not to consume methylxanthines in any form in usual diet. As previously disclosed, there was no information on food habits in 3 out of 38 participants (8%) who did not respond to the food questionnaire in the observational study. Among consumers, males accounted for the 33% whereas females represented the 63%. Among non-consumers, males accounted for 20% whereas females represented 80%.

Next, the pattern of dietary methylxanthines' consumption was translated into caffeine and theobromine content considering the food and beverages reference data as provided in Chapter 1 (see Chapter 1, **Table 18** and **Table 20**, respectively). In this regard, caffeine concentration accounted for 1340 mg/L in coffee, 165 mg/L in tea, 525 mg/kg in chocolate, 108 mg/L in cola soft drinks and 320 mg/L in energy drinks, whereas theobromine accounted for 4630 mg/kg in dark chocolate and 1530 mg/kg in milk chocolate. The serving sizes used for calculations were: 60 mL for coffee, 250 mL for tea, 20 g for chocolate and 330 mL for cola soft drinks and energy drinks, according to reference values published by the *Agència Catalana de Salut Alimentària* on the safety of cafeïne in foods.[368]

Table 57 to **Table 59** detail the concrete responses per each participant, according to genders. **Table 60** summarizes all participant responses. Translation into estimated daily caffeine and theobromine intake is additionally included in each case.

Table 57: Summary of cases: Daily consumption of foods and beverages containing methylxanthines. Males responses

Males		ID	MX consumption	Coffee[1]	Tea	Chocolate[2]	Cola drinks[1]	Energy drinks	Caffeine from all sources	Theobromine from chocolate
				servings/day					mg/day	mg/day
	1	01	Yes	0.0	1.0	0.3	0.0	0.0	58.0	8.9
	2	08	Yes	2.0	0.0	2.0	0.1	0.0	184.0	61.2
	3	10	Yes	0.7	0.0	0.6	0.7	0.0	98.0	38.8
	4	11	NR	.	.	.	.	.	.	.
	5	16	Yes	1.0	0.0	0.0	0.1	0.0	85.0	0.9
	6	17	Yes	1.0	0.0	0.0	0.0	0.0	80.0	0.0
	7	18	Yes	0.0	0.0	0.0	1.0	0.0	36.0	0.0
	8	22	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	9	25	Yes	0.0	0.7	0.3	0.7	0.0	68.0	8.9
	10	26	Yes	0.0	0.0	0.3	0.1	0.0	8.0	25.9
	11	32	Yes	3.0	0.1	0.5	0.1	0.1	282.0	36.7
	12	36	Yes	0.6	0.4	0.8	1.3	0.0	175.0	74.1
Total	N	12	11	11	11	11	11	11	11	11
	Mean	-	-	0.8	0.2	0.4	0.4	0.0	**104.1**	23.2
	SD	-	-	1.0	0.3	0.6	0.5	0.0	**79.2**	26.4
	Median	-	-	**0.6**	**0.0**	**0.3**	**0.1**	**0.0**	85.0	**8.9**
	IQR	-	-	**1.0**	**0.4**	**0.6**	**0.7**	**0.0**	113.0	**38.8**

Missing values for participant ID11

Notes:

1. Coffee and cola drinks account for caffeinated foodstuff. The decaffeinated products have not been considered in these figures.
2. Chocolate accounts for total daily intake, including dark and milk chocolate consumption.

Table 58: Summary of cases: Daily consumption of foods and beverages containing methylxanthines. Females responses

Females		ID	MX consumption	Coffee[1]	Tea	Chocolate[2]	Cola drinks[1]	Energy drinks	Caffeine from all sources	Theobromine from chocolate
				servings/day					mg/day	mg/day
1		02	Yes	3.0	0.4	0.1	0.0	0.0	262.0	6.5
2		03	Yes	0.0	0.0	0.0	3.0	0.0	107.0	0.0
3		04	Yes	1.0	0.1	0.0	0.6	0.0	107.0	0.0
4		06	Yes	0.1	0.0	0.4	0.0	0.0	63.0	13.2
5		07	Yes	3.0	1.0	0.0	2.0	0.3	384.0	0.0
6		09	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7		12	Yes	1.0	1.0	0.0	1.0	0.0	157.0	0.0
8		13	Yes	2.0	0.0	0.5	0.0	0.0	203.0	42.6
9		14	Yes	0.0	0.0	0.0	0.1	0.0	5.0	0.0
10		15	Yes	1.0	0.0	0.0	1.0	0.0	116.0	0.0
11		19	NR	.	.	.	.	.	.	.
12		20	NR	.	.	.	.	.	.	.
13		21	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14		23	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15		24	Yes	0.7	0.1	0.0	0.1	0.0	47.0	0.0
16		27	Yes	1.0	1.0	0.3	0.1	0.0	127.0	6.8
17		28	Yes	0.0	0.0	1.0	0.4	0.0	26.0	30.6
18		29	Yes	1.0	0.0	0.1	0.0	0.0	82.0	5.8
19		30	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20		31	Yes	1.0	0.4	0.0	0.0	0.0	98.0	0.9
21		33	Yes	1.0	0.0	0.0	0.0	0.0	80.0	0.0
22		34	Yes	2.0	0.0	0.3	0.4	0.0	176.0	8.9
23		35	Yes	1.0	0.0	0.0	1.0	0.0	116.0	0.0
24		37	Yes	1.0	0.0	0.0	0.0	0.0	80.0	0.9
25		38	Yes	1.0	0.0	2.0	0.0	0.0	80.0	132.2
Total	N	25	23	23	23	23	23	23	23	23
	Mean	-	-	0.9	0.2	0.4	0.4	0.0	100.7	10.4
	SD	-	-	0.9	0.3	0.6	0.7	0.1	92.4	26.8
	Median	-	-	**1.0**	**0.0**	**0.3**	**0.0**	**0.0**	**82.0**	**0.0**
	IQR	-	-	**1.0**	**0.1**	**0.6**	**0.6**	**0.0**	**101.0**	**6.8**

Missing values for participants ID19 and ID20.

Notes: 1. Coffee and cola drinks account for caffeinated foodstuff. 2. Chocolate accounts for total dark and milk chocolate daily consumption.

Table 59: Summary of cases: Daily consumption of foods and beverages containing methylxanthines. Not disclosed gender responses

Gender not disclosed		ID	MX consumption	Coffee[1]	Tea	Chocolate	Cola drinks[1]	Energy drinks	Caffeine from all sources	Theobromine from chocolate
				servings/day					mg/day	mg/day
	1	05	Yes	1.0	0.0	0.0	0.0	0.0	80.0	0.0
Total	Mean	1	1	1	1	1	1	1	1	1
	SD	5.0	1.0	1.0	0.0	0.0	0.0	0.0	80.0	0.0
	Median	.	.	.	.	.	.	.	.	.
	IQR	5.0	1.0	1.0	0.0	0.0	0.0	0.0	80.0	0.0

Notes:

1. Coffee and cola drinks account for caffeinated foodstuff. The decaffeinated products have not been considered in these figures.

Table 60: Summary of cases: Daily consumption of foods and beverages containing methylxanthines. All participants' responses

All participants		N	MX consumption	Coffee[1]	Tea	Chocolate	Cola drinks[1]	Energy drinks	Caffeine from all sources	Theobromine from chocolate
				servings/day					mg/day	mg/day
Total	N	38	35	35	35	35	35	35	35	35
	Mean	-	-	0.9	0.2	0.3	0.4	0.0	100.9	14.1
	SD	-	-	0.9	0.3	0.5	0.7	1	86.0	26.7
	Median	-	-	**1.0**	**0.0**	**0.0**	**0.1**	**0.0**	**82.0**	**0.9**
	IQR	-	-	**1.0**	**0.1**	**0.3**	**0.7**	**0.0**	**80.0**	**13.2**

Missing values for participants ID11, ID19 and ID20

Notes:

1. Coffee and cola drinks account for caffeinated foodstuff. The decaffeinated products have not been considered in these figures.

Data revealed that the study cohort consumed caffeine on a regular basis (82.0±80.0 mg median caffeine/daily), but almost no theobromine (0.9±13.2 mg median theobromine/daily). The main source of dietary caffeine was coffee, for which 18 out of 35 responders (51%) consumed at least one serving per day. The Mann-Whitney's U test revealed no statistically significant differences in consumption trends between genders for caffeine (U=124.0, Z=-0.092, p=0.926) nor theobromine (U=76.5, Z=-1.945, p=0.052). **Figure 43** and **Figure 44** graphically depict the above-mentioned results.

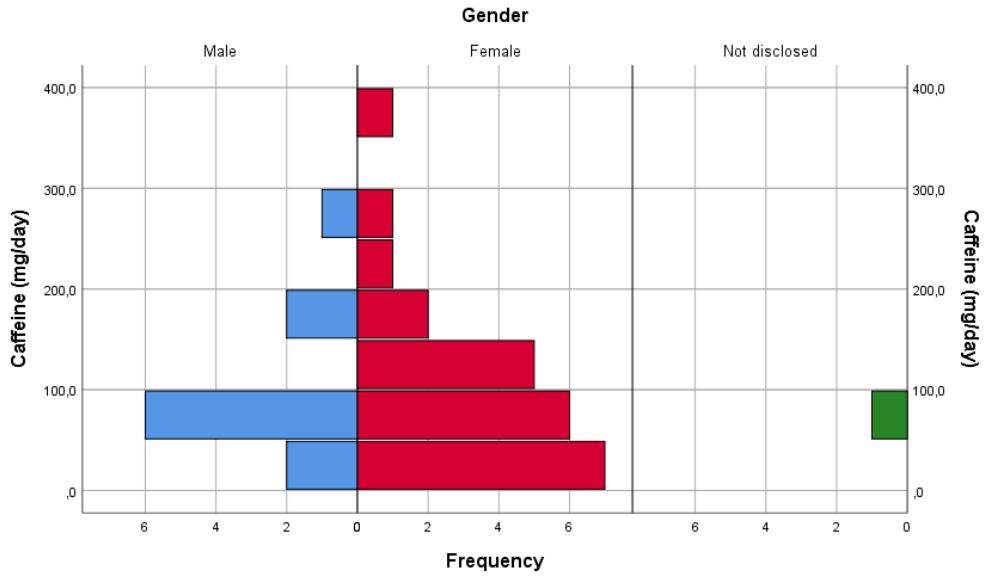


Figure 43: Caffeine consumption distribution among study participants, according to gender.

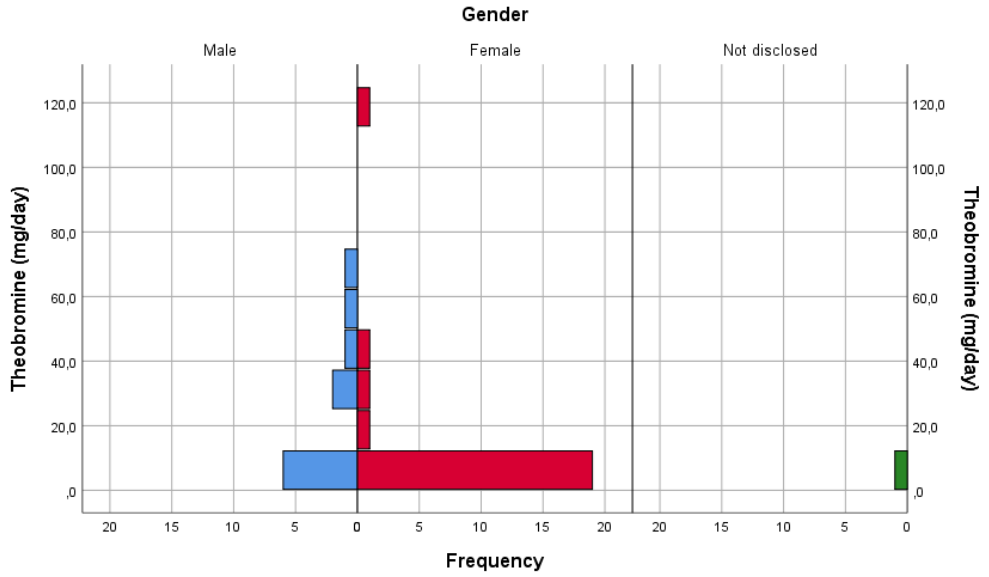


Figure 44: Theobromine consumption distribution among study participants, according to gender.

4.6.4. Health-related quality of life: SF-36 questionnaire results

General health

When asked about their general health, 22 out of 38 participants reported a regular health status (58%), followed by good health status in 13 out of 38 participants (34%). Only 2 out of 38 participants considered they had a very good health status (5%). Neither excellent nor bad health status was pointed out by any participant.

Current health compared to a year ago

When asked about current health compared to a year ago, 21 out of 38 participants (55%) reported a similar health, followed by something worse than a year ago in 10 out of 38 participants (26%). One participant (3%) considered that the current health was worse than one year ago. No participant considered that the current health was better than a year ago. Three participants didn't respond to the question.

Daily activities

Participants were asked to score how DM1 limited them to perform some common daily activities, ranging from no limitation at all to high level of limitations. **Table 61** summarizes the main responses.

Table 61: Daily activities. Limitations

Activity	Yes, it limits me a lot	Yes, it limits me a little	No, it does not limit me at all	NR
Intense efforts	24 (63%)	10 (26%)	3 (8%)	1 (3%)
Moderate efforts	13 (34%)	16 (42%)	8 (21%)	1 (3%)
Take or carry the shopping bag	8 (21%)	23 (61%)	6 (16%)	1 (3%)
Climb several floors up the ladder	16 (42%)	14 (37%)	7 (19%)	1 (3%)
Climb one single floor up the ladder	8 (21%)	14 (37%)	15 (39%)	1 (3%)
Crouch or kneel	14 (37%)	12 (32%)	11 (29%)	1 (3%)
Walk at least one km	12 (32%)	14 (37%)	11 (29%)	1 (3%)
Walk some hundred meters	9 (24%)	11 (29%)	17 (45%)	1 (3%)
Walk one hundred meters	4 (11%)	11 (29%)	22 (58%)	1 (3%)
Bathing or dressing yourself	1 (3%)	12 (32%)	24 (63%)	1 (3%)

NR: No response

In general terms, limitation, either a lot or a little, accounted for the majority of cases, with the exception of walking one hundred meters and bathing or dressing oneself, in which participants mainly disclosed not to experience limitations.

Afterwards, participants were asked to score everyday problems, and how their physical health had limited their work or activities. Only 8 out of 38 participants (21%) reported that their physical health had not limited their work or activities. On the contrary, 28 out of 38 participants (74%) revealed that they had difficulties in everyday work or activities that limited them, in different grades, from almost ever (32%) to sometimes (26%).

Additionally, participants were asked to score how limitations due to physical and emotional health had limited social activities. Only 12 out of 38 participants (32%) reported that their physical and emotional health had not limited their social activities. On the contrary, 25 out of 38 participants (66 %) revealed that their social activities had been limited in different grades.

Physical activity

When asked about physical activity, 22 out of 38 participants (58%) reported to practice some, whereas 16 out of 38 participants (42%) reported to be inactive. Among physical activity practitioners, 14 out of 22 (64%) reported to perform light activities, 7 out of 22 (32%) reported to perform moderate intensity activities, and 1 out of 22 (4%) reported to perform intense activity.

Data collected per each subject in each scale are provided in **Table 62** to **Table 65**, divided into gender. **Figure 45** and **Figure 46** graphically depict the global scores for physical and mental health, respectively, divided into gender.

Table 62: Summary of cases: SF-36 scales and summary scales for males

				ID	PF_Score	RP_Score	BP_Score	GH_Score	VT_Score	SF_Score	RE_Score	MH_Score	PCS_Score	MCS_Score
Gender	**Male**		1	01	40.20	34.81	37.61	35.30	45.85	45.94	44.22	52.82	33.30	52.36
			2	08	29.67	34.81	41.83	26.72	42.72	29.58	32.56	30.30	35.29	33.36
			3	10	48.61	56.85	37.18	60.08	58.33	56.85	55.88	41.56	51.19	52.82
			4	11	40.20	44.61	32.96	38.63	33.36	35.03	36.44	47.19	38.56	39.57
			5	16	44.41	47.06	41.41	18.61	55.21	40.49	48.10	47.19	37.24	49.46
			6	17	54.93	56.85	55.36	41.02	42.72	56.85	40.33	35.93	58.66	36.74
			7	18	46.51	56.85	50.29	35.30	45.85	56.85	55.88	50.01	46.05	53.58
			8	22	46.51	51.96	57.89	46.78	61.46	56.85	55.88	35.93	52.92	50.11
			9	25	46.51	34.81	32.96	31.48	45.85	29.58	32.56	41.56	38.40	36.49
			10	26	35.99	56.85	29.15	26.72	42.72	45.94	40.33	56.64	34.47	50.51
			11	32	48.61	56.85	51.13	29.10	64.58	45.94	55.88	61.27	43.80	60.06
			12	36	57.03	56.85	57.89	52.93	58.33	56.85	44.2	47.19	60.55	46.65
		Total	N	12	12	12	12	12	12	12	12	12	12	12
			Mean	-	**44.93**	49.10	**43.80**	**36.89**	**49.75**	**46.39**	**45.19**	**45.63**	**44.20**	**46.81**
			SD	-	**7.62**	9.56	**10.30**	**11.87**	**9.52**	**10.77**	**9.10**	**9.05**	**9.59**	**8.32**
			Median	-	46.51	**54.41**	41.62	35.30	45.85	45.94	44.22	47.19	41.18	49.79
			IQR	-	8.41	**19.59**	20.29	18.03	15.61	20.45	19.59	14.78	16.71	15.26

Notes:

PF: physical functioning; **RP**: role-physical; **BP**: bodily pain; **GH**: general health; **VT**: vitality (energy/fatigue); **SF**: social functioning; **RE**: role-emotional; **MH**: mental health; **PCS**: physical component summary; **MCS**: mental component summary in SF-36.

Table 63: Summary of cases: SF-36 scales and summary scales for females

				ID	PF_Score	RP_Score	BP_Score	GH_Score	VT_Score	SF_Score	RE_Score	MH_Score	PCS_Score	MCS_Score
Gender	**Female**		1	02	31.78	39.71	32.96	30.53	45.85	45.94	51.99	47.19	28.54	54.86
			2	03	27.57	27.47	29.15	23.28	33.36	24.13	20.89	21.85	30.83	23.20
			3	04	31.78	34.81	33.38	23.38	45.85	24.13	20.89	35.93	33.79	30.70
			4	06	40.20	32.36	46.06	35.30	42.72	45.94	44.22	47.19	36.28	48.37
			5	07	33.88	34.81	37.61	25.76	36.48	45.94	51.99	44.38	28.35	51.03
			6	09	21.26	29.91	24.08	28.15	48.97	29.58	20.89	25.37	28.23	31.62
			7	12	31.78	32.36	33.38	35.30	36.48	40.49	36.44	47.19	30.04	45.23
			8	13	33.88	29.91	41.41	26.72	39.60	45.94	40.33	56.64	27.92	52.39
			9	14	25.47	42.16	29.15	29.10	30.24	29.58	51.99	56.64	22.97	52.43
			10	15	27.57	47.06	33.38	28.15	39.60	35.03	40.33	33.11	34.12	38.61
			11	19	44.41	.	46.90	.	.	.	32.56	.	.	.
			12	20	44.41	39.71	33.38	30.53	48.97	45.94	28.67	47.19	38.60	42.52
			13	21	17.05	37.26	50.29	28.15	33.36	51.40	.	41.56	.	.
			14	23	17.05	27.47	37.61	30.53	39.60	29.58	20.89	44.38	26.01	38.57
			15	24	.	.	.	.	.	.	.	.	.	.
			16	27	31.78	42.16	37.61	32.91	36.48	40.49	55.88	61.27	27.39	58.92
			17	28	19.15	25.02	24.08	23.38	33.36	29.58	55.88	50.01	11.82	56.27
			18	29	19.15	49.51	57.89	30.53	33.36	51.40	55.88	56.64	31.53	58.44
			19	30	46.51	37.26	32.96	41.02	58.33	56.85	32.56	44.38	41.17	47.67
			20	31	29.67	27.47	41.83	32.91	39.60	56.85	55.88	64.09	22.02	67.31
			21	33	31.78	39.71	29.15	50.55	48.85	45.94	51.99	47.19	32.33	54.91
			22	34	19.15	39.71	51.13	32.91	52.09	40.49	55.88	52.82	27.78	60.38
			23	35	54.93	42.16	41.41	41.02	33.36	29.58	55.88	61.17	40.43	49.43
			24	37	54.93	37.26	29.15	32.91	52.09	40.49	32.56	41.56	42.08	39.00
			25	38	50.72	49.51	41.83	45.78	64.58	56.85	55.88	58.46	43.86	62.69
		Total	N	25	24	23	24	23	23	23	23	23	22	22
			Mean	-	**32.74**	**36.73**	**37.32**	**32.12**	**42.31**	**40.96**	42.19	**47.22**	**31.18**	**48.39**
			SD	-	**11.66**	**7.04**	**8.70**	**6.99**	**8.99**	**10.39**	13.52	**10.96**	**7.49**	**11.25**
			Median	-	31.78	37.26	33.38	30.53	39.60	40.49	**48.11**	47.19	30.44	50.23
			IQR	-	16.83	12.25	12.37	7.51	13.27	16.36	**24.29**	12.96	9.18	17.91

Notes:

PF: physical functioning; **RP**: role-physical; **BP**: bodily pain; **GH**: general health; **VT**: vitality (energy/fatigue); **SF**: social functioning; **RE**: role-emotional; **MH**: mental health; **PCS**: physical component summary; **MCS**: mental component summary in SF-36.

Table 64: Summary of cases: SF-36 scales and summary scales for not disclosed gender

			ID	PF_Score	RP_Score	BP_Score	GH_Score	VT_Score	SF_Score	RE_Score	MH_Score	PCS_Score	MCS_Score
Gender Not disclosed		1	05	38.09	25.02	57.89	25.76	27.11	51.40	44.22	61.27	30.59	53.39
	Total	N	1	1	1	1	1	1	1	1	1	1	1
		Mean	-	38.09	25.02	57.89	25.76	27.11	51.40	44.22	61.27	30.9	53.39
		SD	-	.	.	.	.	.	.	.	.	.	.
		Median	-	38.09	25.02	57.89	25.76	27.11	51.40	44.22	61.27	30.9	53.39
		IQR	-	.	.	.	.	.	.	.	.	.	.

Notes:

PF: physical functioning; **RP**: role-physical; **BP**: bodily pain; **GH**: general health; **VT**: vitality (energy/fatigue); **SF**: social functioning; **RE**: role-emotional; **MH**: mental health; **PCS**: physical component summary; **MCS**: mental component summary in SF-36.

Table 65: Summary of cases: SF-36 scales and summary scales for all participants, and comparison in front of minimum and maximum reported values as reviewed by reference [42]

		N	PF_Score	RP_Score	BP_Score	GH_Score	VT_Score	SF_Score	RE_Score	MH_Score	PCS_Score	MCS_Score
Gender All Participants	N	38	37	36	37	36	36	36	36	36	35	35
	Mean	-	**36.84**	**40.53**	**39.98**	**33.53**	**44.37**	43.06	43.24	**47.08**	**35.63**	**47.99**
	SD	-	**11.73**	**10.07**	**9.97**	**9.05**	**10.02**	10.63	11.95	**10.38**	**10.19**	**10.10**
	Median	-	35.99	39.71	37.61	31.48	42.72	**45.94**	**44.22**	47.19	34.12	50.11
	IQR	-	16.84	17.15	13.10	11.91	15.61	**21.82**	**23.32**	15.08	12.82	15.86
Reviewed values[42]	Minimum	-	38	22	51	34	31	56	28	53	ND	ND
	Maximum	-	82	95	82	77	69	93	96	81	ND	ND

Notes:

PF: physical functioning; **RP**: role-physical; **BP**: bodily pain; **GH**: general health; **VT**: vitality (energy/fatigue); **SF**: social functioning; **RE**: role-emotional; **MH**: mental health; **PCS**: physical component summary; **MCS**: mental component summary in SF-36; ND: not disclosed.

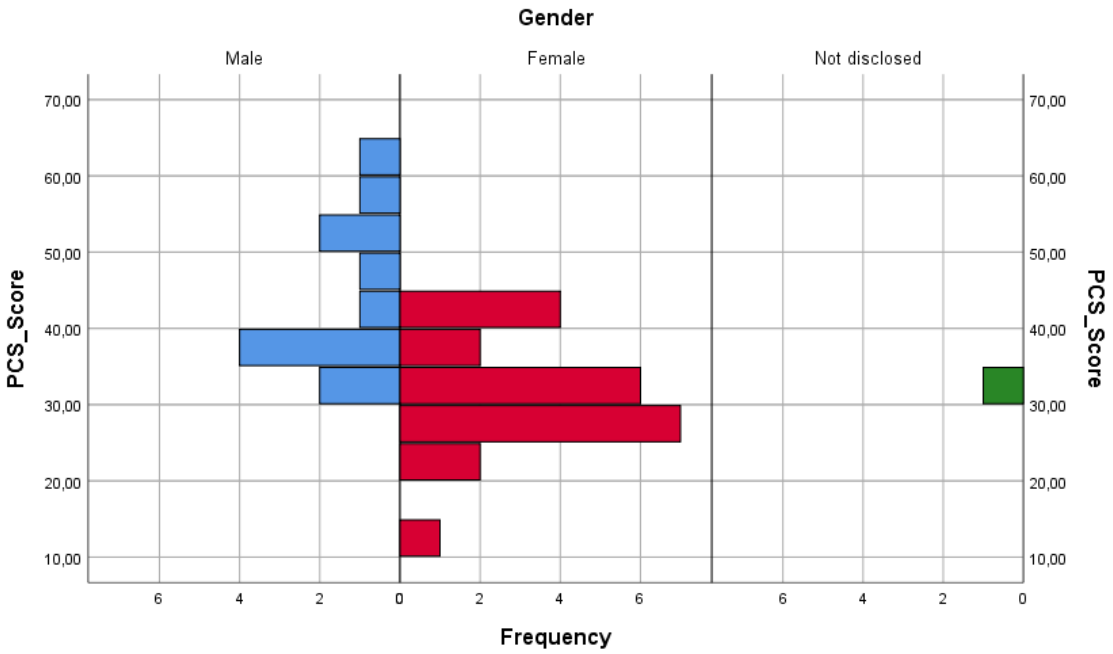


Figure 45: Physical component summary (PCS) score in SF-36 distribution among study participants, according to gender.

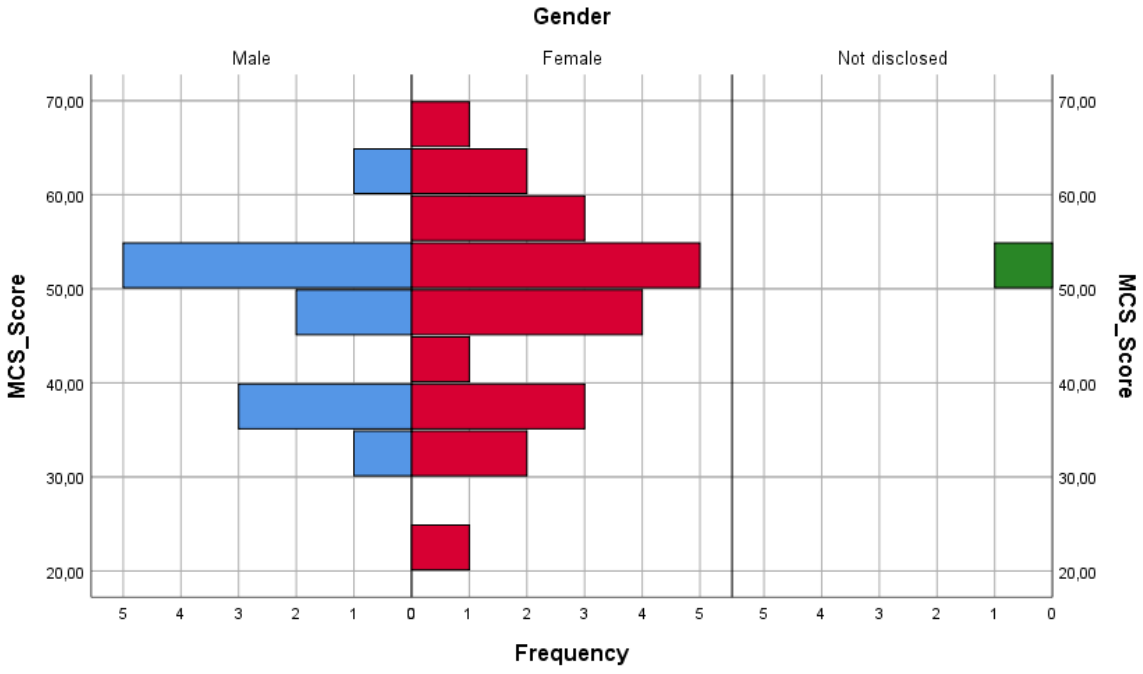


Figure 46: Mental component summary (MCS) score in SF-36 distribution among study participants, according to gender.

In the entire cohort, physical functioning mean scored 36.84±11.73, role-physical scored 40.53±10.07, bodily pain scored 39.98±9.97, general health scored 33.53±9.05, vitality scored 44.37±10.02, social functioning scored 45.94±21.82, role-emotional scored 44.22±23.32 and mental health scored 47.08±10.38, whereas the component summaries for physical and mental performance, PCS and MCS, scored 35.63±10.19 and 47.99±10.10, respectively. The study cohort revealed a moderate HRQoL as measured in SF-36, since all scores were obtained in the low to medium range in the scales. In fact, the majority of the obtained results were in the lower range of the reviewed HRQoL scores in DM1 using the SF-36 questionnaire,[42] illustrating the really limited QoL in DM1.

Regarding gender comparison, some mean scores such as physical functioning, role-physical, vitality and the physical component summary (PCS) were statistically different between males and females (p<0.05) according to the unpaired t-test analysis at 95% confidence interval, whereas bodily pain, general health, social functioning, role-emotional, mental health and the mental component summary (MCS) did not show statistically significant differences between genders (**Table 66**).

Table 66: Mean score comparison of according to gender

Score	Gender	N	Central tendency	Scatter measure	*p*
Physical functioning (PF)	Male	12	44.93†	7.62†	0.002 τ
	Female	24	32.74†	11.66†	
Role-physical (RP)	Male	12	54.41‡	19.59‡	0.002 ◊
	Female	23	36.73†	7.04†	
Bodily pain (BP)	Male	12	43.80†	10.30†	0.056 τ
	Female	24	37.32†	8.70†	
General health (GH)	Male	12	36.89†	11.87†	0.143 τ
	Female	23	32.12†	6.99†	
Vitality (VT)	Male	12	49.75†	9.52†	0.029 τ
	Female	23	42.31†	8.99†	
Social functioning (SF)	Male	12	46.39†	10.77†	0.156 τ
	Female	23	40.96†	10.39†	
Role-emotional (RE)	Male	12	45.19†	9.10†	0.559 ◊
	Female	23	48.11‡	24.29‡	
Mental health (MH)	Male	12	45.63†	9.05†	0.669 τ
	Female	23	47.23†	10.96†	
Physical component summary (PCS)	Male	12	44.20†	9.59†	0.000 τ
	Female	22	31.19†	7.49†	
Mental component summary (MCS)	Male	12	46.81†	8.32†	0.673 τ
	Female	22	48.39†	11.25†	

† (Mean ± SD); ‡ (Median ± IQR); τ sig. (2-tailed) according to unpaired t-test; ◊ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%.

4.6.5. Correlation between HRQoL and dietary methylxanthines

An unpaired two-tailed t-test was used to determine whether the two data sets (participants consuming dietary methylxanthines in front of participants not consuming dietary methylxanthines) were significantly different from each other in terms of the general physical component summary (PCS) and the mental component summary (MCS) scores derived from the SF-36 quality of life questionnaire. To assess the statistical difference, the probability associated with the two-paired t-test was used as the decision parameter, at the common significance level (*p*) of 0.05 (considering a 95% interval of confidence).

Firstly, consumers (most of the studied population) *versus* non-consumers were considered in general, irrespectively of the exact nature of the dietary source of methylxanthines (coffee, tea, chocolate, soft drinks) and the frequency of consumption (see **Table 67**):

Table 67: Comparison of the summary scores from SF-36 in front of dietary methylxanthines consumption

SF-36 summary scores	Gender	Non-consumers		Consumers		*p*
		N	Mean ± SD	N	Mean ± SD	
PCS	All Participants	6	37.58 ± 9.71	29	35.23 ± 10.40	0.614
	Males	2	45.74 ± 10.15	10	43.89 ± 10.01	0.817
	Females	4	33.50 ± 7.50	18	30.67 ± 7.60	0.507
MCS	All Participants	6	41.67 ± 6.68	29	49.29 ± 10.28	0.093
	Males	2	44.84 ± 7.45	10	47.20 ± 8.80	0.732
	Females	4	40.09 ± 6.77	18	50.23 ± 11.34	0.104

Then, comparison was performed among caffeine consumers, divided into low-caffeine consumers and high-caffeine consumers. In this regard, low-caffeine consumers group consisted of those participants that were determined to ingest less than 80 mg/daily, *i.e.* the caffeine equivalent to one single coffee per day, whereas high-consumers group consisted of those participants that were determined to ingest more than 80 mg/daily, *i.e.* the caffeine equivalent to more than one single coffee per day (see **Table 68**):

Table 68: Comparison of the summary scores from SF-36 in front of dietary daily caffeine consumption

SF-36 summary scores	Gender	Caffeine consumption < 80 mg/day		Caffeine consumption > 80 mg/day		*p*
		N	Mean ± SD	N	Mean ± SD	
PCS	All Participants	7	31.90 ± 11.20	22	36.29 ± 10.18	0.340
	Males	4	38.05 ± 5.76	6	49.79 ± 10.73	0.139
	Females	3	23.69 ± 12.24	15	32.06 ± 6.01	0.081
MCS	All Participants	7	50.00 ± 6.44	22	49.07 ± 11.35	0.839
	Males	4	72.67 ± 22.62	6	46.51 ± 10.00	0.781
	Females	3	52.36 ± 3.95	15	49.79 ± 12.36	0.734

Formally, none of the *p*-values resulting from the statistical analysis based on the unpaired t-test showed statistical significance when comparing the SF-36 summary scores in front of the consumption pattern of methylxanthines, at 95% confidence interval ($p>0.05$). **Figure 47** and **Figure 48** graphically depict the physical and mental summary scores, respectively, according to dietary caffeine intake.

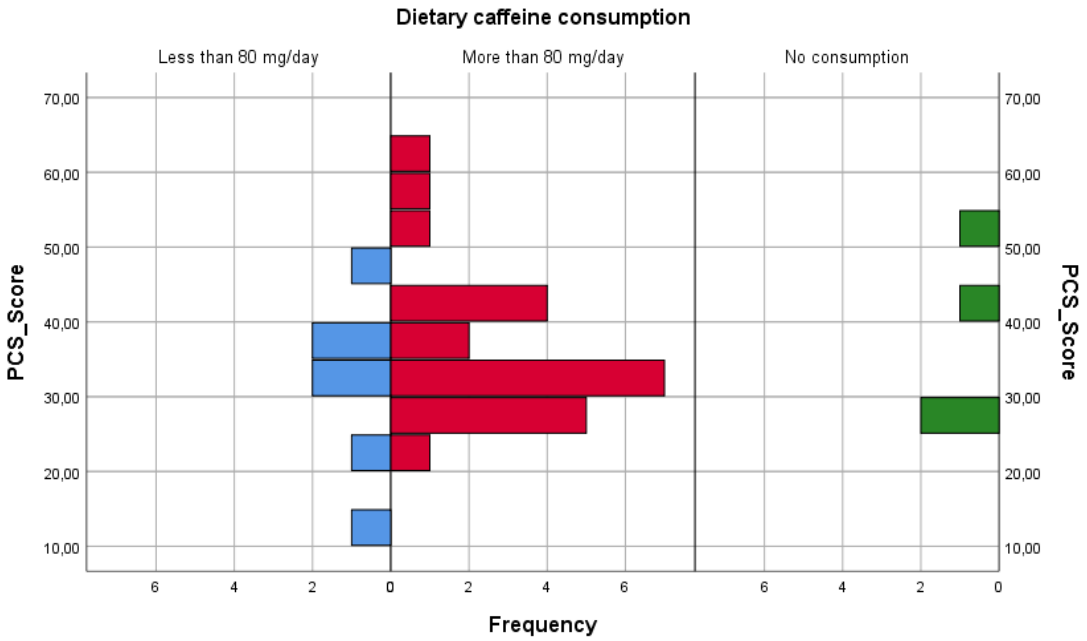


Figure 47: Physical component summary (PCS) score in SF-36 distribution among study participants, according to dietary caffeine consumption.

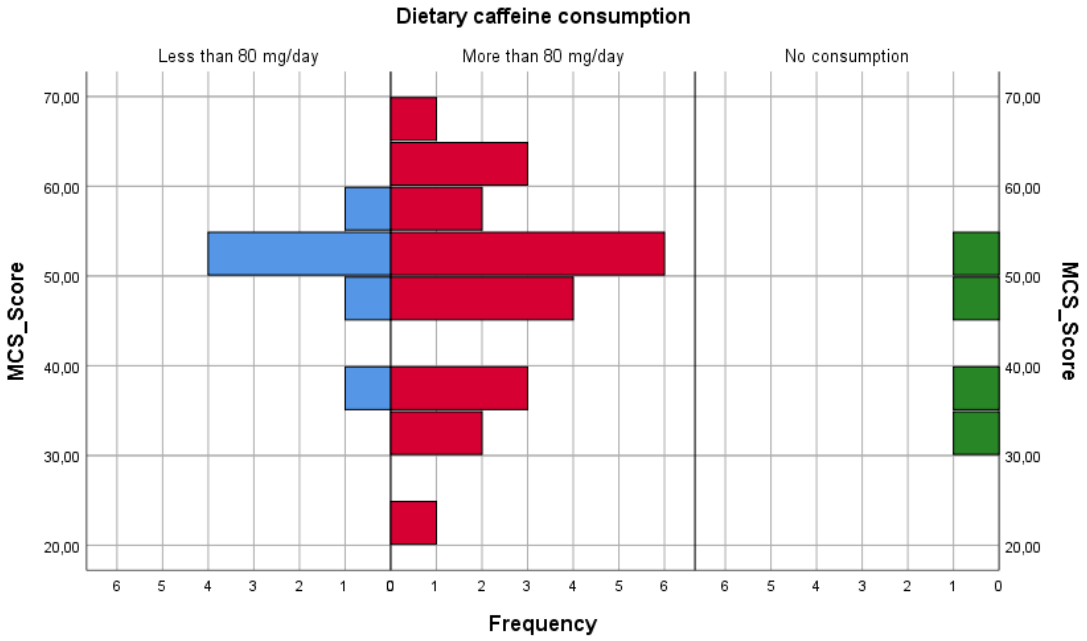


Figure 48: Mental component summary (MCS) score in SF-36 distribution among study participants, according to dietary caffeine consumption.

Finally, Spearman's correlation analysis between the non-parametric consumption of dietary methylxanthines and age, gender, the eight scales and the two summary scales of the SF-36 questionnaire were evaluated in the entire cohort. **Table 69** summarizes these results.

Spearman's correlation analyses showed some statistically significant correlations between dietary methylxanthines consumption and the SF-36 scales related to emotional and mental health, as well as in the mental health component summary score MCS (p<0.05). No

statistically significant correlation was observed in any of the scales measuring physical performance, nor in the physical health component summary score PCS (p>0.05).

Table 69: Comparison of Spearman's correlation between methylxanthines consumption and all SF-36 scores and summary scores

Methylxanthines *vs* SF-36 scores	**N**	**Spearman's correlation (rho)**	***p***
Age	31	-0.259	0.159
Gender	38	-0.050	0.766
Body Mass Index (BMI)	36	-0.058	0.737
Physical Functioning (PSY)	37	0.086	0.611
Role Physical (RP)	36	0.085	0.622
Bodily Pain (BP)	37	0.040	0.814
General Health (GH)	36	-0.112	0.516
Vitality (VT), energy/fatigue (E/F)	36	-0.095	0.581
Social Functioning (SF)	36	-0.041	0.811
Role Emotional (RE)	36	0.420*	**0.011**
Mental Health (MH)	36	0.334*	**0.047**
Physical Health Component Summary (PCS)	35	-0.098	0.577
Mental Health Component Summary (MCS)	35	0.338*	**0.047**

* Correlation is significant at the 0.05 level (2-tailed).

4.7. Concluding remarks

The main goal guiding the research 'Observational study on the quality of life and eating habits in participants with Myotonic Dystrophy type 1 (DM1)' was to evaluate the nutritional habits and potential relationships between dietary methylxanthines' consumption and the QoL in this special population. To the best of our knowledge, there are no preceding studies in this regard.

A total of 38 subjects aged from 18 to 69 years old (mean age, 45±14 years) participated in the observational study MD Project. Female represented 66% whereas male represented 32%. All participants had a previous diagnosis of DM1, and a mean 405±261 $(CTG)_n$ repeat count. Adult onset was the most usual phenotype (47%), followed by congenital myotonic dystrophy (34%), and childhood/juvenile onset (11%). Mean BMI was 24.78±4.45 kg/m^2 (normal weight).

According to the data provided by the participants themselves, the intake of the food groups rice, pasta, bread, vegetables, fruits, legumes, olive oil and nuts did not meet the recommended frequency of consumption in people affected by neuromuscular diseases, as published by the Spanish Federation of Neuromuscular Diseases with the scientific endorsement of the Spanish Society for Endocrinology and Nutrition.[244] For the food groups milk and dairy products, fish and lean meats and eggs, the frequency of consumption did properly fulfilled and even exceed the above-mentioned guidelines, suggesting that, at least in this cohort, protein intake was considered a relevant asset in their nutritional habits.

Regarding the consumption of dietary methylxanthines from food and beverages in the form of coffee, tea, chocolate, caffeinated soft drinks (cola drinks) and energy drinks, 86% of the cohort revealed to consume different products in minor to moderate usual intensity, whereas the 14% did not consume methylxanthines in any form in usual diet. The main source of dietary caffeine was coffee, for which 51% participants consumed at least one serving per day. Accordingly, the study cohort consumed caffeine on a regular basis (82.0±80.0 mg median caffeine/daily), but almost no theobromine (0.9±13.2 mg median theobromine/daily), with very similar trends between genders.

All the SF-36 mean scores were obtained in the central range of the scale (physical functioning 36.84±11.73, role-physical 40.53±10.07, bodily pain 39.98±9.97, general health 33.53±9.05, vitality 44.37±10.02, social functioning 45.94±21.82, role-emotional 44.22±23.32, mental health 47.08±10.38, physical component summaries 35.63±10.19 and mental component summary 47.99±10.10), suggesting moderate levels of quality of life in the study cohort. Almost all these scores are in the lower range of the already reported HRQoL scores in DM1 using the SF-36 tool.

No statistically significant differences were obtained when comparing the SF-36 summary scores in the dietary methylxanthines' consumers *versus* non-consumers. However, interestingly dietary methylxanthines consumption did show statistically significant correlation in front of the SF-36 scales particularly related to emotional and mental health ($p<0.05$).

CHAPTER 5: INTERVENTIONAL CLINICAL TRIAL

Chapter 5 reveals the design, execution and results of the interventional study code NCT04634682 (Effect of MYODM on Quality of Life, Fatigue and Hypersomnia in Patients with Myotonic Dystrophy Type 1), whose objective is to evaluate whether the formulated composition containing *Theobroma cacao* supplemented with caffeine, improves the quality of life of DM1 patients, through the management of excessive daytime sleepiness. Some insights on DM1 patients under COVID-19 pandemic, the time frame in which this study was carried out.

Chapter 5 responds to the specific goal nr 6.

"This project has received funding from the European Union's H2020 research and innovation program under funding agreement No. 875615".

5.1. Introduction and hypothesis

As previously discussed, DM1 is a multisystemic disorder, clinically characterized by progressive muscle atrophy and weakness, cardiomyopathy, insulin resistance and cataracts, among others. **Table 70** summarizes the most frequent clinical manifestations in DM1, as detailed in the study protocol.

Table 70: Most frequent clinical manifestations in DM1

MANIFESTATIONS OF THE MUSCULAR SYSTEM	1. Myotonia 2. Progressive loss of strength 3. Muscle atrophy, especially facial, temporal and neck 4. Involvement of the extensor muscles of the wrist, fingers, and the intrinsic muscles of the hand 5. Weakness of the flexor dorsi of the ankle, which causes what is known as "pendulum foot"
MANIFESTATIONS OF THE RESPIRATORY SYSTEM	1. Respiratory failure due to weakness of the diaphragm and intercostal muscles 2. Risk of pneumonia
MANIFESTATIONS OF THE CARDIOVASCULAR SYSTEM	1. Conduction defects, especially arrhythmias and blocks 2. Mitral valve prolapse 3. Sudden death
MANIFESTATIONS OF THE GASTROINTESTINAL SYSTEM	1. Swallowing problems due to palatal, pharyngeal and lingual involvement 2. Risk of aspiration pneumonia 3. Decreased oesophageal and colonic peristalsis 4. Recurrent intestinal pseudo-obstruction
MANIFESTATIONS OF THE CENTRAL NERVOUS SYSTEM	1. Minor intellectual deficit 2. Apathy 3. Excessive daytime sleepiness
MANIFESTATIONS OF THE REPRODUCTIVE AND ENDOCRINE SYSTEM	1. Frontal baldness 2. Hypogonadism 3. Elevation of liver enzymes 4. Type 2 *diabetes mellitus* 5. Non-alcoholic hepatic steatosis 6. Metabolic syndrome
MANIFESTATIONS OF THE IMMUNOLOGICAL SYSTEM AND TUMOURS	1. Pilomatrixoma Hypogonadism
MANIFESTATIONS OF THE VISION SYSTEM	1. Posterior subcapsular cataracts

The disease leads to substantial physical impairment,[421] which, in combination with the neuropsychological effects of the condition,[422, 423] results in severely restricted social participation and limited quality of life.[424,425]

Daytime sleepiness, defined as excessive sleep during the day, causes significant mortality and morbidity in DM1 because it causes accidents while driving, at work, or at home. The degree of drowsiness can be assessed using specific sleep scales that measure this affectation in patients. Beyond the impairment of nocturnal sleep, DM1 patients also present a dysregulation of the circadian sleep-wake rhythm.[426]

Currently, waking stimulants such as modafinil are now used in routine clinical practice, and medications for the treatment of attention deficit hyperactivity disorder, or ADHD, are sometimes prescribed for daytime sleepiness. These agents induce moderate decreases in the Epworth sleep scale values.[427] However, based on a risk-benefit evaluation performed by EMA's Committee for Medicinal Products for Human Use (CHMP), the European Commission issued a decision in 2011 on the uses of modafinil-containing medicines 'to be restricted to the active of narcolepsy, (…) since the data from the trials in other disorders did not provide strong evidence to support the use of the product', and even discouraged its use in patients with uncontrolled hypertension or irregular heart beat, as well as in children.[428]

It has been demonstrated that caffeine influences the human circadian timing,[429] and reduced consumption of theobromine has been associated with long sleep.[430] Moreover, paraxanthine, the main metabolite of caffeine, has been suggested to be a better wake-promoting agent for hypersomnia associated with neurodegenerative diseases than caffeine or modafinil.[431]

Quality of life (QoL) is a concept related to health defined by the WHO as a state of physical, mental and social well-being from a subjective and temporal assessment. This concept is multidimensional and encompasses different aspects that can be categorized into physical, psychological and social factors (**Table 71**), and whose impact on each patient will depend on individual and social conditions.

Table 71: Most frequent factors related to the limited Quality of life in DM1

PHYSICAL FACTORS:	1. Disabling motor disturbances 2. Fatigue 3. Sexual and urinary disorders 4. Visual disorders 5. Pain
PSYCHOLOGICAL FACTORS:	1. Anxiety disorder 2. Depression 3. Alterations and loss of cognitive functions
SOCIAL FACTORS:	1. Loss or job changes 2. Loss or changes in friends 3. Loss of habits 4. Alterations in social and family relationships

Antonini and col.[432] published a case-control study in which Health-Related Quality of Life (HRQoL) was evaluated in DM1 and its relationships with genetic, clinical, neuropsychological and emotional factors. It took a series of 20 patients with the disease, 11 men and 9 women, who answered the SF-36 questionnaire. In the results, the means of the scores were lower than those of the controls in all dimensions, concluding that HRQoL is seriously affected in DM1 and is negatively affected by the severity and duration of the disease, as well as by

certain cognitive deficits and changes in emotional functioning. The authors propose that therapeutic intervention in this field could contribute to improving the Health-Related Quality of Life in DM1.

Recent epidemiological studies and metaanalysis[433] demonstrated that the consumption of coffee (a natural beverage rich in natural xanthines) is associated with a lower risk or even an improvement of type 2 diabetes mellitus[434,435] and metabolic syndrome (MS).[436] Caffeine has phosphodiesterase inhibitory activity together with adenosine receptor antagonism and is capable of reducing body weight, total serum cholesterol, liver lipid content, adipocyte size, and lipoprotein lipase activity of adipose tissue in rats fed a high-fat diet[437,438,439]

However, arabic coffee and other unfiltered coffee preparations have many other different components, with opposite effects on the risks of metabolic syndrome (MS). With respect to DM1, a commercialized combination of two natural xanthines such as caffeine and theobromine (marketed as MYODM) demonstrated a further increase in MBNL1 levels in DM1 cells,[440,441] thus showing a clear synergistic effect. The composition formulated in MYODM was identified as the optimal relationship between caffeine and theobromine to maximize the amount of MBNL1 and demonstrate activity in most of the *in vitro* efficacy outcome measures as well as in a DM1 fly model (muscle atrophy, climbing and capacity flight, cardiac dysfunction, lifespan and survival).[440,441] Therefore, it was worth to explore whether this methylxanthines' composition could be useful in patients with DM1.

Coffee consumption is associated with a lower risk or even an improvement of type 2 diabetes mellitus and metabolic syndrome (MS).[442] The administration of MYODM with an adequate proportion of caffeine and theobromine could be useful as a nutritional supplementation for patients with DM1, but also, as ingredients are natural stimulants, they could contribute to improving the Health-Related Quality of Life of these patients by contributing to a reduction in their daytime sleepiness.

Traditionally, pharmaceutical drugs have been considered as the sole option to cure and improve the quality of life of a patient in any disease. The QoL is measured by several parameters, such as vitality, pain, general health perception, physical, emotional and social role functioning and mental health. Unfortunately, only a few hundreds of the almost 7,000 rare diseases have some pharmacological treatments available, but this is not the case in DM1.

In the light of the ´Leave No-one Behind´ commitment behind Sustainable Development Goals (SDG), and particularly, SDG10 devoted to reducing inequality, paying attention to the needs of disadvantaged and marginalized populations, the dietary management approach may provide a unique option for rare disease patients. In this regard, the European Commission supported the present research project 'New food for special medical purposes for the nutritional management of Myotonic Dystrophy type 1' (MYODM-FSMP), which was awarded funding in the Horizon 2020 SME Instrument Phase 1 research program (grant agreement nr 875615).[443] The main goal of the pilot project was to compile in human evidences that the developed composition based on caffeine and theobromine and currently marketed in the form of the food supplement MYODM could be upgraded to a food for special medical purposes as a unique approach to nutritionally manage DM1 in order to improve DM1 patients' health-related quality of life, through an interventional pilot clinical trial in these patients.

Accordingly, the 'Research project to evaluate the effect of the MYODM food supplement on quality of life, fatigue and hypersomnia in patients with Myotonic Dystrophy type 1', as a pilot interventional clinical trial, was born.

5.2. DM1 patients under COVID-19 pandemic

On 31 December 2019, while the present project was taking place, the WHO China Country Office was informed of cases of pneumonia from unknown etiology detected in Wuhan City, Hubei Province of China.[444] On 12 January 2020, Chinese authorities determined that the outbreak was caused by a novel coronavirus, named as SARS-CoV-2,[445,446] responsible for a new infectious disease named by WHO as COVID-19.

The viral infection demonstrated capabilities of producing an excessive immune reaction in the host, labelled as 'cytokine storm'.[447] The clinical spectrum of COVID-19 varied from asymptomatic, mild to moderate symptoms including fever, malaise, dry cough, dyspnoea, loss of taste and/or smell, diarrhoea, and vomiting, to clinical conditions characterized by respiratory impairment such as pneumonia, respiratory failure that requests mechanical ventilation and support in an intensive care unit, to multiorgan and systemic manifestations in terms of sepsis, septic shock, and multiple organ dysfunction syndromes (MODS). Severity was associated with pre-existing comorbidities such as cardiovascular disease, diabetes, chronic respiratory disease, oncological diseases, and the like.[448] The pathogenic mechanism that produces pneumonia seems to be particularly complex.

On 11 March 2020, WHO confirmed that COVID-19 could be characterized as a pandemic.[449] On 13 March 2020, WHO stated that Europe had become the epicentre of the pandemic with more reported cases and deaths than the rest of the world combined, apart from the People's Republic of China.

On 14 March 2020, the Spanish Government declared a first state of alarm for the management of the health crisis situation caused by COVID-19.[450] This situation lasted until 21 June 2020. On 25 October 2020, the Spanish Government declared a second state of alarm, again under the pandemic circumstances. This situation was postponed until 09 May 2021,[451] although successive COVID waves impacted Spain and Europe along 2021.

It was promptly recognized that patients suffering neuromuscular diseases were at higher risk of complications in case of SARS-CoV-2 infections. Beside the general risk factors previously mentioned, the respiratory muscle weakness, weakened immune system, obesity and cardiovascular impairment usually found in patients with DM1 could make them more prone to the hyperinflammation situation elicited by SARS-CoV-2.

A part from the scientific literature,[452,453] many Patient Associations and Muscular Networks, such as World Muscle Society,[454] Myotonic Dystrophy Foundation,[455] Muscular Dystrophy Association,[456] European Reference Network for Neuromuscular Diseases,[457] *Federación Española de Enfermedades Neuromusculares*,[458] provided special guidance and recommendations for such a fragile community.

During these extremely complex periods, we kept working on the study design, protocol preparation, submission and evaluation by the Ethical Committee for Clinical Research of the Gipuzkoa Health Area. **Table 72** summarizes the timeline from the project award to the inclusion of the first patient in the light of COVID-19 pandemic situation.

Table 72: Summarized timeline of the MYODM-FSMP project and the interventional pilot clinical trial

Date	Event
06/2019	MYODM-FSMP project Grant Agreement 875615 awarded by the European Commission
09/2019-06/2020	Clinical trial protocol evaluation by the Ethics Committee
03/2020-06/2020	Spanish lockdown due to first Coronavirus SARS-CoV-2 outbreak and COVID-19 pandemic. First primary healthcare and hospital collapses in Spain
06/2020	Protocol RFT-MYO-2020-01 approved by the Ethics Committee
07/2020-10/2020	Contractual formalisms with the Hospital, the Clinical Research Organization (CRO) and insurance company
10/2020-11/2020	Coronavirus SARS-CoV-2 second outbreak in Spain. Second primary healthcare and hospital collapses in Spain.
11/2020	NIH publishes the study protocol RFT-MYO-2020-01 'Effect of MYODM on Quality of Life, Fatigue and Hypersomnia in Patients With Myotonic Dystrophy Type 1' (ClinicalTrials.gov Identifier: NCT04634682)[459]
09/11/2020	Inclusion visit of the first patient in the clinical trial RFT-MYO-2020-01
11/2020-10/2021	MYODM-FSMP clinical trial under active recruitment and follow-up Coronavirus SARS-CoV-2 third, fourth, fifth and sixth outbreaks in Spain
15/10/2021	Last visit of the last patient in the clinical trial RFT-MYO-2020-01

Unfortunately, the COVID-19 pandemic has been impacting the study. Part of the clinical research team was infected in December 2020 and in April 2021. Along those months, some patients declined to attend the follow-up visits and some were dropped-out of the study due to these uncertain times. More details on these very difficult situations and their impact on the clinical trial are further disclosed in the Results section.

5.3. Study goals and Protocol

Clinical trials for foods and supplements have certain differences than pharmaceutical trials,[460,461] since foods tend to have lower risk of adverse side effects. However, the trial had met all ethics standards, in accordance with current Good Clinical Practices as well as specific Guidelines to measure health benefits of foods and food products.

The main goals of the pilot interventional clinical trial 'Research project to evaluate the effect of the food supplement MYODM on quality of life, fatigue and hypersomnia in patients with Myotonic Dystrophy type 1', were defined in order to:

1. Assess the changes in health-related quality of life measured using the Short Form-36 Health Survey (SF-36) and Individualized Neuromuscular Quality of Life (INQoL) in DM1 patients administered with the investigational food product MYODM.

2. Assess whether MYODM reduces daytime sleepiness in patients with DM1.

3. Establish and validate a series of clinically significant criteria to assess the possibility of a future clinical trial to demonstrate the effects of MYODM compared to placebo in patients with DM1.

Study title:

Research Project to Evaluate the Effect of the Food Supplement MYODM on Quality of Life, Fatigue and Hypersomnia in Patients with Myotonic Dystrophy type 1

Protocol code: RFT-MYO-2020-01

Acronym: MYODM PRO

Study population: Adult patients (men and women) diagnosed with DM1.

Design: Research project, monocentric, simple randomized, in patients diagnosed with DM1 whose main objective is to evaluate changes in quality of life, fatigue and hypersomnia of the food supplement MYODM.

We proposed a simple randomized, monocentric, pilot research project, which would include 30 patients, aged> 18 years, diagnosed with DM1.

Duration of study participation

The duration of the study was 6 months and consisted of a baseline visit and a follow-up period.

Patient eligibility was assessed during the Baseline visit. Patients eligible for the study were randomized to one of the two arms of the study: arm dosaged with MYODM (Group A, active arm) or arm not being dosaged with MYODM (Group B, control arm). Randomized patients in group A were dispensed with the investigational food product during the inclusion visit, and started taking the investigational food product the same day. Patients randomized to Group B

followed the same protocol-based assessment schedule as Group A, but without taking the investigational food product MYODM.

All randomized patients, regardless of the group in which they were included, carried out the following face-to-face visits (at the Hospital) in the context of the study: Visit V0 (Baseline), V1 (Month 3) and V2 (Month 6).

Participants were interviewed in the inclusion visit in order to know their baseline consumption pattern of coffee, tea and caffeinated cola soft drinks. Participants were prompted not to modify any aspects in their usual diet during the study.

Study population

The study population consisted of thirty adult patients aged above 18 years old, diagnosed with DM1, who attended the demographic consultation of the Neuromuscular Unit of the Donostia University Hospital and who met the inclusion and exclusion criteria of this research project.

Inclusion and Exclusion criteria

Inclusion criteria:

1) Patients, men and women aged more than 18 years old diagnosed with DM1
2) Patients with the deambulation capacity (use of canes and crutches is allowed)
3) Patients capable of completing all study evaluations appropriately
4) Patients who have given their informed consent to participate in the study (see Annex 3)

Exclusion criteria:

1) Regular intake of plant stanols or other nutritional supplement
2) Daily alcohol consumption score of 4 or more according to Audit C questionnaire (**Figure 49**)
3) Physical or mental incapacity to participate in the study
4) Patients who do not sign the informed consent to participate in the study (see Annex 3)

ALCOHOL SCREENING TOOL

1 unit is typically:

Half-pint of regular beer, lager or cider; 1 small glass of low ABV wine (9%); 1 single measure of spirits (25ml)

UNIT GUIDE

The following drinks have more than one unit:

A pint of regular beer, lager or cider, a pint of strong /premium beer, lager or cider, 440ml regular can cider/lager, 440ml "super" lager, 250ml glass of wine (12%)

The following questions are validated as screening tools for alcohol use

AUDIT- C Questions	Scoring system					Your score
	0	1	2	3	4	
How often do you have a drink containing alcohol?	Never	Monthly or less	2-4 times per month	2-3 times per week	4+ times per week	
How many units of alcohol do you drink on a typical day when you are drinking?	1 -2	3-4	5-6	7-9	10+	
How often have you had 6 or more units if female, or 8 or more if male, on a single occasion in the last year?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
					TOTAL :	

A score of **less than 5** indicates *lower risk drinking* (see overleaf)

Scores of 5+ requires the following 7 questions to be completed:

Figure 49

Medical visits program and evaluations

Table 73 (in the next page) lists all evaluations planned along the clinical trial, wherein 'X' indicates the visits and explorations appointed during the study. Patients should be seen at all visits or as close to the scheduled day as possible.

In addition to the scheduled visits, patients may be given unscheduled visits due to acute illnesses of undetermined cause, dose change, for another reason at the discretion of the investigator. Data collected during unscheduled visits were recorded in the unscheduled visit case report form (CRF).

Visiting windows were ±3 days. If a visit was made outside of its window, all subsequent visits must be in accordance with the original visitation schedule (one month is defined as 30 calendar days).

Normal visiting program

Patients following the normal visiting schedule were required to complete all evaluations as outlined in **Table 73**.

Patients who had their investigational product withdrawn before the scheduled date were asked to continue study visits until the end of the study. These patients would complete an end of administration visit.

End of treatment / End of study / Main part follow-up visits

The End of Study (EOS) visit took place at the end of the active stage, that is, at the time the investigational food product administration stage ended or at the time of premature withdrawal from the study.

The End of Treatment (EOT) visit was not mandatory for all subjects. It applied to those subjects who were prematurely withdrawn from study investigational treatment administration and decided to continue participating in the study.

Table 73: Schedule of visit and evaluations in the pilot clinical trial RFT-MYO-2020-01

Medical visit	**Screening (Day -7 to Day -1)**	**Baseline (Day 0)**	**Week 1**	**Month 3**	**Week 23**	**Month 6 End of Study**
VISIT nr	**1**	**2**		**3**		**4**
Visit window	N.A.	N.A.		±3 days		±3 days
Signature of the Informed Consent	X					
Inclusion/exclusion criteria	X	X				
Demographic data	X					
Physical exploration	X			X		X
Vital signs	X	X		X		X
ECG	X					X
Height, Weight, Waist circumference	X	X		X		X
BMI, muscular mass	X	X		X		X
Motor functioning Walking speed test (10 seconds WT) 6 minutes WT Quantitative muscular testing	X			X		X
Biochemistry blood analysis (12 hours fasting)	X			X		X
Patient reported Outcome (PRO) formularies	X			X		X
WAIS-IV		X				
Previous/concomitant treatment	X	X		X		X
Medical history	X					
Dispensing of study product MYODM		X		X		
Adverse events	X	X		X		X
Deliver / collect the diary of the patient	X	X		X		X
Start daily activity record and dream	X				X	
End daily activity record and dream			X			X

Information to be collected on selection failures

For all patients who signed the informed consent (IC) but had not been included in the next stage, the data collected on the selection stage, demographic data, inclusion / exclusion criteria, and adverse effects was completed in the CRF. Non-serious adverse events would be followed-up by the investigator and recorded only in the source data (medical history).

For patients who signed the informed consent and were included in the active stage of the study, all Adverse Events (AE) that take place after signing the IC were recorded in the CRF in the AE section.

Demographic data / other basic characteristics of the patients

Patient demographics and baseline characteristics to be collected from all patients included: date of birth, sex, race, ethnicity, relevant medical history, physical examination, vital signs, laboratory samples, ECG, previous / concomitant medication, alcoholic habits, MIRS (Muscular Impairment Rating Scale).

At the screening visit, each patient received an actimeter to measure the level of physical activity performed each day and sleep. The registration was carried out continuously in two stages during the study:

- Time 1 - day 1 (corresponds to the Screening visit) - until day 14
- Time 2 - 14 days before the final visit

Physical exploration

A complete physical examination was performed, including an evaluation of the skin, head, neck, lymph nodes, heart, lungs, abdomen, back, neurological function in the visits, as indicated in **Table 73**.

All significant findings that were present prior to signing the Informed Consent were included in the CRF as Relevant Medical History / Current Illnesses. Significant findings that would be made after the signing of the IC and the start of active, that meet the definition of an Adverse Event, would be recorded in the Adverse Events section of the CRF.

Vital signs

Vital signs will include sitting pulse rate, sitting systolic and diastolic blood pressure.

Height, weight, waist circumference, BMI

Height was measured as described in **Table 73**.

Weight and waist circumference was measured as described in **Table 73**.

The body mass index (BMI) would be calculated according to the following formula: BMI = weight [kg] / height [m^2].

The waist circumference was measured using a Gulick tape taken midway between the highest point of the iliac crest and the lowest part of the costal margin of the mid-axillary line.

Motor function

Motor function was measured at each visit using the gait speed test, 6-minute walk test (6 min WT), and manual force using a dynamometer. The three tests are described below.

1. Walking speed test (WT)
   The patient has to walk a distance of 6 meters. The test is carried out in a quiet place without obstacles. The time required to perform the test is measured. A speed of <0.8 m/second is considered abnormal.

2. 6 minute walk test (6MWT)
   The 6MWT is a common measure used in the patient follow-up of various neuromuscular disorders as well as a usual outcome measure in clinical trials testing therapeutic drugs for DM1. By measuring the distance that a patient can walk on a flat surface in a period of 6 minutes, the test evaluates the global and integrated responses of all the systems involved during exercise, including the pulmonary and cardiovascular systems, systemic circulation, peripheral circulation, blood, neuromuscular units, and muscle metabolism.[462]

3. Manual muscle strength test
   Muscle strength is measured using a dynamometer (Camry hydraulic hand dynamometer, model EH101). Force is considered affected if the mean of the 3 measurements is less <30 kg.

Laboratory evaluations

The local laboratory will be used for the analysis of blood samples collected for the analysis of biochemistry and liver function.

Biochemistry

Blood samples for biochemistry were drawn at the scheduled visits indicated in **Table 73**. The evaluated parameters were: electrolytes (Na, K, Cl, bicarbonate, Ca, Mg, P), random glucose, albumin, alkaline phosphatase, creatinine, ALT, AST, GGT, amylase, total bilirubin, conjugated bilirubin, total cholesterol, CRP, triglycerides, HDL, LDL, CK18. Twelve hour fasting blood samples were drawn for lipid profile.

Electrocardiogram

An ECG was performed at the Baseline and V2 visit of the study, according to the Hospital protocol to rule out possible cardiac pathologies.

Patient-reported outcome measures

1. SF-36 Health Questionnaire[416,417]
   The Short Form-36 Health Survey (SF-36) seems to be the ideal instrument for assessing HRQoL in DM1, since it is a quick instrument, easy to administer, with validation studies in Spain, including Spanish population norms.

This is a generic QoL questionnaire. The SF-36 Health Survey is a health survey designed by the Health Institute, New England Medical Center, Boston Massachusetts, which, based on 36 questions, aims to measure eight generic concepts about health, that is, concepts that are not specific to health, a pathology, active group or age, detecting both positive and negative states of physical health and emotional state.

By allowing numerical assessment of different aspects of the health of the person, it becomes an excellent tool for any research related to health.

These 36 questions address different aspects related to the daily life of the person completing the questionnaire. They are grouped and measured in eight sections that are independently valued and give place to eight dimensions (health concepts). In addition to the eight health concepts, the SF-36 includes the general concept of changes in the perception of the current state of health and in that of the previous year.

2. Individualized Neuromuscular Quality of Life (INQoL)[484]
   INQoL is a questionnaire that assesses the health-related quality of life of adults with neuromuscular diseases. A study has analyzed the reliability of the Spanish version of the scale, as an instrument for measuring health-related quality of life in individuals with these disorders.

3. DM1- Activ Questionnaire[491,492]
   It is a 25-item questionnaire that covers a wide range of daily and social activities. The DM1-Activ captures the personal ability to perform a task where the patient is asked to determine if he / she can complete that task independently.[463]

4. Modified Fatigue Impact Scale (MFIS)
   It is the reduced adaptation of the Fatigue Impact Scale (FIS)[464] that assesses the effects of fatigue in the time frame of the last month, and consists of 21 items distributed in three subscales: physical, cognitive and psychosocial. The score is obtained by adding the individual score for each item, which presents five possible responses ranging from 0 = no problem to 4 = extreme problem. The final score ranges from 0 to 84, with higher scores reflecting more severe fatigue. A score of 38 points is established as the cut-off point to define the presence of fatigue or not.[465]
5. Test of the 90 symptoms (Symptom checklist 90 R)[495]
   It is a tool developed to evaluate patterns of symptoms present in individuals and can be used both in community tasks and in clinical diagnosis. Each of the 90 items that make it up is answered on the basis of a five-point scale (0-4). It evaluates the: somatizations, obsessions and compulsions, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, psychoticism.

6. Global Physical Activity Questionnaire (GPAQ) surveillance[500,501]
   To quantify levels of physical activity in the adult population, WHO has developed a Global Physical Activity Questionnaire (GPAQ), which helps countries monitor insufficient physical activity as one of the main factors of risk of suffering from non-communicable diseases. The GPAQ has been integrated into the WHO “progressive” approach, which is applied to the surveillance of the main risk factors for non-communicable diseases.

7. Food consumption frequency questionnaire (short CFCA)[466]
   Semi-quantitative questionnaire on the frequency of food consumption. During the trial, patients should not change their intake of food containing chocolate or cocoa extracts, energy drinks or infusions containing caffeine or other stimulants. Patients were expected to keep a daily feeding log and were expected to be monitored by phone once a week to review dietary compliance. However, in the context of COVID-19 pandemic, this follow-up was not able to be performed, which was translated into a deviation from the protocol.

8. Epworth Sleepiness Scale (ESS)[489]
   The Epworth Sleepiness Scale is a short questionnaire that attempts to measure daytime sleepiness. The researched subject is asked about the frequency (or probability) of falling asleep on a scale of increase that has from 0 to 3, for eight different daily situations, that most people can be involved in, in your daily life, though not necessarily every day. The score of the eight situations is added to obtain a total number. A result between 0 and 6 is considered normal, 7-8 average sleepiness, 9-24 abnormal sleepiness (probably pathological).

9. Neuropsychological evaluation: composed of two tests:
   9.1. California Computerized Assessment Package (CalCap)[505]
   It is a computerized test that allows standardized evaluations of reaction time and the speed of information processing.

   9.2. WAIS-IV (Wechsler Intelligence Scale for Adults-IV)[506]
   It is a psychometric test developed by David Wechsler that provides four scores (Verbal Comprehension, Perceptual Reasoning, Working Memory and Processing Speed), and a fifth one called Total Intelligence Quotient.

Investigational food product

The investigational food product was already manufactured in the form of hard capsules containing theobromine and caffeine as main ingredients and marketed as the food supplement MYODM.

In vitro and *in vivo* experiments had previously demonstrated the ability of the investigational product's composition to increase the levels of expression of MBNL, both MBNL1 and MBNL2 and their effective amount in the nucleus of human myoblasts DM1 (see further details in the Introduction and Chapter 3). The caffeine and theobromine composition in the investigational food product had demonstrated a synergistic effect on the increase of MBNL1 in transcription and protein level, after administration to human DM1 myoblasts. When administered to DM1 model animals, the corresponding composition of the investigational food product increased their survival, improved muscle area, cardiac dysfunction, and locomotor dysfunction in these experimental animals. Therefore, the investigational product was able to rescue DM1-related phenotypes *in vivo* and globally improved the lifespan of DM1 model individuals fed with the product (see further details in Chapter 3).

Caffeine and theobromine as ingredients in the investigational food product

The main ingredients of the investigational food product are the methylxanthines theobromine and caffeine, formulated to the optimal composition and doses obtained during the research and development of said product (see further details in Chapter 3). No other food contains the ratio of theobromine and caffeine formulated in the investigational product (see further details in Chapter 1).

Caffeine as an ingredient in the investigational food product

The investigational food product contained 20 mg of caffeine per capsule. Three capsules per day were evaluated in the study, which entailed 60 mg caffeine. Accordingly, the daily intake of the investigational food product provided 0.9 mg/kg/day caffeine in an individual adult weighing 70 kg. EFSA discloses that caffeine single doses up to 3 mg/kg/day for a 70 kg adult (equivalent to 200 mg of caffeine in a single intake) or habitual caffeine doses up to 5.7 mg/kg/day (equivalent to up to 400 mg of caffeine per day intake) are safe in healthy adult population[300] (see Chapter 2 and 3 for further details).

Dispensing of the investigational product

The investigational food product was presented in boxes containing 90 capsules, equivalent to one month intake. At scheduled visits, the study staff dispensed one box of the investigational food product to each patient. The patient number and date of dispensing were recorded on the box.

Instructions for the prescription and administration of the investigational food product

During the study, capsules of the investigational food product should be taken three times per day (morning, noon and afternoon), preferably at the same time each day. The capsules can be taken with or without food. The first dose of the investigational product was administered at the Donostia University Hospital before 10 a.m. The product is kept at room temperature.

Missed dose readjustment

If the patient forgets to take a dose at some point during the study, he/she will take the next one when appropriate. Taking a double dose is not advised at any time.

Discontinuation or withdrawal

Investigational food product under study may be interrupted or withdrawn based on the judgement of the investigator and the overall clinical evaluation, including:

- Withdrawal of informed consent
- Any violation of the protocol that results in a significant risk to patient safety
- Breach of study active
- Loss of tracking
- Adverse event
- Abnormal laboratory values

Patients can voluntarily withdraw from the study for any reason and at any time. They can be considered withdrawn if they express an intention to withdraw, or fail to return to visits, or are lost to follow-up for any reason.

If premature withdrawal occurs for any reason, the investigator should make every effort to determine the primary reason for the premature withdrawal of the patient from the study and record this information on the appropriate page of the CRF. All changes in the dose must be recorded in the CRF in the section "Dose administration record".

Study completion

A participant was considered to have completed the study when all study visits were completed. The study would be considered completed when all patients had completed the study or were withdrawn prematurely from the study.

Patient numbering

Each patient was uniquely identified by a Patient Number that was composed of the center's initials (HUD) and a sequential number assigned by the investigator. Once assigned to a patient, the Patient Number was not reused.

When the patient had signed the informed consent form, the investigator assigned the patient the following sequential number. If for any reason the patient could not be treated, the sponsor was notified within two days that the patient was not treated and the reason why the patient was not treated will be entered in the CRF.

Investigational food product allocation, randomization

At the baseline visit, all patients eligible to participate in the study were randomized using the simple randomization list generated with the WINPEPI computer program to one of the two arms in the study.

Access to the scrambling codes was restricted. The randomization was carried out by a member of the research team who was not involved in the clinical evaluation part. The clinical evaluators did not have access to the randomization lists.

Patients were explained it was necessary to keep hidden from the evaluating physician at all times whether or not they were taking the investigational food product, to maintain the blindness of the study.

Data analysis

The data was collected in the corresponding questionnaires in prospective form. Once the study was finished, after recording the data of the last patient included, the database was closed and the statistical analysis was carried out. Firstly, the content of the database was exported to a file with a suitable format so that it can be imported into the format used for the statistical analyzes. Methodology for the statistical analysis was previously disclosed in the ‘Methodology’ section.

Ethical issues

The study was carried out in accordance with the requirements expressed in the regulation currently in force, as previously disclosed in the 'Methodology' section.

Source documents

Source documents are defined as original documents, data and records, which may include hospital or medical records, results of clinical tests, subject assessment diaries or lists, and other records, recorded data on automatic instruments, microfiche, photographic negatives, microfilm or magnetic media and X-rays. The data collected during this study should be recorded in the appropriate source documents.

The researcher or the hospital will allow the activities related to the study of monitoring, auditing, review by the CEIm and inspection by the regulatory authorities, allowing direct access to source documents and data.

Data collection

The research staff entered the data required by the protocol into a paper case report form (CRF) designed for this project. Subsequently, all the data was properly collected in an Excel file.

The investigator documented the patient data in his/her own patient files, which served as the source data for the study.

All data entered in the CRF is supported by the source documentation.

The investigator or an authorized member of his team would make the necessary corrections in the CRF. All information changes, along with the date and the person making the correction, are available through the system's audit trail. The reason for any correction made was presented.

Changes in the protocol

All modifications made to this protocol must be communicated to the Ethics Committee that carried out its evaluation and to the corresponding authorities, as appropriate. In the case of relevant modifications, those that affect fundamental aspects of the study protocol such as objectives, methods or ethical aspects, were submitted again to the evaluation of the CEIm that reported favourably on it and the corresponding authorities, as appropriate, and would request administrative authorization for said amendment. Notification to the CEIm would suffice for the rest of the amendments, justifying the reason why it is not considered relevant.

Follow-up/final report

The definitive closure of the study was carried out once all the data of the last patient included in the study was completed. After closing the database, the statistical analysis was carried out and the final report was presented with the results.

5.4. Patient-reported outcome measures

The primary outcome in a clinical trial is the most important issue for evaluating the effect of an intervention or active. However, additional secondary outcome measures may be additionally included for evaluating further aspects of the intervention that could render interesting information on the effects of the investigational food product. Both primary and secondary outcomes may be a single or several measures.

A systematic review on the most common measures of general HRQoL in patients with adult-onset DM1 mainly rendered the 36-Item Short Form Health Survey (SF-36) and the Individualized Neuromuscular Quality of Life Questionnaire (INQoL) as the main tools, which could be complemented with additional surveys to research for further information.[42] Therefore, in the present study, the selected outcome measures were:

Primary Outcome Measures:

1. Change in Individualized Neuromuscular Quality of Life (INQoL) Mean Scores [Time Frame: Screening, Month 3, Month 6]

   Scores from the self-administered INQoL questionnaire will be compared at the start of the study (Month 0) and at the end (Month 6) among the MYODM administered group and the control group. Scores range from 0-100, with closer to 100 being a better outcome.

2. Change in Individualized Short Form-36 (SF-36) Mean Scores [Time Frame: Screening, Month 3, Month 6]

   Scores from the self-administered SF-36 questionnaire will be measured at the start of the study (Month 0), and at the end (Month 6) among patients in the MYODM administered group and control group. Mean scores range from 0 (minimum) - 100 (maximum) with higher mean scores reflecting better outcomes.

3. Change in Epworth Sleepiness Scale (ESS) Scores [Time Frame: Screening, Month 3, Month 6]

   ESS score range is 0-24; lower ESS scores indicate less daytime sleepiness; higher ESS scores indicate more severe sleepiness.

Secondary Outcome Measures:

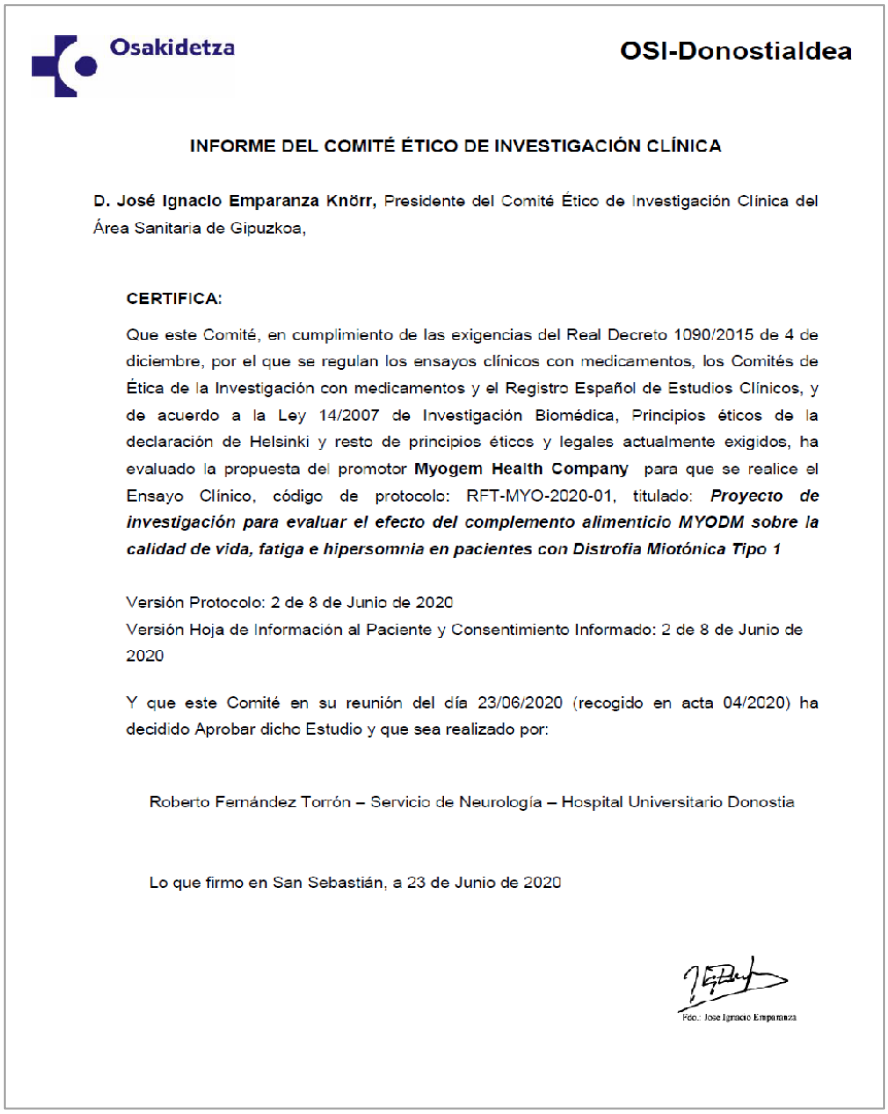
1. Change in Physical activity and daytime sleepiness measured with GeneActiv actimeter [Time Frame: Screening, Baseline, Month 6]

Additional Outcome Measures:

Besides the selected outcomes, additional health-related quality of life questionnaires were included in the study in order to collect as much information as possible from the interventional part. Some of them were specific for neuromuscular diseases whereas some others were more general health-related. The background and main characteristics of each questionnaire are disclosed as supplementary material in the Annex 5 section.

5.5. Ethics Committee Approval

The official communication from the Ethics Committee in Clinical Research from the Sanitary Area Gipuzkoa on the approval of the Protocol RFT-MYO-2020-1 title 'Research project to evaluate the effect of the MYODM food supplement on quality of life, fatigue and hypersomnia in patients with Myotonic Dystrophy type 1', was received in June 2020 (**Figure 50**).



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INFORME DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

D. José Ignacio Emparanza Knörr, Presidente del Comité Ético de Investigación Clínica del Área Sanitaria de Gipuzkoa,

CERTIFICA:

Que este Comité, en cumplimiento de las exigencias del Real Decreto 1090/2015 de 4 de diciembre, por el que se regulan los ensayos clínicos con medicamentos, los Comités de Ética de la Investigación con medicamentos y el Registro Español de Estudios Clínicos, y de acuerdo a la Ley 14/2007 de Investigación Biomédica, Principios éticos de la declaración de Helsinki y resto de principios éticos y legales actualmente exigidos, ha evaluado la propuesta del promotor **Myogem Health Company** para que se realice el Ensayo Clínico, código de protocolo: RFT-MYO-2020-01, titulado: ***Proyecto de investigación para evaluar el efecto del complemento alimenticio MYODM sobre la calidad de vida, fatiga e hipersomnia en pacientes con Distrofia Miotónica Tipo 1***

Versión Protocolo: 2 de 8 de Junio de 2020
Versión Hoja de Información al Paciente y Consentimiento Informado: 2 de 8 de Junio de 2020

Y que este Comité en su reunión del día 23/06/2020 (recogido en acta 04/2020) ha decidido Aprobar dicho Estudio y que sea realizado por:

Roberto Fernández Torrón – Servicio de Neurología – Hospital Universitario Donostia

Lo que firmo en San Sebastián, a 23 de Junio de 2020

Fdo.: José Ignacio Emparanza

Figure 50: Ethics Committee in Clinical Research from the Sanitary Area Gipuzkoa approval to the Protocol RFT-MYO-2020-1

5.6. Patient enrolment and execution

The study population consisted of 30 patients, both men and women, adults aged above 18 years and diagnosed with DM1, who attended the demographic consultation of the Neuromuscular Unit of the Donostia University Hospital and who met the inclusion and exclusion criteria of this research project, as previously disclosed.

Patients were selected in order to be representative of the DM1 patient community, by including subjects with low, moderate and high affectation, either considering their number of CTG repetitions or their clinical evaluation and muscular impairment rating scale (MIRS).

Each patient was uniquely identified by a Patient Number that was composed of the center's initials (HUD) and a sequential number assigned by the investigator. Once assigned to a patient, the Patient Number was not reused. For the purpose of the present disclosure, patients were anonymized and identified by a Patient_ID number, from ID01 to ID30.

Patient eligibility was assessed during the Baseline visit. Patients eligible for the study were randomized to one of the two arms of the study: active arm (participants being administered with the investigational food product, MYODM) or control arm (participants not being administered with the investigational food product, MYODM). Therefore, fifteen patients were assigned to the active arm and fifteen patients were assigned to the control arm.

At the baseline visit, all patients eligible to participate in the study were randomized using the simple randomization list generated with the WINPEPI computer program to one of the arms of the study, in 1:1 ratio.

Randomized patients in the active arm were dispensed with the investigational food product and began taking MYODM the same day. Patients randomized to the control arm followed the same protocol-based assessment as scheduled as for the active arm, but without taking MYODM. At the baseline visit all patients were provided with the investigational food product and the questionnaires.

As previously disclosed, at the time this experimental research was performed, the entire globe was immersed in the COVID-19 pandemic. This very complex situation seriously affected the normal evolution of wordwide common life, and without any doubt, the normal evolution of this particular clinical trial, wherein:

1. Some of the subjects in the study were infected by SARS-CoV-2. Therefore, they were dropped out from the follow-up visits and controls.
2. The Hospital was functioning under the pressure of a very high healthcare demand in other medical areas, reallocating the professionals where more needed.
3. Part of the investigator’s team at the Hospital was infected as well, and some of the visits were performed by alternative medical colleagues not sufficiently familiarized with the protocol.
4. Non infected patients were really afraid to attend the Hospital for their follow-up visits. For this reason, some of them discontinued the study.

Overall, six patients (20% of the entire cohort) did not properly fulfil or continue the study after the inclusion visit. In the control arm, three out fifteen (20%) refused to continue, the same figures as in the active arm (three out fifteen, 20%) that abandoned the study.

The main reasons for the dropped out or missing data in the above-mentioned patients are disclosed in **Table 74**:

Table 74: Dropped out patients in the study cohort

Arm	Patient ID	Dropped out?	Comments
Control	03	Yes	No data from main questionnaires. COVID difficulties at the Hospital
	13	Yes	Scared by potential COVID infection at the Hospital
	15	Yes	Scared by potential COVID infection at the Hospital
Active	02	Yes	Adverse events reported (somnolence)
	05	Yes	COVID infection
	22	Yes	Incomplete adherence to active (less than 50%, according to capsules reconciliation)

5.7. Study results

5.7.1. Statistics

Descriptive and exploratory analysis

Generalities of the statistical analysis are already disclosed in the Methodology section. However, due to its relevance in the interventional study, we disclose herein an extended description thereof.

The statistical study focused on the descriptive and exploratory analysis of the data collected throughout the study. Beyond the categorical variables arm, ID, gender, age, inheritance and MIRS, the quantitative continuous variables: body weight, body mass index, biochemistry profile and scores from the previously mentioned questionnaires were evaluated.

Accordingly, in the descriptive analysis, the distribution of each variable was analyzed at each time point (baseline or inclusion visit, month 3 follow-up visit and month 6 end of study visit, as defined in the study protocol), checking that all the data had values within the expected range, and that there were no logical inconsistencies between variables, or impossible data. If any of these circumstances occurred, the information registered in the database was corrected by comparing the information with that of the clinical history.

As a result of the descriptive analysis, the estimators of the variables were obtained using the most appropriate statistic for the nature and scale of measurement in each case.

Data was used as such, without any transformation, except for the analysis of differences between time points (changes from baseline to month 3 follow-up or changes from baseline to

month 6 end of study), wherein normalization was performed as the differences between the evaluated time point and the baseline situation.

Assumptions of parametric tests (normally distributed data, homogeneity of variance, independence and the like) were confirmed by means of the Shapiro-Wilk normality test (p>0.05) and Levene's homogeneity test (p>0.05), respectively, for each variable at each time point. For those variables which the parametric assumptions could not be met, non-parametric tests were used instead.

Descriptive results are represented by the measures of central tendency and scatter measures of the scores of the questionnaires' domains carried out. In this regard, the statistics mean ± standard deviation (SD) summarizes results for parametric variables, whereas median ± interquartile range (IQR) summarizes results for non-parametric variables. The parametric or non-parametric statistics that better describe each result are expressed in **bold** in the tables along this chapter.

The mean or median differences between the changes from baseline visit to the month 3 visit, and between the changes from baseline to the month 6 end of study visit *between* arms, were studied using parametric (unpaired Student's t-test) or non-parametric (independent samples Mann-Whitney's U test), when appropriate.

The mean or median differences between the changes from baseline visit to the month 3 visit, and/or between the changes from baseline visit to the month 6 end of study visit *within* arms were studied using parametric (paired Student's t-test) or non-parametric (Wilcoxon signed-rank test), when appropriate.

The results obtained in the descriptive study were expected to be used as part of an exploratory study that allows establishing new hypotheses about possible effects of the investigational product. In the exploratory study, correlations between variables were analyzed using Spearman's correlation test.

For the estimation of the sample size, the statistical tool G*Power was used to compute statistical power.[467,468] Considering as an example the parametric unpaired t-test median comparison between groups (*i.e.,* control arm and active arm), and the conventional 80% statistical power threshold, a sample size *ca*. 42 participants (21 per group) would be eventually the minimum required to detect a large effect size, whereas for the detection of medium or small effect size variations, the study cohort should be composed of 102 participants (51 per group) or 620 participants (310 per group), respectively.

For the purposes of effect size convention, Cohen's definition was considered (d=0.8 large effect size, d=0.5 medium effect size, d=0.2 small effect size),[469] wherein effect size (Cohen's d) is defined as the magnitude of the difference between groups, and the absolute effect size is the difference between the average, or mean, outcome of two different intervention groups. Effect size is considered the practical significant measure in a study, whereas the p-value is considered the statistical measure.[470]

However, given the exploratory nature of the pilot study and the intrinsic characteristics of the rare disease, the sample size of the study was defined based on the expected difficulty and length of recruitment, the cost of the clinical testing and the available financial resources to be allocated to the project. In this regard, the sample size for the pilot study was defined as 30 patients, which were finally divided into two arms (15 participants per arm) to fulfil the requirements for the inclusion of a control group and eventual approval from the Ethics Committee.

It is worth mentioning that clinical trials in rare diseases usually involve cohorts with less participants than clinical trials in prevalent diseases, due to the intrinsic characteristics thereof. In this regard, even in the orphan medicinal products clinical trials field, one third of the trials evaluated during the decade from 2000 to 2010 involved less than 100 participants, whereas more than half of the trials involved 100–200 participants, according to EMA public information.[471]

Throughout the study, 95% confidence was considered when determining statistical significance ($p<0.05$), defined as the probability that the observed difference between two groups is due to chance and not to sampling variability ($p>0.05$).

Taking into account the limitations in the study sample size, both statistical significance and effect size are discussed in the interpretation of the statistical analysis shown herein.

5.7.2. General data

A total of 30 subjects aged from 28 to 72 years old (mean age, 46.3±9.9) participated in this study. In the control arm mean age was 47.9±13.2 years, and 44.7±5.0 years in the active arm. Female (mean age, 46.4±12.8) represented the 37%, whereas male (mean age, 46.4±8.2) represented the 63%. Patients were mostly overweight. General data is summarized in **Table 75**. All participants had a previous diagnosis of DM1.
Mean CTG repeats were 562±101 in the control arm and 644±109 in the active arm, as detailed in **Table 76**.

Table 75: Summary of cases. General data at inclusion visit

Arm			Patient ID	Gender	Age (years)	Weight (kg)	BMI (kg/m^2)	BMI Class
Control	1		01	M	49	69.0	22.28	NW
	2		03	F	57	79.0	25.50	OW
	3		04	M	39	80.0	24.70	NW
	4		06	F	38	88.1	34.40	Obese class I
	5		07	M	52	96.0	30.80	Obese class I
	6		09	M	43	62.5	21.40	NW
	7		10	F	53	82.0	34.58	Obese class I
	8		11	M	35	70.0	22.84	NW
	9		12	M	64	108.0	39.19	Obese class II
	10		13	F	72	78.0	32.89	Obese class I
	11		14	M	61	98.0	36.89	Obese class II
	12		15	F	28	96.0	35.26	Obese class II
	13		20	M	42	79.0	29.20	OW
	14		21	F	28	55.0	19.49	NW
	15		24	M	58	83.0	28.72	OW
	Total	N	15	15	15	15	15	15
		Mean	.	.	**47.9**	**81.6**	**29.21**	OW
		SD	.	.	**13.2**	**14.2**	**6.24**	-
Active	1		02	F	45	112.0	53.30	Obese class III
	2		05	M	42	67.0	22.40	NW
	3		08	M	49	64.7	25.60	OW
	4		16	M	34	112.0	38.30	Obese class II
	5		17	M	43	101.0	29.51	OW
	6		18	M	45	77.0	27.55	OW
	7		19	F	46	71.0	28.80	OW
	8		22	F	44	51.0	21.23	NW
	9		23	M	46	92.0	30.74	Obese class I
	10		25	M	46	83.0	28.06	OW
	11		26	M	52	88.0	27.80	OW
	12		27	M	45	63.0	21.00	NW
	13		28	M	37	84.4	24.90	NW
	14		29	F	43	78.0	30.85	Obese class I
	15		30	F	54	73.6	30.40	Obese class I
	Total	N	15	15	15	15	15	15
		Mean	-	-	**44.7**	**82.0**	29.98	OW
		SD	-	-	**5.0**	**16.0**	8.23	-
		Median	-	-	45	78.0	**28.06**	-
		IQR	-	-	5	21.0	**5.55**	-
Total	N		30	30	30	30	30	-
	Mean		-	-	**46.3**	**81.4**	29.29	OW
	SD		-	-	**9.9**	**15.7**	7.02	-
	Median		-	-	45	79.5	**28.76**	-
	IQR		-	-	11	23.3	**9.03**	-

Gender: M: Male; F: Female

Body mass index (BMI): NW: normal weight, OW: overweight

Table 76: Summary of cases. Genetic data at inclusion visit

Arm			Patient ID	Onset	Inheritance	CTGn (n)
Control	1		01	Adult	Paternal	1500
	2		03	Adult	Paternal	500
	3		04	Young	Paternal	333
	4		06	Unknown (childhood?)	Maternal	500
	5		07	Adult	Paternal	667
	6		09	Young	Paternal	1000
	7		10	Adult	Paternal	333
	8		11	Young	Paternal	833
	9		12	Partial	Paternal	50
	10		13	Adult	Paternal	80
	11		14	Adult	Paternal	267
	12		15	Unknown (childhood?)	Maternal	1000
	13		20	Young	Maternal	667
	14		21	Young	Paternal	267
	15		24	Adult	Paternal	433
	Total	N	15	15	15	15
		Mean	-	-	-	**562**
		SD	-	-	-	**101**
Active	1		02	Childhood	Maternal	1400
	2		05	Childhood	Maternal	1000
	3		08	Adult	Paternal	140
	4		16	Young	Paternal	667
	5		17	Adult	Paternal	167
	6		18	Adult	Paternal	500
	7		19	Young	Maternal	833
	8		22	Adult	Paternal	667
	9		23	Adult	Unknown	1333
	10		25	Adult	Paternal	333
	11		26	Adult	Paternal	65
	12		27	Adult	Paternal	500
	13		28	Young	Paternal	667
	14		29	Adult	Paternal	750
	15		30	Adult	Paternal	.
	Total	N	15	15	15	14
		Mean	-	-	-	**644**
		SD	-	-	-	**109**
Total	N		30	30	30	29
	Mean		-	-	-	**602**
	SD		-	-	-	**73**

Regarding muscular impairment rating scale (MIRS), median score was very similar in both arms, mild to moderate proximal weakness (**Table 77**). The MIRS is an ordinal five-point rating scale useful to monitor major stages of DM1 progression, to study the natural history of the disease, and to identify homogeneous groups of patients for clinical trials.[463]

Table 77: Summary of cases – Muscular impairment rating scale (MIRS) at inclusion visit

				Patient_ID	MIRS
Arm	**Control**	1		01	4- Severe proximal weakness
		2		03	3- Mild to moderate proximal weakness
		3		04	3- Mild to moderate proximal weakness
		4		06	3- Mild to moderate proximal weakness
		5		07	4- Severe proximal weakness
		6		09	4- Severe proximal weakness
		7		10	2-Distal weakness
		8		11	3- Mild to moderate proximal weakness
		9		12	0- No muscular impairment
		10		13	0- No muscular impairment
		11		14	4- Severe proximal weakness
		12		15	3- Mild to moderate proximal weakness
		13		20	3- Mild to moderate proximal weakness
		14		21	2- Distal weakness
		15		24	3- Mild to moderate proximal weakness
		Total	N	15	15
			Median	-	**3**
			IQR	-	**2**
	Active	1		02	3- Mild to moderate proximal weakness
		2		05	3- Mild to moderate proximal weakness
		3		08	4- Severe proximal weakness
		4		16	3- Mild to moderate proximal weakness
		5		17	4- Severe proximal weakness
		6		18	3- Mild to moderate proximal weakness
		7		19	4- Severe proximal weakness
		8		22	3- Mild to moderate proximal weakness
		9		23	4- Severe proximal weakness
		10		25	3- Mild to moderate proximal weakness
		11		26	1- Minimal signs
		12		27	3- Mild to moderate proximal weakness
		13		28	4- Severe proximal weakness
		14		29	4- Severe proximal weakness
		15		30	4- Severe proximal weakness
		Total	N	15	15
			Median	-	**3**
			IQR	-	**3**
	Total	N		30	30
		Median		-	**3**
		IQR		-	**1**

All participants had a complex clinical history of comorbidities and comedication, that are detailed in **Table 78** (control arm) and **Table 79** (active arm). These background situations were certainly challenging along the study.

Table 78: Comorbidities and comedication in the control arm

Patient ID	Comorbidities	Comedication
01	Asthma, atrial flutter, subclinical hypothyroidism, haemorrhoids, elevated transaminases	Sotapor
03	Total thyroidectomy, elevated transaminases, blepharitis, low back pain, sterolemia	Eutirox, Urbasone
04	Chronic obstructive pulmonary disease	None
06	Pneumothorax, low back pain	Omeprazole
07	Anxiety, fistula, low back pain, loss of hearing not otherwise specified, neck pain	None
09	Elevated transaminases, hypertriglyceridemia	Omeprazole
10	Dairy allergy	COVID vaccine
11	Diabetes	Lantus
12	Hypertension, hyperlipidemia, double aortic injury, vitamin D deficiency, obesity, cardiac arrhythmia, flutter	Sintrom, gemfibrozil, valsartan, pantoprazole, bisoprolol
13	Depression	Lormetazepam, esomeprazol
14	Hyperlipidemias, obstinate sleep apnea, atrioventricular block 1 degree, lower back pain, type 2 diabetes	Zinc Citrate, Vitamin D, Advancet + Acidophilus, Cholestril
15	Hypercholesterolemia	Implanon, Ibuprofen
20	None	Buscapina, fluvastine
21	Reynaud's syndrome, right wrist osteochondroma	None
24	Hyperlipidemia, obesity, hyperkalemia, low back pain, type II diabetes	None

Table 79: Comorbidities and comedication in the active arm

Patient ID	Comorbidities	Comedication
02	Hyperlipidemia, obesity, grade 1 atrioventricular block, diabetes, total thyroidectomy, low back pain, hearing loss, hepatic steatosis, arterial hypertension	Metformin, HCTZ, Lisinopril, Ezetrol, Euthyrox, Budesonide, Tramadol
05	Kidney failure, folate deficiency	Ezetrol, testosterone
08	Hypercholesterolemia, atrial arrhythmia, neck pain, sleep apnea	None
16	Hypothyroidism, hypertriglyceridemia, jaw fracture, hepatic steatosis, epigastralgia	Plantago ovata, Cholestyramine
17	Renal colic, mixed hyperlipidemia, polyglobulia, diabetes	Magnesium
18	Allergic rhinitis, acute pericarditis, epigastralgia	None
19	Hyperlipidemia, anxiety, migraine, deafness, neoplasm of renal pelvis, gonalgia (knee pain)	None
22	None	Ibuprofen
23	Hypertriglyceridemia, low back pain, type II diabetes, spondyloarthritis, ventricular tachycardia, cataracts, anaemia	Pantoprazole, Glimepiride, Metformin / Pioglitazone, Dapagliflozin, Ticagrelor, Bisoprolol, Atorvastatin
25	Chronic liver disease, esophagitis	None
26	Bilateral hip osteoarthritis, elevated transaminases, lumbar disc herniation	Torasemide
27	Grade 1 atrioventricular block, hypertriglycemia, bronchitis	Amoxicillin
28	Low back pain	None
29	Bilateral cataract	Folic Acid, Vitamin D
30	Bilateral cataract, type II diabetes, anaemia, vertigo, anxiety, vitamin B12 deficiency, herniated disc, nocturnal respiratory failure, cardiac aneurysm	Toujeo, Metformin, Trulicity, Tranxilium, Vitamin D

Summary statistics of baseline parameters at the inclusion visit are represented in tables below, detailed into gender and arm:

Table 80: Statistics of age per gender and arm

Gender	Study cohort			Control arm			Active arm		
	N	Age (range)	Age (mean±SD)	N	Age (range)	Age (mean±SD)	N	Age (range)	Age (mean±SD)
All	30 (100%)	28-72	46±10	15 (50%)	28-72	48±13	15 (50%)	34-54	45±5
Female	11 (37%)	28-72	46±13	6 (40%)	28-72	46±18	5 (33%)	43-54	46±4
Male	19 (63%)	34-64	46±8	9 (60%)	35-64	49±10	10 (67%)	34-52	43±5

Table 81: Statistics of CTG_n repetitions per gender and arm

Gender	Study cohort			Control arm			Active arm		
	N	CTG_n (range)	CTG_n (mean±SD)	N	CTG_n (range)	CTG_n (mean±SD)	N	CTG_n (range)	CTG_n (mean±SD)
All	29* (97%)	50-1500	602±396	15 (50%)	50-1500	562±392	14* (47%)	65-1400	644±409
Female	10* (33%)	80-1400	633±386	6 (40%)	80-1000	447±313	4* (29%)	667-1400	912±332
Male	19 (64%)	50-1500	585±410	9 (60%)	50-1500	639±667	10 (71%)	65-1333	537±401

*One female missing case for unknown CTG_n repetitions (3% of the entire cohort)

Table 82: Statistics of weight (in Kg) per gender and arm at inclusion visit

Gender	Study cohort			Control arm			Active arm		
	N	Weight (range)	Weight (mean±SD)	N	Weight (range)	Weight (mean±SD)	N	Weight (range)	Weight (mean±SD)
All	30 (100%)	51-112	81.38±15.75	15 (50%)	55-108	81.57±14.19	15 (50%)	51-112	81.18±17.67
Female	11 (37%)	51-112	78.52±17.01	6 (40%)	55-96	79.63±13.82	5 (33%)	51-112	77.12±22.07
Male	19 (63%)	62-112	83.03±15.14	9 (60%)	62-108	82.83±15.12	10 (67%)	63-112	83.21±15.98

Table 83: Statistics of body mass index (BMI, in kg/m^2) per gender and arm at inclusion visit

Gender	Study cohort			Control arm			Active arm		
	N	BMI (range)	BMI (mean±SD)	N	BMI (range)	BMI (mean±SD)	N	BMI (range)	BMI (mean±SD)
All	30 (100%)	19.49-53.30	29.86±7.02	15 (50%)	19.49-39.19	29.21±6.24	15 (50%)	21.00-53.30	29.36±7.94
Female	11 (37%)	19.49-53.30	31.52±8.96	6 (40%)	19.49-36.25	30.53±6.42	5 (33%)	21.23-53.30	32.92±12.04
Male	19 (63%)	21.00-39.19	27.99±5.47	9 (60%)	21.40-39.19	28.45±6.38	10 (67%)	21.00-38.30	27.59±4.83

BMI (Quetelet index) ranges: underweight (<19), normal (19-24.9), overweight (25-29.9), obese (30-39.9), morbidly obese (>40)

Weight and body mass index

Weight and height were measured at the inclusion visit (time zero), at the follow-up visit (month 3) and at the final visit (month 6). Patients ID02, ID05 and ID22 in the active arm, as well as patient ID03, ID13 and ID15 in the control arm dropped out of the study. Height was not measured for patient ID06 in the month 3 visit, and therefore BMI was not calculated for this patient in the follow-up visit. Available and missing data is shown below in **Table 84** and **Table 85**.

Table 84: Weight data from baseline to the end of study (summary of cases)

				Patient_ID	Weight (kg) Baseline	Weight (kg) Month 3	Weight (kg) Month 6
Arm	**Control**	1		01	69.0	69.0	68.0
		2		03	79.0	78.4	.
		3		04	80.0	81.6	82.5
		4		06	88.1	89.8	89.4
		5		07	96.0	95.0	92.0
		6		09	62.5	62.2	62.2
		7		10	82.0	84.0	83.0
		8		11	70.0	72.5	70.7
		9		12	108.0	109.5	109.5
		10		13	78.0	.	.
		11		14	98.0	100.0	100.0
		12		15	96.0	100.8	.
		13		20	79.0	81.8	80.0
		14		21	55.0	54.8	53.0
		15		24	83.0	81.8	81.7
		Total	N	15	15	14	12
			Mean	-	**81.6**	**82.9**	**81.1**
			SD	-	**14.2**	**15.3**	**16.0**
	Active	1		02	112.0	.	.
		2		05	67.0	.	.
		3		08	64.7	63.0	65.0
		4		16	112.0	115.5	111.0
		5		17	101.0	100.5	96.0
		6		18	77.0	77.0	75.0
		7		19	71.0	72.0	72.1
		8		22	51.0	51.3	.
		9		23	92.0	93.0	90.5
		10		25	83.0	84.5	83.0
		11		26	88.0	90.0	91.0
		12		27	63.0	.	60.5
		13		28	84.4	87.0	86.0
		14		29	78.0	81.3	79.7
		15		30	73.6	75.8	77.0
		Total	N	15	15	12	12
			Mean	-	**81.2**	**82.6**	**82.2**
			SD	-	**17.7**	**16.9**	**13.9**
	Total	N		28	30	26	24
		Mean		-	**81.38**	**82.8**	**81.6**
		SD		-	**15.8**	**15.8**	**14.7**

Table 85: BMI data from baseline to the end of study (summary of cases)

				Patient_ID	BMI Baseline	BMI Month 3	BMI Month 6
Arm	**Control**	1		01	22.28	22.30	22.00
		2		03	25.50	25.30	.
		3		04	24.70	25.20	25.60
		4		06	34.40	.	34.90
		5		07	30.80	30.50	29.80
		6		09	21.40	21.30	21.40
		7		10	34.58	35.40	34.00
		8		11	22.84	23.50	23.10
		9		12	39.19	39.70	39.70
		10		13	32.89	.	.
		11		14	36.89	37.60	37.60
		12		15	35.26	37.00	.
		13		20	29.20	30.00	29.00
		14		21	19.49	19.40	19.00
		15		24	28.72	28.30	28.30
		Total	N	15	15	13	12
			Mean	-	**29.21**	**28.88**	**28.73**
			SD	-	**6.24**	**6.78**	**6.75**
	Active	1		02	53.30	.	.
		2		05	22.40	.	.
		3		08	25.60	24.90	25.70
		4		16	38.30	39.50	37.96
		5		17	29.51	29.40	28.05
		6		18	27.55	28.30	28.28
		7		19	28.80	29.20	29.25
		8		22	21.23	21.40	.
		9		23	30.74	31.10	30.24
		10		25	28.06	28.60	28.10
		11		26	27.80	28.40	28.70
		12		27	21.00	.	20.20
		13		28	24.90	25.00	25.00
		14		29	30.85	32.20	32.00
		15		30	30.40	31.60	32.00
		Total	N	15	15	12	12
			Mean	-	**29.36**	**29.13**	**28.79**
			SD	-	**7.94**	**4.50**	**4.33**
	Total	N		28	30	25	24
		Mean		-	**29.29**	**29.00**	**28.76**
		SD		-	**7.02**	**5.68**	**5.55**

BMI is expressed in kg/m^2

Along the study, the mean weight and the mean body mass index were mostly maintained in general terms in both arms (control and active). **Figure 51** illustrates these minor changes, wherein almost all results at baseline and month 6 visit lie along the line representing the y=x equation.

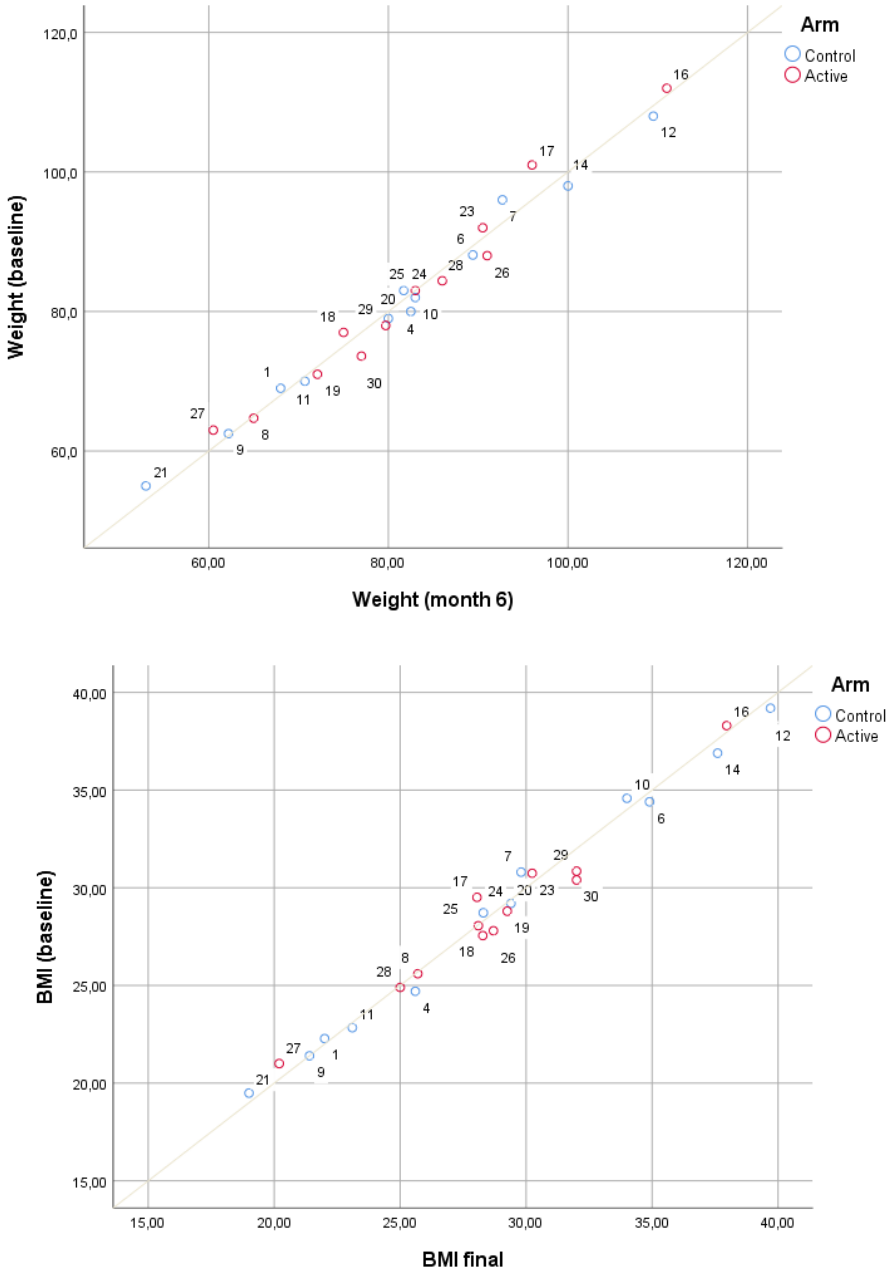


Figure 51: Correlations between weight (expressed in kg) and body mass index comparison (expressed in kg/m^2) from baseline to end of visit, respectively, according to arms. Patient ID labels have not been included in the graphics for better clarity.

The results of the unpaired t-test test did not revealed significant differences between the changes in body weight in the active arm (mean -0.1±2.4 kg) from changes in body weight in the control arm (mean 0.2±1.7 kg) at the end of study, $t(22)=0.776$, $p=0.776$ two-tailed, effect size $r=0.10$. The same conclusion was obtained when the arms were divided into gender, non-statistically differences for males ($p=0.337$) nor for females ($p=0.193$), although the trend to body weight increase was medium effect size ($r=0.62$) in the female gender, as illustrated in **Figure 52**.

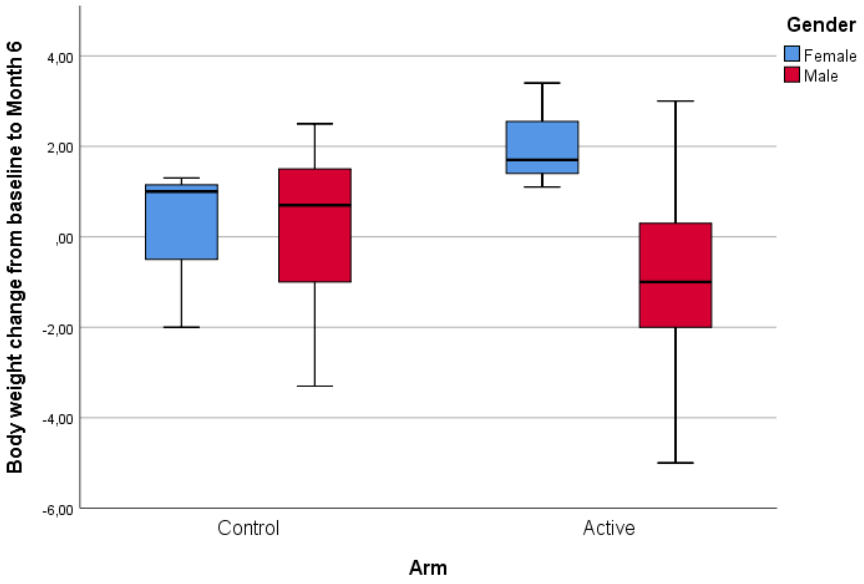


Figure 52: Changes in weight (expressed in kg) from baseline to month 6 end of study visit, according to arms, divided into gender. Labels correspond to Patient ID.

Then, the unpaired test was used to compare the distribution of changes in body mass index from baseline to month 6. The changes in the active arm (mean 0.2±0.9 kg/m^2) did not differ significantly from the changes in body mass index in the control arm (mean 0.0±0.6 kg/m^2) at the end of study, p=0.651, effect size (r)=0.01. However, when the arms were divided into gender, changes in females BMI turned into almost statistically significant (p=0.059) and exhibit a high effect size (r)=0.79, as illustrated in **Figure 53**. None of these effects were obtained in the male subgroup (p>0.05, r=0.1).

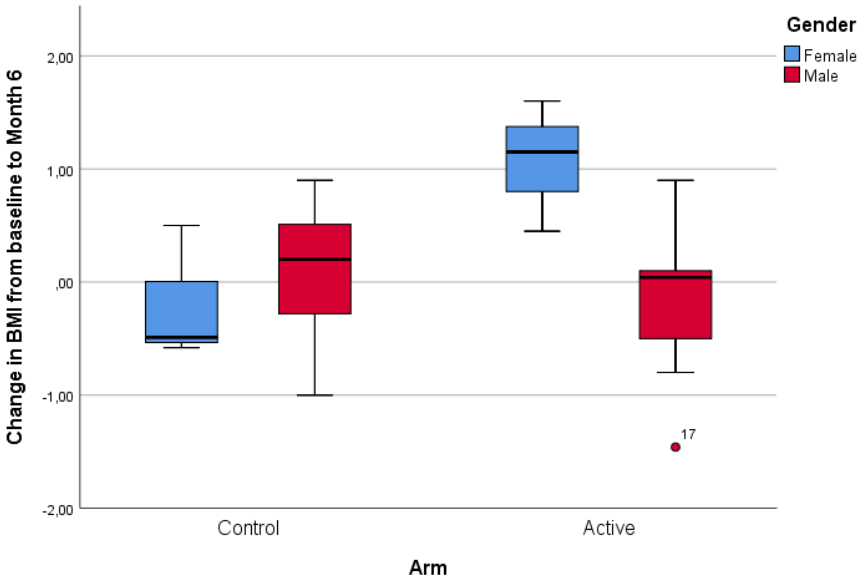


Figure 53: Changes in body mass index (expressed in kg/m^2) from baseline to month 6 end of study visit, according to arms, divided into gender. Labels correspond to Patient ID.

5.7.3. Consumption of dietary methylxanthines

Data discussed along Chapter 4 revealed consumption habits of dietary methylxanthines in the DM1 community. According to the observational MD Project data, 86% of the responders in the cohort consumed methylxanthines, especially caffeine on a regular basis (median 82.0±80.0 mg caffeine/day), but almost no theobromine (median 0.9±13.2 mg theobromine/day), with very similar trends between genders. The main source of dietary caffeine was coffee, for which 51% responders consumed at least one serving per day.

Certainly, this previous knowledge pointed to the possibility that, especially dietary caffeine, could behave as an interfering or confusing factor in the interpretation of the results from the present interventional study. Accordingly, a rational possibility would have been to include caffeine consumption as an exclusion criteria in the clinical trial protocol. However, the present study did not intend to nutritionally intervene nor modify the usual diet of the participants, and previously disclosed data from the MD Project had already demonstrated that dietary caffeine from food and beverages *per se* did not improve the health-related quality of life in DM1 patients, as measured by the SF-36 tool. Moreover, dietary caffeine restriction could significantly tight the target population that could benefit from the present research, and at the same time, could limit the recruiting capacity in a population that was already limited for the fact that DM1 is a rare disease and some other additional issues experienced in the pandemic times when this research was conducted. Therefore, caffeine intake from food and beverages was not limited in the study cohort, but dealt within.

Participants were interviewed in the inclusion visit in order to know their usual consumption pattern of coffee, tea and caffeinated cola soft drinks. Due to the fact that chocolate and energy drinks were residually consumed according to the results from the observational study, they were not included in the survey. Next, the pattern of dietary methylxanthines' consumption was translated into caffeine as established in Chapter 4, considering the reference data as provided in Chapter 1 (see Chapter 1, **Table 18** and **Table 20**, respectively). In this regard, caffeine concentration accounted for 1340 mg/L in coffee, 165 mg/mL in tea and 108 mg/mL in cola soft drinks. The serving sizes used for calculations were: 60 mL for coffee, 250 mL for tea and 330 mL for cola soft drinks, as previously explained in Chapter 4.

Table 86 details the responses collected per each participant in the inclusion visit and includes the conversion into estimated daily caffeine intake from dietary sources. Baseline data revealed that 67% participants in the control arm and 60% in the active arm consumed caffeinated foods and beverages on a regular basis, accounting for median 36.0±152.5 mg caffeine/day in the control arm and median 80.0±122.0 mg caffeine/day in the active arm.

Table 86: Summary of cases: Daily consumption of foods and beverages containing methylxanthines. All participants' responses

Arm			Patient_ID	Gender	MX consumption	Coffee	Tea	Soft drinks	Dietary caffeine
						(servings/day)			(mg/day)
Control	1		01	M	No	0.0	0.0	0.0	0.0
	2		03	F	Yes	2.0	0.0	0.0	161.0
	3		04	M	Yes	0.0	0.0	1.0	36.0
	4		06	F	Yes	1.0	0.0	0.0	80.0
	5		07	M	Yes	1.0	0.0	0.1	85.0
	6		09	M	No	0.0	0.0	0.0	0.0.
	7		10	F	Yes	0.0	3.5	0.0	144.0
	8		11	M	Yes	2.0	0.0	0.0	161.0
	9		12	M	No	0.0	0.0	0.0	0.0
	10		13	F	Yes	0.1	0.0	0.0	0.0
	11		14	M	NR	.	.	.	.
	12		15	F	NR	.	.	.	.
	13		20	M	Yes	0.0	0.0	0.1	3.0
	14		21	F	Yes	0.4	0.0	0.0	30.0
	15		24	M	Yes	3.0	0.0	0.0	241.0
	Total	N	15	15	15	13	13	13	13
		Mean	-	-	-	0.7	0.3	0.1	72.4
		SD	-	-	-	1.0	1.0	0.3	80.8
		Median	-	-	-	**0.1**	**0.0**	**0.0**	**36.0**
		IQR	-	-	-	**1.5**	**0.0**	**0.0**	**152.5**
Active	1		02	F	Yes	1.0	0.0	0.1	85.0
	2		05	M	Yes	1.0	0.4	0.0	95.0
	3		08	M	Yes	1.0	0.0	0.0	80.0
	4		16	M	Yes	0.0	0.0	1.0	36.0
	5		17	M	No	0.0	0.0	0.0	0.0
	6		18	M	No	0.0	0.0	0.0	0.0
	7		19	F	Yes	1.0	1.0	0.0	122.0
	8		22	F	No	0.0	0.0	0.0	0.0
	9		23	M	Yes	1.0	0.0	0.0	80.0
	10		25	M	Yes	4.0	0.0	0.3	332.0
	11		26	M	Yes	2.5	0.0	0.0	201.0
	12		27	M	No	0.0	0.0	0.0	0.0
	13		28	M	No	0.0	0.0	0.0	0.0
	14		29	F	Yes	4.5	0.0	0.0	362.0
	15		30	F	No	0.0	0.0	0.0	0.0
	Total	N	15	15	15	15	15	15	15
		Mean	-	-	-	1.1	0.1	0.1	92.9
		SD	-	-	-	1.5	0.3	0.3	118.8
		Median	-	-	-	**1.0**	**0.0**	**0.0**	**80.0**
		IQR	-	-	-	**1.0**	**0.0**	**0.0**	**122.0**
Total	N		30	30	30	27	28	28	28
	Mean		-	-	-	0.9	0.2	0.1	83.4
	SD		-	-	-	1.3	0.7	0.3	101.6
	Median		-	-	-	**0.2**	**0.0**	**0.0**	**58.0**
	IQR		-	-	-	**1.0**	**0.0**	**0.0**	**138.5**

Missing values for participants ID14 and ID15.

Figure 54 illustrates intake distribution between caffeine containing food and beverages' consumers and non consumers in both arms at the inclusion visit, whereas **Figure 55** represents the estimated amount of dietary caffeine expressed in mg/day, which was calculated as previously disclosed. **Figure 56** and **Figure 57** represent the above-mentioned dietary caffeine distribution in both arms according to gender.

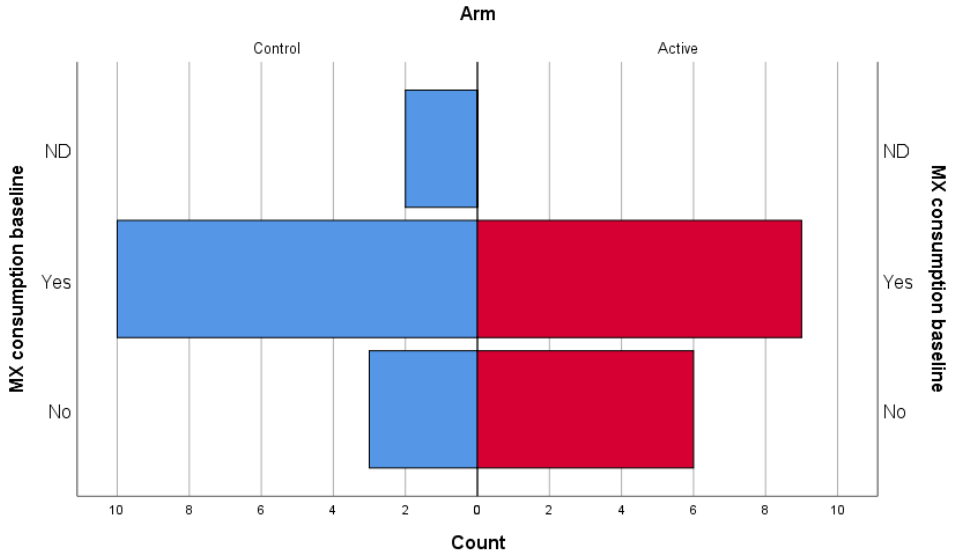


Figure 54: Methylxanthines' consumption pattern at inclusion visit, in the entire cohort, divided into arms.

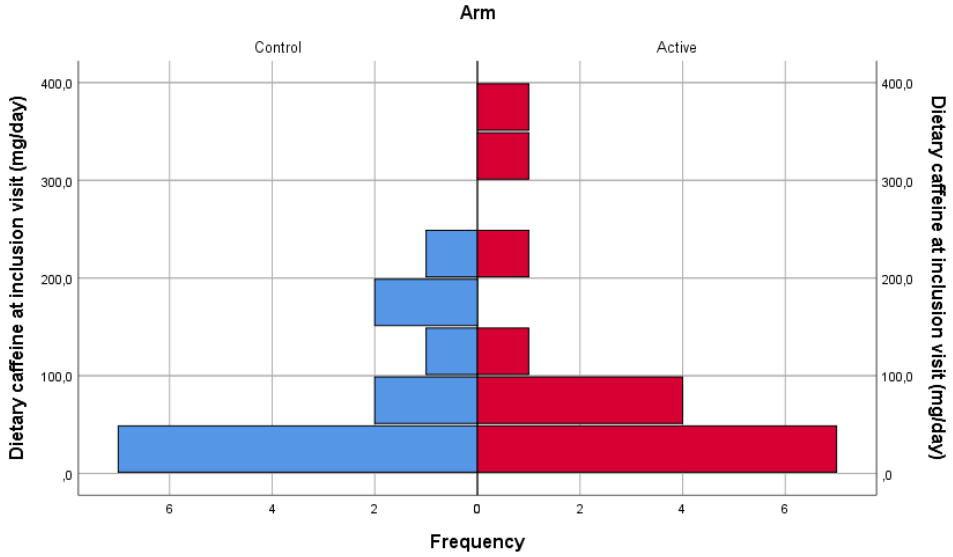


Figure 55: Dietary caffeine intake at inclusion visit, in the entire cohort, divided into arms.

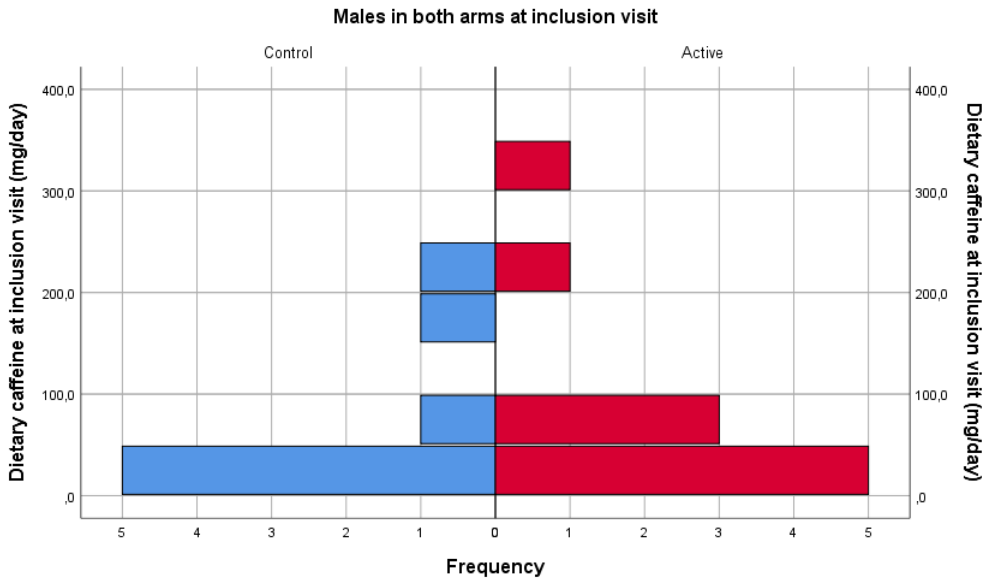


Figure 56: Males consumption of dietary caffeine intake at inclusion visit, divided into arms.

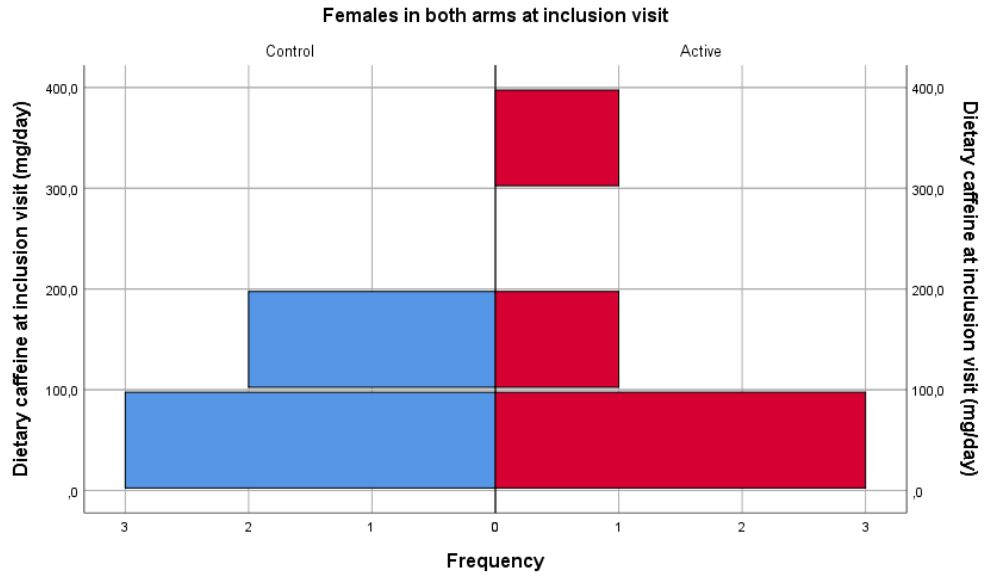


Figure 57: Females consumption of dietary caffeine intake at inclusion visit, divided into arms.

The statistical comparison in basal dietary caffeine consumption between arms was performed using the Mann-Whitney's U test. The distribution of dietary caffeine intake in the active arm (median 80.0±122.0) did not differ significantly from dietary caffeine intake in the control arm (median 36.0±152.5) at the inclusion visit, U=97.00, Z=-0.024, p=0.981, effect size r=0.00. The same conclusion was obtained when the arms were divided into gender, with calculated effect sizes r=-0.03 both in females and males.

Nevertheless, due to the fact that some participants dropped out along the study for the reasons already disclosed in **Table 74**, these initial figures changed from the baseline visit to

the end of the study visit. In particular, Patients ID03, ID13 and ID15 from the control arm, and patients ID02, ID05 and ID22 from the active arm did not complete the protocol schedule. According to the responses provided at month 3 and month 6, the participants that continued the study did not significantly change their consumption habits, as they were prompted to. Therefore, at the end of the study, 80% participants in the control arm and 70% in the active arm consumed caffeinated foods and beverages on a regular basis, accounting for median 36.0±144.0 mg caffeine/day in the control arm and median 58.0±181.3 mg caffeine/day in the active arm.

Figure 58 illustrates intake distribution between caffeine-containing food and beverages' consumers and non consumers in both arms at the end of study visit, whereas **Figure 59** represents the estimated amount of dietary caffeine expressed in mg/day, which was calculated as previously disclosed. **Figure 60** and **Figure 61** represent the above-mentioned dietary caffeine distribution in both arms according to gender.

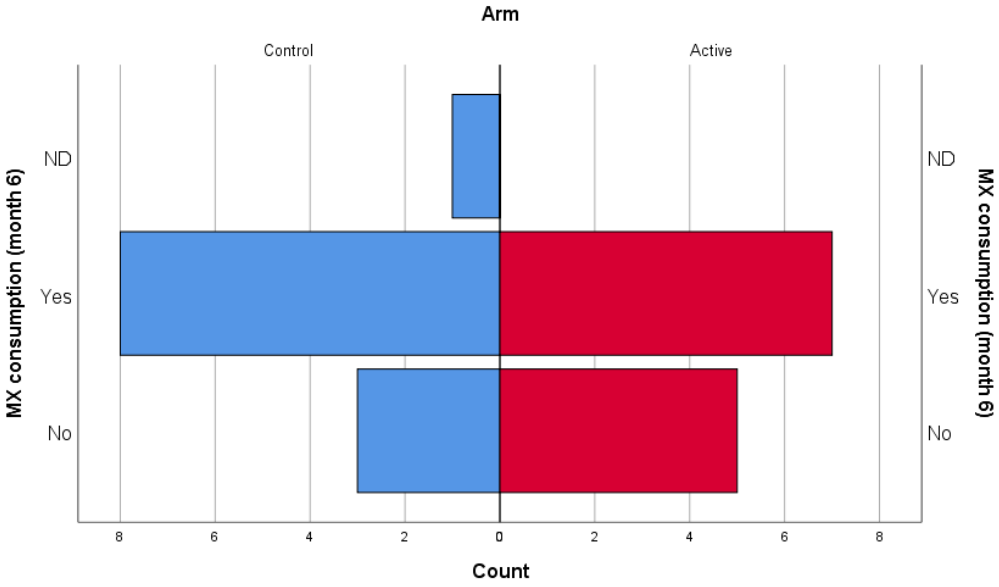


Figure 58: Methylxanthines' consumption pattern at month 6 visit, in the entire cohort, divided into arms, control (C) and active (A). Missing values for dropped out patients ID: 02 (A), 03 (C), 05 (A), 13 (C), 15 (C) and 22 (A).

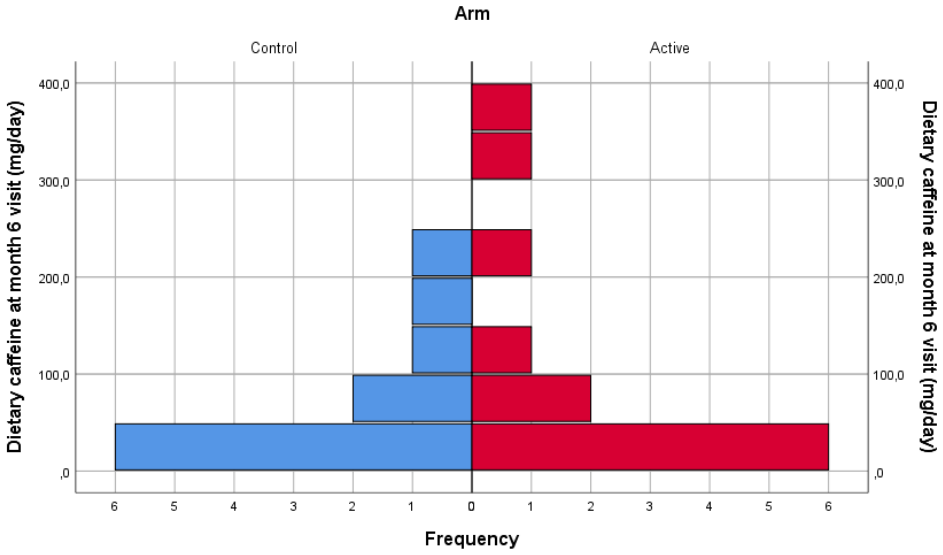


Figure 59: Dietary caffeine intake at month 6 visit, in the entire cohort, divided into arms.

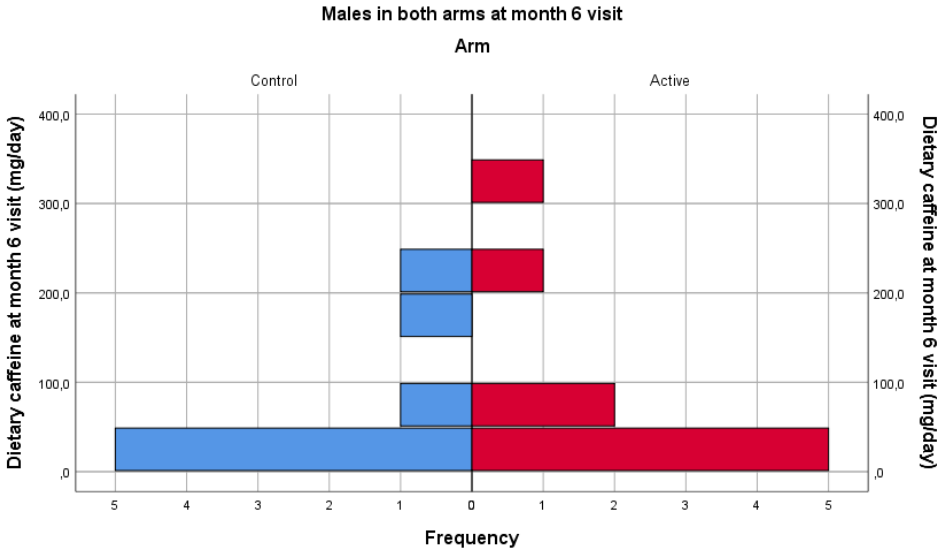


Figure 60: Males consumption of dietary caffeine intake at month 6 visit, divided into arms.

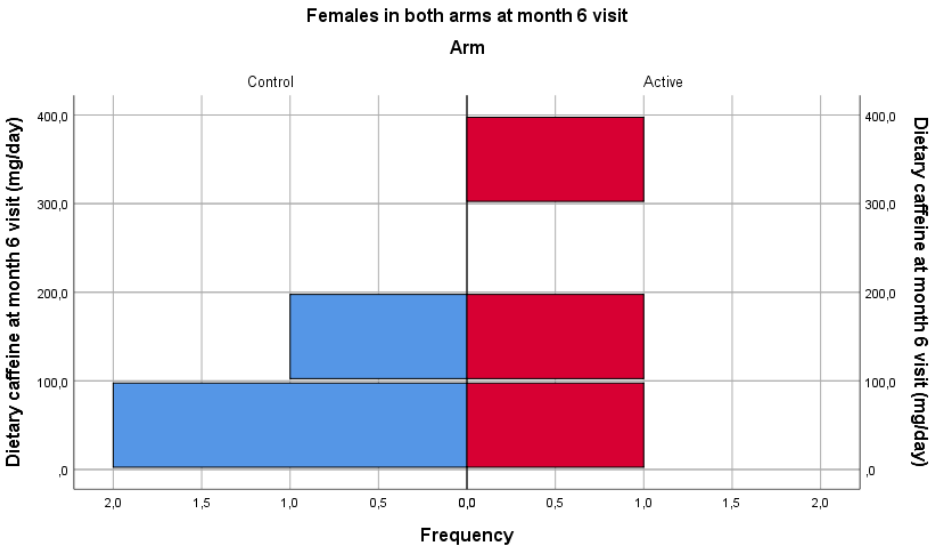


Figure 61: Females consumption of dietary caffeine intake at month 6 visit, divided into arms.

As calculated at the inclusion visit as well as at the baseline situation, the statistical comparison in dietary caffeine consumption between arms was additionally performed at the month 6 end of study visit using the independent samples Mann-Whitney's U test. At that time point, the distribution of dietary caffeine intake in the active arm (median 58.0±181.3) did not differ from dietary caffeine intake in the control arm (median 36.0±144.0), U=66.0, Z=0.00, p=1.000, effect size r=0.00. Same conclusions were obtained from the analysis at baseline and month 6 when the arms were divided into gender.

Taken all together, and in the light of the results from the comparison analysis between arms and even considering gender of the participants per arm, either at the inclusion visit and at the end of study visit, the background dietary caffeine intake was not statistically different and the effect size of the differences measured for each condition was negligible between the two study arms along the present clinical trial. Therefore, the amount of dietary caffeine routinely consumed by the study participants revealed not to behave as an interfering or confusing factor in the interpretation of the results discussed herein.

In this regard, the decision to maintain the background nutritional habits of the participants in the study (and especially the dietary caffeine issue) in order to avoid induced changes in the participants' diet seemed to have been properly addressed.

5.7.4. Biochemistry

Biochemistry analyses were intended to evaluate parameters as cholesterol profile (including total cholesterol, high density cholesterol (HDL), low density cholesterol (LDL) and triglycerides), liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), creatinine (CK), cytokeratin-18 fragments (CK18), alkaline phosphatase, amylase, total bilirubin, conjugated bilirubin), as well as electrolytes (Na, K, Cl, bicarbonate, Ca, Mg, P), glucose, albumin, C-reactive protein (CRP), and the like.

From the complete biochemistry data set, dyslipidemia (defined as elevated total or low-density lipoprotein (LDL) cholesterol levels, or low levels of high-density lipoprotein (HDL) cholesterol levels), as well as the hepatic profile, are particularly key parameters to monitor in DM1 patients, in order to control the risk of associated diseases related to, for instance, cardiovascular morbidity.[472]

Biochemistry data suffered from a significant number of missing values at the month 3 visit which was associated with the impact of one of the several COVID-19 outbreaks that spanked Spain throughout the present clinical trial. Several factors contributed to the lack of the full data in both groups, mostly affecting the active arm:

- Some patients were scared of getting infected at the hospital and declined to attend the blood extraction appointment.
- Laboratories were saturated by the biochemistry analysis of the extraordinary amount of COVID patients that were being attending at the Hospital at that time.
- Most of the investigator's team at the Hospital was also infected by SARS-CoV-2 during the study. In spite of being partially substituted by other colleagues, some unlucky deviations from the protocol occurred.

Table 87 discloses the participants' biochemistry baseline profile at inclusion visit (V0), whereas **Table 88** and **Table 89** detail the corresponding biochemistry profile at month 3 (V1) and month 6 visits (V2), respectively. Fortunately, month 6 visit, which temporally matched with an inter-outbreak period, collected a considerable amount of data, further disclosed herein.

Table 87: Biochemistry data at inclusion visit V0 (baseline, summary of cases)

				Patient_ID	TCH_V0 mg/dl	HDL_V0 mg/dl	LDL_V0 mg/dl	TG_V0 mg/dl	AST_V0 U/L	ALT_V0 U/L	GGT_V0 U/L	CK_V0 mg/dl
Arm	**Control**	1		01	175	53	106	78	34	38	206	73
		2		03	280	69	179	159	18	18	58	.
		3		04	142	44	78	96	45	45	33	.
		4		06	208	51	119	189	35	48	78	428
		5		07	222	38	140	218	37	18	64	401
		6		09	229	55	128	55	109	117	266	536
		7		10	165	45	90	45	26	34	18	144
		8		11	270	52	170	238	23	42	62	255
		9		12	168	31	137	406	22	17	14	185
		10		13	169	55	88	132	17	13	24	86
		11		14	207	40	123	218	37	60	51	239
		12		15	183	51	107	124	38	30	16	.
		13		20	190	38	116	181	28	44	.	167
		14		21	137	53	59	76	22	11	11	126
		15		24	324	54	223	235	34	34	296	.
		Total	N	15	15	15	15	15	15	15	14	11
			Central tendency	-	**205†**	**49†**	**124†**	**163†**	**30‡**	**40‡**	**56‡**	**240†**
			Scatter measure	-	**53†**	**9†**	**42†**	**94†**	**15‡**	**35‡**	**93‡**	**152†**
	Active	1		02	339	62	206	354	30	69	394	304
		2		05	207	50	124	163	54	64	174	137
		3		08	346	77	222	235	66	81	311	335
		4		16	173	31	63	394	32	96	101	266
		5		17	274	46	228	419	38	49	297	.
		6		18	209	49	115	223	49	59	309	310
		7		19	.	.	.	.	.	26	.	189
		8		22	147	50	81	80	20	23	67	.
		9		23	117	32	37	238	23	30	70	174
		10		25	232	42	142	241	38	73	72	454
		11		26	225	61	152	60	26	33	92	114
		12		27	226	37	123	336	44	64	101	.
		13		28	221	36	129	279	31	32	17	.
		14		29	214	54	121	196	36	28	22	.
		15		30	198	53	73	53	23	34	26	.
		Total	N	15	14	14	14	14	14	15	14	9
			Central tendency	-	**223†**	**49†**	**130†**	**234†**	**36†**	**51†**	**147†**	**254†**
			Scatter measure	-	**63†**	**13†**	**58†**	**118†**	**13†**	**23†**	**127†**	**110†**
	Total	N		30	29	29	29	29	29	30	28	20
		Central tendency		-	**214†**	**49†**	**127†**	**197†**	**33‡**	**45‡**	**116†**	**246†**
		Scatter measure		-	**58†**	**11†**	**50†**	**110†**	**18‡**	**43‡**	**115†**	**131†**

† (Mean ± SD); ‡ (Median ± IQR)

Table 88: Biochemistry data at month 3 follow-up visit V1 (summary of cases). Remarkably, many data is missing at this time point due to the second COVID-19 outbreak in Spain.

				Patient_ID	TCH_V1 mg/dl	HDL_V1 mg/dl	LDL_V1 mg/dl	TG_V1 mg/dl	AST_V1 U/L	ALT_V1 U/L	GGT_V1 U/L	CK_V1 mg/dl
Arm	**Control**	1		01	178	49	109	100	24	31	169	.
		2		03	302	71	200	157	30	25	56	.
		3		04	143	38	88	83	32	33	27	.
		4		06	.	.	.	.	.	.	.	.
		5		07	188	37	105	228	35	19	49	434
		6		09	266	51	162	268	56	76	223	559
		7		10	169	46	103	98	27	31	19	121
		8		11	237	56	151	149	27	38	56	.
		9		12	164	30	134	405	22	18	17	.
		10		13	.	.	.	.	.	.	.	.
		11		14	191	37	137	271	38	69	60	180
		12		15	.	.	.	.	.	.	.	.
		13		20	.	.	.	.	.	.	.	.
		14		21	152	67	65	101	24	13	11	125
		15		24	.	.	.	.	.	.	.	.
		Total	N	15	10	10	10	10	10	10	10	5
			Central tendency	-	**199†**	**48†**	**125†**	**186†**	**32†**	**35†**	**49‡**	**284†**
			Scatter measure	-	**52†**	**13†**	**39†**	**105†**	**10†**	**21†**	**127‡**	**200†**
	Active	1		02	.	.	.	.	.	.	.	.
		2		05	.	.	.	.	.	.	.	.
		3		08	.	.	.	.	.	.	.	.
		4		16	.	.	.	.	.	.	.	.
		5		17	.	.	.	.	.	.	.	.
		6		18	203	50	120	165	55	79	406	385
		7		19	284	51	122	376	25	22	.	126
		8		22	.	.	.	.	.	.	.	.
		8		23	143	38	39	332	19	33	92	.
		10		25	223	42	136	225	42	80	78	.
		11		26	.	.	.	.	.	.	.	.
		12		27	234	38	122	371	18	21	49	.
		13		28	.	.	.	.	.	.	.	.
		14		29	.	.	.	.	.	.	.	.
		15		30	192	63	91	191	24	26	23	41
		Total	N	15	6	6	6	6	6	6	5	3
			Central tendency	-	**213†**	**47†**	**105†**	**277†**	**31†**	**44†**	**130†**	**184†**
			Scatter measure	-	**47†**	**10†**	**36†**	**94†**	**15†**	**28†**	**157†**	**179†**
	Total	N		30	16	16	16	16	16	16	15	8
		Central tendency		-	**204†**	**48†**	**118†**	**220†**	**31†**	**38†**	**49‡**	**246†**
		Scatter measure		-	**49†**	**12†**	**38†**	**108†**	**12†**	**23†**	**204‡**	**187†**

† (Mean ± SD); ‡ (Median ± IQR)

Table 89: Biochemistry data at month 6 visit V2 (summary of cases)

				Patient_ID	TCH_V2 mg/dl	HDL_V2 mg/dl	LDL_V2 mg/dl	TG_V2 mg/dl	AST_V2 U/L	ALT_V2 U/L	GGT_V2 U/L	CK_V2 mg/dl
Arm	**Control**	1		01	186	59	114	66	42	50	206	66
		2		03	.	.	.	.	.	.	.	.
		3		04	151	39	89	114	34	35	23	304
		4		06	214	43	120	257	25	31	54	255
		5		07	.	.	.	.	.	.	.	.
		6		09	246	47	135	320	66	73	187	628
		7		10	141	42	73	131	33	55	18	118
		8		11	.	.	.	.	.	.	.	.
		9		12	181	33	149	407	21	20	16	109
		10		13	.	.	.	.	.	.	.	.
		11		14	257	36	160	305	43	68	66	155
		12		15	.	.	.	.	.	.	.	.
		13		20	211	41	171	206	36	62	58	123
		14		21	142	50	70	112	21	12	10	87
		15		24	307	59	175	363	25	22	216	.
		Total	N	15	10	10	10	10	10	10	10	9
			Central tendency	-	**204†**	**45†**	**126†**	**228†**	**35†**	**43†**	**54‡**	**123‡**
			Scatter measure	-	**55†**	**9†**	**39†**	**119†**	**14†**	**22†**	**110‡**	**182‡**
	Active	1		02	.	.	.	.	.	.	.	.
		2		05	.	.	.	.	.	.	.	.
		3		08	356	86	230	198	48	59	296	350
		4		16	120	32	43	227	29	79	124	140
		5		17	224	42	182	424	28	182	34	340
		6		18	.	.	.	.	.	.	.	.
		7		19	282	52	230	485	34	49	209	128
		8		22	.	.	.	.	.	.	.	.
		9		23	110	34	33	216	26	34	69	230
		10		25	231	45	139	233	35	73	85	523
		11		26	177	44	109	122	28	32	165	165
		12		27	229	40	141	240	22	20	43	114
		13		28	234	33	153	241	26	30	23	173
		14		29	203	46	113	222	26	17	20	229
		15		30	184	51	81	259	24	25	20	80
		Total	N	15	11	11	11	11	11	11	11	11
			Central tendency	-	**214†**	**44‡**	**132†**	**233‡**	**28‡**	**34‡**	**69‡**	**225†**
			Scatter measure	-	**69†**	**17‡**	**66†**	**43‡**	**8‡**	**48‡**	**142‡**	**132†**
	Total	N		30	21	21	21	21	21	21	21	21
		Central tendency		-	**209†**	**43‡**	**129†**	**245†**	**29‡**	**42‡**	**56‡**	**160‡**
		Scatter measure		-	**61†**	**13‡**	**54†**	**110†**	**11‡**	**40‡**	**134‡**	**177‡**

† (Mean ± SD); ‡ (Median ± IQR)

An unpaired t-test was conducted to compare the background cholesterol profile between arms (TCH, HDL, LDL and TG). The analysis revealed that there were no significant differences (p>0.05, ns) in any of these parameters between the control arm and the active arm, at the baseline visit.

For the background comparison between arms of the hepatic profile parameters (AST, ALT, GGT) and CK, a Mann-Whitney's U test revealed that only GGT was statistically different between arms at the baseline visit (p=0.043, two-tailed; effect size r=0.38), being significantly elevated in the active arm in front of the control arm.

In the light of the previous information, we calculated the changes between the month 3 follow-up visit and baseline visit (cholesterol profile$_{M3}$-cholesterol profile$_{baseline}$, **Table 90**), and between the month 6 end of study visit and the baseline visit (cholesterol profile$_{M6}$-cholesterol profile$_{baseline}$, **Table 91**), respectively, in both arms. Moreover, we calculated the changes between the month 3 follow-up visit and baseline visit (hepatic profile$_{M3}$-hepatic profile$_{baseline}$), and between the month 6 end of study visit and the baseline visit (hepatic profile$_{M6}$-hepatic profile$_{baseline}$), respectively, in both arms (data not shown for the hepatic profile, since no relevant findings were obtained therein).

Table 90: Results from changes in cholesterol biochemistry profile from baseline to month 3 follow-up visit

Arm			Patient_ID	Changes in cholesterol profile from baseline to month 3 follow-up visit			
				TCH	HDL	LDL	TG
Control	1		01	3	-4	3	22
	2		03	22	2	21	-2
	3		04	1	-6	10	-13
	4		06	.	.	.	.
	5		07	-34	-1	-35	10
	6		09	37	-4	34	213
	7		10	4	1	13	53
	8		11	-33	4	-19	-89
	9		12	-4	-1	-3	-1
	10		13	.	.	.	.
	11		14	-16	-3	14	53
	12		15	.	.	.	.
	13		20	.	.	.	.
	14		21	15	14	6	25
	15		24	.	.	.	.
	Total	N	15	10	10	10	10
		Central tendency	-	**-0.5**†	**0.2**†	**4.4**†	**16.0**‡
		Scatter measure	-	**22.7**†	**5.7**†	**19.7**†	**58.0**‡
Active	1		02	.	.	.	.
	2		05	.	.	.	.
	3		08	.	.	.	.
	4		16	.	.	.	.
	5		17	.	.	.	.
	6		18	-6	1	5	-58
	7		19	.	.	.	.
	8		22	.	.	.	.
	9		23	26	6	2	94
	10		25	-9	0	-6	-16
	11		26	.	.	.	.
	12		27	.	.	.	.
	13		28	.	.	.	.
	14		29	.	.	.	.
	15		30	-6	10	18	138
	Total	N	15	4	4	4	4
		Mean	-	**1.3**	**4.3**	**4.8**	**39.5**
		SD	-	**16.60**	**4.6**	**10.0**	**91.8**
Total	N		30	14	14	14	14
	Central tendency		-	**-1.5**‡	**1.4**†	**4.5**†	**30.6**†
	Scatter measure		-	**28.0**‡	**5.6**†	**17.1**†	**77.8**†

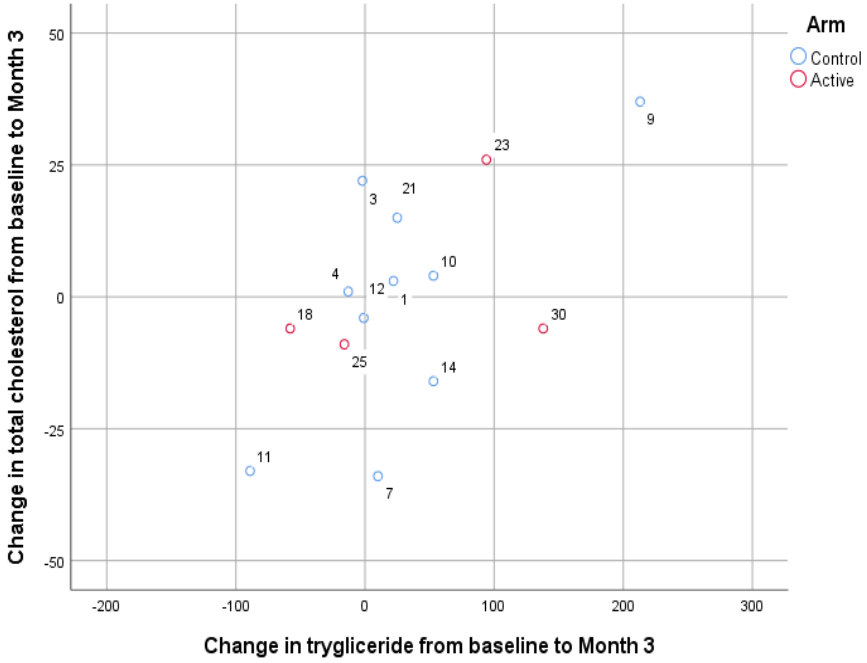
Values expressed in mg/dl. † (Mean ± SD); ‡ (Median ± IQR)

Table 91: Results from changes in cholesterol biochemistry profile from baseline to month 6 end of study visit

Arm			Patient_ID	Changes in cholesterol profile from baseline to month 6 end of study visit			
				TCH	HDL	LDL	TG
Control	1		01	11	6	8	-12
	2		03	.	.	.	.
	3		04	9	-5	11	18
	4		06	6	-8	1	68
	5		07	.	.	.	.
	6		09	17	-8	7	265
	7		10	-24	-3	-17	86
	8		11	.	.	.	.
	9		12	13	2	12	1
	10		13	.	.	.	.
	11		14	50	-4	37	87
	12		15	.	.	.	.
	13		20	21	3	55	25
	14		21	5	-3	11	36
	15		24	-17	5	-48	128
	Total	N	15	10	10	10	10
		Mean	-	**9.1**	**-1.5**	**7.7**	**70.2**
		SD	-	**20.3**	**5.1**	**27.7**	**81.2**
Active	1		02	.	.	.	.
	2		05	.	.	.	.
	3		08	10	9	8	-37
	4		16	-53	1	-20	-167
	5		17	-50	-4	-46	5
	6		18	.	.	.	.
	7		19	.	.	.	.
	8		22	.	.	.	.
	9		23	-7	2	-4	-22
	10		25	-1	3	-3	-8
	11		26	-48	-17	-43	62
	12		27	3	3	18	-96
	13		28	13	-3	24	-38
	14		29	-11	-8	-8	26
	15		30	-14	-2	8	206
	Total	N	15	10	10	10	10
		Mean	-	**-15.8**	**-1.6**	**-6.6**	**-6.9**
		SD	-	**25.3**	**7.2**	**23.7**	**98.3**
Total	N		30	20	20	20	20
	Mean		-	**-3.4**	**-1.6**	**0.6**	**31.7**
	SD		-	**25.7**	**6.1**	**26.1**	**96.2**

Values expressed in mg/dl

Figure 62 graphically summarizes changes in total cholesterol and changes in triglycerides at month 3 and month 6 visits. Beyond the less data collected at month 3, data at month 6 suggest that only participants from the active arm simultaneously decrease TC and TG (bottom left quadrant) whereas only participants from the control arm simultaneously increase their TC and TG along the study (top right quadrant).



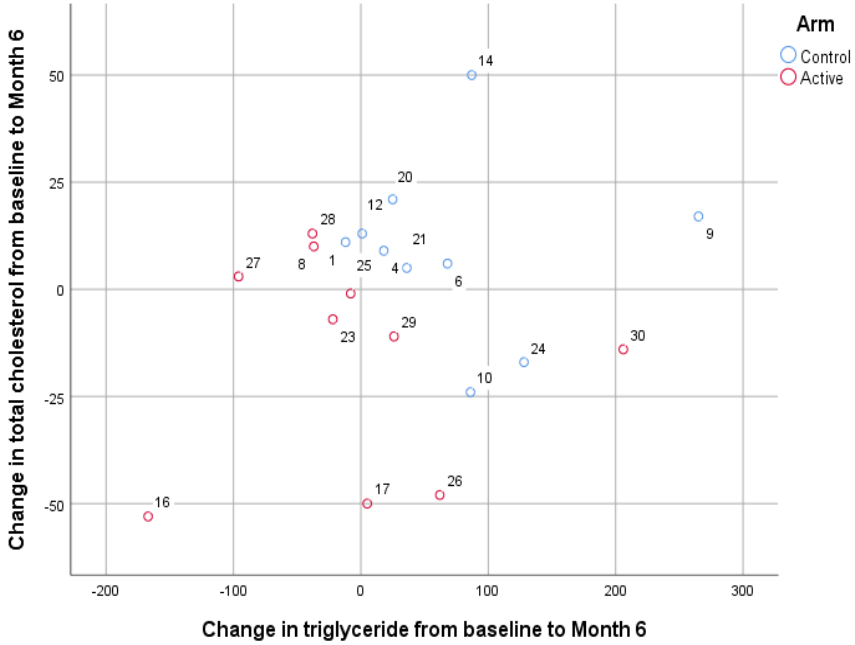


Figure 62: Comparison of the changes in total cholesterol and changes in triglyceride levels at baseline at month 3 follow-up visit (upper) and at the end of the study (bottom) in the entire cohort. Labels correspond to Patient ID

Table 92 discloses the comparison on the changes in the biochemistry profile values in the month 3 follow-up visit from the baseline visit ($biochemistry_{M3}$-$biochemistry_{baseline}$), and in the month 6 end of study visit from the baseline visit ($biochemistry_{M6}$-$biochemistry_{baseline}$), in both arms. In this regard, negative values correspond to situations in which the corresponding parameter decreased from baseline to the analyzed time point, whereas positive values correspond to situations in which the biochemistry parameter increased from the baseline background value.

Table 92: Statistical analysis of changes in biochemistry values along the study from baseline

Changes from:	Control arm		Active arm		*p*-value	Effect size
	N	Central tendency ± scatter measure	N	Central tendency ± scatter measure		
Baseline to Month 3						
TCH	10	-0.5 ± 22.7 †	4	1.3 ± 16.6 †	0.892 $^{\tau}$	0.04
HDL	10	0.2 ± 5.7 †	4	4.2 ± 6.0 †	0.235 $^{\tau}$	0.34
LDL	10	4.4. ± 19.7 †	4	4.8 ± 10.0 †	0.974 $^{\tau}$	0.00
Triglycerides	10	25.0 ± 54.0 ‡	4	72.0 ± 79.3 †	1.000 $^{\Diamond}$	0.00
AST	10	0.0 ± 14.0 ‡	4	0.3 ± 4.0 †	0.355 $^{\Diamond}$	-0.25
ALT	10	-4.7 ± 14.2 †	5	3.6 ± 10.9 †	0.274 $^{\tau}$	0.30
GGT	10	0.0 ± 40.0 ‡	4	8.3 ± 12.7 †	0.047 $^{\Diamond\circ}$	-0.53
CK	5	-5.4 ± 37.0 †	2	6.0 ± 97.6 †	1.000 $^{\Diamond}$	0.00
Baseline to Month 6						
TCH	10	9.1 ± 20.3 †	10	-15.8 ± 25.3 †	**0.026** $^{\tau}$	**0.47**
HDL	10	-1.5 ± 5.1 †	10	-1.6 ± 7.1 †	0.972 $^{\tau}$	0.00
LDL	10	7.7 ± 27.7 †	10	-6.6 ± 23.7 †	0.230 $^{\tau}$	0.28
Triglycerides	10	70.2 ± 81.2 †	10	-6.9 ± 98.3 †	**0.072** $^{\tau}$	**0.41**
AST	10	-1.0 ± 18.0 ‡	10	0.3 ± 3.0 †	0.324 $^{\Diamond}$	0.22
ALT	10	-2.0 ± 19.5 †	11	4.0 ± 45.7 †	0.663 $^{\tau}$	0.10
GGT	9	0.0 ± 12.0 ‡	10	2.0 ± 9.8 †	0.595 $^{\Diamond}$	0.12
CK	8	-44.0 ± 75.1 †	6	0.7 ± 77.9 †	0.293 $^{\tau}$	0.30

Values expressed in mg/dl. † (Mean ± SD); ‡ (Median ± IQR); $^{\tau}$ sig. (2-tailed) according to unpaired t-test; $^{\Diamond}$ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%

$^{\circ}$ GGT values were already statistically different between arms at the baseline visit.

5.7.4.1. Cholesterol profile results

The exploratory analysis shown in **Table 92** revealed statistically significant differences (*p*=0.026) and medium-size effect (*r*=0.47) between the control arm and the active arm from the analysis conducted based on the unpaired t-test, when comparing the total cholesterol levels from baseline to the end of study, at 95% confidence interval, in the full set of patients (see **Figure 63**):

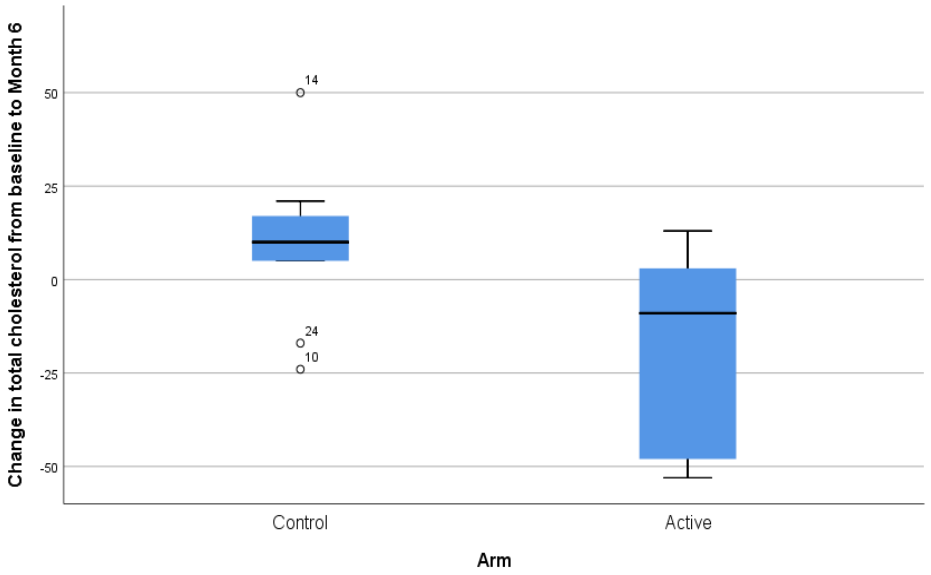


Figure 63: Comparison of the changes in total cholesterol levels from baseline to month 6 time points in the control arm and the active arm. Labels correspond to Patient ID

This unexpected but certainly interesting finding conducted us to in depth explore the presence of potential gender effects in a further step forward. In this regard, the same above-mentioned analysis was performed now taking into consideration patients' gender. Results are shown in **Figure 64** and **Table 93**:

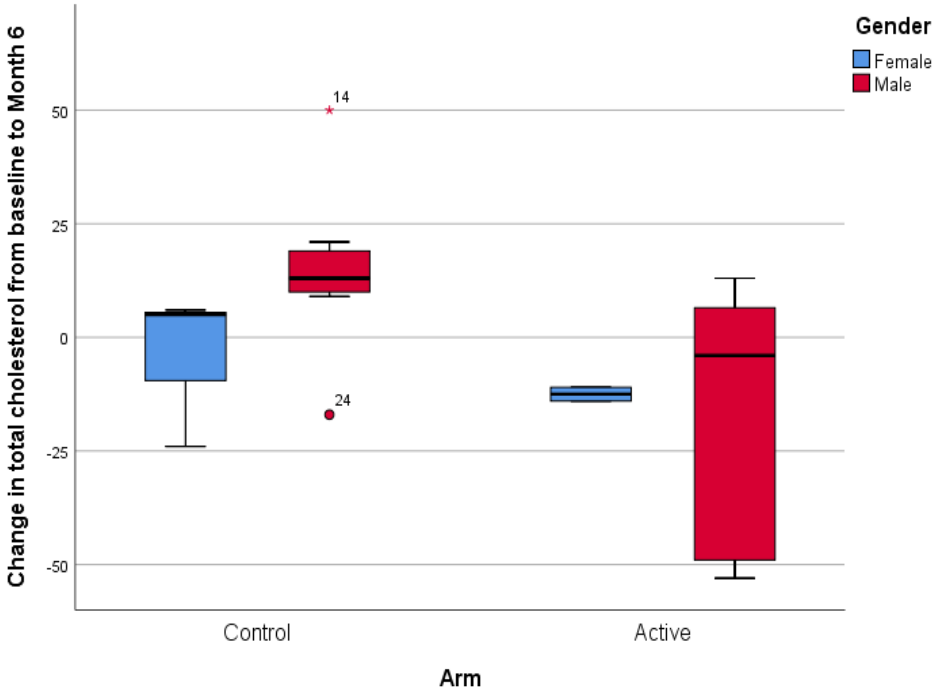


Figure 64: Comparison of the changes in total cholesterol levels from baseline to month 6 time points in the control arm and the active arm, according to gender. Labels correspond to Patient ID

Table 93: Statistical analysis of gender effects in changes in total cholesterol values along the study

Changes in total cholesterol from baseline to Month 6:	Control arm		Active arm		*p*-value	Effect size
	N	Mean ± SD	N	Mean ± SD		
All participants	10	9.1 ± 20.3 †	10	-15.8 ± 25.3 †	**0.026** $^{\tau}$	**0.47**
Male	7	14.9 ± 19.8 †	8	-16.6-± 28.6†	**0.030** $^{\tau}$	**0.56**
Female	3	-4.3 ± 17.0†	2	-12.5 ± 2.1 †	0.567 $^{\tau}$	0.35

Values expressed in mg/dl. $^{\tau}$ sig. (2-tailed) according to unpaired t-test; CI 95%

Based on these results from the unpaired t-test, there was a statistically significant decrease in cholesterol levels from baseline to month 6 between the participants when comparing the control arm (whose change in cholesterol levels, in fact, had increased: mean=9.1 mg/dl, SD=20.3 mg/dl) and the participants in the active arm (that certainly revealed cholesterol levels decrease: mean=-15.8 mg/dl, SD=25.3 mg/dl), $t(18)=2.43$, $p=0.026$, two-tailed. The magnitude of this difference had medium effect size ($r=0.47$), revealing substantial practical effects.

When considering participants' gender, males clearly promote these figures, since according to the same statistical test, there was a statistically significant difference in cholesterol levels from baseline to month 6 between the male group in the control arm (that increased: mean=14.9 mg/dl, SD=19.8 mg/dl) and the participants in the active arm (that decreased: mean=-16.6 mg/dl, SD=28.6 mg/dl), $t(12)=2.44$, $p=0.030$, two-tailed, medium effect size ($r=0.56$). The equivalent trend was not observed in the female group, for which the decrease in cholesterol levels from baseline to month 6 between the control arm (mean=-4.3 mg/dl, SD=17.0 mg/dl) and the participants in the active arm (mean=-12.5 mg/dl, SD=2.1 mg/dl), $t(3)=0.64$, did not reach a statistically significant effect ($p>0.05$, two-tailed), probably associated to the tiny sample set in the female subgroup.

Figure 65 graphically depicts the comparisons in total cholesterol scores per Patient ID according to arms at the end of the study. Results above the y=x equation correspond to Patient ID whose total cholesterol score at month 6 decreased from total cholesterol score at baseline, *i.e.,* those for whom total cholesterol levels had decreased along the study. Results lying on the y=x equation correspond to Patient ID whose total cholesterol levels did not change along the study. Finally, results lying below the y=x equation correspond to Patient ID whose cholesterol scores increased from the corresponding scores at baseline, *i.e.,* those for whom total cholesterol levels had increased along the study.

The decrease in total cholesterol levels from baseline values was clearly evident in the active arm participants ID16, ID17 and ID26.

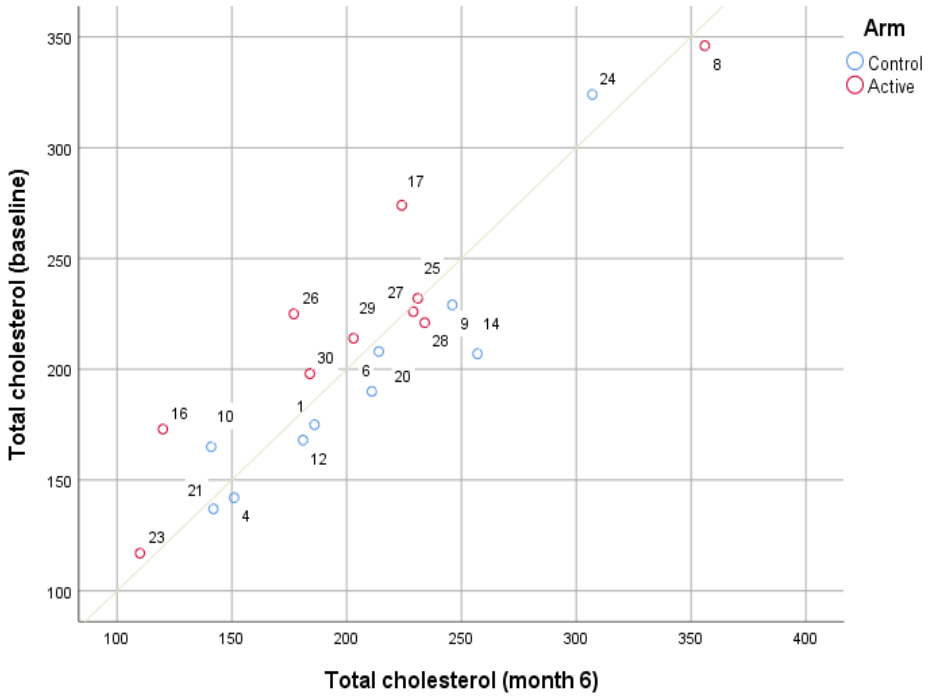


Figure 65: Comparison of the total cholesterol scores at baseline and month 6 time points in the study cohort. Labels correspond to Patient ID

Data from the clinical histories (see **Table 78** and **Table 79**) revealed that some of the participants were under active pharmacological treatment for controlling hypercholesterolemia (ID14 in the control arm and ID05, ID16 and ID23 in the active arm) or hypertriglyceridemia (ID12 in the control arm). Therefore, in order to avoid any confusion effect due to the concomitant drug treatment, we then decided to evaluate the changes in biochemistry profile when the above-mentioned patients were completely excluded from the statistical analysis. **Table 94**, **Figure 66** and **Figure 67** show these results:

Table 94: Statistical analysis of gender effects in changes in total cholesterol values excluding certain participants (*)

Changes in total cholesterol from baseline to Month 6:	**Control arm**		**Active arm**		***p*-value**	**Effect size**
	N	**Central tendency ± scatter measure**	**N**	**Central tendency ± scatter measure**		
All participants	8	3.5 ± 15.9 †	8	-12.2 ± 24.5 †	0.149 $^{\tau}$	0.38
Male	5	8.2 ± 14.9 †	6	1.0 ± 59.0 ‡	0.144 $^{\Diamond}$	**-0.44**
Female	3	-4.3 ± 17.0 †	2	-12.5 ± 2.1 †	0.567 $^{\tau}$	0.31

Values expressed in mg/dl. † (Mean ± SD); ‡ (Median ± IQR); $^{\tau}$ sig. (2-tailed) according to unpaired t-test; $^{\Diamond}$ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%. (*) Exclusion of participants ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A).

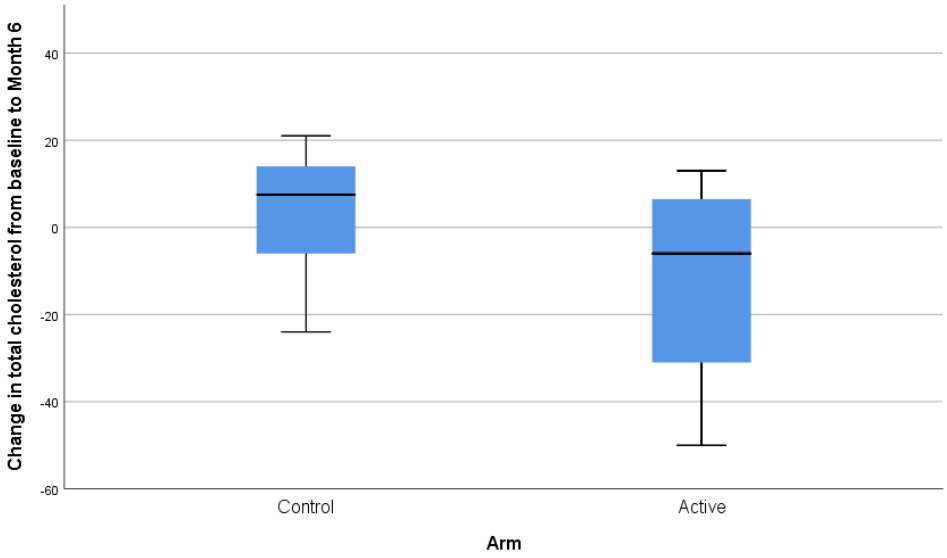


Figure 66: Comparison of the changes in total cholesterol levels from baseline to month 6 time points when participants ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A) were excluded from the statistical analysis

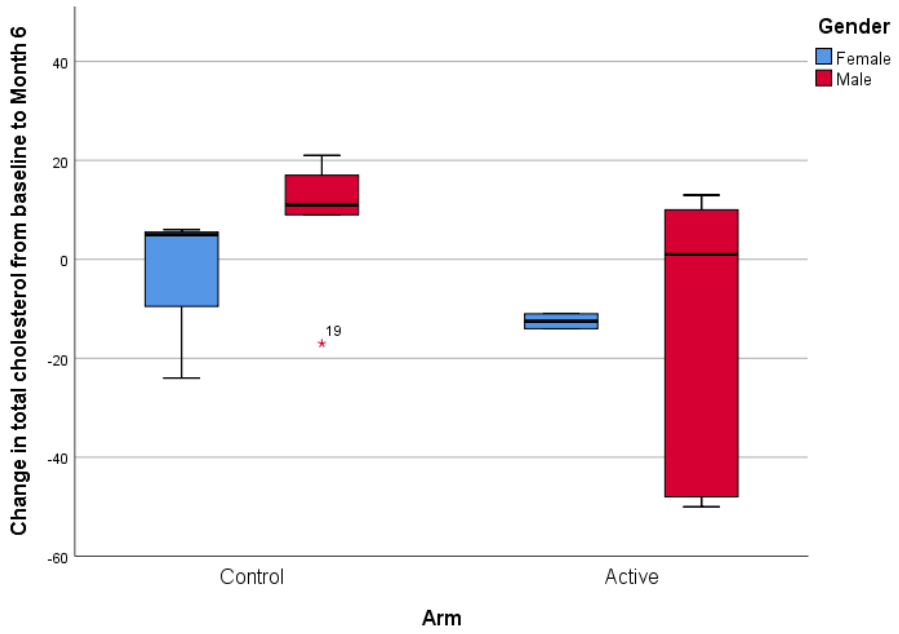


Figure 67: Comparison of the changes in total cholesterol levels from baseline to month 6 time points according to gender when participants ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A) were excluded from the statistical analysis

According to the results from the unpaired t-test, even when only participants without any active pharmacological treatment to manage antihypercholesterolemia and antihypertriglyceridemia were considered, mean values again revealed a decrease in total cholesterol levels from baseline to month 6 when comparing the control arm (3.5 ± 15.9 mg/dl) and the active arm (-12.2 ± 24.5 mg/dl). Although formally it was not statistically significant, this trend exhibited a medium effect size.

When considering participants' gender, both males and females values suggested a decrease although the sample size could not provide enough statistical power. In the males group, the calculated effect size (*r*=-0.44) confirmed the already mentioned reduction, revealing substantial practical effects.

5.7.4.2. Triglyceride profile results

The equivalent analysis was conducted for the changes in triglyceride levels (as obtained in **Table 92**), for all participants irrespectively of gender, and then considering gender analysis. Again, gender-underlying effects arose, as shown in **Table 95**.

Table 95: Statistical analysis of gender effects in changes in triglyceride values along the study

Changes in triglyceride levels from baseline to Month 6:	**Control arm**		**Active arm**		***p*-value**	**Effect size**
	N	**Mean ± SD**	**N**	**Mean ± SD**		
All participants	10	70.2 ± 81.2 †	10	-6.9 ± 98.3 †	**0.072** [τ]	**0.41**
Male	7	73.1 ± 98.2 †	8	-37.6 ± 68.7 †	**0.024** [τ]	**0.59**
Female	3	63.3 ± 25.3 †	2	116.0 ± 127.3 †	1.000 [◊ &]	0.00

Values expressed in mg/dl. [τ] sig. (2-tailed) according to unpaired t-test; [◊] Asymp. Sig. (2 tailed) according to Mann-Whitney's U test ([&]Levene's test for equality of variance not met); CI 95%.

Based on the results from the unpaired t-test, there was a significant decrease in triglyceride levels from baseline to month 6 between the participants when comparing the control arm (whose change in triglyceride levels, in fact, had increased: mean=70.2 mg/dl, SD=81.2 mg/dl) and the participants in the active arm (that certainly revealed triglyceride levels decrease: mean=-6.9 mg/dl, SD=98.3 mg/dl), *t*(18)=1.91. Although the difference did not reach statistical significance (*p*=0.072, two-tailed), the magnitude of this difference had medium effect size (*r*=0.41), revealing substantial practical effects.

When considering participants' gender, again males clearly promote these figures, since according to the same statistical test, there was a statistically significant difference in triglyceride levels from baseline to month 6 between the male group in the control arm (that increased: mean=73.1 mg/dl, SD=98.2 mg/dl) and the participants in the active arm (that decreased: mean=-37.6 mg/dl, SD=68.7 mg/dl), *t*(13)=2.56, *p*=0.024, two-tailed, medium effect size (*r*=0.59). The equivalent trend was not observed in the female group, for which the Mann-Whitney's U test must be applied instead of the unpaired t-test. In this regard, females revealed an increase in triglyceride levels from baseline to month 6 between the control arm (mean=63.3 mg/dl, SD=25.3 mg/dl) and the participants in the active arm (mean=116.0 mg/dl, SD=127.3 mg/dl), *Z*=0.0, that did not reach a statistically significant effect (*p*>0.05, two-tailed), probably associated to the tiny sample set in the female subgroup (effect size, *r*=0.0). **Figure 68** and **Figure 69** graphically illustrate these findings.

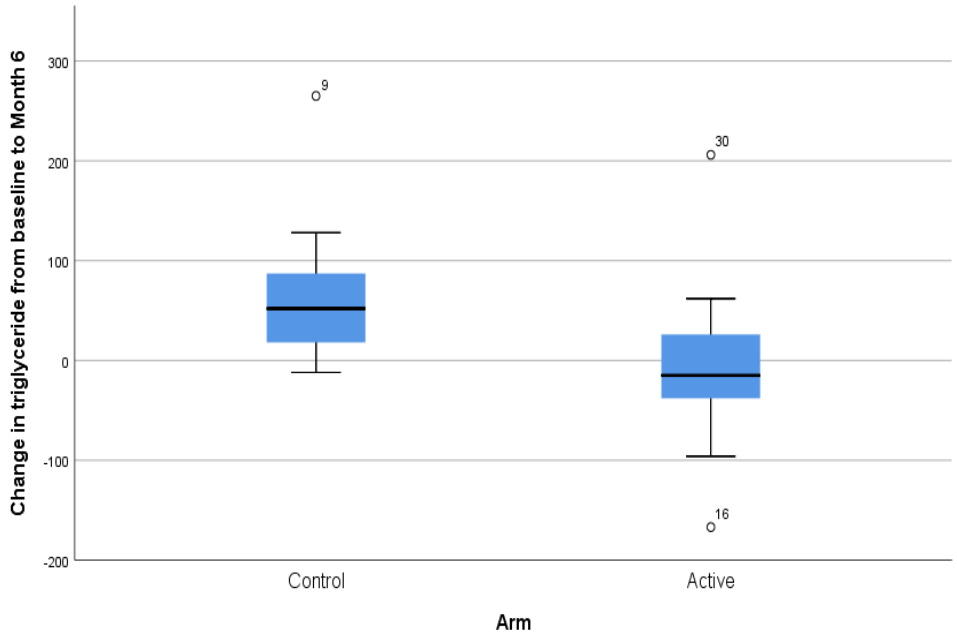


Figure 68: Comparison of the changes in triglyceride levels from baseline to month 6 time points in the control arm and the active arm. Labels correspond to Patient ID

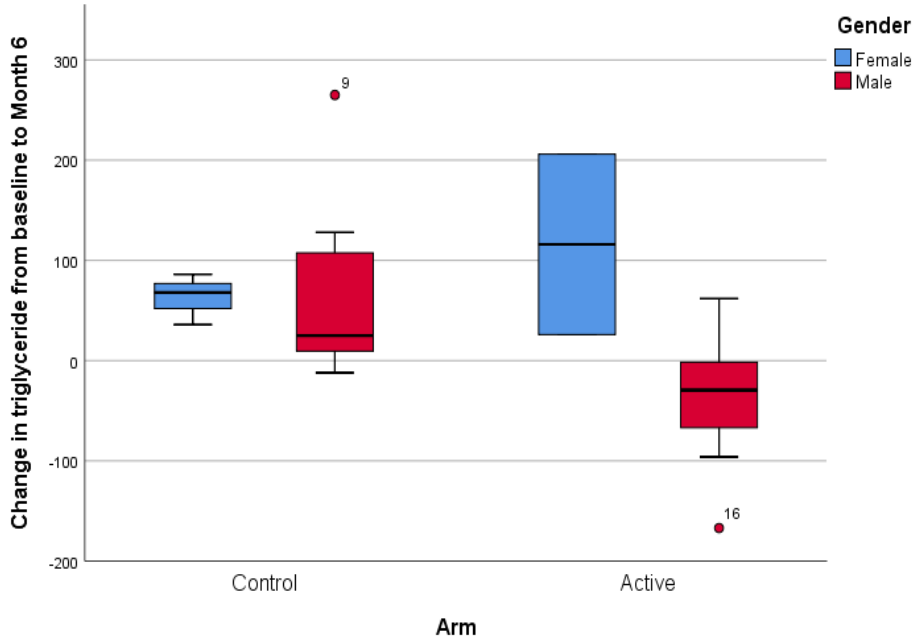


Figure 69: Comparison of the changes in triglyceride levels from baseline to month 6 time points in the control arm and the active arm, according to gender. Labels correspond to Patient ID

Then, the analysis was performed at the individual participant level. **Figure 70** graphically depicts the comparisons in triglyceride scores per Patient ID according to arms at the end of the study. Results above the y=x equation correspond to Patient ID whose triglyceride score at month 6 decreased from triglyceride score at baseline, *i.e.,* those for whom triglyceride levels had decreased along the study. Results lying on the y=x equation correspond to Patient ID whose total triglyceride levels did not change along the study. Finally, results lying below the

y=x equation correspond to Patient ID whose triglyceride levels increased from the corresponding values at baseline, *i.e.,* those for whom triglyceride levels had increased along the study.

The decrease in triglyceride levels from baseline values was evident in the active arm participants ID16, ID27 and ID28, whereas the majority of participants in the control arm had experienced an increase in triglyceride levels along the study. It is worth mentioning that only participants ID16 and ID23 (males) were under active pharmacological treatment for controlling their hypercholesterolemia and hypertriglyceridemia, respectively (reference is made to **Table 79**).

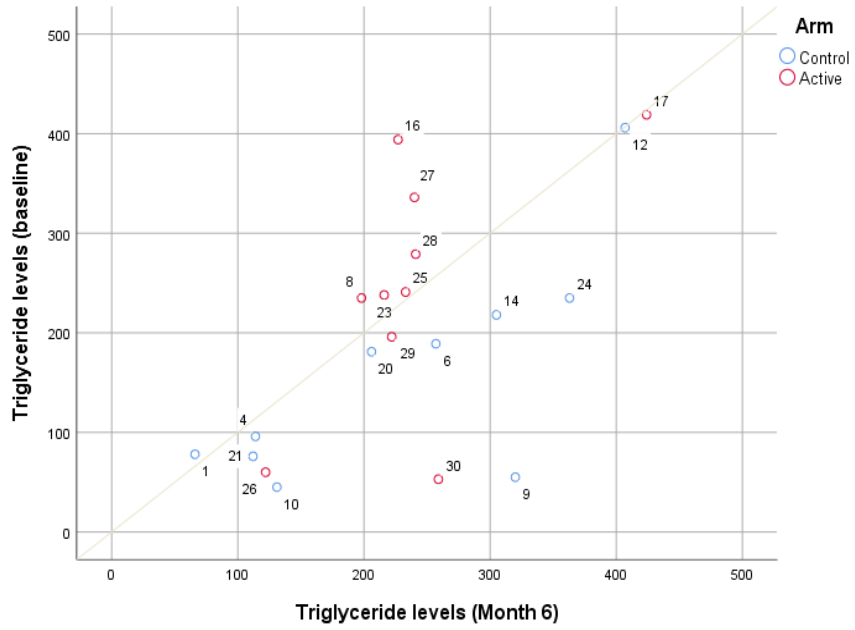


Figure 70: Comparison of the triglyceride scores at baseline and month 6 time points in the study cohort. Labels correspond to Patient ID

Again, in order to avoid any confusion effect due to the concomitant antihypercholesterolemia and antihypertriglyceridemia drug treatment, we evaluated the changes in triglyceride profile when patients (ID12 and ID14 in the control arm, and ID05, ID16 and ID23 in the active arm) were completely excluded from the statistical analysis. It is worth mentioning that in fact only participant ID12 was under antihypertriglyceridemic active treatment. However, in consistency with the previous total cholesterol analysis, we considered the same sample set. **Table 96**, **Figure 71** and **Figure 72** show these results:

Table 96: Statistical analysis of gender effects in changes in triglyceride values excluding certain participants

Changes in triglyceride from baseline to Month 6:	**Control arm**		**Active arm**		***p*-value**	**Effect size**
	N	**Mean ± SD**	**N**	**Mean ± SD**		
All participants	8	76.8 ± 87.7 †	8	15.0 ± 90.5 †	0.187 $^{\tau}$	0.35
Male	5	84.8 ± 113.7 †	6	-18.7 ± 52.7 †	0.076 $^{\tau}$	**0.55**
Female	3	63.3 ± 25.3 †	2	116.0 ± 127.3 †	0.505 $^{\tau}$	0.14

Values expressed in mg/dl. $^{\tau}$ sig. (2-tailed) according to unpaired t-test; CI 95%. Participants excluded: ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A) excluded.

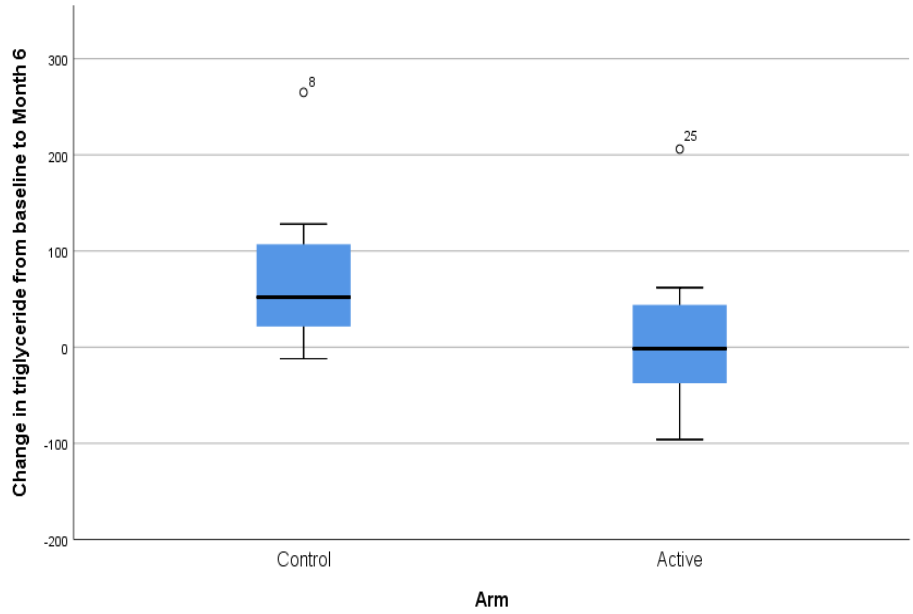


Figure 71: Comparison of the changes in triglyceride levels from baseline to month 6 time points when participants ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A) were excluded from the analysis

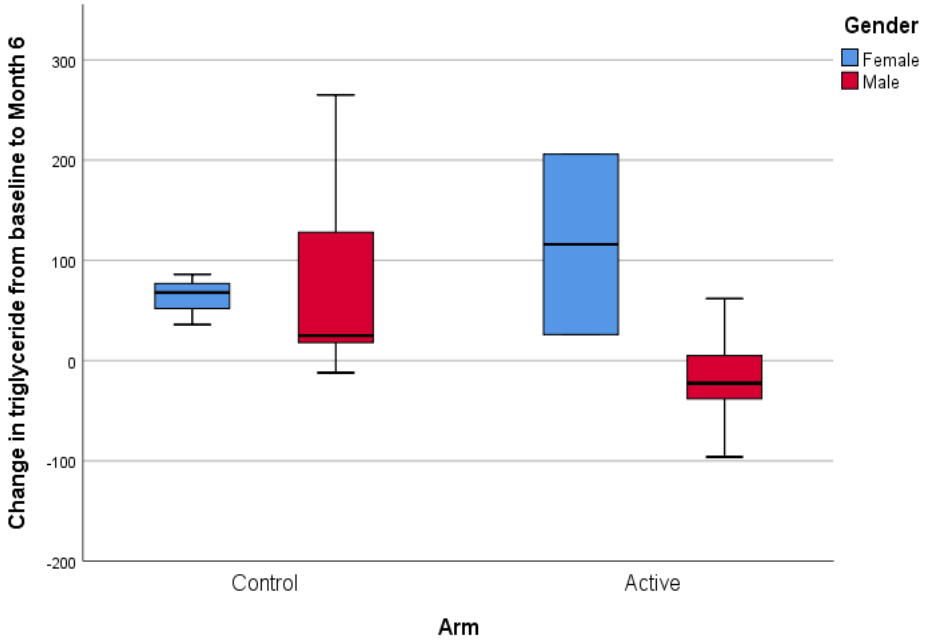


Figure 72: Comparison of the changes in triglyceride levels from baseline to month 6 time points according to gender when participants ID05 (A), ID12 (C), ID14 (C), ID16 (A) and ID23 (A) were excluded from the analysis

According to the results from the unpaired t-test, even when only participants without any pharmacological active treatment to manage antihypercholesterolemia and antihypertriglyceridemia were considered, mean values again revealed a decrease in triglyceride levels from baseline to month 6 when comparing the control arm (76.8 ± 87.7 mg/dl) and the active arm (15.0 ± 90.5 mg/dl). Although they were not statistically different, the reduction trend exhibited a medium effect size.

When considering participants' gender, males evidenced this decrease from the start to the end of the study. The mean change values in the control arm (84.8 ± 113.7 mg/dl) and the mean change values in the active arm (-18.7 ± 52.7 mg/dl) did not formally reach statistical significance (p=0.076), although the calculated effect size (r=0.55) revealed substantial practical effects that should be further explored.

No further differences between arms or gender were obtained when computing the equivalent analysis in the case of HDL and LDL parameters in the biochemistry profile.

Regarding the state of the art on the effects of cocoa components on lipid levels, some meta-analyses suggest beneficial effects of dark chocolate/cocoa products on total and LDL cholesterol and no major effects on HDL and TG in short-term intervention trials, although the administered amounts of cocoa (>30 g in some cases) are certainly not recommended in a healthy diet.[473] Other studies point out that 500 mg/day theobromine during four weeks was not effective in reducing HDL in subjects although it reduced CH in participants whose phenotype was characterized by low HDL and high TG,[474] whereas some other authors conclude that cocoa supplemented with theobromine up to 1000 mg/day during four weeks increased HDL.[475]

Recently, the mechanism by which caffeine regulates cholesterol metabolism and how caffeine may protect against cardiovascular disease risk has been elucidated and demonstrated in healthy human subjects at 400 mg oral doses.[476] Moreover, theobromine and paraxanthine as well as other xanthine-related compounds exhibited similar effects, suggesting that xanthine scaffold is 'a potent starting point for the development of compounds capable of mitigating cardiovascular risk'.[476] Remarkably, the single 400 mg oral doses evaluated in the referenced study were far away from the regular low doses of the caffeine and theobromine investigational food product evaluated herein, for which some potential trends in cholesterol and triglycerides' lowering effect when administered at regular low doses along time deserve further evaluations.

5.7.4.3. Patient ID16 profile results

Even having excluded participant ID16 from the analysis, we were interested in exploring his clinical history. It is worth mentioning that in patient ID16 (male, 34 years, active arm, **Table 75**), body weight ranged from 112.0 kg to 111.0 kg (**Table 84**), body mass index ranged from 38.30 kg/m^2 to 37.96 kg/m^2, was classified as obese class II (**Table 85**). His mean methylxanthines' dietary intake was one caffeinated cola drink daily (**Table 86**).

In terms of the total cholesterol and triglyceride levels, the registries available in the hospital for patient ID16 when this manuscript was being prepared covered from January 2017 to February 2022. It is worth mentioning that patient ID16 participated in the present study from 26th November 2020 (inclusion visit V1) to 26th May 2021 (end of study visit V2). The cholesteramide treatment (**Table 79**) started on 30th April 2021, and was still under treatment in April 2022, when the present statistical analysis was done.

Along this time frame, patient ID16's cholesterol levels ranged between the *ca*. 210 mg/dl top level in December 2017 and August 2020, to the bottom level *ca*. 120 mg/dl in June 2021. It is

worth mentioning that the largest decrease that patient ID16 had experienced along the four years cholesterol track records stored in the Hospital took place in the period covering the present clinical trial. As per February 2022, patient ID16's cholesterol levels had increased again to 180 mg/dl, even though he continued under the cholesteramide treatment.

Regarding the triglyceride levels monitored in the same period for patient ID16, they ranged from *ca.* 550 mg/dl top level in January 2017, to the bottom level *ca*. 120 mg/dl in April 2019. Although the profile exhibited certain increases and decreases along the entire period, the second most prominent decrease in patient ID16's triglyceride levels occurred between December 2020 and June 2021, the period covering the present clinical trial.

Further research should be done to identify which intrinsic characteristics in patient ID16 could be associated with the findings disclosed herein.

5.7.5. Results from the primary outcome measures

Note: Data analysis for the INQoL patient-reported primary outcome measure is currently ongoing and will be reported in due time, somewhere else beyond this Doctoral Thesis.

5.7.5.1. Quality of life: SF-36 questionnaire

One of the main outcomes of the present clinical trial was based on the perception the participants were able to quantify on the changes in their quality of life along the six months study timeframe. As previously discussed, the quality of life of people affected by DM1 is quite limited in general terms. Particularly, the participants in both arms of the present study suffered from several complex comorbidities (reference is made to **Table 78** and **Table 79**), that seriously affected their daily life.

In the present study, quality of life assessment was based on the SF-36 item health survey, covering eight health concepts: physical functioning (PF), bodily pain (BP), role limitations due to physical health problems (RP), role limitations due to personal or emotional problems (RE), emotional well-being (EW), social functioning (SF), energy/fatigue (E/F), and general health perceptions (GH). A ninth item related to the perceived change in health (HC) was also included.[477]

Data collected per each subject in each SF-36 scale at the baseline visit (**Table 97** and **Table 98**) as well as at the month 6 end of study visit (**Table 99** and **Table 100**) are provided below. It is worth mentioning that, while at the baseline time visit the 30 patients completed the questionnaire (100% response), only 12 patients in the control arm (80%) and 11 patients in the active arm (73%) responded to the questionnaire at the follow-up visit. At the end of the study, responders were 10 in both arms (67% each).

The data from the transformed variable 'changes in physical health' and 'changes in emotional health' at the month 6 end of study visit from the baseline visit are disclosed in **Table 101** and **Table 102**, respectively. The statistical analysis of 'changes in the SF-36 scores' and the statistical analysis of 'sum of changes in the grouped variables' physical health and emotional health are disclosed in **Table 103** and **Table 104**, respectively. The study results are further discussed after the above-mentioned table set.

Table 97: Results from SF-36 scales related to physical health at baseline visit (summary of cases)

Arm			Patient ID	SF-36 scores (physical health scales) at baseline visit				
				PF_V0	RP_V0	BP_V0	GH_V0	HC_V0
Control	1		01	10.0	0.0	45.0	30.0	25.0
	2		03	60.0	100.0	100.0	55.0	50.0
	3		04	50.0	100.0	47.5	25.0	25.0
	4		06	35.0	50.0	12.5	55.0	50.0
	5		07	35.0	0.0	22.5	20.0	.0
	6		09	15.0	0.0	45.0	50.0	25.0
	7		10	70.0	100.0	80.0	70.0	50.0
	8		11	85.0	100.0	100.0	25.0	25.0
	9		12	85.0	100.0	100.0	55.0	75.0
	10		13	45.0	100.0	55.0	50.0	25.0
	11		14	45.0	100.0	67.5	65.0	50.0
	12		15	55.0	100.0	35.0	50.0	75.0
	13		20	100.0	100.0	90.0	75.0	50.0
	14		21	65.0	0.0	12.5	75.0	50.0
	15		24	40.0	50.0	.0	15.0	25.0
	Total	N	15	15	15	15	15	15
		Mean	-	**53.0**	66.7	**54.2**	**47.7**	**40.0**
		SD	-	**25.3**	45.0	**34.3**	**20.1**	**20.7**
		Median	-	50.0	**100.0**	47.5	50.0	50.0
		IQR	-	35.0	**100.0**	67.5	40.0	25.0
Active	1		02	10.0	100.0	47.50	50.0	50.0
	2		05	50.0	100.0	22.5	30.0	25.0
	3		08	70.0	100.0	100.0	30.0	25.0
	4		16	75.0	100.0	100.0	50.0	50.0
	5		17	5.0	100.0	87.5	100.0	35.0
	6		18	60.0	100.0	90.0	50.0	50.0
	7		19	5.0	100.0	77.5	25.0	25.0
	8		22	90.0	100.0	90.0	60.0	50.0
	9		23	50.0	100.0	62.5	75.0	50,0
	10		25	90.0	100.0	57.5	35.0	25.0
	11		26	35.0	0.0	90.0	35.0	25.0
	12		27	85.0	100.0	10.0	50.0	25.0
	13		28	35.0	100.0	77.5	50.0	25.0
	14		29	25.0	100.0	35.0	35.0	50.0
	15		30	0.0	0.0	80.0	15.0	25,0
	Total	N	15	15	15	15	15	15
		Mean	-	**45.7**	86.7	66.0	**43.3**	**34.0**
		SD	-	**32.2**	35.2	31.8	**23.1**	**12.0**
		Median	-	50.0	**100.0**	**77.5**	35.0	25.0
		IQR	-	65.0	**0.0**	**55.0**	20.0	25.0
Total	N		30	30	30	30	30	30
	Mean		-	49.3	76.7	60.1	**45.5**	37.0
	SD		-	**28.7**	41.0	33.0	**21.4**	16.9
	Median		-	50.0	**100.0**	**65.0**	50.0	**25.0**
	IQR		-	38.8	**50.0**	**58.1**	27.5	**25.0**

Table 98: Results from SF-36 scales related to emotional health at baseline visit (summary of cases)

Arm			Patient ID	SF-36 scores (emotional health scales) at baseline visit			
				RE_V0	E/F_V0	EW_V0	SF_V0
Control	1		01	100.0	35.0	56.0	62.5
	2		03	100.0	40.0	12.0	87.5
	3		04	100.0	25.0	72.0	50.0
	4		06	100.0	35.0	80.0	87.5
	5		07	66.7	40.0	60.0	50.0
	6		09	100.0	60.0	72.0	75.0
	7		10	100.0	50.0	76.0	100.0
	8		11	66.7	70.0	84.0	100.0
	9		12	100.0	75.0	84.0	100.0
	10		13	66.7	40.0	52.0	50.0
	11		14	100.0	60.0	88.0	100.0
	12		15	100.0	50.0	92.0	50.0
	13		20	100.0	90.0	100.0	100.0
	14		21	100.0	75.0	80.0	62.5
	15		24	100.0	45.0	76.0	75.0
	Total	N	15	15	15	15	15
		Mean	-	93.3	**52.7**	72.3	76.7
		SD	-	13.8	**18.4**	21.2	21.1
		Median	-	**100.0**	50.0	**76.0**	**75.0**
		IQR	-	**0.0**	30.0	**24.0**	**50.0**
Active	1		02	100.0	30.0	48.0	87.5
	2		05	100.0	65.0	84.0	75.0
	3		08	100.0	70.0	92.0	100.0
	4		16	100.0	100.0	30.0	88.0
	5		17	100.0	60.0	92.0	100.0
	6		18	100.0	35.0	84.0	87.5
	7		19	100.0	55.0	92.0	100.0
	8		22	100.0	70.0	88.0	100.0
	9		23	.0	80.0	56.0	62.5
	10		25	100.0	80.0	100.0	87.5
	11		26	0.0	35.0	76.0	25.0
	12		27	100.0	55.0	72.0	100.0
	13		28	100.0	45.0	64.0	100.0
	14		29	33.3	30.0	56.0	75.0
	15		30	100.0	15.0	68.0	87.5
	Total	N	15	15	15	15	15
		Mean	-	82.2	**55.0**	**73.5**	85.0
		SD	-	37.5	**24.0**	**19.7**	20.2
		Median	-	**100.0**	55.0	76.0	**87.5**
		IQR	-	**0.0**	35.0	36.0	**25.0**
Total	N		30	30	30	30	30
	Mean		-	87.8	**53.8**	72.9	80.9
	SD		-	28.3	**20.7**	20.1	20.7
	Median		-	**100.0**	52.5	**76.0**	**87.5**
	IQR		-	**0.0**	35.0	**29.0**	**37.5**

Table 99: Results from SF-36 scales related to physical health at month 6 visit (summary of cases)

Arm			Patient ID	SF-36 scores (physical health scales) at month 6 visit				
				PF_V2	RP_V2	BP_V2	GH_V2	HC_V2
Control	1		01	.	.	.	.	.
	2		03	.	.	.	.	.
	3		04	90.0	100.0	80.0	25.0	50.0
	4		06	55.0	100.0	32.5	50.0	25.0
	5		07	20.0	0.0	22.5	5.0	0.0
	6		09	20.0	25.0	67.5	65.0	25.0
	7		10	80.0	100.0	100.0	70.0	75.0
	8		11	75.0	100.0	90.0	0.0	25.0
	9		12	80.0	0.0	100.0	55.0	75.0
	10		13	.	.	.	.	.
	11		14	.	.	.	.	.
	12		15	.	.	.	.	.
	13		20	100.0	100.0	100.0	85.0	50.0
	14		21	65.0	0.00	0.0	55.0	25.0
	15		24	25.0	75.00	0.0	15.0	0.0
	Total	N	15	10	10	10	10	10
		Mean	-	**61.0**	60.0	**59.3**	**47.5**	**35.0**
		SD	-	**29.8**	47.4	**41.5**	**25.9**	**26.9**
		Median	-	70.0	**87.5**	73.8	52.5	25.0
		IQR	-	58.75	**100.0**	83.1	43.8	37.5
Active	1		02	.	.	.	.	.
	2		05	.	.	.	.	.
	3		08	55.0	100.0	100.0	35.0	50.0
	4		16	70.0	75.0	90.0	20.0	25.0
	5		17	5.0	100.0	100.0	70.0	50.0
	6		18	.	.	.	.	.
	7		19	5.0	0.0	52.5	30.0	25.0
	8		22	.	.	.	.	.
	9		23	15.0	75.0	67.5	70.0	75.0
	10		25	100.0	100.0	67.5	60.0	50.0
	11		26	25.0	0.0	0.0	55.0	0.0
	12		27	85.0	100.0	100.0	60.0	50.0
	13		28	25.0	50.0	22.5	30.0	25.0
	14		29	10.0	100.0	47.5	60.0	25.0
	15		30	.	.	.	.	.
	Total	N	15	10	10	10	10	10
		Mean	-	**39.5**	70.0	**64.8**	**49.0**	**37.5**
		SD	-	**35.2**	40.5	**34.7**	**18.4**	**21.2**
		Median	-	25.0	**87.5**	67.5	57.5	37.5
		IQR	-	65.0	**62.5**	58.8	32.5	25.0
Total	N		30	20	20	20	20	20
	Mean		-	50.3	65.0	62.0	**48.3**	36.3
	SD		-	33.6	43.2	37.3	**21.5**	23.6
	Median		-	**55.0**	**87.5**	**67.5**	55.0	**25.0**
	IQR		-	**60.0**	**93.8**	**75.0**	33.8	**25.0**

Table 100: Results from SF-36 scales related to emotional health at month 6 visit (summary of cases)

Arm			Patient ID	SF-36 scores (emotional health scales) at month 6 visit			
				RE_V2	E/F_V2	EW_V2	SF_V2
Control	1		01	.	.	.	.
	2		03	.	.	.	.
	3		04	100.0	50.0	76.0	100.0
	4		06	100.0	40.0	64.0	75.0
	5		07	33.3	20.0	72.0	50.0
	6		09	100.0	55.0	100.0	87.5
	7		10	100.0	85.0	88.0	87.5
	8		11	100.0	75.0	92.0	100.0
	9		12	100.0	70.0	80.0	87.5
	10		13	.	.	.	.
	11		14	.	.	.	.
	12		15	.	.	.	.
	13		20	100.0	95.0	100.0	100.0
	14		21	100.0	75.0	72.0	75.0
	15		24	100.0	45.0	76.0	87.5
	Total	N	15	10	10	10	10
		Mean	-	93.3	**61.0**	**82.0**	85.0
		SD	-	21.1	**22.9**	**12.4**	15.4
		Median	-	**100.0**	62.5	78.0	**87.5**
		IQR	-	**0.0**	33.8	22.0	**25.0**
Active	1		02	.	.	.	.
	2		05	.	.	.	.
	3		08	100.0	50.0	88.0	100.0
	4		16	100.0	35.0	68.0	100.0
	5		17	100.0	70.0	96.0	100.0
	6		18	.	.	.	.
	7		19	100.0	35.0	88.0	100.0
	8		22	.	.	.	.
	9		23	100.0	50.0	80.0	75.0
	10		25	100.0	70.0	80.0	100.0
	11		26	0.0	20.0	56.0	0.0
	12		27	100.0	65.0	80.0	87.5
	13		28	100.0	50.0	64.0	87.5
	14		29	100.0	30.0	72.0	75.0
	15		30	.	.	.	.
	Total	N	15	10	10	10	10
		Mean	-	90.0	**47.5**	**77.2**	82.5
		SD	-	31.6	**17.4**	**12.2**	30.7
		Median	-	**100.0**	50.0	80.0	**93.8**
		IQR	-	**0.0**	32.5	21.0	**25.0**
Total	N		30	20	20	20	20
	Mean		-	91.7	**54.3**	**79.6**	83.8
	SD		-	26.2	**21.0**	**12.2**	23.7
	Median		-	**100.0**	50.0	80.0	**87.5**
	IQR		-	**0.0**	33.8	16.0	**25.0**

Table 101: Results from SF-36 scales related to changes in physical health from baseline to month 6 end of study visit

Arm			Patient ID	Changes in SF-36 scores (physical health scales) from baseline to month 6 end of study visit				
				PF	RP	BP	GH	HC
Control	1		01	.	.	.	.	.
	2		03	.	.	.	.	.
	3		04	40.0	0.0	32.5	0.0	25.0
	4		06	20.0	50.0	20.0	-5.0	-25.0
	5		07	-15.0	0.0	0.0	-15.0	0.0
	6		09	5.0	25.0	22.5	15.0	0.0
	7		10	10.0	0.0	20.0	0.0	25.0
	8		11	-10.0	0.0	-10.0	25.0	0.0
	9		12	-5.0	-100.0	0.0	0.0	0.0
	10		13	.	.	.	.	.
	11		14	.	.	.	.	.
	12		15	.	.	.	.	.
	13		20	0.0	0.0	10.0	10.0	0.0
	14		21	0.0	0.0	-12.5	-20.0	-25.0
	15		24	-15.0	25.0	0.0	0.0	-25.0
	Total	N	15	10	10	10	10	10
		Mean	-	**3.0**	0.0	**8.3**	**1.0**	-2.5
		SD	-	**17.1**	39.1	**15.0**	**13.3**	18.4
		Median	-	0.0	**0.0**	5.0	0.0	**0.0**
		IQR	-	23.8	**25.0**	23.1	18,8	**31.3**
Active	1		02	.	.	.	.	.
	2		05	.	.	.	.	.
	3		08	-15.0	0.0	0.0	-15.0	0.0
	4		16	-5.0	-25.0	2.5	-80.0	-10.0
	5		17	0.0	0.0	10.0	20.0	0.0
	6		18	.	.	.	.	.
	7		19	0.0	-100.0	-37.5	-30.0	-25.0
	8		22	.	.	.	.	.
	9		23	-35.0	-25.0	10.0	35.0	50.0
	10		25	10.0	0.0	-22.5	25.0	25.0
	11		26	-10.0	0.0	-10.0	5.0	-25.0
	12		27	0.0	0.0	22.5	10.0	25.0
	13		28	-10.0	-50.0	-12.5	-5.0	-25.0
	14		29	-15.0	0.0	-32.5	45.0	0.0
	15		30	.	.	.	.	.
	Total	N	15	10	10	10	10	10
		Mean	-	**-8.0**	-20.0	**-7.0**	**1.0**	**1.5**
		SD	-	**12.3**	32.9	**19.6**	**36.4**	**25.2**
		Median	-	-7.5	**0.0**	-5.0	7.5	0.0
		IQR	-	15.0	**31.3**	35.0	46.3	50.0
Total	N		30	20	20	20	20	20
	Mean		-	**-2.5**	-10.0	**0.6**	**1.0**	-0.5
	SD		-	**15.5**	36.6	**18.7**	**26.7**	21.6
	Median		-	-2.5	**0.0**	0.0	0.0	**0.0**
	IQR		-	17.5	**18.8**	29.4	31.3	**43.8**

Table 102: Results from SF-36 scales related to changes in emotional health from baseline to month 6 end of study visit

Arm			Patient ID	Changes in SF-36 scores (emotional health scales) from baseline to month 6 end of study visit			
				RE	E/F	EW	SF
Control	1		01	.	.	.	.
	2		03	.	.	.	.
	3		04	0.0	25.0	4.0	50.0
	4		06	0.0	5.0	-16.0	-12.5
	5		07	-33.4	-20.0	12.0	0.0
	6		09	0.0	-5.0	28.0	12.5
	7		10	0.0	35.0	12.0	-12.5
	8		11	33.3	5.0	8.0	0.0
	9		12	0.0	-5.0	-4.0	-12.5
	10		13	.	.	.	.
	11		14	.	.	.	.
	12		15	.	.	.	.
	13		20	0.0	5.0	0.0	0.0
	14		21	0.0	0.0	-8.0	12.5
	15		24	0.0	0.0	0.0	12.5
	Total	N	15	10	10	10	10
		Mean	-	0.0	**4.5**	**3.6**	5.0
		SD	-	15.7	**15.5**	**12.3**	18.8
		Median	-	**0.0**	2.5	2.0	**0.0**
		IQR	-	**0.0**	15.0	17.0	**25.0**
Active	1		02	.	.	.	.
	2		05	.	.	.	.
	3		08	0.0	-20.0	-4.0	0.0
	4		16	0.0	-65.0	38.0	12.0
	5		17	0.0	10.0	4.0	0.0
	6		18	.	.	.	.
	7		19	0.0	-20.0	-4.0	0.0
	8		22	.	.	.	.
	9		23	100.0	-30.0	24.0	12.5
	10		25	0.0	-10.0	-20.0	12.5
	11		26	0.0	-15.0	-20.0	-25.0
	12		27	0.0	10.0	8.0	-12.5
	13		28	0.0	5.0	0.0	-12.5
	14		29	66.7	0.0	16.0	0.0
	15		30	.	.	.	.
	Total	N	15	10	10	10	10
		Mean	-	16.7	**-13.5**	**4.2**	**-1.3**
		SD	-	36.0	**22.7**	**18.3**	**12.4**
		Median	-	**0.0**	-12.5	2.0	0.0
		IQR	-	**16.7**	28.8	26.0	24.6
Total	N		30	20	20	20	20
	Mean		-	8.3	**-4.5**	**3.9**	1.9
	SD		-	28.4	**21.1**	**15.2**	15.8
	Median		-	**0.0**	0.0	2.0	**0.0**
	IQR		-	**0.0**	23.8	16.0	**25.0**

Table 103: Statistical analysis of changes in SF-36 questionnaire scores from baseline to the end of study

Changes from baseline to Month 6 visit	Control arm		Active arm		*p*-value	Effect size
	N	Central tendency ± scatter measure	N	Central tendency ± scatter measure		
Physical health						
SF-36 PF Physical functioning	10	3.0 ± 17.0 †	10	-8.0 ± 12.3 †	0.115 $^{\tau}$	0.36
SF-36 RP Limitation to physical health	10	0.0 ± 25.0 ‡	10	0.0 ± 31.3 ‡	0.089 $^{\lozenge}$	-0.43
SF-36 BP Bodily pain	10	8.3 ± 15.0 †	10	-7.0 ± 19.6 †	0.067 $^{\tau}$	0.42
SF-36 GH General health	10	1.0 ± 13.3 †	10	1.0 ± 36.4 †	0.595 $^{\lozenge\&}$	-0.12
Emotional health						
SF-36 RE Limitation due to emotional problems	10	0.0 ± 0.0 ‡	10	0.0 ± 16.7 ‡	0.280 $^{\lozenge}$	-0.24
SF-36 E/F Energy/fatigue (vitality)	10	4.5 ± 15.5 †	10	-13.5 ± 22.7 †	0.053 $^{\tau}$	0.44
SF-36 EW Emotional well-being	10	3.6 ± 12.3 †	10	4.2 ± 18.3 †	0.932 $^{\tau}$	0.02
SF-36 SF Social functioning	10	0.0 ± 25.0 ‡	10	-1.3 ± 12.4 ‡	0.530 $^{\lozenge}$	-0.14
Health change						
SF-36 HC Health change	10	0.0 ± 31.0 ‡	10	1.5 ± 25.2 †	0.874 $^{\lozenge}$	-0.03

† (Mean ± SD); ‡ (Median ± IQR); $^{\tau}$ sig. (2-tailed) according to unpaired t-test; $^{\lozenge}$ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; ($^{\&}$Levene's test for equality of variance not met); CI 95%

Table 104: Statistical analysis of sum of changes in SF-36 questionnaire scores from baseline to the end of study

Changes from baseline to Month 6 visit	Control arm		Active arm		*p*-value	Effect size
	N	Mean ± SD	N	Mean ± SD		
Physical health						
Sum of changes in physical scores in SF-36	10	12.25 ± 57.7	10	-34.0 ± 64.6	0.108 $^{\tau}$	0.37
Emotional health						
Sum of changes in emotional scores in SF-36	10	13.1 ± 36.6	10	6.1 ± 51.0	0.728 $^{\tau}$	0.08

$^{\tau}$ sig. (2-tailed) according to unpaired t-test; CI 95%

Formally, none of the eight HRQoL SF-36 scales based exhibited statistical significant differences when comparing changes in each single physical health and emotional health scale at the month 6 end of study final visit from the baseline situation (p > 0.05, ns, 95% CI), although values in the active arm suggested certain tendency to even worsening. **Figure 73** and **Figure 74** graphically illustrate the comparison in the grouping variables 'sum of changes' in physical and emotional health scores, respectively:

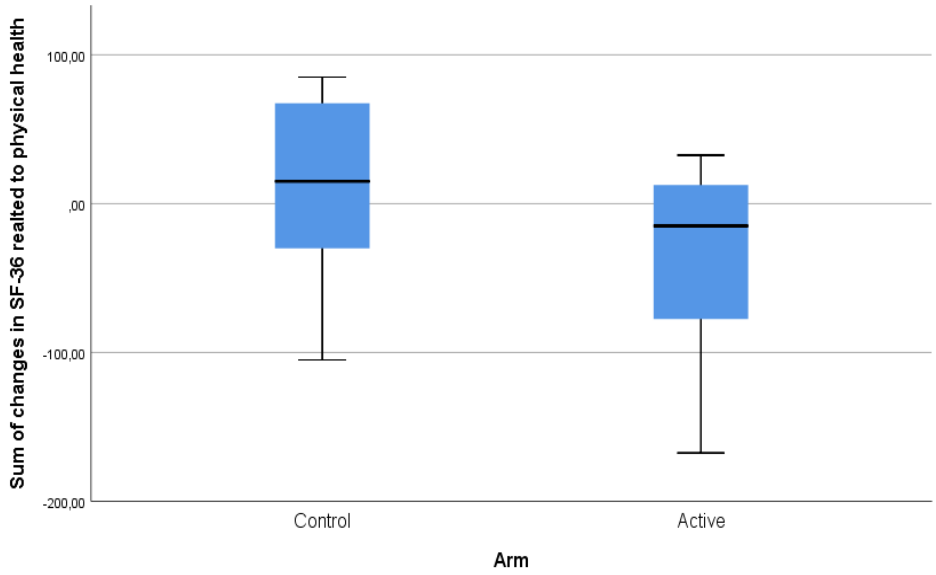


Figure 73: Comparison of the sum of changes in SF-36 scores related to physical health between month 6 and baseline time points in the study arm

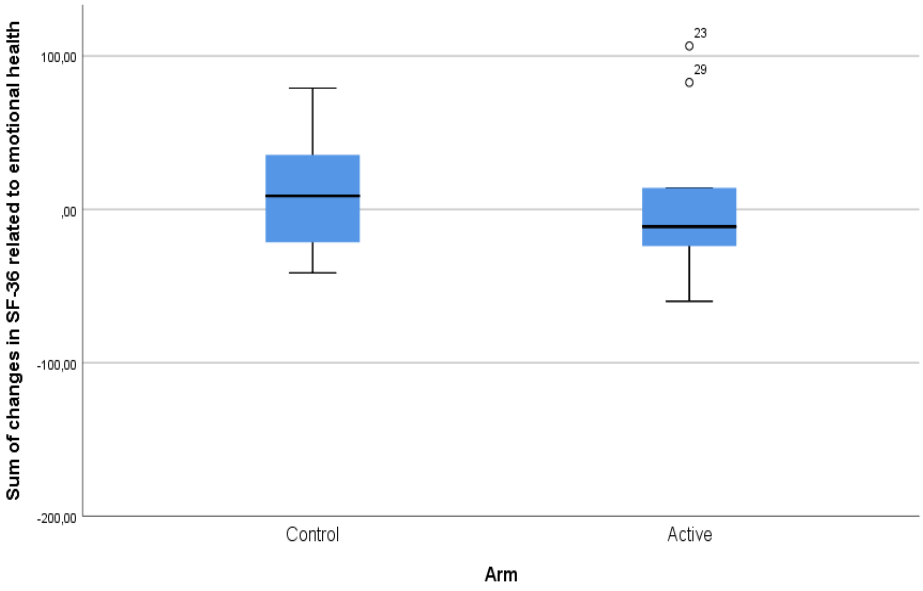


Figure 74: Comparison of the sum of changes in SF-36 scores related to emotional health between month 6 and baseline time points in the study arm. Labels correspond to Patient ID

Figure 75 and **Figure 76** illustrate the analysis of the grouping variables 'sum of changes' in physical and emotional health, respectively, according to gender.

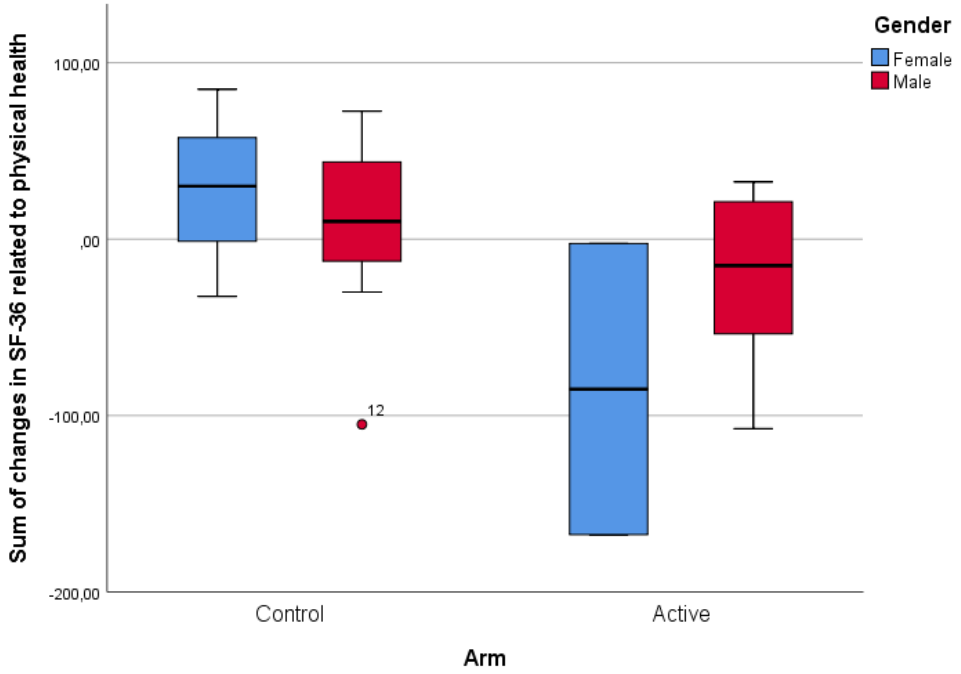


Figure 75: Comparison of the sum of changes in SF-36 scores related to physical health between month 6 and baseline time points in the study arm, divided into gender. Labels correspond to Patient ID

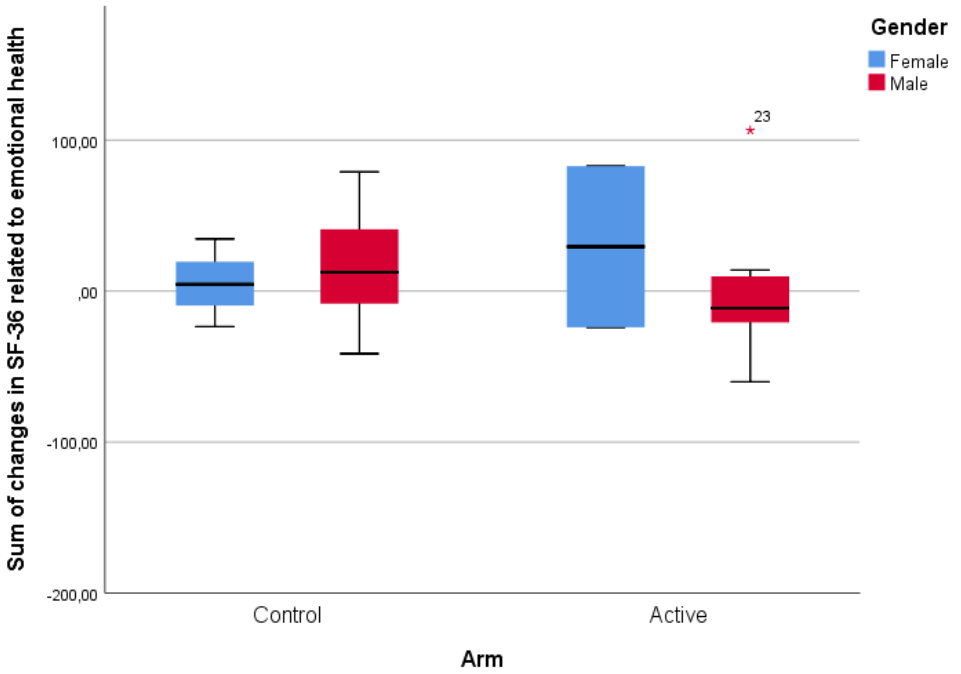


Figure 76: Comparison of the sum of changes in SF-36 scores related to emotional health between month 6 and baseline time points in the study arm, divided into gender. Labels correspond to Patient ID

An unpaired t test was conducted to evaluate the existence of any gender effect in the grouping variables 'sum of changes' in physical and emotional health. No statistically significant differences from baseline to month 6 in the male group nor in the female group was revealed when comparing the gender effect in the control arm nor in the active arm (p>0.05), although the apparently decrease in the sum of changes in the SF-36 scores related to physical health in the females group in the active arm reached a medium effect size r=0.65. This effect should be further evaluated in a larger female cohort.

It is worth mentioning that the present study was fully conducted during the COVID-19 pandemic that hit the world and particularly impacted Spain. After having overcome a severe lockdown in spring 2020, several outbreaks took place along the clinical trial from the inclusion of the first patient in November 2020 through the end of the study in October 2021. Some patients in the study were infected by SARS-CoV-2, and most of them were afraid of being infected.

Recent post-pandemic studies have demonstrated the negative impact of COVID-19 pandemic on physical and emotional health and quality of life of patients with neuromuscular diseases, as well as a slight worsening in their disease state.[478,479] Under these very complex circumstances, the subjective perception of any improvement in the quality of life may certainly be undervalued. Ideally, HRQoL outcome should be measured beyond the post-pandemic era.

5.7.5.2. Epworth sleepiness scale

Epworth sleepiness scale measures daytime sleepiness in a Likert scale (0 = would *never* doze, 1 = *slight* chance of dozing, 2 = *moderate* change of dozing, 3 = *high* chance of dozing), for eight different daily situations, to obtain a total score ranging from 0 to 24. A result between 0 and 6 is considered normal, 7-8 average sleepiness, 9-24 abnormal/pathological sleepiness.

Participants filled the Epworth sleepiness scale questionnaire at the inclusion visit, month 3 follow-up and month 6 end of study visit time points. Data provided by participants' responses is detailed in **Table 105**, whereas the changes from baseline to month 3 and from baseline to month 6 for the entire cohort computed as differences in scores between each above-mentioned time point are disclosed in **Table 106**.

Firstly, we compared the background ESS scores between arms, in order to explore whether some differences could arise from the beginning of the study. The statistical comparison in baseline sleepiness' data between arms was performed using the Mann-Whitney's U test, which revealed no statistically significant differences in the baseline daytime sleepiness in the active arm from the control arm at the inclusion visit (p>0.05, ns), although the effect size was (r)=0.30. When the arms were divided into gender, no statistically significant differences in females nor in males were revealed as well, according to the results from the unpaired t test and the Mann-Whitney's U test, respectively (p>0.05 in both genders). It is worth mentioning that in the males subgroup, median ± IQR background ESS values accounted for 6.11 ± 4.14 in the control arm and a median score 7.50 ± 8.0 in the active arm (effect size r=0.38), whereas mean ± standard deviation ESS scores in the females group accounted for 7.80 ± 3.70 in the control arm and 9.80 ± 3.90 in the active arm.

Based on this background data, provided that the daily sleepiness was not statistically significant into the two arms at the start of the present study, and that caffeine was determined not to behave as confusion factor between arms either at the start nor at the end of the study, the exploratory analysis on the variation in the Epworth's sleepiness scale from the data collected from patients as well as effect sizes along the study is further discussed herein.

Table 105: Results from Epworth sleepiness scales obtained from study participants at each time point

				Patient_ID	Epworth sleepiness scale (ESS)		
					Baseline	Month 3	Month 6
Arm	**Control**	1		01	3	3	.
		2		03	3	5	.
		3		04	9	.	10
		4		06	9	7	9
		5		07	7	10	5
		6		09	3	5	2
		7		10	13	10	5
		8		11	4	3	4
		9		12	4	3	4
		10		13	.	.	.
		11		14	15	.	.
		12		15	8	11	.
		13		20	2	2	2
		14		21	6	6	7
		15		24	8	11	12
		Total	N	15	14	12	10
			Mean	-	**6.7**	**6.3**	**6.0**
			SD	-	**3.9**	**3.4**	**3.4**
	Active	1		02	6	.	.
		2		05	8	.	.
		3		08	7	5	6
		4		16	24	2	3
		5		17	5	4	5
		6		18	16	10	12
		7		19	6	8	8
		8		22	10	.	.
		9		23	6	.	2
		10		25	10	7	.
		11		26	5	6	1
		12		27	12	5	6
		13		28	4	5	4
		14		29	15	16	14
		15		30	12	11	15
		Total	N	15	15	11	11
			Mean	-	**9.8**	**7.2**	**6.9**
			SD	-	**5.6**	**3.9**	**4.8**
	Total	N		30	29	23	21
		Central tendency		-	**7.0**‡	**6.7**†	**6.5**†
		Scatter measure		-	**8.0**‡	**3.6**†	**4.1**†

† (Mean ± SD); ‡ (Median ± IQR);

Table 106: Results from Epworth sleepiness scales. ESS changes from baseline situation

				Patient_ID	ESS change from baseline at Month 3 visit	ESS change from baseline at Month 6 visit
Arm	**Control**	1		01	0.0	.
		2		03	2.0	.
		3		04	.	1.0
		4		06	-2.0	0.0
		5		07	3.0	-2.0
		6		09	2.0	-1.0
		7		10	-3.0	-8.0
		8		11	-1.0	0.0
		9		12	-1.0	0.0
		10		13	.	.
		11		14	.	.
		12		15	3.0	.
		13		20	0.0	0.0
		14		21	0.0	1.0
		15		24	3.0	4.0
		Total	N	15	12	10
			Mean	-	**0.5**	-0.5
			SD	-	**2.1**	3.1
			Median	-	0.0	**0.0**
			IQR	-	4.0	**2.0**
	Active	1		02	.	.
		2		05	.	.
		3		08	-2.0	-1.0
		4		16	-22.0	-21.0
		5		17	-1.0	0.0
		6		18	-6.0	-4.0
		7		19	2.0	2.0
		8		22	.	.
		9		23	.	-4.0
		10		25	-3.0	.
		11		26	1.0	-4.0
		12		27	-7.0	-6.0
		13		28	1.0	0.0
		14		29	1.0	-1.0
		15		30	-1.0	3.0
		Total	N	13	11	11
			Mean	-	-3.4	-3.3
			SD	-	6.8	6.5
			Median	-	**-1.0**	**-1.0**
			IQR	-	**7.3**	**5.0**
	Total	N		30	23	21
		Mean		-	-1.4	-1.9
		SD		-	5.2	5.3
		Median		-	**0.0**	**0.0**
		IQR		-	**3.0**	**4.0**

Figure 77 graphically depicts the comparisons in ESS scores per Patient ID according to arms at the end of the study compared from the baseline situation. Results above the y=x equation correspond to Patient ID whose ESS score at month 6 decreased from ESS score at baseline, *i.e.,* those for whom drowsiness had decreased along the study. Results lying on the y=x equation correspond to Patient ID whose ESS scores did not change along the study. Finally, results lying below the y=x equation correspond to Patient ID whose ESS scores increased from ESS scores at baseline, *i.e.,* those for whom drowsiness had increased along the study. Remarkably, participant ID16 led again the most prominent decrease as in previous observations.

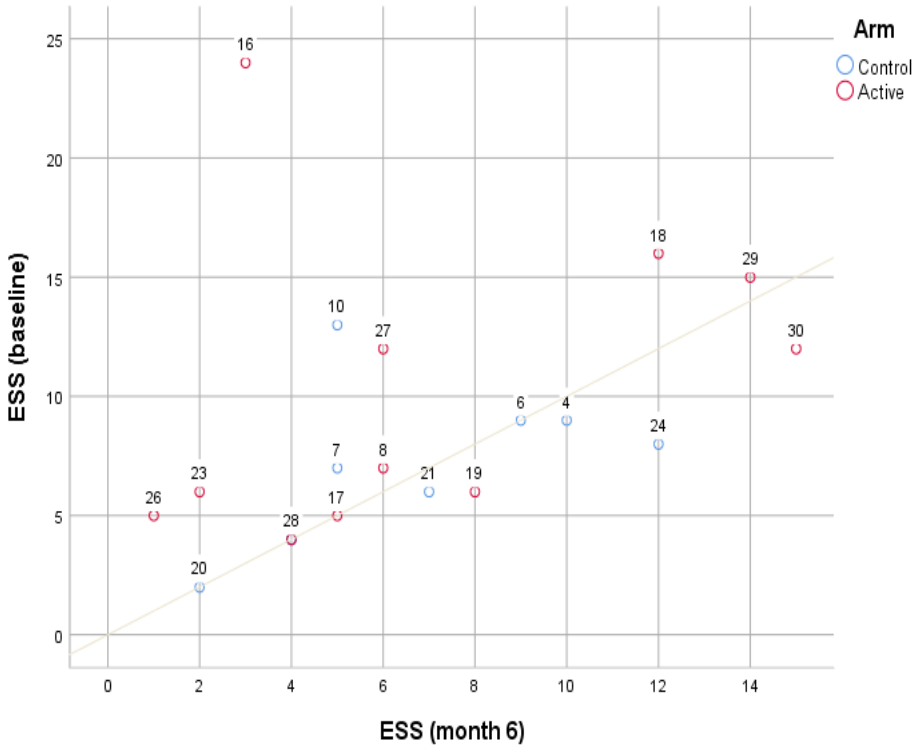


Figure 77: Comparison of the ESS scores at baseline and month 6 time points in both arms. Labels correspond to Patient ID. Patient labels' ID 11, 12 and 28 are overlapped

Table 107 discloses the comparison on the changes in Epworth's sleepiness scale values between the month 3 follow-up visit and baseline visit (ESS_{M3}-$ESS_{baseline}$), and between the month 6 end of study visit and the baseline visit (ESS_{M6}-$ESS_{baseline}$), respectively, within both arms and divided into gender. In this regard, negative values correspond to situations in which somnolence decreased from baseline to the analyzed time point, whereas positive values correspond to situations in which somnolence increased from the baseline background value.

Table 107: Statistical analysis of changes in ESS questionnaire from baseline to study time points

Changes from:	Control arm		Active arm		*p*-value	Effect size
	N	Central tendency ± scatter measure	N	Central tendency ± scatter measure		
Baseline at Month 3						
All patients	12	0.11 ± 2.15 †	11	-1.00 ± 7.25 ‡	0.072 ◊	-0.37
Males	7	0.86 ± 1.77 †	8	-2.00 ± 8.00 ‡	**0.027** ◊	**-0.57**
Females	5	0.00 ± 2.55 †	3	0.67 ± 1.53 †	0.700 τ	0.16
Baseline at Month 6						
All patients	10	0.00 ± 2.00 ‡	11	-1.00 ± 5.00 ‡	0.198 ◊	-0.28
Males	7	0.28 ± 1.89 †	8	-4.00 ± 6.00 ‡	**0.021** ◊	**-0.60**
Females	3	-2.33 ± 4.93 †	3	1.33 ± 2.08 †	0.301 τ	0.51

† (Mean ± SD); ‡ (Median ± IQR); τ sig. (2-tailed) according to unpaired t-test; ◊ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%

At month 6, end of the study, participants in the active arm experienced a decrease in Epworth's sleepiness scale (median score -1.00 ± 5.00) when compared to the participants in the control arm (median score 0.00 ± 2.00), although this difference was not significant U=37.00, *Z*=-1.286, *p*=0.198, effect size *r*=-0.28. Interestingly, a very similar trend could be also seen at month 3, when comparing the changes from baseline to the follow-up visit, wherein participants in the active arm experienced a decrease in Epworth's sleepiness scale (median score -1.00 ± 7.25) when compared to the participants in the control arm (median score 0.11 ± 2.15), although this difference was not significant U=37.00, *Z*=-1.797, *p*=0.072, effect size *r*=-0.37.

However, when the same analysis was conducted based on participants' gender, males but not females revealed to lead this trend. Indeed, at month 6, end of the study visit, males in the active arm had experienced a more pronounced decrease in Epworth's sleepiness scale (median score -4.00 ± 6.00) when compared to the males in the control arm (median score 0.28 ± 1.89), which was statistically significant U=8.50, *Z*=-2.309, *p*=0.021, effect size *r*=-0.60 (**Figure 79**). Again, this trend was already observed even earlier, at month 3 visit, when comparing the changes from baseline to the follow-up visit, wherein participants in the active arm experienced a decrease in Epworth's sleepiness scale (median score -2.00 ± 8.00) when compared to the participants in the control arm (median score 0.86 ± 1.77), for which the difference was statistically significant U=9.00, *Z*=-2.213, *p*=0.027, effect size *r*=-0.57 (**Figure 78**). It is noteworthy that participant ID16 had experienced the most relevant change in ESS along the study, surprisingly in a similar trend as already observed in the cholesterol data (reference is made to **Figure 69**, biochemistry section).

On the contrary, the trend in the female subgroup seemed to behave completely differently, and no statistically differences were revealed when exploring the female participants' changes in somnolence along the study, although the sample set was really small and this trend should be further evaluated in a larger cohort.

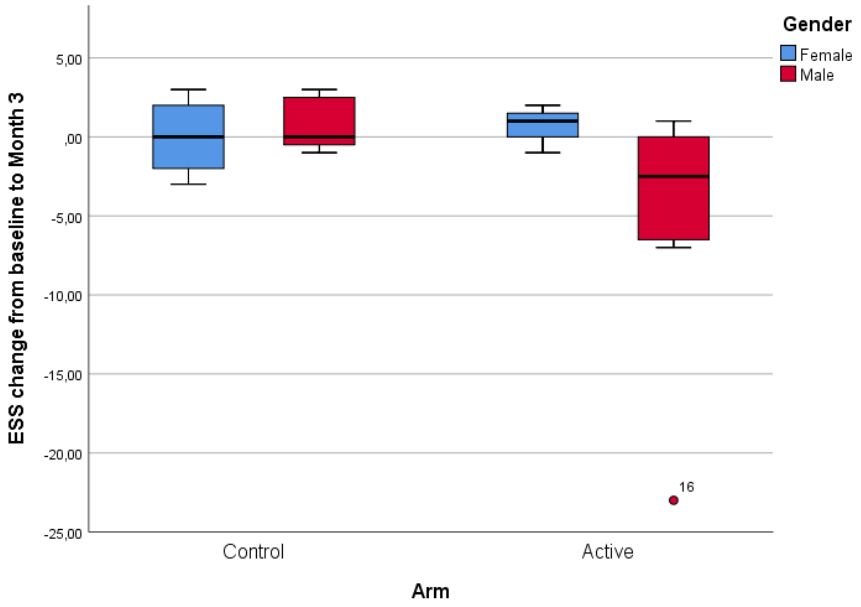


Figure 78: Comparison of the changes in ESS scores between month 3 and baseline time points in the control arm. Labels correspond to Patient ID

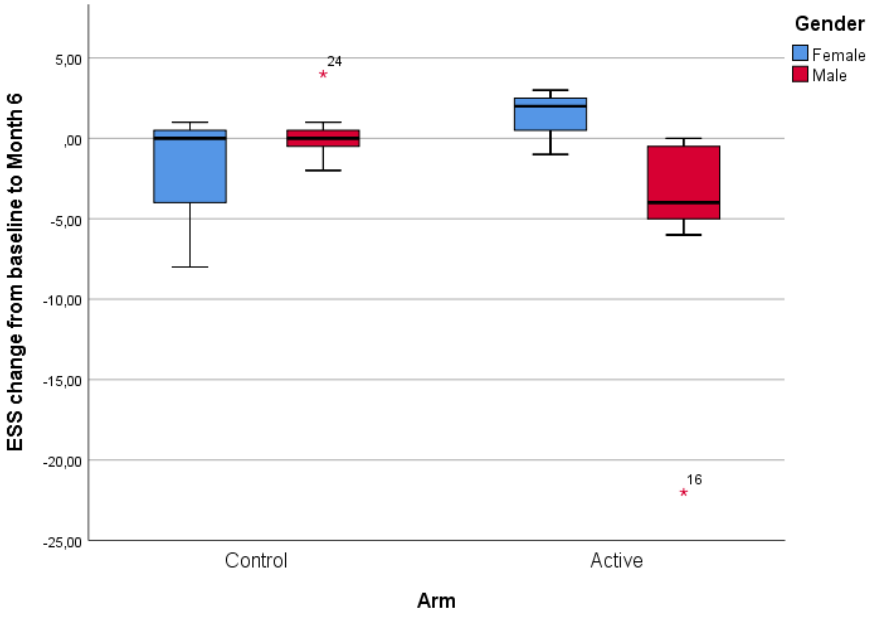


Figure 79: Comparison of the changes in ESS scores between month 6 and baseline time points in the control arm. Labels correspond to Patient ID

Interestingly, males seemed to be more sensitive to the decrease in somnolence than females, at both time points. The correlation analysis exploring the potential relationship between changes in ESS at month 3 and gender, as well as the potential relationship between changes in ESS at month 6 and gender were investigated using Spearman's correlation test (**Table 108**).

Table 108: Correlation analysis of gender on changes in ESS questionnaire along the study

Spearman's rho (all participants)	Gender effect in control arm		Gender effect in active arm	
	Correlation coefficient	p	Correlation coefficient	p
ESS changes at M3	0.199	0.534	-0.555	0.076
ESS changes at M6	0.118	0.746	-0.662	**0.041**

p: Asymp. Sig. (2 tailed) according to Spearman's test; CI 95%

Results revealed a medium/large correlation in the active arm at both changes at month 3 and changes at month 6 (r=-0.555 and r=-0.662, respectively), which was statistically significant for the changes at month 6 (p=0.041). Negative signs suggest correlation in different directions in front of gender. On the contrary, in the control arm, correlation coefficients were revealed to be small (r<0.20), non-statistically significant, and positively signed.

Regarding the findings related to gender effects, our analysis did not exclude the extraordinary sleepiness reduction observed in patient ID16 (male), as well as the fact that the sample set in the female group was probably too low for statistical purposes. Therefore, in a further step forward, we decided to explore the changes in ESS in the absence of the participant ID16 scores.

Figure 80 and **Figure 81** illustrate the data distribution in change in the ESS from baseline to month 3 and from baseline to month 6 in both arms, divided into gender, whereas **Table 109** summarizes the results from the statistical analysis when participant ID16 was fully excluded from the statistical analysis. For purposes of entire cohort comparison (when participant ID16 was still included in the analysis), reference is made to **Figure 78**, **Figure 79** and **Table 107**.

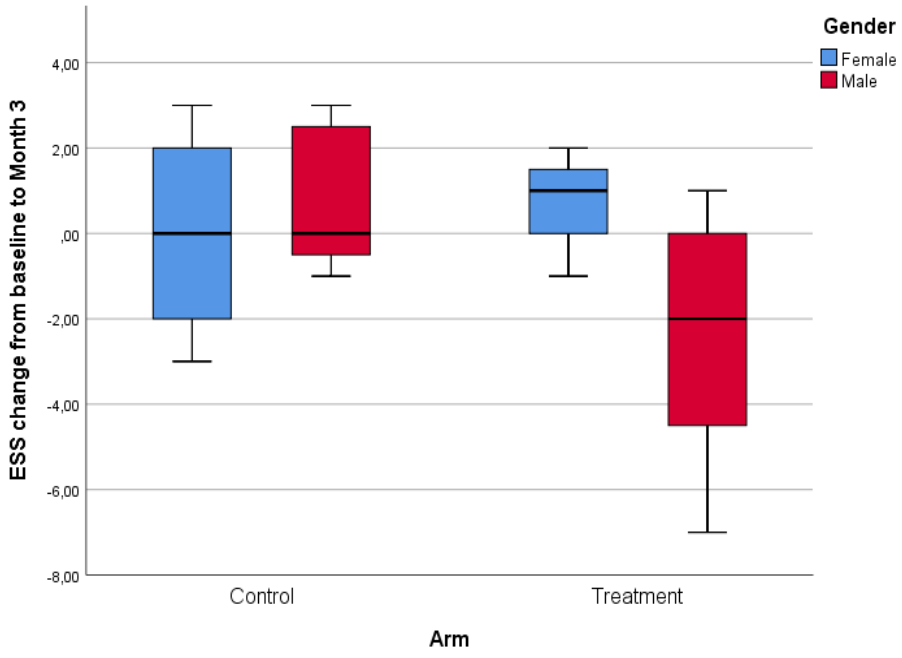


Figure 80: Comparison of the changes in ESS scores between month 3 and baseline time points in the control arm when patient ID16 was excluded from the analysis

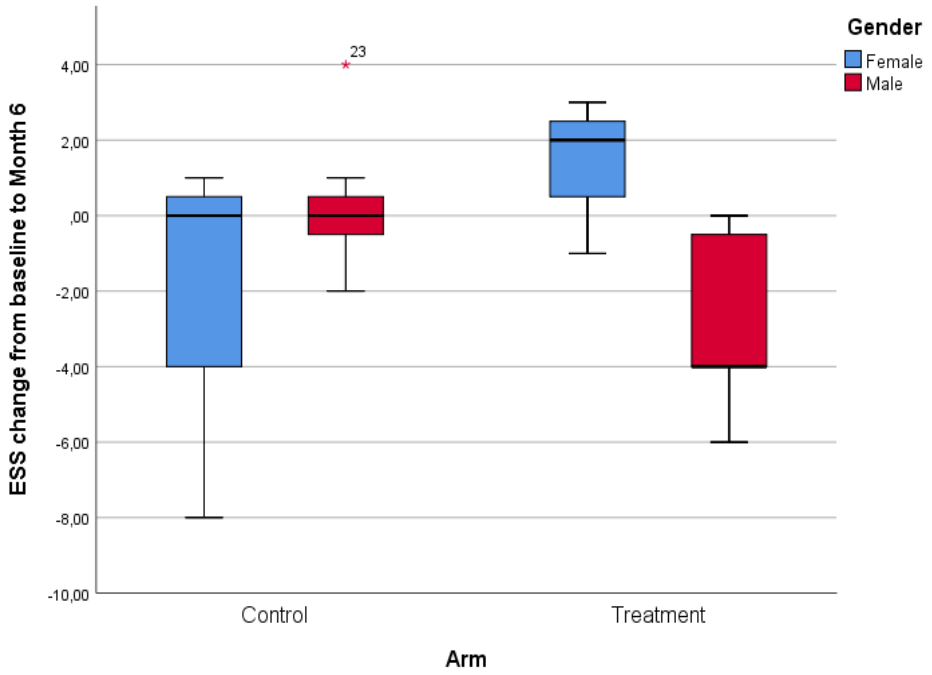


Figure 81: Comparison of the changes in ESS scores between month 6 and baseline time points in the control arm when patient ID16 was excluded from the analysis

Table 109: Statistical analysis of changes in ESS questionnaire from baseline to study time points (participant ID16 excluded)

Changes from:	Control arm		Active arm		*p*-value	Effect size
	N	Central tendency ± scatter measure	N	Central tendency ± scatter measure		
Baseline at Month 3						
All patients	12	0.50 ± 2.07 †	10	-1.50 ± 3.06 †	0.084 $^{\tau}$	0.38
Males	7	0.86 ± 1.77 †	7	-2.43 ± 3.15 †	**0.033** $^{\tau}$	**0.57**
Females	5	0.00 ± 2.55 †	3	0.67 ± 1.53 †	0.700 $^{\tau}$	0.16
Baseline at Month 6						
All patients	10	0.00 ± 2.25 ‡	10	-1.50 ± 2.92 †	0.318 $^{\lozenge}$	0.22
Males	7	0.29 ± 1.89 †	7	-2.71 ± 2.36 †	**0.022** $^{\tau}$	**0.60**
Females	3	-2.33 ± 4.93 †	3	1.33 ± 2.08 †	0.301 $^{\tau}$	0.50

† (Mean ± SD); ‡ (Median ± IQR); $^{\tau}$ sig. (2-tailed) according to unpaired t-test; $^{\lozenge}$ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%. Participant ID16 excluded.

When evaluating the changes at month 3 from baseline once participant ID16 was excluded from the statistical analysis, the independent t-test revealed that participants in the active arm experienced a decrease in Epworth's sleepiness scale (mean score -1.50 ± 3.06) when compared to the participants in the control arm (mean score 0.50 ± 2.07), although this difference was not statistically significant $t(20)=1.822$, $p=0.084$, two-tailed, effect size ($r=0.38$).

However, when gender was considered, males data but not females, revealed that when comparing participants in the active in front of participants in the control arm, changes in the ESS at month 3 from baseline experienced a statistically significant decrease in the active arm (mean score -2.43 ± 3.15) in front of the control arm (mean score 0.86 ± 1.77), $t(12)=2.402$, $p=0.033$, two-tailed, medium effect size ($r=0.57$).

When evaluating the changes at month 6 from baseline, these trends were confirmed. The results from the independent Mann-Whitman's U test revealed that participants in the active arm experienced a decrease in Epworth's sleepiness scale (mean score -1.50 ± 2.92) when compared to the participants in the control arm (mean score 0.00 ± 2.25), although this difference was not significant U=37.0, Z=-0.999, p=0.318, two-tailed, effect size (r=0.22).

Again, when gender was considered, males data but not females, revealed that when comparing participants in the active in front of participants in the control arm, changes in the ESS at month 6 from baseline experienced a statistically significant decrease in the active arm (mean score -2.71 ± 2.36) in front of the control arm (mean score 0.29 ± 1.89), t(12)=2.625, p=0.022, two-tailed, medium effect size (r=0.60).

In summary, through the descriptive evaluation of the data, we observed a statistically significant decrease in the changes in ESS from baseline to the month 6 end of study, which was already statistically significant at month 3 follow-up visit, in the male subgroup but not in the entire cohort. Providing that the ESS questionnaire is a measure of excessive daytime sleepiness, these results point out to the fact that male participants in the study decreased their daytime somnolence at statistical and practical significant levels.

Regarding the gender effect, our analysis has demonstrated that even if the extraordinary sleepiness reduction observed in patient ID16 (male) was considered as an outlier and removed from the analysis set, results and conclusions remain consistent and statistically significant.

From the data shown herein, the exploratory evaluation of excessive daytime sleepiness measured through the changes in the Epworth sleepiness scale revealed a statistically significant decrease of -4.0±5.25 score in the eight male patients allocated in the active group (**Table 107**), that turned into a decrease of -2.71±2.36 score in the seven male patients allocated in the active group if participant ID16 is potentially considered an outlier and excluded from the data set (**Table 109**) when the investigational food product based on caffeine and theobromine was daily dosaged during six month (24 weeks). In comparison, the corresponding values in ESS changes scored 0.28±1.89 and 0.29 ± 1.89, respectively, in male patients in the control group. Although the potential presence of a placebo effect could not be disregarded, the fact that both arms shared a similar and considerable daily consumption pattern of dietary methylxanthines somehow attenuated this effect.

It is worth mentioning that modafinil, the wake-promoting drug to treat narcolepsia, promoted a statistically significant reduction from 13.25 to 7.75 in ESS score (change value -5.5) in nine DM1 patients administered with 200 to 400 mg/daily during approximately 16 weeks.[480] Interestingly, in sleep-deprived healthy adults, modafinil 400 mg/day improved mood, fatigue, sleepiness and cognition to the same extent as caffeine 600 mg/day.[481] Remarkably, the investigational food product based on caffeine and theobromine evaluated herein provided just 57 mg caffeine daily plus 109 mg theobromine (mean) daily.

Due to the relevant impact of excessive daytime sleepiness in DM1, the trends in ESS scores from baseline to the study time points in both arms and gender deserve a more detailed

evaluation in larger cohorts and dosing periods to deepen in the findings in the excessive daytime sleepiness.

5.7.6. Results from additional outcome measures

5.7.6.1. Six minutes walk test scale (6MWT)

The 6MWT is a common measure used in the patient follow-up of various NMD, and it is a usual outcome measure in clinical trials testing therapeutic drugs for DM1. By measuring the distance that a patient can walk on a flat surface in a period of six minutes, the test evaluates the global and integrated responses of all the systems involved during exercise, including the pulmonary and cardiovascular systems, systemic and peripheral circulation, blood, neuromuscular units, and muscle metabolism.[482] The walking distance measured in the study cohort at each time point is disclosed in **Table 110**.

Table 110: Results from 6MWT scale. Comparison from baseline visit to the end of study visit

				Patient_ID	6MWT (m)		
					Baseline	Month 3	Month 6
Arm	**Control**	1		01	280.0	.	.
		2		03	160.0	282.0	.
		3		04	401.0	460.0	481.0
		4		06	307.0	325.0	331.0
		5		07	259.0	75.0	.
		6		09	323.0	270.0	309.0
		7		10	387.0	386.0	360.0
		8		11	455.0	484.0	465.0
		9		12	455.0	450.0	450.0
		10		13	314.0	.	.
		11		14	.	.	.
		12		15	391.0	.	.
		13		20	368.0	415.0	375.0
		14		21	472.0	538.0	495.0
		15		24	347.0	360.0	345.0
		Total	N	15	14	11	9
			Mean	-	**351.4**	**367.7**	**401.2**
			SD	-	**85.9**	**128.4**	**71.2**
	Active	1		02	120.0	.	.
		2		05	268.0	.	.
		3		08	358.0	363.0	370.0
		4		16	375.0	391.0	400.0
		5		17	.	.	.
		6		18	329.0	345.0	368.0
		7		19	265.0	292.0	235.0
		8		22	427.0	463.0	.
		9		23	210.0	90.0	255.0
		10		25	400.0	420.0	450.0
		11		26	.	.	.
		12		27	331.2	458.0	480.0
		13		28	.	.	.
		14		29	316.0	323.0	352.0
		15		30	268.0	299.0	272.00
		Total	N	15	12	10	9
			Mean	-	**305.6**	**344.4**	**353.6**
			SD	-	**85.6**	**108.1**	**85.4**

First of all, the statistical comparison in 6MWT results between both arms at baseline was performed using unpaired t test. Under both conditions, the distribution of the distance walked during six minutes in the active arm (351.4 ± 85.9 m) did not significantly differ from the distance walked during six minutes in the control arm (305.6 ± 85.6 m) at the inclusion visit (p>0.05, ns), effect size r=0.27 in the entire cohort. When the arms were divided into gender, no statistically differences were obtained in the control arm *versus* the active arm, although it is worth to mention that female from the control arm scored 338.5 ± 106.2 m whereas female from the active arm scored 279.2 ± 110.5 m (effect size, r=0.29). A similar situation was observed in the males subgroup, for which the control arm scored 361.0 ± 73.6 m whereas male from the active arm scored 324.5 ± 65.5 m (effect size, r=0.27). Therefore, formally there were no statistically differences in the mean distance that patients from both arms were able to walk for a period of six minutes at the inclusion visit.

Figure 82 graphically depicts the comparisons in 6MWT scores per Patient ID according to arms at the end of the study compared from the baseline situation. Results above the y=x equation correspond to Patient ID whose scores at month 6 had decreased from the corresponding score at baseline, *i.e.,* those for whom mobility had decreased along the study. Results lying on the y=x equation correspond to Patient ID whose scores did not change along the study. Finally, results lying below the y=x equation correspond to Patient ID whose scores had increased from the baseline situation, *i.e.,* those for whom mobility had increased along the study.

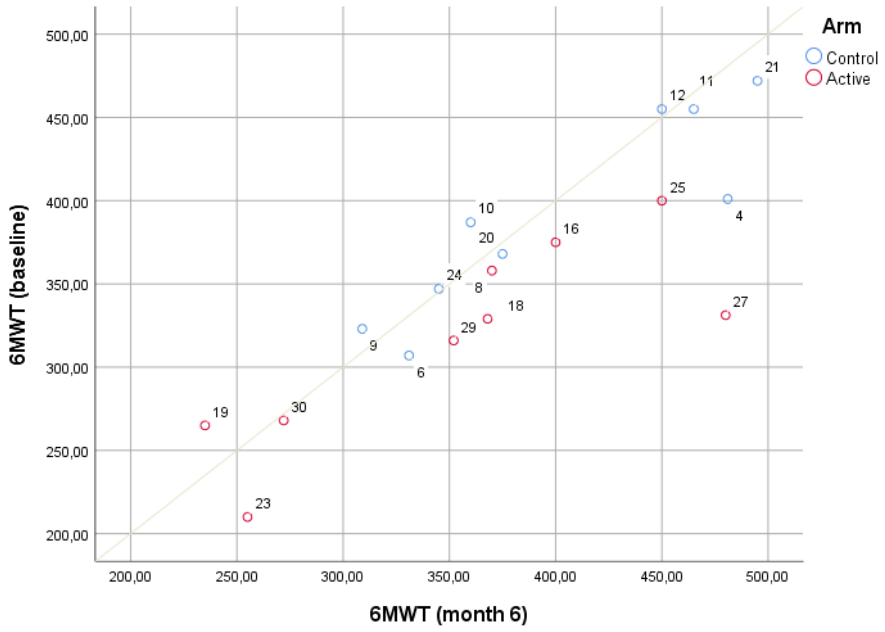


Figure 82: Comparison of the 6MWT distances (expressed in meters) at baseline and month 6 time points in the both arms. Labels correspond to Patient ID

The exploratory analysis on the variation in 6MWT scale from the data collected along the study is provided in **Table 111**, showing the normalized data on the changes in 6MWT scale values between the month 3 follow-up visit and baseline visit ($6MWT_{M3}$-$6MWT_{baseline}$), and between the month 6 end of study visit and the baseline visit ($6MWT_{M6}$-$6MWT_{baseline}$), respectively, within both arms.

Table 111: Results from 6MWT. Walking distance changes from baseline situation (values expressed in meters)

				Patient_ID	6MWT change from baseline at Month 3 visit	6MWT change from baseline at Month 6 visit
Arm	**Control**	1		01	.	.
		2		03	122.0	.
		3		04	59.0	80.0
		4		06	18.0	24.0
		5		07	-184.0	.
		6		09	-53.0	-14.0
		7		10	-1.0	-27.0
		8		11	29.0	10.0
		9		12	-5.0	-5.0
		10		13	.	.
		11		14	.	.
		12		15	.	.
		13		20	47.0	7.0
		14		21	66.0	23.0
		15		24	13.0	-2.0
		Total	N	15	11	9
			Mean	-	**10.1**	**10.7**
			SD	-	**78.6**	**30.8**
	Active	1		02	.	.
		2		05	.	.
		3		08	5.0	12.0
		4		16	16.0	25.0
		5		17	.	.
		6		18	16.0	39.0
		7		19	27.0	-30.0
		8		22	36.0	.
		9		23	-120.0	45.0
		10		25	20.0	50.0
		11		26	.	.
		12		27	126.8	148.8
		13		28	.	.
		14		29	7.0	36.0
		15		30	31.0	4.0
		Total	N	15	10	9
			Mean	-	16.5	**36.6**
			SD	-	59.4	**48.8**
			Median	-	**18.0**	36.0
			IQR	-	**64.0**	39.5
	Total	N		30	21	18
		Mean		-	**13.1**	**23.7**
		SD		-	**68.5**	**41.8**

Figure 83 and **Figure 84** graphically illustrate the differences further disclosed in **Table 112**.

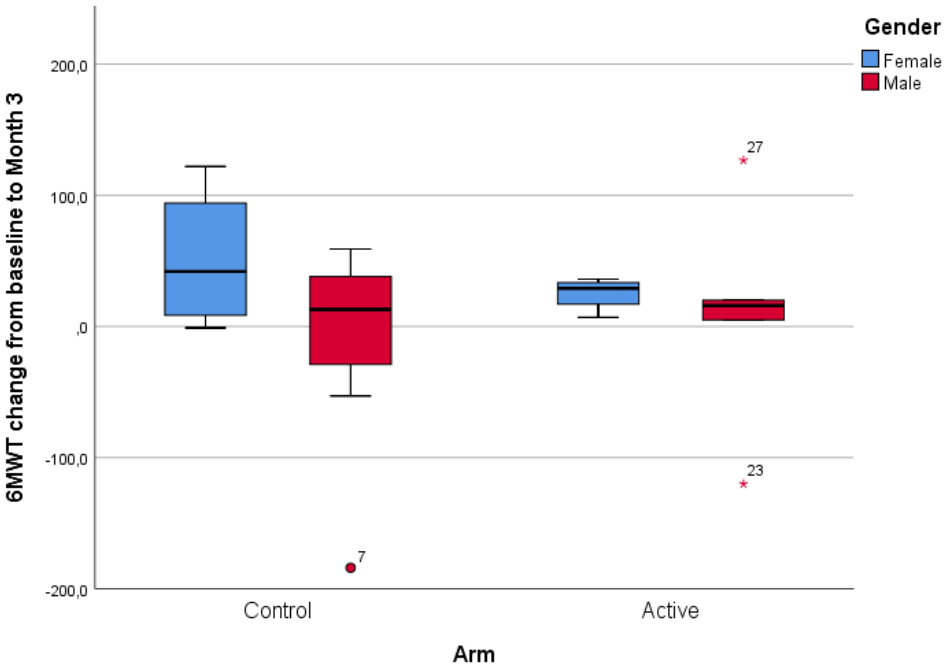


Figure 83: Comparison of the changes in 6MWT scores between month 3 and baseline time points in the control arm. Labels correspond to Patient ID

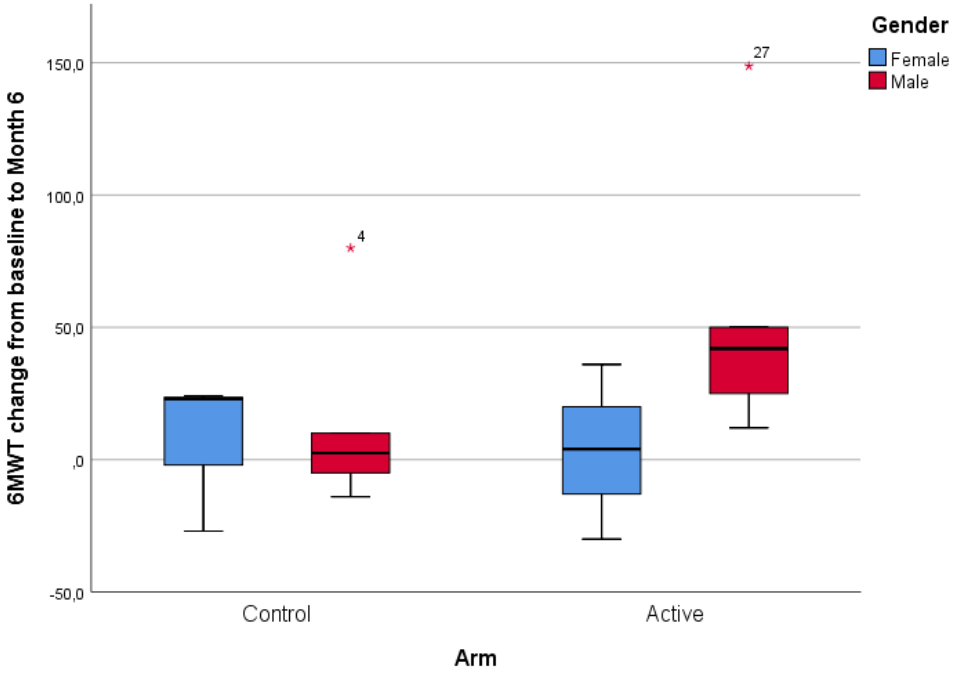


Figure 84: Comparison of the changes in 6MWT scores between month 6 and baseline time points in the control arm. Labels correspond to Patient ID

Table 112 discloses the comparison on the changes in 6MWT scale values between the month 3 follow-up visit and baseline visit ($6MWT_{M3}$-$6MWT_{baseline}$), and between the month 6 end of study visit and the baseline visit ($6MWT_{M6}$-$6MWT_{baseline}$), respectively, within both arms, and additionally divided into gender. In this regard, negative values correspond to situations in which the distance walked from baseline to the analyzed time point decreased, whereas positive values correspond to situations in which walking distance increased from the baseline background value.

Table 112: Statistical analysis of changes in 6MWT measure from baseline to study time points

Changes from:	Control arm		Active arm		*p*-value	Effect size
	N	Central tendency ± scatter measure	N	Central tendency ± scatter measure		
Baseline at Month 3						
All patients	11	10.1±78.6 †	10	18.0±64.0 ‡	0.944 ◊	-0.01
Males	7	-13.4±83.8 †	6	10.6±78.4 †	0.606 τ	0.16
Females	4	51.3±54.9 †	4	25.3±12.7 †	0.392 τ	0.35
Baseline at Month 6						
All patients	9	10.7±30.8 †	9	36.6±48.8 †	0.196 τ	0.32
Males	6	2.5±34.8 ‡	6	42.0±52.9 ‡	**0.037** ◊	**-0.60**
Females	3	23.0±0.0 ‡	3	3.3±33.0 †	0.827 ◊	-0.09

† (Mean ± SD); ‡ (Median ± IQR); τ sig. (2-tailed) according to unpaired t-test; ◊ Asymp. Sig. (2 tailed) according to Mann-Whitney's U test; CI 95%.

As extracted from **Table 112**, and revealed by the results of the unpaired t test, at the end of the study, the changes in the mean distance walked by the participants in the active arm as measured by the 6MWT experienced an increase (36.6±48.8 m) when compared to the changes in the mean distance walked by the participants in the control arm (10.7±30.8 m), which although was not statistically significant t(16)=-1.350, $p>0.05$, it represented a medium-sized effect $r=0.32$.

Interestingly, males seemed to be more sensitive to this effect than females, at both time points. Particularly, the changes in the median distance walked as measured by the 6MWT scale by males in the active arm at the end of the study period improved up to 42.0±52.9 m in comparison to the changes in males from the control arm, whose median changes scored 2.5±34.8 m after the month 6 time point. As revealed by the Mann-Whitman's U test, these differences were statistically significant $U=5.0$, $Z=-2.082$, $p=0.037$, two-tailed, and exhibited a medium-to-large effect size ($r=-0.60$). It is worth mentioning that the analysis at month 3 follow-up visit in males changes in 6MWT scored for just 10.6±78.4 m with an effect size $r=0.16$. When the comparison was done between changes in 6MWT from baseline to month 3 and changes in 6MWT from baseline to month 6, the Wilcoxon signed rank test revealed a statistically significant different in males in the active arm ($Z=-2.201$, $p=0.028$) which exhibited a large effect ($r=0.90$). In the six active male cases, the changes in 6MWT from baseline to month 6 ranked positively, *i.e.* were superior to the changes in 6MWT from baseline to month 3, suggesting some accumulative effect along the study period. When the same analysis was

performed on males in the control arm, no statistical differences nor relevant effect size were observed. Interestingly, even excluding participants under metformin treatment (ID23 and ID30) from the analysis, the statistically significant differences in males between arms at the end of the study persisted, in medium to large effect.

As in the previous analysis, again females behave completely differently than males. In this regard, the females in the active arm experienced more moderate and non-statistically significant differences than males: the increase in the changes in mean distance walked as measured by the 6MWT scale by females at the end of the study period reached 3.3±33.0 m with an effect size *r*=-0.09, whereas in the same group the increase at month 3 follow-up visit scored for 25.3±12.7 m with an effect size *r*=0.35.

Last but not least, we intended to explore the changes in the 6MWT scale beyond the potential gender effects. For this purpose, the muscular impairment rating scale (MIRS) was considered as a valuable ordinal variable to evaluate. **Figure 85** and **Figure 86** illustrate the comparison.

At month 3 follow-up visit (**Figure 85**), there was an evident worsening in the walking distance measured in the control arm for the patients impaired with severe proximal weakness, with no remarkable differences between the MIRS' subgroups in both arms.

However, the situation clearly evolved at month 6 end of study visit. As depicted in **Figure 86**, especially patients impaired with mild to moderate proximal weakness but even patients impaired with severe proximal weakness demonstrated a remarkable increase in their capacity to walk more distance than at their respective baseline situation. A similar difference was certainly not measured in the control arm at any MIRS' subgroup, although the sample was not enough for statistical tests measurements.

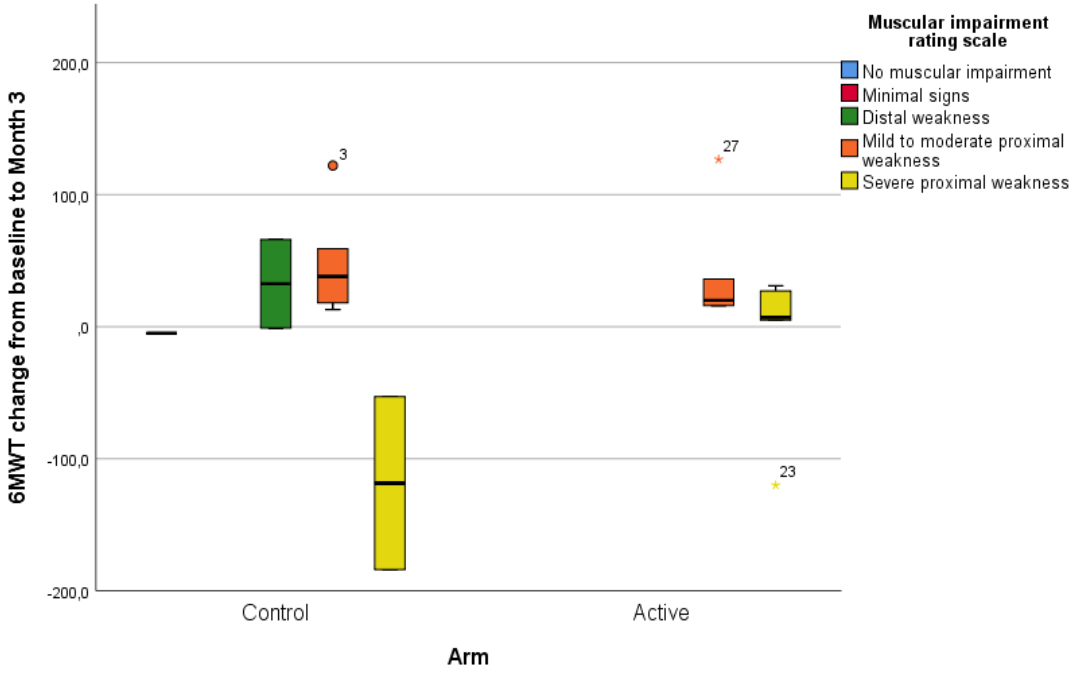


Figure 85: Comparison of the changes in 6MWT scores between month 3 and baseline time points in the control arm and active arm according to MIRS values

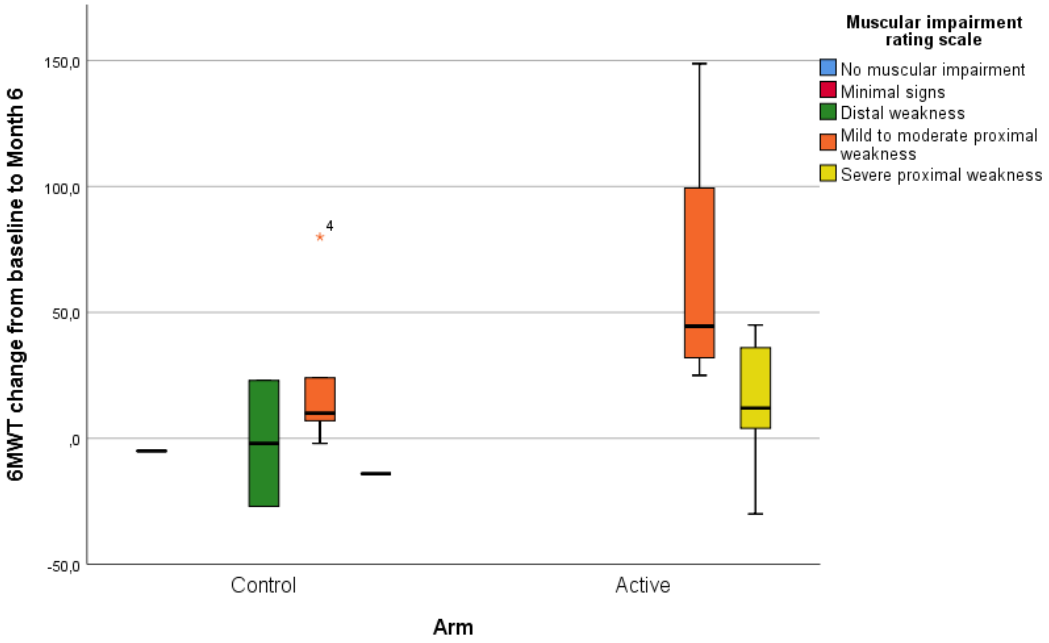


Figure 86: Comparison of the changes in 6MWT scores between month 6 and baseline time points in the control arm and active arm according to MIRS values

In summary, the main finding in the changes in the 6MWT measurement performed through the descriptive analysis of the data revealed a statistically significant (p=0.037) increase in the walking distance of in the six male patients allocated in the active group (42.0±52.9 m) when the investigational food product based on caffeine and theobromine MYODM was daily dosaged (57 mg caffeine daily plus 109 mg theobromine daily) during six month (24 weeks), in comparison to the walking distance gained by the six male patients in the control group (2.5±34.8 m). The difference reached a medium-to-large size effect (r=0.60)

It is worth mentioning that metformin, the anti-diabetic drug under repurposing for the improvement of mobility in DM1 patients, promoted a statistically significant gaining distance of 32.9±32.7 m in 6MWT in the nine patients allocated in the active group when administered at 3 g/day dosaging during 52 weeks, in comparison to the 3.7±32.4 m gained by the fourteen patients in the placebo group.[483]

Further studies in larger cohorts, blinded designs and dosaging periods should be conducted to deepen in the findings disclosed herein in the 6-minute walk test.

5.7.7. Analysis of correlations among study variables

Last but not least, a Spearman Rank Order Correlation analysis was performed in order to evaluate the strength and direction of the relationships among a selected set of baseline study variables, including arm, gender, age, number of CTG repeats, body mass index, the muscular impairment rating scale and dietary caffeine intake, in front of the evaluated changes in total cholesterol, triglyceride levels, the Epworth sleepiness scale scores and 6-minute walk test distance. The analysis was performed in the entire cohort data set (**Table 113**), as well as for males (**Table 114**) and females (**Table 115**) subgroups. The strength of the relationship was interpreted according to Cohen's guidelines[469] (small correlation: $0.29 \geq r > 0.10$; medium correlation: $0.49 \geq r > 0.30$; large correlation: $1.0 \geq r > 0.50$; wherein r ranges from -1.0 to 1.0, the sign revealing positive or negative correlation, and 0 states for no correlation at all).

The entire cohort (**Table 113**) revealed a medium-to-large negative correlation between study arm and changes in total cholesterol levels from baseline to the end of the study (r=-0.494, n=20, *p*=0.027, 24% shared variance) and between study arm and changes in triglyceride levels from baseline to the end of the study (r=-0.503, n=20, *p*=0.024, 25% shared variance). Gender revealed a medium-to-large negative correlation in the changes in triglyceride levels along the study (r=-0.451, n=20, *p*=0.046, 20% shared variance), wherein changes in triglyceride levels along the study revealed a large negative correlation between the changes in the distance walked as per the 6MWT from baseline to the end of the study (r=-0.643, n=15, *p*=0.010, 41% shared variance) which was statistically significant at the 99% confidence interval.

When gender was defined, the analysis of the males subgroup in the study cohort (**Table 114**) revealed large negative correlations between study arm and changes in total cholesterol levels from baseline to the end of the study (r=-0.604, n=15, *p*=0.017, 36% shared variance), between study arm and changes in triglyceride levels from baseline to the end of the study (r=-0.650, n=15, *p*=0.009, 42% shared variance), between study arm and changes in ESS scores from baseline to the end of the study (r=-0.617, n=15, *p*=0.014, 38% shared variance) and positive correlation between study arm and changes in 6MWT from baseline to the end of the study (r=0.628, n=12, *p*=0.029, 39% shared variance). All these correlations were statistically significant at 95% confidence interval, but correlation between arm and changes in triglyceride levels along the study in the males subgroup was statistically significant at 99% confidence interval. No further correlations were obtained in the males subgroup.

No statistically significant relationship was obtained when the same correlation analysis was performed in the females subgroup (**Table 115**).

Table 113: Analysis of correlations among selected study variables in the entire cohort (All participants)

Spearman's rho		Baseline data						Change from baseline to Month 6			
Entire cohort		**Gender**	**Age**	**CTG_n**	**BMI**	**MIRS**	**Dietary caff.**	**TCH**	**TG**	**ESS**	**6MWT**
Arm	rho	0.069	-0.108	0.120	-0.073	0.276	0.005	**-0.494***	**-0.503***	-0.288	0.396
	p	0.716	0.570	0.535	0.701	0.140	0.982	**0.027***	**0.024***	0.206	0.104
	N	30	30	29	30	30	28	20	20	21	18
Gender	rho	-	-0.004	-0.087	-0.228	0.195	-0.118	0.250	**-0.451***	-0.265	0.318
	p	-	0.983	0.653	0.226	0.302	0.549	0.287	**0.046***	0.246	0.198
	N	-	30	29	30	30	28	20	20	21	18
Age	rho	-	-	**-0.399***	0.262	-0.039	0.086	-0.002	0.320	-0.011	-0.370
	p	-	-	**0.032***	0.161	0.838	0.663	0.992	0.170	0.964	0.131
	N	-	-	29	30	30	28	20	20	21	18
CTG_n	rho	-	-	-	-0.104	**0.451***	0.050	0.106	-0.145	-0.085	-0.041
	p	-	-	-	0.591	**0.014***	0.805	0.666	0.553	0.723	0.877
	N	-	-	-	29	29	27	19	19	20	17
BMI	rho	-	-	-	-	-0.072	0.192	-0.220	0.105	-0.210	-0.245
	p	-	-	-	-	0.707	0.327	0.352	0.659	0.362	0.328
	N	-	-	-	-	30	28	20	20	21	18
MIRS	rho	-	-	-	-	-	-0.091	0.204	-0.057	0.119	0.005
	p	-	-	-	-	-	0.645	0.389	0.810	0.609	0.986
	N	-	-	-	-	-	28	20	20	21	18
Dietary caffeine baseline	rho	-	-	-	-	-	-	-0.426	0.179	-0.032	0.002
	p	-	-	-	-	-	-	0.069	0.462	0.891	0.993
	N	-	-	-	-	-	-	19	19	21	18
TCH change from baseline to M6	rho	-	-	-	-	-	-	-	0.031	0.273	-0.075
	p	-	-	-	-	-	-	-	0.897	0.289	0.791
	N	-	-	-	-	-	-	-	20	17	15
TG change from baseline to M6	rho	-	-	-	-	-	-	-	-	0.389	**-0.643****
	p	-	-	-	-	-	-	-	-	0.123	**0.010****
	N	-	-	-	-	-	-	-	-	17	**15**
ESS change from baseline to M6	rho	-	-	-	-	-	-	-	-	-	-0.353
	p	-	-	-	-	-	-	-	-	-	0.165
	N	-	-	-	-	-	-	-	-	-	17

Notes:

*. Correlation is significant at the 0.05 level (2-tailed). 95% CI

**. Correlation is significant at the 0.01 level (2-tailed). 99% CI

Table 114: Analysis of correlation among selected study variables in the entire cohort (Males group)

Spearman's rho		Baseline data					Change from baseline to Month 6			
Males		**Age**	**CTG_n**	**BMI**	**MIRS**	**Dietary caff.**	**TCH**	**TG**	**ESS**	**6MWT**
Arm	rho	-0.232	-0.116	-0.058	-0.021	0.033	**-0.604***	**-0.650****	**-0.617***	**0.628***
	p	0.340	0.636	0.814	0.931	0.896	**0.017***	**0.009****	**0.014***	**0.029***
	N	19	19	19	19	18	15	15	15	12
Age	rho	-	-0.445	0.357	0.007	0.173	0.140	0.365	0.003	-0.193
	p	-	0.056	0.133	0.978	0.493	0.619	0.181	0.992	0.548
	N	-	19	19	19	18	15	15	15	12
CTG_n	rho	-	-	-0.386	0.357	-0.100	0.122	-0.196	-0.210	-0.084
	p	-	-	0.103	0.134	0.694	0.665	0.485	0.452	0.794
	N	-	-	19	19	18	15	15	15	12
BMI	rho	-	-	-	-0.072	0.169	-0.161	0.025	-0.084	-0.245
	p	-	-	-	0.771	0.502	0.567	0.930	0.766	0.443
	N	-	-	-	19	18	15	15	15	12
MIRS	rho	-	-	-	-	-0.272	0.248	-0.080	-0.022	0.008
	p	-	-	-	-	0.275	0.373	0.777	0.938	0.979
	N	-	-	-	-	18	15	15	15	12
Dietary caffeine baseline	rho	-	-	-	-	-	-0.466	0.163	0.091	0.129
	p	-	-	-	-	-	0.093	0.578	0.748	0.690
	N	-	-	-	-	-	14	14	15	12
TCH change from baseline to M6	rho	-	-	-	-	-	-	0.281	0.315	-0.406
	p	-	-	-	-	-	-	0.311	0.319	0.244
	N	-	-	-	-	-	-	15	12	10
TG change from baseline to M6	rho	-	-	-	-	-	-	-	0.507	-0.600
	p	-	-	-	-	-	-	-	0.092	0.067
	N	-	-	-	-	-	-	-	12	10
ESS change from baseline to M6	rho	-	-	-	-	-	-	-	-	-0.442
	p	-	-	-	-	-	-	-	-	0.173
	N	-	-	-	-	-	-	-	-	11

Notes:

*. Correlation is significant at the 0.05 level (2-tailed). 95% CI

**. Correlation is significant at the 0.01 level (2-tailed). 99% CI

Table 115: Analysis of correlation among selected study variables in the entire cohort (Females group)

Spearman's rho		Baseline data					Change from baseline to Month 6			
Females		**Age**	**CTG_n**	**BMI**	**MIRS**	**Dietary caff.**	**TCH**	**TG**	**ESS**	**6MWT**
Arm	rho	0.058	0.642*	-0.115	0.736**	-0.035	-0.289	0.000	0.488	-0.098
	p	0.866	0.046*	0.735	0.010**	0.923	0.638	1.000	0.326	0.854
	N	11	10	11	11	10	5	5	6	6
Age	rho	-	-0.293	-0.032	-0.080	-0.067	-0.800	0.700	0.200	-0.543
	p	-	0.412	0.926	0.815	0.853	0.104	0.188	0.704	0.266
	N	-	10	11	11	10	5	5	6	6
CTG_n	rho	-	-	0.419	0.751*	0.378	0.000	-0.400	0.300	-0.100
	p	-	-	0.228	0.012*	0.316	1.000	0.600	0.624	0.873
	N	-	-	10	10	9	4	4	5	5
BMI	rho	-	-	-	-0.097	0.202	-0.300	0.200	-0.714	0.143
	p	-	-	-	0.778	0.575	0.624	0.747	0.111	0.787
	N	-	-	-	11	10	5	5	6	6
MIRS	rho	-	-	-	-	0.277	0.000	0.000	0.494	0.031
	p	-	-	-	-	0.439	1.000	1.000	0.320	0.954
	N	-	-	-	-	10	5	5	6	6
Dietary caffeine baseline	rho	-	-	-	-	-	-0.100	-0.600	-0.771	0.143
	p	-	-	-	-	-	0.873	0.285	0.072	0.787
	N	-	-	-	-	-	5	5	6	6
TCH change from baseline to M6	rho	-	-	-	-	-	-	-0.500	0.300	0.700
	p	-	-	-	-	-	-	0.391	0.624	0.188
	N	-	-	-	-	-	-	5	5	5
TG change from baseline to M6	rho	-	-	-	-	-	-	-	0.300	-0.800
	p	-	-	-	-	-	-	-	0.624	0.104
	N	-	-	-	-	-	-	-	5	5
ESS change from baseline to M6	rho	-	-	-	-	-	-	-	-	-0.314
	p	-	-	-	-	-	-	-	-	0.544
	N	-	-	-	-	-	-	-	-	6

Notes:

* Correlation is significant at the 0.05 level (2-tailed). 95% CI

** Correlation is significant at the 0.01 level (2-tailed). 99% CI

In summary, this independent correlation analysis confirmed the trends in gender effects already obtained along the present study, suggesting that at least in this cohort, the intake of the investigational food product could especially benefit males in terms of certain reduction in total cholesterol levels, triglyceride levels and excessive daytime sleepiness, as well as to an increase of walking abilities and mobility. Further studies in larger cohorts, dosing periods and study design should be conducted to confirm the results obtained in the present pilot clinical trial.

5.8. Concluding remarks

The main hypothesis guiding the present pilot clinical trial is that the administration of the investigational food product containing caffeine and theobromine developed herein could be useful in the nutritional management of DM1, by at least contributing to an improvement in the quality of life, as measured by either an improvement in the self-reported Health-Related Quality of Life and/or through the reduction of drowsiness and excessive daytime sleepiness.

Accordingly, the 'Effect of the MYODM food supplement on quality of life, fatigue and hypersomnia in patients with Myotonic Dystrophy type 1' (NCT04634682) was conceived as a monocentric six month randomized pilot interventional clinical trial to test these hypothesis. The patients were randomly assigned to one of the two study arms: the active arm receiving the investigational food product containing caffeine and theobromine developed herein, and the control arm as a parallel group to follow the same evaluation program. Primary outcome measures were based on changes in SF-36 HRQoL and ESS questionnaires' scores from baseline visit to the end of study visit. Concomitant patient outcome measures included biochemistry profile and mobility evaluation through 6MWT scales, among other additional outcome measures.

A total of 30 subjects aged from 28 to 72 years old (mean age, 46.3±9.9) participated in this study. Female represented 37% whereas male represented 63%. Mean CTG repeats in the study cohort was 602±73, whereas muscular impairment rating scale (MIRS) was 3.0±1.1, corresponding to mild to moderate proximal weakness. All patients had a previous diagnosis of DM1 and fulfilled inclusion and exclusion criteria. All patients in both arms had a very complex history of comorbidities and comedications.

It is worth mentioning that the investigational food product was well tolerated by the great majority of patients. Adverse events that could be related with the investigational food product were mainly absent in the majority of participants along the study, with the single exceptions of participant ID02 (who reported somnolence and was dropped out before the month 3 visit) and participant ID17 (who reported certain anxiety at month 6 visit, although completed the study).

By the time this experimental research was performed, the entire globe was immersed in the COVID-19 pandemic. This very complex situation seriously affected the normal evolution of the pilot clinical trial, wherein some of the study participants were infected by SARS-CoV-2 and dropped out, part of the investigator's team at the Hospital were infected as well, the hospital suffered of a very high healthcare demand in other medical areas, non infected patients were really afraid to attend the Hospital for their follow-up visits and for this reason, some of them dropped-out, as well. Overall, six participants (20% of the entire study cohort) did not properly fulfil or fully continue the study after the inclusion visit. In the control arm, three out fifteen (20%) refused to continue. In the active arm, three out fifteen (20%) abandoned the study, as well.

The negative impact of COVID-19 pandemic on the quality of life in neuromuscular patients has been extensively discussed in literature. Recent post-pandemic studies have demonstrated the negative impact of COVID-19 pandemic on physical and emotional health and quality of life of

patients with neuromuscular diseases. Accordingly, the measurement of the primary outcome measure 'changes in HRQoL according to SF-36 questionnaire' must be interpreted in the light of these extraordinarily challenging circumstances, especially for such fragile and complex patients. In this regard, changes in SF-36 scales were deemed to be non-statistically different when comparing self-reported participants' perception from baseline to the end of study visit in the control arm and in the active arm. Ideally, HRQoL outcome should be measured beyond the post-pandemic era.

Regarding the primary outcome measure 'changes in daytime sleepiness according to changes in ESS scores', participants in the active arm experienced a decrease in Epworth's sleepiness scale when compared to the participants in the control arm. Although this difference was not statistically significant in the entire cohort, when considering participant's gender, males in the active arm had experienced a more pronounced decrease in Epworth's sleepiness scale (median score -4.00 ± 5.25) than males in the control arm (median score 0.28 ± 1.89), which was statistically significant with a medium-to-large effect size. Even under the consideration of Patient ID16 as a potential outlier, males but not females, revealed that changes in the ESS from baseline to month 6 had experienced a statistically significant decrease in the active arm (mean score -2.71 ± 2.36) in front of the control arm (mean score 0.29 ± 1.89) with a medium-to-large effect size. Interestingly, this effect was not observed for females in the active arm, although this subset was probably too tiny for statistical purposes.

Regarding the concomitant motor function evaluation, the 6MWT scale revealed that, at the end of the study, changes in the mean distance walked by male in the active arm had experienced an statistically significant increase from the baseline visit (median increase 42.0±52.9 m) in front of the males in the control arm (median increase 2.5±34.8 m), whereas the same trend was not observed in females. Further studies in larger cohorts should be conducted to reveal unambiguous gender trends.

Last but not least, even when only participants without any pharmacological active treatment to manage antihypercholesterolemia and antihypertriglyceridemia comorbidities were considered, some trends in the lower total cholesterol and triglyceride levels in the active arm, in front of the control arm, especially in males, deserve further future evaluation.

As previously disclosed in the Introduction section herein, gender is a controversial source of variation in caffeine pharmacokinetics. In this regard, whereas some studies disclose that gender has no effect on caffeine pharmacokinetics in men and women,[187] some other studies conclude that males and females differ in their responses to caffeine, being males more prone to respond to caffeine at lower doses than females.[188]

From the data shown herein, the overview of the Epworth sleepiness and 6MWT scales' results suggest that the controlled and properly dosaged over time administration of caffeine and theobromine in the formulated investigational food product developed herein, could provide significant improvements in diminishing excessive daytime sleepiness and increasing walked distance, at least in adult DM1 male.

Even though the present study suffers from certain limitations, mainly the small number of participants and lack of placebo double-blinded design, it also demonstrated relevant

strengths, such as the complete tolerability of the investigational food product in front of a complex history of comorbidities and comedications, and the practical absence of adverse events.

The results achieved in the Epworth sleepiness scale primary outcome measure and the motor function through the 6MWT concomitant outcome measure provide preliminary evidences that encourage further clinical trials in larger cohorts and dosaging periods in blinded designs to improve our understanding of the investigational food product effects and gender differences, if any, ideally to be performed beyond the era of COVID-19 pandemic.

Future analysis will be performed on the data collected from the questionnaires that have not been disclosed herein, which will be reported somewhere else in due time.

CONCLUSIONS

Nutritional aspects in DM1

1. There are no official nutritional guidelines for people living with DM1. Although scarce, scientific literature suggests that there is a nutritional inadequacy in the DM1 community, wherein dietary imbalances, reduced physical activity and socioeconomic factors lead to overweight, obesity and an increased risk of developing non-communicable diseases.
2. The direct introduction of methylxanthines from food and beverages in the nutritional management of DM1: i) is limited by the natural source content, ii) could further promote nutritional imbalances, overfeeding and serious concerns, and iii) must be limited in the context of a healthy diet and nutritional adequacy in DM1.

Regulatory framework

3. Even formulated in dosage form to be taken in small measured unit quantities, food supplements and food for special medical purposes are considered foods according to the current European regulation in force. The investigational methylxanthine-based food product developed herein could be launched as a food supplement, but must comply with the food for special medical purposes' regulation when claimed for the nutritional management of DM1. Accordingly, this was the strategy and the roadmap defined in the present development project.

Product development

4. The binary composition containing caffeine and theobromine in 1:2 molar ratio, equivalent to 1:1.85 weight/weight ratio, showed the highest and most synergistic effect in the screening cascade comprising *in vitro* and *in vivo* assays in *Drosophila melanogaster* DM1 models. Said selected composition: i) increased MBNL1 and MBNL2 protein levels *in vitro*, ii) rescued the functional climbing and flying capacity in the DM1 model, iii) improved the muscular area quantification, iv) rescued almost all the parameters measuring heart dysfunction, v) improved survival and life expectancy of the dystrophic model flies, when compared to the control flies, and vi) complied with current regulatory constraints in force. Accordingly, it was selected as the final composition in the methylxanthine-based formulated product developed herein.

5. In order to avoid the potential patent infringement of third parties' intellectual property rights on food products containing methylxanthines, the form of unit dosage form (tablets, capsules or sachets) was finally chosen.
6. Particularly, the capsule was selected as the unit dosage form of the product, wherein the administration schedule for human dosaging consisted of three capsules per day containing the formulated product, equivalent to 60 mg caffeine and 111 mg theobromine per day.

Evaluation of the relationship between dietary methylxanthines and HRQoL in DM1: Observational clinical study

7. The clinical study 'Observational study on the quality of life and eating habits in participants with Myotonic Dystrophy type 1 (DM1)', MD Project, code CEI 17/50, revealed that participant's intake of the food groups rice, pasta, bread, vegetables, fruits, legumes, olive oil and nuts did not comply with the frequency of consumption recommended by ASEM/FEEN in people affected by neuromuscular diseases. In the case of the food groups milk and dairy products, fish and lean meats and eggs, the frequencies of consumption were adequately met and even exceeded the aforementioned guidelines, confirming that protein intake was considered the most relevant asset in their nutritional habits.
8. Methylxanthine consumption habits in the study cohort were mainly caffeine (at least one coffee serving per day on a regular basis, equivalent to approximately 82.0 mg median caffeine/day), while theobromine consumption was almost negligible (less than 1 mg median theobromine/day).
9. Mean HR-QoL SF-36 scores revealed moderate levels of quality of life for the entire cohort, regardless of their pattern of dietary methylxanthine consumption.

Evaluation of the effect of the investigational food product in HRQoL in DM1: Interventional pilot clinical study (NCT04634682)

10. The investigational food product tested in the interventional pilot clinical trial NCT04634682 to assess quality of life, fatigue and hypersomnia was well tolerated by the vast majority of patients, despite the entire cohort suffering from a very complex history of comorbidities and comedications.
11. The results of the primary outcome measures of the Epworth sleepiness scales and 6MWT suggest that controlled, dosaged administration of caffeine and theobromine over time in

the investigational food product developed herein could provide significant improvements in decreasing excessive daytime sleepiness and increasing walking distance, at least in adult men with DM1. Further studies should be conducted to unequivocally reveal gender effects, ideally to be conducted beyond the COVID-19 pandemic era.

12. The data on changes in total cholesterol and triglyceride levels may suggest a downward trend over the course of the study. Although not formally statistically significant, they deserve further evaluation in larger cohorts ideally to be conducted beyond the COVID-19 pandemic era.
13. Changes in the SF-36 HR-QoL scales were considered to be non-statistically different when comparing participants' self-reported perception from baseline to the final study visit in the control arm and the active arm. It should be noted that these results should be framed in light of the negative impact of the COVID-19 pandemic on the quality of life of the frail and complex neuromuscular patients, which has been widely discussed and demonstrated in the literature. Consequently, the measurement of changes in health-related quality of life endpoint should be better determined beyond the post-pandemic era.

Scientific substantiation of investigational food products based on methylxanthines as a novel nutritional management approach in Myotonic Dystrophy type 1

14. In light of the results disclosed herein, the rationale for the specific composition, formulation and human data documenting the use of the investigational food product for the dietary management of DM1 patients have been fully provided. This scientific information can substantiate the dossier of the investigational food product as ‘food for special medical purposes for the dietary management of Myotonic Dystrophy type 1’, complying with EFSA recommendations in the context of Article 3 of Regulation (EU) No 609/2013.
15. Ultimately, the main conclusion of the present study is that the investigational food product based on the methylxanthines caffeine and theobromine in the present form of food supplement can be upgraded to become a food for special medical purposes to nutritionally manage DM1.

ANNEXES

ANNEX 1: Patient informed consent of the observational study MD Project

Estudio observacional de calidad de vida y hábitos alimentarios en pacientes con Distrofia Miotónica tipo 1 (DM1)

Promotor:

Investigador principal:
Nombre y apellidos y cargo
Institución
Dirección
Teléfono
Correo electrónico

Información para el paciente

Estimado paciente;

El objetivo de esta hoja de información consiste en informarla sobre este estudio en el cual se le invita a participar.

Cuando haya leído y comprendido toda la información, en el caso que decida participar en el estudio se le pedirá que firme un formulario de consentimiento informado de participación.

¿Cuáles son los objetivos del estudio?

Los objetivos principales son conocer y evaluar la calidad de vida de los pacientes afectados por DM1 y colectar información sobre hábitos alimenticios, actividad física, uso de medicamentos y condiciones general de salud de los pacientes que sufren de distrofia miotónica; con el fin de establecer y evaluar relaciones entre dieta y calidad de vida, por entender aquellos factores que afectan de manera positiva y aquellos que les afectan de manera negativa.

La DM1 es clasificada como “enfermedad rara” y por lo tanto no existe un número elevado de potenciales pacientes que puedan participar en este ensayo, aunque con un mínimo de 100 pacientes podemos obtener unos resultados estadísticamente significativos .Todos o la mayoría de los pacientes elegidos serán miembros de alguna asociación de pacientes (a nivel regional o nacional) de DM1 y cumplirán con todos los criterios de inclusión

Versión número: 01 de 15/05/2017

¿Por qué me ofrecen participar a mí?

Se invita a participar porque usted es un adulto al cual se le ha diagnosticado DM1.

¿Cuáles son los beneficios esperables?

Por la naturaleza del estudio es posible que con su participación no obtenga ningún beneficio; del mismo modo no se espera ningún riesgo por su participación.

Al tratarse de uno estudio observacional su práctica clínica y su tratamiento médico no van a verse afectados.

¿Cuáles son las modalidades de realización de estudio?

El estudio prevé la realización de una sola visita durante la cual se expondrá el proyecto y se darán las instrucciones necesarias para rellenar los cuestionarios adjuntos.

La compilación de la encuesta necesitará menos de media hora.

¿Cuáles son las modalidades de consentimiento?

Su participación es totalmente voluntaria. Usted puede decidir de no participar sin necesidad de explicar los motivos y sin ninguna repercusión en su diaria práctica clínica.

¿Se publicarán los resultados de este estudio?

Todos los entes participantes recibirán informes oficiales semestralmente y por toda la duración del estudio.

Además, los resultados podrán ser comunicados a la Comunidad Científica a través de Congresos y/o publicaciones, o usados con otros fines de carácter científico.

Todos los datos que impliquen la identificación de los participantes no serán revelados.

¿Cómo va a ser protegida la confidencialidad de mis datos?

Los datos recogidos en los cuestionarios y aquellos relativos a su enfermedad y/o su historia clínica serán tratados de forma totalmente anónima.

El Promotor se compromete a su custodia, tratamiento, cesión y comunicación de acuerdo con la Ley Orgánica de protección de datos de carácter personal 15/1999 de 13 de diciembre.

Versión número: 01 de 15/05/2017

Conforme a esta ley, usted puede ejercer el derecho de acceso para modificar y cancelar modificación, oposición y cancelación de datos, para lo cual deberá dirigirse a su médico.

Requisitos éticos y legales del ensayo

La realización de ese estudio ha sido aprobada por el Comité ético de la Associació Catalana d'Hospitals. El Promotor se compromete al cumplimiento de todas las normas éticas en cuanto a ensayos clínicos observacionales de la Ley del Medicamento.

Este estudio de observación se lleva a cabo de conformidad con la Declaración de Helsinki sobre la investigación con seres humanos. El plan de observación de este estudio se le presentó para su revisión, en los casos necesarios, a un comité de ética independiente y no se hizo ninguna observación con respecto a este estudio. No obstante, el investigador de su centro se responsabilizará íntegramente de este estudio clínico de observación.

GRACIAS POR EL INTERÉS MOSTRADO EN ESTE ESTUDIO DE OBSERVACIÓN

Versión número: 01 de 15/05/2017

DECLARACIÓN DE CONSENTIMIENTO

Nombre y apellidos del paciente :_____

Nombre del médico que facilita la información :_____

Centro del estudio :_____

Confirmo mediante mi firma que me han informado de forma completa y exhaustiva sobre el carácter de este estudio, sobre su importancia y sobre el alcance del estudio, tanto mediante la explicación verbal del médico como por escrito, con la hoja de información para el paciente.

Me han entregado la hoja de información para el paciente y la declaración de consentimiento.

He tenido la oportunidad de formular preguntas y se me ha asegurado que también se me responderá cualquier duda posterior que pueda surgirme al respecto. Asimismo, se me ha informado de que, cuando firme la declaración de consentimiento, se me entregará una copia de la hoja de información para el paciente y de la declaración de consentimiento. El investigador se quedará con el original.

Confirmo mediante mi firma mi voluntad de participar en este estudio.

Soy consciente de que puedo revocar en cualquier momento mi consentimiento para participar en este estudio sin que tenga que dar explicaciones y sin que ello suponga un perjuicio para mí.

Este apartado debe firmarlo el paciente en el momento en que otorgue su consentimiento.

Fecha ..... Firma .....

A partir de la información escrita que se ha facilitado a los participantes del estudio, he informado al paciente sobre los objetivos y sobre la realización del estudio.

Este apartado debe firmarlo el médico que explique la información al paciente en el momento en que el paciente otorgue el consentimiento.

Fecha ..... Firma .....

Versión número: 01 de 15/05/2017

ANNEX 2: Observational study MD Project survey

Encuesta Paciente

El principal objetivo de MD Project es investigar los hábitos alimentarios de la comunidad de personas afectadas por Distrofia Miotónica y su calidad de vida.

Esta novedosa investigación trata de profundizar en la correlación entre los hábitos alimentarios de dicha comunidad y su calidad de vida, con el fin de entender aquello que les afecta de manera positiva y aquello que les afecta de manera negativa.

La información recogida será de gran utilidad, no sólo para poder explorar nuevos aspectos relacionados con la Distrofia Miotónica, sino también para programar políticas alimentarias eficaces, encaminadas a la prevención y tratamiento de los problemas que puedan plantearse en dicha población.

Para compilar esta encuesta necesitará menos de media hora de su tiempo, y sus respuestas serán completamente anónimas.

**** Recordamos que no existen respuestas correctas o incorrectas, y que las preguntas son propuestas únicamente al fin del conocimiento científico.***

1

CUESTIONARIO DE SALUD
SF-36 (Versión 2)

Versión Española de SF-36v2™ Health Survey 1996, 2000
Adaptada por J Alonso y cols 2003.

Este instrumento ha superado los estándares de calidad del **Medical Outcome Trust** y de la Red Cooperativa para la Investigación en Resultados de Salud y Servicios Sanitarios (**Red IRYSS**).
El cuestionario y su material de soporte están disponibles en BiblioPRO, la biblioteca virtual de la Red IRYSS (www.rediryss.net).

Su Salud y Bienestar

Por favor conteste las siguientes preguntas.
Algunas preguntas pueden parecerse a otras pero cada una es diferente.
Tómese el tiempo necesario para leer cada pregunta, y marque con una ☐ la casilla que mejor describa su respuesta.

1. En general, usted diría que su salud es:

☐ Excelente ☐ Muy Buena ☐ Buena ☐ Regular ☐ Mala

2. ¿Cómo diría usted que es su salud actual, comparada con la de hace un año?:

☐ Mucho mejor ahora que hace un año ☐ Algo mejor ahora que hace un año ☐ Más o menos igual que hace un año ☐ Algo peor ahora que hace un año ☐ Mucho peor ahora que hace un año

2

3. Las siguientes preguntas se refieren a actividades o cosas que usted podría hacer en un día normal. Su salud actual, ¿le limita para hacer esas actividades o cosas? Si es así, ¿cuánto?

	Sí, me limita mucho	Sí, me limita un poco	No, no me limita nada
a Esfuerzos intensos, tales como correr, levantar objetos pesados, o participar en deportes agotadores	☐	☐	☐
b Esfuerzos moderados, como mover una mesa, pasar la aspiradora, jugar a los bolos o caminar más de 1 hora	☐	☐	☐
c Coger o llevar la bolsa de la compra	☐	☐	☐
d Subir varios pisos por la escalera	☐	☐	☐
e Subir un solo piso por la escalera	☐	☐	☐
f Agacharse o arrodillarse	☐	☐	☐
g Caminar un kilómetro o más	☐	☐	☐
h Caminar varios centenares de metros	☐	☐	☐
i Caminar unos 100 metros	☐	☐	☐
j Bañarse o vestirse por sí mismo	☐	☐	☐

4. Durante las 4 últimas semanas, ¿con qué frecuencia ha tenido alguno de los siguientes problemas en su trabajo o en sus actividades cotidianas, a causa de su salud física?

	Siempre	Casi siempre	Algunas veces	Sólo alguna vez	Nunca
a ¿Tuvo que reducir el tiempo dedicado al trabajo o a sus actividades cotidianas?	☐	☐	☐	☐	☐
b ¿Hizo menos de lo que hubiera querido hacer?	☐	☐	☐	☐	☐
c ¿Tuvo que dejar de hacer algunas tareas en su trabajo o en sus actividades cotidianas?	☐	☐	☐	☐	☐
d ¿Tuvo dificultad para hacer su trabajo o sus actividades cotidianas (por ejemplo, le costó más de lo normal)?	☐	☐	☐	☐	☐

5. Durante las 4 últimas semanas, ¿con qué frecuencia ha tenido alguno de los siguientes problemas en su trabajo o en sus actividades cotidianas, a causa de algún problema emocional (como estar triste, deprimido o nervioso)?

	Siempre	Casi siempre	Algunas veces	Sólo alguna vez	Nunca
a ¿Tuvo que reducir el tiempo dedicado al trabajo o a sus actividades cotidianas por algún problema emocional?	☐	☐	☐	☐	☐
b ¿Hizo menos de lo que hubiera querido hacer por algún problema emocional?	☐	☐	☐	☐	☐
c ¿Hizo su trabajo o sus actividades cotidianas menos cuidadosamente que de costumbre, por algún problema emocional?	☐	☐	☐	☐	☐

6. Durante las 4 últimas semanas, ¿hasta qué punto su salud física o los problemas emocionales han dificultado sus actividades sociales habituales con la familia, los amigos, los vecinos u otras personas?

☐ Nada ☐ Un Poco ☐ Regular ☐ Bastante ☐ Mucho

7. ¿Tuvo dolor en alguna parte del cuerpo durante las 4 últimas semanas?

☐ No, ninguno ☐ Si, muy poco ☐ Si, un poco ☐ Si, moderado
☐ Si, mucho ☐ Si, muchísimo

8. Durante las 4 últimas semanas, ¿hasta qué punto el dolor le ha dificultado su trabajo habitual (incluido el trabajo fuera de casa y las tareas domésticas)?

☐ Nada ☐ Un Poco ☐ Regular ☐ Bastante ☐ Mucho

9. Las preguntas que siguen se refieren a cómo se ha sentido y cómo le han ido las cosas durante las 4 últimas semanas. En cada pregunta responda lo que se parezca más a cómo se ha sentido usted. Durante las últimas 4 semanas ¿con qué frecuencia...

	Siempre	Casi siempre	Algunas veces	Sólo alguna vez	Nunca
a Se sintió lleno de vitalidad?	☐	☐	☐	☐	☐
b Estuvo muy nervioso?	☐	☐	☐	☐	☐
c Se sintió tan bajo de moral que nada podía animarle?	☐	☐	☐	☐	☐
d Se sintió calmado y tranquilo?	☐	☐	☐	☐	☐
e Tuvo mucha energía?	☐	☐	☐	☐	☐
f Se sintió desanimado y deprimido?	☐	☐	☐	☐	☐
g Se sintió agotado?	☐	☐	☐	☐	☐
h Se sintió feliz?	☐	☐	☐	☐	☐
i Se sintió cansado?	☐	☐	☐	☐	☐

10. Durante las 4 últimas semanas, ¿con qué frecuencia la salud física o los problemas emocionales le han dificultado sus actividades sociales (como visitar a los amigos o familiares)?

☐ Siempre ☐ Casi siempre ☐ Algunas Veces ☐ Solo alguna vez ☐ Nunca

11. Por favor diga si le parece CIERTA o FALSA cada una de las siguientes frases:

	Totalmente cierta	Bastante cierta	No lo sé	Bastante Falsa	Totalmente falsa
a Creo que me pongo enfermo más fácilmente que otras personas	☐	☐	☐	☐	☐
b Estoy tan sano como cualquiera	☐	☐	☐	☐	☐
c Creo que mi salud va a empeorar	☐	☐	☐	☐	☐
d Mi salud es excelente	☐	☐	☐	☐	☐

A. Condición de Salud

En esta sección se le hacen unas preguntas sobre su estado general de salud.

A.1. Peso

_____ Kg

A. Sexo

☐ Hombre ☐ Mujer

A.2. Altura

_____ cm

A.A Edad

_____ años

A.3. Tipología de DM

☐ DM1 Forma congénita ☐ DM1 Aparición en adolescencia ☐ DM1 Aparición en edad Adulta
☐ DM2 ☐ Desconocida/ No sé

A.4. Edad en la cual empezó a notar los síntomas

Edad_____

-o-

☐ Aún no he notado los Síntomas

A.5. Diagnosis Médica con Test Genético

☐ Si ☐ No

A.5.1. En caso afirmativo, usted se acuerda del número de repeticiones

☐ Sí, mi número de repeticiones es_____
☐ Si pero no recuerdo el número exacto de repeticiones
☐ No lo he recibido

A.6. ¿Practica Actividad Física?

☐ Si ☐ No

A.6.1. En caso afirmativo, describa la tipología de actividad que hace

☐ Ligera (Pasear, caminar, ir en bicicleta…) ☐ Moderada (Correr, nadar, gimnasia..) ☐ Intensa (Entrenamiento Deportivo, fútbol, baloncesto ..)

A.6.2. ¿Y cuántas veces por semana?

☐ 1 - 2 veces ☐ 3 - 5 veces ☐ > 5 veces

A.6.3. ¿Durante cuánto tiempo?

☐ 10 - 20 min ☐ 21 - 30 min ☐ 31 - 40 min ☐ 41 - 50 min ☐ > 51 min

A.7. ¿Fuma cigarrillos, puros o en pipa?

☐ Si ☐ No

A.7.1. En caso afirmativo, ¿cuántos al día? _____

A.8. ¿Toma alguna droga?

☐ Si ☐ No

En caso afirmativo nos las podría enumerar

_____ _____

_____ _____

B. Terapias

Se le van a hacer unas preguntas sobre cualquier tipología de terapia y/o tratamiento que usted está siguiendo y su opinión sobre el grado de satisfacción, con el fin de saber cuánto útiles/efectivos han sido para usted.

TERAPIA				Nombre del Tratamiento	Grado de Satisfacción				Formulación y Dosis
B.1. TRATAMIENTOS FARMACOLÓGICOS									
Tratamiento de la Miotonía (dificultad a relajar los músculos)	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
Tratamiento por Somnolencia diurna y/o Fatiga	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
Tratamiento de Problemas Gastrointestinales relacionados con DM	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
Tratamiento de problemas respiratorios y cardiovasculares	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
B.2. TERAPIAS COMPLEMENTARIAS									
Vitaminas y/o Minerales	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
Complemento Alimenticio o Nutracéutico	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
B.3. TERAPIAS ALTERNATIVAS									
Remedio Homeopático o Tratamiento Natural	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
Medicina no convencional	Si	No	No sé		Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	
					Para nada Satisfecho	Poco Satisfecho	Mediamente Satisfecho	Muy Satisfecho	

B.4. Fisioterapia

☐ Si ☐ No

B.4.1. En caso afirmativo, ¿cuántas veces por semana?

☐ 1 - 2 veces ☐ 3 - 5 veces ☐ > 5 veces

B.4.2. ¿Por cuánto tiempo?

☐ 10 - 20 min ☐ 21 - 30 min ☐ 31 - 40 min ☐ 41 - 50 min ☐ > 51 min

C. Hábitos Alimentarios

Esta es la sección específicamente dedicada a sus costumbres alimentarias

Se le van a hacer unas preguntas sobre el número de veces que consume una serie de alimentos y bebidas, para obtener unos datos sobre la frecuencia de consumo de estos últimos. Describa todos los alimentos y bebidas que usted consume, teniendo en cuenta cualquier tipo de comida (almuerzo, comida, cena, merienda, etc...) y su frecuencia de consumo.

- Si usted consume estos alimentos muy frecuentemente, rellene la casilla *Número de veces por día*.

- Si usted consume estos alimentos menos frecuentemente, entonces rellene la casilla *Número de veces por semana*.

- Si usted consume estos alimentos raramente, rellene la casilla *Número de veces por mes*.

Unidades = las unidades consumidas

Tamaño de la Ración= se refiere al tamaño de la porción (Plato, cucharada, etc...) consumida que puede ser:
P= pequeña *M*= mediana *G*= grande

C.1. ¿Sigue usted alguna dieta particular?

☐ Si ☐ No

En caso afirmativo, ¿puede describirla?

_____

_____

_____

ALIMENTO		Veces /día	Veces /semana	Veces /mes	Tamaño de la ración			Unidades
C.2. Cereales y derivados								
a. Cereales de Desayuno (plato)					P	M	G	
b. Pasta (plato)					P	M	G	
c. Arroz (plato)					P	M	G	
d. Galletas (unidades)								
e. Pan (Rebanadas)	Blanco							
	Integral							
	Con Cereales							
C.3. Leche y derivados								
a. Leche (Vasos)	Entero				P	M	G	
	Semi-Desnatada				P	M	G	
	Desnatada				P	M	G	
b. Yogur (Unidad)	Entero							
	Desnatado							
	Enriquecido							
c. Queso (plato)	Fresco				P	M	G	
	Curado				P	M	G	
	Cremoso				P	M	G	
C.4. Azúcares y derivados								
a. Azúcares (cucharas)	Azúcar							
	Miel							
	Edulcorantes Artificiales							
C.5. Aceites y grasas								
a. Aceite (cucharas)	Oliva							
	Girasol							
	Otros (Maíz, palma, coco etc.)							
b. Mantequilla (porción cafetería)								
c. Margarina (porción cafetería)								

ALIMENTO		Veces /día	Veces /semana	Veces /mes	Tamaño de la ración			Unidades
C.6. Verduras y hortalizas								
a. Verduras y hortalizas	Calabacín				P	M	G	
	Calabaza				P	M	G	
	Brócoli				P	M	G	
	Berenjena				P	M	G	
	Champiñones y Setas				P	M	G	
	Espinacas				P	M	G	
	Zanahoria				P	M	G	
	Pimiento (rojo y verde)				P	M	G	
	Espárragos				P	M	G	
	Avocado							
	Ensaladas, rúcula, canónigo				P	M	G	
	Ajo (dientes)							
	Cebolla				P	M	G	
	Tomate				P	M	G	
	Alcachofas				P	M	G	
C.7. Leguminosas								
a. Garbanzos					P	M	G	
b. Judías					P	M	G	
c. Lentejas					P	M	G	
d. Habas					P	M	G	
e. Guisantes					P	M	G	
f. Soja					P	M	G	
C.8. Frutas								
a. Frutas	Fresa							
	Arándano							
	Uva							
	Mora							
	Piña							
	Manzana							
	Mandarina							
	Pera							
	Naranja							
	Kiwi							
	Banana							
b. Frutos Secos	Almendras				P	M	G	
	Castañas				P	M	G	
	Avellanas				P	M	G	
	Cacahuetes				P	M	G	
	Piña				P	M	G	
	Nueces				P	M	G	
	Piñones				P	M	G	
	Pistachos				P	M	G	
c. Conservas de Frutas	Fruta en Almíbar				P	M	G	
	Mermelada				P	M	G	

	Membrillo y Pastas de Fruta				P	M	G	

ALIMENTOS		Veces /día	Veces /semana	Veces /mes	Tamaño de la ración			Unidades
C.9. Huevos								
a. Huevos								
C.10. Carnes y productos cárnicos (plato)								
a. Carne Blanca	Pollo				P	M	G	
	Pavo				P	M	G	
	Conejo				P	M	G	
	Pato				P	M	G	
b. Carne Roja	Vacuno				P	M	G	
	Cerdo				P	M	G	
	Cordero				P	M	G	
	Cacería				P	M	G	
c. Embutidos y otros derivados Cárnicos	Vísceras				P	M	G	
	Callos o tripa				P	M	G	
	Embutidos				P	M	G	
C.10.10 Pescados (plato)								
a. Pescado graso y semigraso	Anchoa				P	M	G	
	Anguila				P	M	G	
	Salmón				P	M	G	
	Atún				P	M	G	
	Emperador				P	M	G	
	Verdel				P	M	G	
b. Pescado Blanco	Gallo				P	M	G	
	Rape				P	M	G	
	Merluza				P	M	G	
	Lenguado				P	M	G	
	Bacalao				P	M	G	
C.11. Moluscos, cefalópodos y crustáceos (plato)								
a. Cigalas, gambas, langostinos y camarones					P	M	G	
b. Cangrejos					P	M	G	
c. Mejillones					P	M	G	
d. Almejas					P	M	G	
e. Erizos de Mar					P	M	G	
f. Calamares, chopitos y sepias					P	M	G	
C.12. Otros								
a. Helados					P	M	G	
b. Pizza					P	M	G	
c. Dulces					P	M	G	
d. Chocolate	Chocolate negro > 70%				P	M	G	
	Chocolate negro (50-70%)				P	M	G	
	Chocolate Blanco				P	M	G	
	Chocolate con Leche				P	M	G	

BEBIDAS		Veces /día	Veces /semana	Veces /mes	Tamaño del Vaso M= Vaso estándar 200ml			Unidades
C.13. Zumos								
a. Zumos recién exprimidos	Naranja				P	M	G	
	Pomelo				P	M	G	
	Limón				P	M	G	
b. Zumos envasados	Frutos Rojos				P	M	G	
	ACE				P	M	G	
	Otros				P	M	G	
C.14. Café y Derivados								
a. Expreso					P	M	G	
b. Café con leche					P	M	G	
c. Cappuccino					P	M	G	
d. Café instantáneo					P	M	G	
e. Café Americano					P	M	G	
f. Descafeinado					P	M	G	
C.15. Té e Infusiones								
a. Té Verde					P	M	G	
b. Té Rojo					P	M	G	
c. Té Negro					P	M	G	
d. Otras Infusiones					P	M	G	
C.16. Bebidas Energéticas								
a. RedBull, Monster, Rockstar					P	M	G	
b. Otras					P	M	G	
C.17. Refrescos								
a. Coca Cola					P	M	G	
b. Coca Cola (sin cafeína)					P	M	G	
c. 7Up					P	M	G	
d. Sprite					P	M	G	
e. Fanta					P	M	G	
f. Schweppes					P	M	G	
C.18. Bebidas Alcohólicas								
a. Cerveza					P	M	G	
b. Sidra, cava					P	M	G	
c. Licores					P	M	G	
d. Vino					P	M	G	

D. Preguntas generales

En esta sección se le harán una serie de preguntas acerca de informaciones de carácter más general.

D.1. Número de visitas anuales al médico

☐ 1 - 2 visitas ☐ 3 - 5 visitas ☐ > 5 visitas ☐ Ninguna

D.2. Número de visitas anuales al especialista

☐ 1 - 2 visitas ☐ 3 - 5 visitas ☐ > 5 visitas ☐ Ninguna

D.3. Número de análisis y controles anuales

☐ 1 - 2 visitas ☐ 3 - 5 visitas ☐ > 5 visitas ☐ Ninguna

D.4. Número de hospitalizaciones anuales

☐ 1 - 2 veces ☐ 3 - 5 veces ☐ > 5 veces ☐ Ninguna

D.5. Participación en conferencias o eventos

☐ Si ☐ No

D.6. Seguimiento de las noticias y actualizaciones sobre la enfermedad, nuevas curas posibles, eventos, artículos científicos, etc.

☐ Si ☐ No

D.7. ¿A través de qué herramienta se mantiene actualizado?

☐ Páginas de Asociaciones ☐ Páginas de empresas ☐ Su Especialista ☐ Internet
☐ Ninguna de éstas

D.7.1. Si no es a través de ninguna de éstas, ¿nos podría explicar cómo usted se mantiene actualizado?

_____

_____

_____

FIN

Muchas gracias por su ayuda

ANNEX 3: Patient informed consent of the interventional study MYODM-FSMP Project

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Título del estudio	PROYECTO DE INVESTIGACION PARA EVALUAR EL EFECTO DEL COMPLEMENTO ALIMENTICIO MYODM SOBRE LA CALIDAD DE VIDA, FATIGA E HIPERSOMNIA EN PACIENTES CON DISTROFIA MIOTONICA TIPO 1
Acrónimo	**MYODM PRO**
Promotor	**Myogem Health Company, S.L.** Carrer Álvarez de Castro, 63, 08100 Mollet del Vallès, Barcelona, España
Investigador Principal	**Roberto Fernández Torron** Hospital Universitario Donostia Paseo Dr. Begiristain, S/N, 20014, San Sebastián, Guipúzcoa, España Tel: 943 00 70 27

La presente **HOJA DE INFORMACIÓN AL PACIENTE Y CONSENTIMIENTO INFORMADO (HIP/CI)** consta de tres partes:

1. Hoja de información del paciente (para compartir con usted información sobre la investigación)
2. Consentimiento informado (destinado a las firmas si acepta participar en el estudio)
3. Revocación del consentimiento informado

Nos dirigimos a usted para informarle sobre un proyecto de investigación en el que se le invita a participar.

El proyecto ha sido aprobado por el **Comité Ético de Investigación Clínica de Gipuzkoa**.

Nuestra intención es que usted reciba la información correcta y suficiente para que pueda decidir si acepta o no participar en este proyecto. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir.

Además, puede consultar con las personas que considere oportuno.

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1. HOJA DE INFORMACIÓN PARA EL PACIENTE

Participación voluntaria

Debe saber que su participación en este estudio es voluntaria y que puede decidir NO participar. Si decide participar, puede cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su atención sanitaria.

¿Cuál es el objetivo del estudio?

La distrofia miotónica tipo 1 (DM1) o enfermedad de Steinert, es caracterizado clínicamente por una atrofia muscular progresiva y debilidad, cardiomiopatía, resistencia a la insulina y cataratas, entre otros. Es la forma más común de distrofia muscular en adultos.

La enfermedad conduce a un deterioro físico sustancial, que, en combinación con los efectos neuropsicológicos de la afección, resulta en una participación social severamente restringida.

MYODM es una combinación comercializada de dos xantinas naturales como la cafeína y la teobromina que ha demostrado un aumento adicional de los niveles de MBNL1 en las células DM1 mostrando un claro efecto sinérgico, por lo que consideramos que este complemento alimenticio sería útil como terapia en pacientes con DM1. Además, como hablamos de un excitante natural podría contribuir en la disminución de la somnolencia diurna de estos pacientes y contribuir a mejorar la Calidad de la Vida Relacionada con la Salud de estos pacientes.

¿Porque me ofrecen a mí participar en este estudio?

Le están invitando a participar en este estudio porque esta diagnosticado con distrofia miotónica tipo 1 (DM1) o enfermedad de Steinert y consideramos que podría cumplir los criterios para participar en este proyecto.

¿En qué consiste mi participación?

Si usted decide participar en este proyecto de investigación se le pedirá que acuda a un total de 3 visitas al centro del estudio durante los próximos 6 meses. Estas visitas incluirán: una visita

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basal, una vista en el Mes 3 y una visita final. Estimamos que cada visita durara en torno a 1 hora.

El médico del estudio le explicará los requisitos para participar en el estudio y lo que es necesario para cumplirlos.

Visita de selección:

En la primera visita se realizarán los procedimientos siguientes para determinar si es apto para participar en el proyecto de investigación:

- Tendrá que firmar y fechar el documento de consentimiento informado
- Se le randomizar en uno de los brazos del estudio. Esto significa que aleatoriamente podrá es ser incluido en el brazo que tomara el suplemento alimenticio u en el brazo control.

Independientemente del brazo en cual será incluido se le realizaran las siguientes pruebas:

- Se repasarán sus antecedentes médicos y sus datos demográficos
- Se le tomará la tensión, se le pesará y se le medirá
- El médico le realizará una exploración física completa
- Se le realizará un electrocardiograma
- El médico y el personal del estudio le harán una serie de preguntas sobre su alimentación, sueño y se le pedirá que rellene una serie de cuestionarios sobre su calidad de vida actual, ansiedad, depresión.
- Se le extraerá sangre para realizar una analítica.
- Se le entregara un actimetro (un dispositivo tipo reloj que mide los pasos que realiza durante el día y su sueño)

Si usted será incluido en el brazo activo (el que recibe el "tratamiento") Se le dispensará el complemento alimenticio MYODM que usted deberá tomar todos los días tres veces al día durante 6 meses.

A partir de esta visita acudirá al Hospital universitario Donostia cada 3 para las revisiones que se muestran a continuación en la tabla.

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Visita	SCR (día-7-dia-1)	BL (día 0)	Semana 1	Visita Mes 3	Semana 23	Visita Mes 6 EOT
VISITA nº	1	2		3		4
Ventana de visita	N.A.	N.A.		+/- 3 días		+/- 3 días
Firma del Consentimiento informado	X					
Criterios de inclusión/exclusión	x	X				
Datos demográficos	x					
Exploración física	X			X		X
Constantes vitales	X	X		X		X
ECG	X					X
Altura, Peso, Circunferencia cintura	X	X		X		X
BMI, masa muscular	X	X		X		X
Función motora - *Test de velocidad de la marcha* - *6 min WT* - *Prueba muscular cuantitativa*	X			X		X
Análisis bioquímico de la sangre (en ayunas 12 horas)	X			X		X
Formularios PRO	X			X		X
WAIS-IV		X				
Tratamiento previo / concomitante	X	X		X		X
Historia medica	X					
Dispensación del producto del estudio		X		X		
Acontecimientos adversos	X	X		X		X
Entregar/recoger el diario del paciente	X	X		X		X
Inicio registro actividad diaria y sueño	X				X	
Fin registro actividad diaria y sueño			X			X

¿Qué riesgos o inconvenientes tiene participar?

Se trata de un proyecto de investigación en cual usted tomara un complemento alimenticio llamado MYODM cuyos principales componentes son cafeína y teobromina.

MYODM contiene 20 mg de **cafeína** por cápsula. La toma diaria de tres cápsulas equiespaciadas conlleva una ingesta máxima de 60 mg.

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MYODM cumple con la legislación vigente en materia de contenido de **cafeína** en complementos alimenticios, para los que se aceptan como máximo una cantidad diaria de 80 mg. La toma de una cápsula de MYODM (con sus 20 mg de **cafeína**), supone una ingesta de 0.28 mg/kg en un individuo de 70 Kg de peso.

La Agencia de Seguridad Alimentaria Europea (EFSA), en sus informes de expertos indica que en la población sana en general, son seguras dosis individuales de cafeína de hasta 3 mg/kg para un adulto de 70 Kg (equivalente a 200 mg de cafeína en una sola ingesta). En general, dosis habituales de cafeína hasta 400 mg/día se consideran seguras en adultos. Por tanto, la EFSA indica como segura una dosis diez veces superior a la cantidad de **cafeína** en una cápsula de MYODM.

El producto comercializado y empleado en el presente proyecto ha sido fabricado por la CMO que cumple con GMP Elaborados Dietéticos SA (Eladiet), en nombre de Myogem Health Company.

La teobromina es un extracto del árbol de cacao estimulante del sistema nervioso central.

Hasta el momento no se han notificado efectos adversos relacionados con MYODM.

Los riesgos de la extracción de sangre de una vena son: molestias en el lugar del pinchazo, posibles hematomas e hinchazón en el lugar del pinchazo; rara vez infección y con poca frecuencia desvanecimiento durante el procedimiento.

No se le proporcionarán los resultados de los análisis de sangre y orina. Solo el investigador y sus colaboradores conocerán dicha información. Sin embargo, tiene derecho a solicitar dicha información sobre los resultados de los mismos si así lo desea.

La exploración física y neurológica incluye una evaluación habitual o similar a la que se le suele realizar en las consultas de Neurología.

¿Qué ocurrirá con las muestras de sangre obtenidas?

Las muestras de sangre serán analizadas en el contexto del estudio. La muestra se enviará una vez extraída al Laboratorio del Hospital Universitario Donostia para ser analizada. En el caso de que haya remanente de muestra esta se destruirá según los protocolos del laboratorio.

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¿Obtendré algún beneficio para participar?

Es posible que no reciba ningún beneficio directo por participar en el estudio. En cualquier caso, con su participación proporcionara información nueva que podría beneficiar a otros sujetos en el futuro. Si decide no participar en este proyecto, o si se suspende el proyecto mientras está participando seguirá recibiendo la atención medica habitual para la Distrofia Miotónica Tipo 1.

¿Recibiré la información que se obtenga del estudio?

Si usted lo desea, se le facilitará un resumen de los resultados del estudio cuando estén disponibles. Algunos resultados pueden no tener una interpretación clara, si desea disponer de ellos, le serán proporcionados directamente por los médicos de la investigación junto con un adecuado asesoramiento.

¿Cómo se protegerá la confidencialidad de mis datos?

Su privacidad es muy importante para nosotros y en consecuencia el equipo investigador del estudio hará todos los esfuerzos posibles para protegerla. El estudio cumple lo establecido en el REGLAMENTO (UE) 2016/679 DEL PARLAMENTO EUROPEO Y DEL CONSEJO de 27 de abril de 2016 relativo a la protección de las personas físicas en lo que respecta al tratamiento de datos personales y a la libre circulación de estos datos y a la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales que deroga la Ley Orgánica 15/1999, de 5 de diciembre, de protección de datos personales.

En general en un estudio, los datos personales se refieren a los distintos tipos de información recogida o procesada después de que se otorgue el consentimiento informado y normalmente incluye lo siguiente:

- Información general sobre usted (definida por la ley de protección de datos), como su nombre, dirección y su fecha de nacimiento
- Su información del estudio, que significa su información clínica recogida durante el estudio, como sus datos sobre la enfermedad, la información obtenida de las evaluaciones del estudio o del análisis de sus muestras biológicas, la medicación que está tomando habitualmente
- Sus muestras biológicas

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Sus muestras y los datos del estudio sólo se utilizarán para investigación. Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no incluya información que pueda identificarle, y sólo su médico del estudio/colaboradores podrá relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a persona alguna salvo excepciones en caso de urgencia médica o requerimiento legal. Los datos personales serán tratados por Osakidetza – Servicio Vasco de Salud. No se cederán datos a terceros, salvo obligación legal. Los datos sobre usted se recogerán en un fichero de investigación responsabilidad de la institución y se tratarán en el marco de su participación en este estudio.

Es posible que los datos del presente estudio se utilicen para otras investigaciones futuras en combinación con datos de otros estudios según proceda o con datos obtenidos en otros centros. El investigador principal adoptará las medidas pertinentes para garantizar la protección de su privacidad y no permitirá que sus datos se crucen con otras bases de datos que pudieran permitir su identificación.

Las peticiones de otros investigadores para acceder a las muestras codificadas y a los datos de la investigación clínica se revisarán científicamente conforme a los criterios establecidos por el Investigador principal del estudio (Dr. Roberto Fernández Torron).

Además de los derechos de acceso, modificación, oposición y cancelación de datos, ahora también puede limitar el tratamiento de datos que sean incorrectos, solicitar una copia o que se trasladen a un tercero (portabilidad) los datos que usted ha facilitado para el estudio. Para ejercitar sus derechos, diríjase al investigador principal del estudio (Dr. Roberto Fernández Torron)

Si usted **decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos**, pero sí se utilizarán los que ya se hayan recogido para garantizar la validez de la investigación y cumplir con los deberes legales.

Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

El Investigador Principal es el responsable del tratamiento de sus datos y se compromete a cumplir con la normativa de protección de datos en vigor. Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no se incluya información que pueda identificarle, y sólo su médico del estudio/colaboradores podrá relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a ninguna otra persona salvo a las autoridades sanitarias, cuando así lo requieran o en casos de urgencia médica. Los Comités de Ética de la Investigación, los representantes de la Autoridad Sanitaria en materia de inspección y el personal autorizado por el Promotor, únicamente podrán acceder para

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comprobar los datos personales, los procedimientos del estudio clínico y el cumplimiento de las normas de buena práctica clínica (siempre manteniendo la confidencialidad de la información). El Investigador Principal está obligado a conservar los datos recogidos para el estudio al menos hasta 25 años tras su finalización. Posteriormente, su información personal solo se conservará por el centro para el cuidado de su salud y por el Investigador Principal para otros fines de investigación científica si usted hubiera otorgado su consentimiento para ello y si así lo permite la ley y requisitos éticos aplicables.

Si realizáramos transferencia de sus datos codificados fuera de la UE a las entidades de nuestro grupo, a prestadores de servicios o a investigadores científicos que colaboren con nosotros, los datos del participante quedarán protegidos con salvaguardas tales como contratos u otros mecanismos por las autoridades de protección de datos. Podrá ampliar información en materia de protección de datos en la siguiente dirección web: *http://www.osakidetza.euskadi.eus/protecciondatos*.

¿Qué sucederá si cambio de opinión?

Su participación en este proyecto es voluntaria. No está obligado a participar y puede interrumpir su participación en cualquier momento sin que ello suponga ninguna desventaja ni pérdida de las prestaciones a las que tenga derecho. Si decide abandonar el estudio antes de la última visita de este, informe al médico y siga sus instrucciones. Sería útil que explicase el motivo de la retirada, aunque no está obligado en hacerlo. Podrá seguir recibiendo la atención médica habitual sin perjuicio alguno y podrá participar si así lo desea en futuras investigaciones.

Por otro lado, debería ser informado que el médico del estudio o el promotor podrán retirarle del estudio por su propia seguridad aun cuando usted quiera continuar; por ejemplo:

- Si necesita medicación adicional que podría interferir con MYODM
- Si sufre una lesión relacionada con el estudio
- Si no sigue las normas del estudio
- Si se produce un acontecimiento adverso grave
- Si se produce una variación clínicamente significativa en un resultado analítico
- Si es mujer y se queda embarazada

En el caso de que se interrumpa su participación en el estudio antes del tiempo se le pedirá que continúe en el estudio para un seguimiento de seguridad.

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¿Tendré algún gasto si decido participar?

No es previsible que la participación en este proyecto le suponga ningún gasto. El complemento alimenticio se le suministrará de forma gratuita. No se le cobrará nada por ninguno de los procedimientos que se realicen en el marco del proyecto. No se le reembolsarán los gastos de desplazamiento, aparcamiento etc.

No recibirá ningún beneficio económico por participar en el proyecto.

¿Quién financia esta investigacion?

Myogem Health Company, S.L. es la empresa que organiza y financia el presente estudio. Este proyecto ha recibido financiación del programa de investigación e innovación H2020 de la Unión Europea bajo el acuerdo de financiación No 875615.

Myogem Health Company, S.L remunerará al médico del estudio y al centro del estudio para cubrir los costes derivados de la realización de este proyecto. Su participación en este proyecto puede contribuir al desarrollo de productos comerciales a partir de los que el promotor puede obtener algún beneficio económico. No tendrá ningún derecho sobre las patentes o descubrimientos que surjan en esta investigación, ni recibirá ningún beneficio económico.

¿Estaré asegurado mientras participe en el estudio?

Si sufre algún daño como consecuencia de su participación en el estudio, tendrá derecho a recibir una indemnización de acuerdo con la legislación vigente en España. El médico le explicará cómo puede obtener una copia de estas normas.

El promotor del estudio tiene contratada una póliza de responsabilidad civil de conformidad con la legislación vigente (Real decreto 1090/2015) que cubrirá los gastos e indemnizaciones en caso de daños para la salud o lesiones que pudieran producirse en relación con su participación en el proyecto, siempre que no sean resultado de la propia enfermedad en estudio o de la evolución de la misma como consecuencia de la ineficacia del tratamiento.

Myogem Health Company, S.L, ha contratado una póliza de seguro con

W.R. Berkley Europe AG, número de póliza **2031836**.

Le informamos de que su participación en este proyecto podría modificar los términos y condiciones generales y particulares (cobertura) de sus pólizas de seguro (vida, salud, accidente etc). En consecuencia, le recomendamos que se ponga en contacto con su compañía de seguros para comprobar si su participación en este estudio podría afectar a su póliza de seguro actual.

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¿Quién me puede dar más información sobre el Estudio?

Para más información puede contactar con:

Dr (a) _____ _____al teléfono: _____

o con la coordinadora del estudio_____,

al teléfono: _____.

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2. CONSENTIMIENTO PARA LA REALIZACIÓN DEL PROYECTO DE INVESTIGACIÓN

Título del estudio	PROYECTO DE INVESTIGACION PARA EVALUAR EL EFECTO DEL COMPLEMENTO ALIMENTICIO MYODM SOBRE LA CALIDAD DE VIDA, FATIGA E HIPERSOMNIA EN PACIENTES CON DISTROFIA MIOTONICA TIPO 1
Acrónimo	**MYODM PRO**
Promotor	**Myogem Health Company, S.L.** Carrer Álvarez de Castro, 63, 08100 Mollet del Vallès, Barcelona, España
Investigador Principal	**Roberto Fernández Torron** Hospital Universitario Donostia Paseo Dr. Begiristain, S/N, 20014, San Sebastian, Guipuzkoa, España Tel: 943 00 70 27

Yo.....con DNI..... declaro que he leído la Hoja de Información al paciente, de la que se me ha entregado una copia. He recibido información sobre las características del estudio, así como los posibles beneficios y riesgos que puedo esperar, los derechos que puedo ejercitar, y las previsiones sobre el tratamiento de datos y muestras. He recibido suficiente información sobre el estudio.

Sé que se mantendrá en secreto mi identidad y que se identificarán mis muestras con un sistema de codificación. Soy libre de revocar mi consentimiento en cualquier momento y por cualquier motivo, sin tener que dar explicación y sin que repercuta negativamente sobre cualquier tratamiento médico presente o futuro.

Yo doy mi consentimiento para que se utilicen mis muestras y los datos clínicos asociados como parte de **este proyecto de investigación** y su uso posterior en otras investigaciones relacionadas con la Distrofia Miotónica Tipo 1.

Consiento en participar voluntariamente.

Al firmar el presente formulario de consentimiento, no he renunciado a ninguno de mis derechos legales.

Fecha:..... Firma del paciente.....

Fecha:..... Firma representante legal (si procede).....

Nombre representante legal:

12

Relación con el paciente:

Constato que he explicado las características de las condiciones de conservación y seguridad que se aplicarán a la muestra y a los datos clínicos conservados.

Nombre del clínico responsable

_____

Fecha: .....

Firma:.....

13

3. Revocación del consentimiento informado

(Para ser utilizado en participantes que quieren retirarse del proyecto)

Título del estudio	PROYECTO DE INVESTIGACION PARA EVALUAR EL EFECTO DEL COMPLEMENTO ALIMENTICIO MYODM SOBRE LA CALIDAD DE VIDA, FATIGA E HIPERSOMNIA EN PACIENTES CON DISTROFIA MIOTONICA TIPO 1
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Quiero RETIRAR mi consentimiento a participar en el estudio aquí descrito y entiendo que esta decisión NO afectará a ningún tratamiento o mi relación con el centro.

Nombre del participante _____ Fecha _____

Firma _____

ANNEX 4: Scientific publications

This doctoral thesis has referenced the following scientific communications:

Poster communications

International Myotonic Dystrophy Consortium (IDMC-11, 2017). “Natural xanthines rescue myotonic dystrophy-like phenotypes in *Drosophila* and increase MBNL expression levels”. San Francisco, EEUU.

International Myotonic Dystrophy Consortium (IDMC-12, 2019). “Natural xanthines caffeine and theobromine rescue myotonic dystrophy-like features in cell and animal models of DM”. Gothenburg, Sweden.

Indexed articles

Pascual-Gilabert M, López-Castel A, Artero R. Myotonic dystrophy type 1 drug development: A pipeline toward the market. *Drug Discov Today*. 2021;26(7):1765-1772.

Pascual-Gilabert M, López-Castel A, Artero R. The myotonic dystrophy type 1 drug development pipeline: 2022 edition. *Drug Discov Today*. 2022. Manuscript under preparation.

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Myotonic dystrophy type 1 drug development: A pipeline toward the market

SPECIAL FEATURE

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Myotonic dystrophy type 1 (DM1) is a multisystemic neuromuscular genetic disease with an estimated prevalence of approximately at least half a million individuals based on its vast ethnic variation. Building upon a well-known physiopathology and several proof-of-concept therapeutic approaches, herein we compile a comprehensive overview of the most recent drug development programs under preclinical and clinical evaluation. Specifically, close to two dozen drug developments, eight of which are already in clinical trials, explore a diversity of new chemical entities, drug repurposing, oligonucleotide, and gene therapy-based approaches. Of these, repurposing of tideglusib, mexiletine, or metformin appear to be therapies with the most potential to receive marketing authorization for DM1.

Keywords: Myotonic dystrophy; Drug development; Repurposing drug; Antisense oligonucleotide; Gene therapy; Clinical trial

Introduction

The past two decades have witnessed the generation of breakthrough knowledge regarding the molecular causes and conceptual approaches to treating neuromuscular disorders. The consequence has been the approval of the first oligonucleotide-based drug treatments for Duchenne muscular dystrophy (DMD; eteplirsen and golodirsen) and spinal muscular atrophy (SMA; nusinersen) [1], and the first gene therapy (Zolgensma; SMA), which complement more traditional small-molecule therapeutic options (e.g., risdiplam; SMA) [2]. Next in line with at least one therapeutic option might be Myotonic dystrophy type 1 (DM1). DM1 is a life-threatening chronic disease with symptoms in the neuromuscular, cardiac, and central nervous systems, and compared with most neuromuscular diseases, its clinical presentation is very variable [3,4]. DM1 can affect newborns (congenital DM1) to older adults, with around at least half a million patients worldwide based on the reported prevalence estimation of 1 in 3,000–8,000 people [3,5].

A repeat length >50 units of the repetitive trinucleotide sequence (CTG)n in the 3′-untranslated region (UTR) of the DM1 protein kinase (*DMPK*) gene causes DM1. Mutant *DMPK* transcripts in skeletal muscle, heart, and brain tissue are retained in the cell nucleus in microscopically visible ribonuclear foci, which are the most prominent histopathological hallmark of the disease. CUG expansions fold into stable double-stranded stem-loop structures with U-U mismatches with a strong affinity for proteins of the Muscleblind-like (MBNL) family, which are sequestered and become depleted and unable to perform their normal function. MBNL loss of function contributes significantly to DM1 phenotypes [6]. Likewise, stress responses triggered by the toxic *DMPK* RNA cause the stabilization/activation of MBNL antagonists CELF1 and hnRNPA1 [6]. Together, these proteins function as developmental switches, and their imbalance results in the abnormal persistence of fetal patterns of alternative splicing in adult tissues, termed 'spliceopathy', which affects hundreds of genes, some of which explain specific symptoms in

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DM1. Various signaling pathways also have a role in DM1 muscle pathogenesis, including impaired protein kinase B (AKT), activated autophagy and ubiquitin–proteasome activity, AMP-activated protein kinase (AMPK) downregulation, and activated glycogen synthase kinase 3 beta (GSK3β) [7]. Taken together, this reasonably detailed knowledge of the molecular pathophysiology of DM1, availability of cell and mouse preclinical models, and the exploration of several proof-of-concept therapeutic strategies have culminated in a promising drug development pipeline for DM1.

Drug development programs

Herein, we summarize existing drug development programs in DM1 encompassing advanced candidates from the preclinical stage to human clinical trials. Therapies are further classified into three broad categories of small molecules, oligonucleotide-based therapies, and gene therapies, encompassing mechanisms of action targeting from root cause disease events up to specific clinical symptoms (Fig. 1).

Small-molecule drugs

In this category, we have mainly identified repurposed pre- and investigational new drugs (INDs), which were initially developed to treat a different condition, compared with only one new chemical entity (NCE) IND with first therapeutic use in DM1. Given that drug-repurposing candidates have already demonstrated safety in humans, they can be tested in patients more quickly and at less cost and lower risk than for entirely new drugs, which explains why they are closest to reaching market authorization (Table 1).

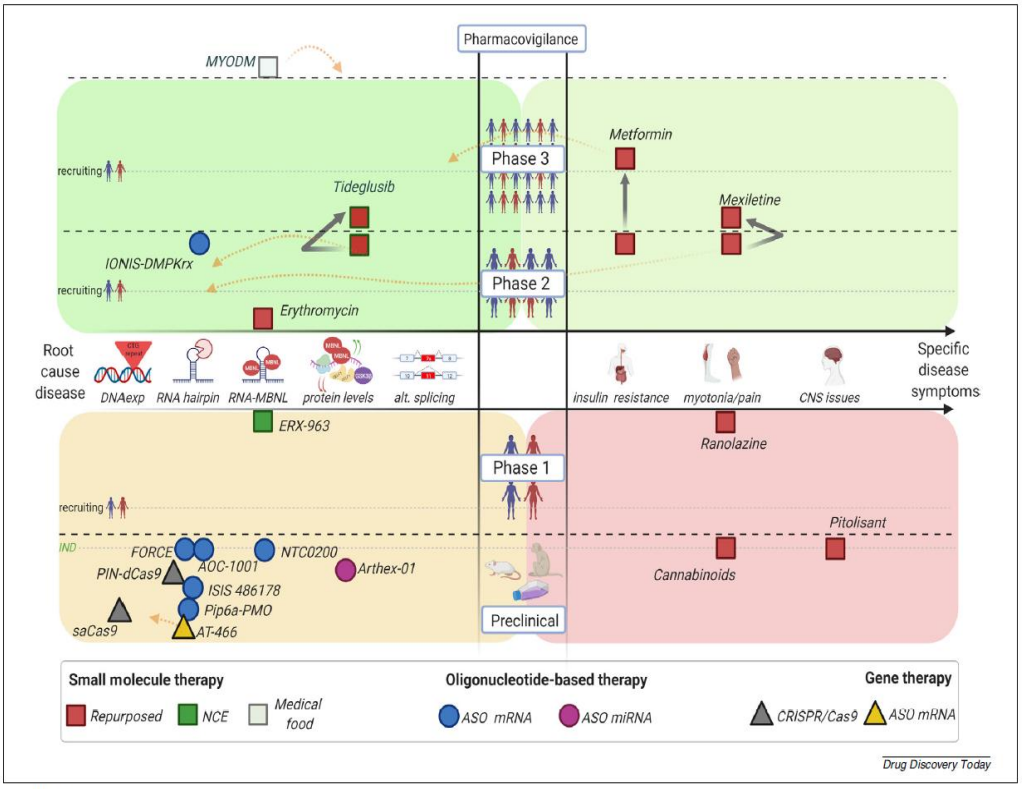

FIGURE 1

Graphical representation of the preclinical and clinical DM1 drug candidate pipeline. The middle X-axis indicates the step targeted within the DM1 physiopathological model (left side: the root causes of disease; right side: specific clinical disease symptoms), while the middle Y-axis shows the level of drug evaluation from preclinical to market authorization. Black discontinued lines separate clinical phases, while grey discontinued lines indicate human recruiting timing for each clinical phase. The green discontinued line denotes the IND milestone at the end of the preclinical evaluation phase. Drug candidates are represented in different shapes according to the type of therapy and colours for specific types of molecules. Black solid arrows display progression of those drug candidates evaluated in more than one clinical trial. Orange discontinued arrows indicate the description of additional mechanisms of action proposed for the drug candidate (see text and tables for detailed description and references) (Created with BioRender.com).

Levels of GSK3β are elevated in DM1 skeletal muscles [8], leading to the proposal that resulted in a new therapeutic target to prevent myopathy in DM1 [9]. **Tideglusib** is a marine-derived GSK3β inhibitor initially developed to treat Alzheimer's disease [10]; it causes a significant correction of GSK3β and CELF1 levels and reduces expression of toxic *DMPK* RNA in normal and congenital DM1 (CMD) myoblasts [11]. GSK3β is abnormally high in the HSA^{LR} mouse model (Box 1), and tideglusib was found to improve functional parameters, such as muscle weakness and myotonia; in the muscles and brain of the DMSXL mouse (Box 1), as a model of CMD, the drug improved postnatal survival, weight, and neuromotor activity [8,11]. These results supported the potential of tideglusib to target early events of the disease in congenital and adult forms of DM1 and on skeletal muscle and brain defects via multiple pathways. Orally administered tideglusib, developed by AMO-Pharma, has already completed Phase II trials on patients with CMD and childhood-onset DM1, where most patients reported improved CNS and clinical neuromuscular symptoms [12]. Given the favorable pharmacokinetic (PK) and clinical risk/benefit profiles, an extended Phase II/III clinical trial has been announced.

Inhibition of the interaction between the toxic CUG hairpin and the MBNL1 splicing factor is one of the most common ongoing therapeutic avenues in DM1 [13]. The most-advanced lead compounds, **MYD-0124** (erythromycin) and **ERX-963**, can bind to the hairpin RNA structure with high selectivity, decreasing foci formation and rescuing mis-splicing in DM1 cell and mouse models. A Phase II clinical trial sponsored by Osaka University Hospital is currently testing the oral administration of erythromycin in adult patients with DM1 [14], uncovered as an anti-DM1 candidate after a targeted screen for RNA-binding potential of US Food and Drug Administration (FDA)-approved antibiotics [15]. **ERX-963** is a rationally designed NCE that can strongly bind pathogenic expanded CUG repeats (CUGexp) but not normal repeats, limiting off-target effects [16] (Expansion Therapeutics, www.expansionrx.com/dm1/#research). ERX-963 recently finished a Phase I clinical trial in adult patients with DM1, according to the sponsor Expansion Therapeutics.

Among candidates targeting specific disease symptoms, the most advanced is **metformin**, a first-line agent for type 2 diabetes mellitus. The drug has been suggested to treat the insulin resistance phenotype in patients with DM1 [17] and promote alternative splicing correction via AMPK-dependent and independent mechanisms [18]. More recently, metformin was also reported to improve mitochondrial dysfunction and impaired metabolism in DM1 fibroblasts and prevent cancer risk, thus suggesting ample therapeutic benefits in DM1 [19,20]. A completed Phase II clinical trial assessed oral metformin, revealing a signif-

TABLE 1

Myotonic dystrophy drug candidates currently in clinical trials.

Phase	Clinical trial	Status	Clinical trial information	Results
Mexiletine (repurposed small molecule): Orphan Drug Designation by FDA and EMA				
III	NCT04624750	Not yet recruiting: 2020–2024	Safety, efficacy, and steady-state PK of mexiletine in paediatric patients with myotonic disorders. Sponsor: Lupin, Ltd	Not posted
II	NCT01406873	Completed: 2011–2018	Effects of mexiletine on ambulation, myotonia, muscle function, strength, pain, gastrointestinal functioning, cardiac conduction, and quality of life in DM1. Sponsor: University of Rochester	[24]
Metformin (repurposed small molecule)				
III	2018–000692-32	Ongoing: 2019–2021	Efficacy of metformin on motility and strength in DM1. Sponsor: Tor Vergata	Not posted
II	2013–001732-21	Completed: 2013–2017	Randomized, double-blind, placebo-controlled Phase II study of metformin in patients with DM1. Sponsor: Centre d'Etude des Cellules Souches/Istem	[21]
Tideglusib (repurposed small molecule): Orphan Drug Designation by FDA				
II/III	NCT03692312	Not yet recruiting: 2020–2022	Randomized, multicenter, double-blind, placebo-controlled, Phase II/III study of patients (aged 6–16 years) with congenital DM1. Sponsor: AMO Pharma Ltd	Not posted
II	NCT02858908	Completed: 2016–2018	Safety, efficacy and PK of tideglusib in treatment of adolescents and adults with congenital and juvenile-onset DM1. Sponsor: AMO Pharma Ltd	[12]
Erythromycin (repurposed small molecule)				
II	jRCT2051190069	Recruiting: 2019–ongoing	Blinded, placebo-controlled study to assess the safety, tolerability, and efficacy of MYD-0124 in adult patients with DM1. Sponsor: Hospital Osaka University	Not posted
IONIS-DMPKRx (ISIS 598769) (ASO)				
I/II	NCT02312011	Completed: 2014–2016	Safety, tolerability, and PK of multiple escalating doses of ISIS 598769 administered subcutaneously to adult patients with DM1. Sponsor: IONIS-Biogen	Not posted
ERX-963 (new chemical small molecule)				
I	NCT03959189	Completed: 2019–2020	Safety, tolerability, and potential reduction of excessive daytime sleepiness/hypersomnia and improvement of cognitive function in patients with DM1. Sponsor: Expansion Therapeutics, Inc.	Not posted
Ranolazine Ralexa™ (repurposed small molecule)				
I	NCT02251457	Completed: 2014–2017	Preliminary data to determine safety and efficacy of ranolazine on symptoms of DM1. Sponsor: Ohio State University/Gilead Sciences	[28]
Caffeine and theobromine formulation *MYODM*™ (natural compounds)				
N/A	NCT04634682	Recruiting: 2020–2021	Effect of food supplement MYODM™ on excessive daytime sleepiness and quality of life in adults with DM1. Sponsor: Myogem Health Company, S.L.	Not posted

[a]N/A, not applicable.

Box 1 Mouse preclinical models.

HSA^{LR} mouse model

The HSA^{LR} mouse model is a homozygous transgenic mouse that includes a genomic fragment of the human skeletal actin gene to express 250 CTG repeats in the 3′-UTR of the gene. It shows myotonia and muscle weakness, centrally located nuclei in muscle fibers, and alternative splicing defects [64].

DM200 mouse model

DM200 mice overexpress a GFP-DMPK 3′-UTR (CTG)200 transgene carrying a DMPK 3′-UTR with 200 CTG repeats under the control of a doxycycline-responsive human DMPK promoter. The model reproduces myotonic dystrophy cardinal features, including myotonia, cardiac conduction abnormalities, histopathology, and RNA splicing defects [65].

DMSXL mouse model

The DMSXL mouse model is a homozygous mouse line carrying >700 CTG repeats, derived from the DM300-328 transgenic mouse line, which carries 45 kb of human genomic sequence from the DM1 locus [66]. The model shows mild splicing defects but ribonuclear foci, growth retardation, histology alterations, muscle weakness and atrophy, and myotonia in skeletal muscles [67,68]. It has been proposed as a study model for brain function alterations typical of DM1 [69].

icant improvement in mobility in a 6-min walk test (6MWT) associated with an increase in total mechanical power and a suggestive effect on gait parameters [21]. These encouraging results supported a replication study in a multicenter Phase III clinical trial sponsored by the Tor Vergata University of Rome.

Additional molecules in development address the symptomatic management of DM1 features, such as myotonia, chronic pain, or daytime sleepiness, namely **mexiletine**, **ranolazine**, **cannabinoids** and **pitolisant**.

Mexiletine is an antiarrhythmic medicine used to reduce or prevent myotonia through blocking sodium channels involved in the contraction and relaxation of muscles [22]. Mexiletine was orally administered to evaluate the managing of myotonia symptoms in adult patients with DM1 in two Phase II clinical trials [23,24] that reached similar results indicating a positive effect on handgrip myotonia in ambulatory patients but no significant benefit on 6MWT. Given that mexiletine was safe over a 9-year follow-up study [25], Lupin Ltd is evaluating this drug in a Phase III clinical trial in children and adolescent patients with DM1 under the name NaMuscla, already approved for nondystrophic myotonic disorders [26]. Interestingly, mexiletine has been reported to downregulate *DMPK* mRNA levels, pointing to additional activity through the DM1 disease pathway [27].

In addition to mexiletine, ranolazine, which acts by enhancing the slow inactivation of sodium channels, is a therapeutic alternative targeting similar DM1 molecular defects, as concluded from a completed Phase I clinical trial [28].

Similar to myotonia, attempts to treat myalgia and other muscular complaints led to the evaluation of cannabinoids (**cannabidiol** and **tetrahydrocannabinol** or CBD/THC, respectively) in DM1, driven by the presence of cannabinoid receptors in muscles [29] and by modulation of both central and peripheral pain pathways [30,31]. In this regard, Nexien Biopharma recently filed a patent covering methods and compositions for treating patients with myotonic dystrophy (DM1 and DM2) with oral formulations of CBD and THC [32].

On a similar theme, pitolisant, a stimulant drug that antagonizes histamine H3 receptors to treat excessive daytime sleepiness in patients with narcolepsy, will be evaluated by Harmony Biosciences to treat the same symptom in DM1. This is a clinical hallmark in most patients and one of the most frequent non-muscular symptoms contributing to a reduced quality of life [33]. A repurposing program with a Phase II clinical trial in adult patients with DM1 was announced for the first half of 2021.

Finally, a complementary therapeutic strategy for DM1 comes from evaluating methylxanthines, natural alkaloid products commonly used as mild stimulants and bronchodilators [34]. A synergistic combination of **theobromine and caffeine** (commercialized by Myogem Health Company as **MYODM^{TM}**) showed the ability to reduce the number of foci aggregates per cell and increase MBNL1 at the transcript and protein level [35,36]. Data on *Drosophila* DM1 flies rendered a recovery of survival and cardiac and locomotor dysfunction (M. Pascual-Gilabert et al., unpublished 2021). The clinical trial NCT04634682 is evaluating the effect of MYODM^{TM} on the quality of life, fatigue, and hypersomnia in adult patients with DM1 as a novel nutritional management strategy. MYODM^{TM} is expected to be upgraded into a food for special medical purposes or medical food [European Food Safety Authority (EFSA) and FDA classification, respectively, for foods specifically intended for the nutritional management of patients, under medical supervision].

Oligonucleotide-based therapeutics

RNA-based therapies can target any gene in the genome and, thus, are particularly well suited to address the diversity among rare genetic diseases. The promise of oligonucleotide-based drugs is starting to be realized with the increasing pace at which these drugs reach authorization, with at least ten already approved for clinical use [2]. In DM1, chief approaches have been designing antisense oligonucleotides (ASOs) able to degrade *DMPK* transcripts via the activation of RNase-H machinery in the nucleus (i.e., gapmers) or able to prevent MBNL1 sequestration by CUG repeats (or displace prebound MBNL1 proteins) by an occupancy-based mechanism (i.e., mixmers) (reviewed in [37]). In contrast to their high specificity, oligonucleotide-based therapies suffer from poor delivery to the muscle tissues, which, in the case of DM1, is further aggravated because cells do not display compromised membrane integrity that could facilitate cell uptake [38].

Screening of >3000 ASOs led IONIS to identify the **IONIS-DMPKRx** (ISIS 598769) gapmer-type candidate; the first and still single ASO evaluated in a clinical trial for the treatment of DM1 [39,40]. IONIS-DMPKRx produces significant *DMPK* mRNA degradation through base pairing with a specific 3′-UTR gene sequence outside the repeat tract. The drug did not generate safety concerns, being well tolerated even at the highest dose tested (600 mg) (Myotonic Dystrophy Foundation Annual Conference; www.myotonic.org/digital-academy/ionis-pharmaceuticals-industry-updates-drug-development-2018-mdf-annual-conference). Still, IONIS concluded that insufficient drug reached the muscles (Muscular Dystrophy Association, Ionis Reports Set-

back on DMPKRx Program for Myotonic Dystrophy, https://strongly.mda.org/ionis-reports-setback-dmpkrx-program-myotonic-dystrophy) to elicit a significant therapeutic response on the functional and biological end-points set up in the trial (Table 1). The company announced a program to enhance the potency of future DM1 candidates in the skeletal and heart muscle through Ligand-Conjugated Antisense (LICA) technology [41].

IONIS also reported preclinical data for **ISIS 486178** (Table 2), a different gapmer ASO designed to target the 3′-UTR region of the *hDMPK* transcript [42]. In cynomolgus monkeys, ISIS 486178 reduced *DMPK* expression by 70% in several muscles and 50% in cardiac tissue, with no skeletal muscle or cardiac toxicity after 13 weeks [42]. Importantly, this was also the first ASO reported to reach the heart with demonstrated benefit on molecular and functional alterations in the cardiac conduction defects of the DM200 mouse model (Box 1), thus suggesting ASOs as a viable option for treating cardiac pathology in DM1 [43]. This molecule also showed relevant activity in the DMSXL mouse model in terms of body-weight increase, muscle strength, and muscle histology, whereas no overt toxicity was detected [44].

Conjugation of therapeutic ASOs to carrier delivery systems, such as peptides or antibodies, is an active field to enhance their skeletal muscles and heart uptake. In this regard, **AOC1001** is a conjugate of a small interfering (si)RNA degrading *DMPK* with a proprietary monoclonal antibody against the transferrin receptor 1 (TfR1) protein. Developed by Avidity Biosciences, the company recently stated that preclinical results confirmed the ability of AOC1001 to deliver siRNAs to muscle cells, boosting the reduction in *DMPK* mRNA levels in a durable and dose-dependent manner (Avidity Biosciences, www.aviditybiosciences.com/programs). Although details regarding the level of *DMPK* reduction, *in vivo* drug administration route, doses, or drug safety for AOC1001 have not been disclosed, Avidity has announced a Phase I/II clinical trial by the end of 2021 (Table 2).

Using a similar approach, Dyne Therapeutics is developing the **FORCE-DMPK ASO candidate**, which involves the conjugation of a proprietary ASO designed to reduce *DMPK* RNA

TABLE 2

Myotonic dystrophy drug candidates in preclinical stages of development.

Preclinical status	Preclinical information	Refs
Cannabinoids CBD/THC (small molecule/repurposing)		
IND-enabling phase	Management of chronic neuropathic pain, myotonia, and myalgia. Pilot survey in DM1, DM2, and congenital myotonia, with two patients per disease. Sponsor: Nexien Biopharma (https://nexienbiopharma.com/news)	[30–32]
Pitolisant; Wakix (small molecule/repurposing)		
IND-enabling phase: Phase II by 2021	Orally available to and able to cross the blood–brain barrier in animal models. Sponsor: Harmony Biosciences (www.harmonybiosciences.com/science)	[57]
ISIS 486178 (oligonucleotide-based therapy)		
Lead optimization	Systemic treatment resulting in myotonia and cardiac conduction improvement, correction of splicing defects, reduction in RNA foci, and redistribution of MBNL1. DMSXL and DM200 mice models. Sponsor: Ionis Pharmaceuticals	[43,44,58,59]
AOC1001 (oligonucleotide-based therapy)		
IND enabling phase: Phase I/II by 2021	Delivered to muscle cells reduces levels of *DMPK* mRNA in a durable, dose-dependent manner. AOC™ platform. Sponsor: Avidity Biosciences (www.aviditybiosciences.com/)	[60]
FORCE-DMPK candidate (oligonucleotide-based therapy)		
IND enabling phase	*In vivo* dose-dependent correction of splicing and myotonia in the HSA^{LR} model after single low dose. *DMPK* mRNA reduction in mouse and nonhuman primates. FORCE™ platform. Sponsor: Dyne Therapeutics (www.dyne-tx.com/pipeline/)	[61]
Pip6a-PMO (oligonucleotide-based therapy)		
Lead optimization	Intravenously injected in HSA^{LR}. Nuclear foci reduction, MBNL1 redistribution, splicing, and myotonia correction. Sponsor: Oxford University	[45]
NTC0200 (oligonucleotide-based therapy)		
IND enabling phase	*In vitro* ability to target and open up aberrant DM1-linked secondary RNA structure in mutant transcript, thereby displacing sequestered splice proteins. PATrOL™ platform. Sponsor: Neubase (www.neubasetherapeutics.com/pipeline/)	[46]
Arthex-01 (oligonucleotide-based therapy)		
Lead optimization: start of Phase I/II by 2022	Subcutaneous injection in HSA^{LR}. Enhanced MBNL1/2 protein levels, recovered missplicing, muscle strength, and myotonia. Long-lasting activity of antagomiR-23b for up to 45 days. Sponsor: Arthex Biotech (https://arthexbiotech.com/pipeline/)	[49,50]
AAV-PIN-dCas9 (gene therapy)		
Lead optimization: start of IND-enabling studies by 2021	Intramuscular/systemic delivery in adult and neonatal HSA^{LR}. RNA-targeting Cas9 lasted for up to 3 months. Elimination of RNA foci, reversal of splicing biomarkers, and myotonia. Sponsor: Locanabio (https://locanabio.com/pipeline/)	[56,62]
AT-466 (gene therapy)		
Lead optimization	AAV delivery to overcome the biodistribution limitations of ASO-based therapies. Sponsor: Audentes (www.audentestx.com/our-approach/)	[63]
AAV-CRISPR-Cas9 (gene therapy)		
Preclinical proof of concept	*In vivo* genome editing for DM1: deletion of CTG repeat tract leading to reduction in nuclear foci in muscle fibres after intramuscular injection of SaCas 9 and sgRNA rAAV9 vectors in DMSXL mice. Sponsor: Genethon-INSERM	[55]

POST-SCREEN (GREY)

levels in the nucleus by RNase H cleavage with a TfR1-binding Fab for effective delivery to muscles. According to Dyne's preclinical results in mouse models, the FORCE-DMPK candidate reached potent *Dmpk* RNA knockdown in skeletal muscles (70–78%) and durable ASO muscle distribution on its proprietary wild-type hTfR1 mouse (up to 12 weeks) as well as in nonhuman primates. *Dmpk* knockdown was also observed in the heart, with an acceptable toxicity profile for blood, kidney, and liver function parameters. For therapeutic parameters, the FORCE candidate rescued mis-splicing and myotonia to near-normal levels after a single low dose in HSA^{LR} mice within 14 days after administering a single low dose (R. Subramanian et al., unpublished 2021).

An alternative to enhance muscle and heart uptake of ASOs is conjugation with cell-penetrating peptides (CPPs). The **Pip6a-PMO-CAG7**, a conjugate that combines a CPP moiety (Pip6a) and an ASO with morpholino chemistry-targeting repeats, promotes an occupancy-based mechanism for MBNL1 displacement from the toxic CUG repeat. *In vivo*, CPP-conjugated CAG7 PMO displayed significant improvement of oligonucleotide delivery into the striated muscles of HSA^{LR} mice following systemic administration, which promoted reversion of myotonia and splicing defects that were long-lasting and involved most of the altered transcriptome [45].

The **NTC0200** (or Compound A) preclinical candidate developed by NeuBase Therapeutics explores a peptide-nucleic acid ASO identified through the proprietary platform PATrOL™. The resultant compound is a short, flexible, and selective peptide-nucleic acid sequence proved *in vitro* to discriminate the pathogenic CUG expansions from the wild-type transcripts, opening up CUG RNA secondary structures in the mutant transcript and displacing sequestered MBNL proteins [46]. Recent communication from the company on social networks announced long-lasting correction of global levels of mis-spliced transcripts in the HSA^{LR} transgenic mouse after a well-tolerated single intravenous injection.

There is ample evidence that limited MBNL protein function contributes to DM1. Given that the endogenous genes remain normal in patients with DM1, a therapeutic gene modulation approach to compensate for their depletion appears feasible. Building upon previous proof-of-concept reports [47,48], this therapeutic avenue is being tested based on the identification of miR-23b and miR-218 as natural endogenous translational repressors of *MBNL1/2* genes. Treatment with antimiRs, a class of chemically engineered ASOs complementary to their cognate target miRNA, significantly upregulated MBNL1/2 protein levels and improved molecular defects in cell models and functional muscle defects in the HSA^{LR} mouse model. Additionally, no safety concerns were observed in treatments *in vivo* [49,50]. ARTHEx Biotech is working on the preclinical evaluation of the antimiR lead candidate **(Arthex-01)** against miR-23b or miR-218 with planned clinical trials by the beginning of 2022 (Table 2).

Gene therapy approaches

The most straightforward approach to overcome the limited biodistribution of AONs is to promote its endogenous expression using gene therapy vectors. Adeno-associated viruses (AAVs) are being evaluated for drug development in several muscular dystrophies [51], and with recent success with Zolgensma gene replacement approach in SMA. Benefits of this avenue in DM1 were initially disclosed with a proof-of-concept approach for an AAV-delivered RNAi in the HSA^{LR} mice [52]. Audentes is taking a similar approach with the AT466 candidate, a vectorized ASO-like design targeting the reduction of toxic *DMPK* RNA levels in cells from patients with DM1 by RNA degradation or exon skipping (or both simultaneously) mechanisms of action, both clinically already validated in studies with ASOs (Audentes, www.audentestx.com/innovative-therapies). Preclinical studies are underway to determine the optimal construct for AT466 (Table 2).

CRISPR/Cas9 genetic scissors have also emerged as innovative approaches to treat DM1 by targeting the removal of the expansions at the DNA level, preventing their transcription, or targeting degradation of the toxic RNA, therefore effectively targeting the underlying DM1 etiology [53,54]. The fundamental requirement for genome editing by CRISPR is a purified Cas9 nuclease and a guide RNA (gRNA) targeting the desired genome sequence with an adjacent protospacer motif (PAM sequence). The endonuclease cuts both strands of the chromosomal DNA, making genome modifications possible through nonhomologous end joining or homologous-driven repair.

More recently, CRISPR-Cas9 has emerged as a multifunctional platform for sequence-specific gene expression regulation. Engineered nuclease-deficient Cas9, termed dCas9, when fused to effector domains with distinct regulatory functions, enables the CRISPR-Cas9 system to be repurposed as a general platform for RNA-guided DNA targeting without cleavage activity. Two approaches (Table 2) are moving through preclinical stages, making use of vectorized AAV administration.

The approach reported by Lo Scrudato et al. [55] at Généthon demonstrated the excision of long CTG repeats and reduced pathological RNA foci within tibialis anterior muscle in the DMSXL mouse model after a single intramuscular injection of recombinant AAV vectors expressing CRISPR-SaCas9 components.

On a related approach, but targeting the toxic RNA molecules, Locanabio is developing AAV-vectors encoding PIN-dCas9 (a dCas9 fused to the PIN RNA endonuclease) and a single-gRNA targeting CUG repeats, which, in intramuscular and systemic administration in adult and neonatal HSA^{LR} mice, showed up to 3 months of expression of the RNA-targeting Cas9. The treatment eliminated RNA foci, redistributed the MBNL1 factor, reversed dysregulated splicing biomarkers and transcriptional profiles, and ameliorated myotonia in skeletal muscle without significant toxicity signs, thus demonstrating potential clinical utility [56]. Future studies in large animals will be needed to assess further the safety, dose levels, and immunosuppression regimens required for safe and long-term treatments.

Concluding remarks and future challenges

We anticipate that patients with DM1 will have at least one therapeutic option targeting the genetic cause of the disease within the next few years. To reach this goal, it is imperative to maintain ongoing close collaboration between the academic and pharma-

ceutical sectors and to keep developing new and better preclinical models for hypothesis testing and drug evaluation. In this regard, it is vital to develop human 3D skeletal muscle models using tissue engineering to overcome the numerous limitations of 2D cultured myoblasts. These bioengineered microtissues would better recapitulate structural and molecular DM1 phenotypes as the gene expression changes associated with the CUG toxic RNA, and provide platforms to test drug activity and uptake under conditions closer to *in vivo*. Additional mammal models outside the murine paradigm would also significantly speed up preclinical validation in support for clinical programs. Overall, over the past decade, the DM1 community has smoothly transitioned from basic to translational research with the common goal of improving the lives of patients.

Note added in proofs

Since the information described in this paper was completed and accepted for publication, Expansion Therapeutics has disclosed the name of the **ERX-963** lead compound as flumazenil (online talk: https://youtu.be/Nf7AUdTvC9k). Instead of a new chemical entity able to bind the CUG expanded repeat, as described through the paper and suggested on the previous limited available data, **ERX-963** is a repurposed drug targeting the GABA receptor in the brain that showed no significant improvement in DM1 daytime sleepiness through the NCT03959189 clinical trial.

Declaration of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: R.A. is an inventor in patent PCT/EP2017/073685, currently licensed to Arthex Biotech, of which he is a co-founder and scientific consultant. R.A. and M.P-G. are inventors in patents WO2016075285A1 and WO2016075288A1, currently licensed to Myogem Health Company. M.P-G. is a former member of the Board of Directors and shareholder of Myogem Health Company. A.L-C. is a member of the Arthex Board of Directors.

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ANNEX 5: Supplementary material

1. Individualized Neuromuscular Quality of Life (INQoL)

In 2007, researchers at King's College (London)[484] developed a specific scale for muscle disease patients, named as Individualized Neuromuscular Quality of Life (INQoL). INQoL is the HRQoL questionnaire that assesses the health-related quality of life of adults with neuromuscular diseases (NMD).

INQoL consists of 45 questions within 10 sections, scheduled as follows:

Table 116: Structure of questions and scores in INQoL

Section	Impact	Scores
1	Key muscle disease symptoms	Weakness
2		Myotonia
3		Pain
4		Fatigue
5	Particular areas of life	Activities
6		Independence
7		Relationships
8		Emotions
9		Body image
10	Effects of treatment	-

Responses to items looking at symptoms and the impact of NMD on life domains are weighted by the perceived importance of the item, assigned by the participant. The final score is presented as a percentage of the maximum detrimental impact. Therefore, a higher percentage indicates greater impact.[484]

The questionnaire has been used as a quality of life outcome measure in several muscular diseases, including but not limited to, myotonic dystrophy type 1.[485,486]

The Spanish version of the questionnaire has been properly validated.[487] According to authors,[487] it takes approximately fifteen minutes to complete the questionnaire.

2. SF-36 Health Questionnaire

The Short Form-36 Health Survey (SF-36) was already disclosed in the previous observational study. SF-36 is considered an ideal instrument for assessing Health-Related Quality of Life (QoL) in general, and in DM1 in particular, since it is a quick instrument, easy to administer, with validation studies in Spain, including Spanish population norms. Reference is made to Chapter 4 herein.

3. Epworth sleepiness scale (ESS)

Impaired sleep or excessive daytime sleepiness was scored by DM1 patients as the fifth most limiting symptom in their quality of life by patients themselves in a DM1 survey.[488]

Symptoms related to daytime sleepiness include tiredness, weariness or difficulties concentrating or paying attention during the day, need or desire to sleep during the day, sudden sleep attacks, and is perceived by DM1 patients among the most important limiting aspects in their quality of life. Patients suffering excessive daytime sleepiness present higher levels of depression, tend to be less sociable, active, empathetic, motivated and have lower quality of life. Accordingly, their employment rate and economic resources could be seriously impacted.

The Epworth Sleepiness Scale was developed in 1991 as a short questionnaire that attempts to measure daytime sleepiness.[489] The researched subject is asked about the frequency (or probability) of falling asleep on a scale of increase in a four levels scales ranging from 0 to 3 in a Likert scale (0 = would *never* doze, 1 = *slight* chance of dozing, 2 = *moderate* change of dozing, 3 = *high* chance of dozing), for eight different daily situations, that most people can be involved in, in daily life, though not necessarily every day.

The score of the eight situations is added to obtain a total score ranging from 0 to 24. A higher score is correlated to a major somnolence impact on daily life. A result between 0 and 6 is considered normal, 7-8 average sleepiness, 9-24 abnormal sleepiness (probably pathological).

The Spanish version of the questionnaire has been properly validated.[490]

Epworth Sleepiness Scale

Name: _____ Today's date: _____

Your age (Yrs): _____ Your sex (Male = M, Female = F): _____

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired?

This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently try to work out how they would have affected you.

Use the following scale to choose the **most appropriate number** for each situation:

0 = would **never** doze
1 = **slight chance** of dozing
2 = **moderate chance** of dozing
3 = **high chance** of dozing

It is important that you answer each question as best you can.

Situation	**Chance of Dozing (0-3)**
Sitting and reading	
Watching TV	
Sitting, inactive in a public place (e.g. a theatre or a meeting)	
As a passenger in a car for an hour without a break	
Lying down to rest in the afternoon when circumstances permit	
Sitting and talking to someone	
Sitting quietly after a lunch without alcohol	
In a car, while stopped for a few minutes in the traffic	

THANK YOU FOR YOUR COOPERATION

Figure 87: The ESS Questionnaire

4. DM1- ACTIV$^{C©}$ Questionnaire

It is a 25-item questionnaire developed in 2010[491] and improved in 2015[492] at Maastricht University Medical Centre, which covers a wide range of daily and social activities. The DM1-ACTIV$^{C©}$ captures the personal ability to perform a task where the patient is asked to determine if he / she can complete that task independently. Interestingly, the scale is able to differentiate among various levels of disability. The questionnaire is scored in a 0–100 interval range (a higher score indicates higher capacity). According to the authors, it takes approximately 3 minutes to complete the questionnaire, which is disclosed in **Figure 88** (extracted from reference [492]).

	Item	Impossible to perform (0)	Able to perform, but with difficulty (1)	Able to perform, without difficulty (2)
	Are you able to...			
1	eat soup?	☐	☐	☐
2	visit family or friends?	☐	☐	☐
3	care for your hair and body?	☐	☐	☐
4	dress your lower body?	☐	☐	☐
5	wash your upper body?	☐	☐	☐
6	take a shower?	☐	☐	☐
7	wash your lower body?	☐	☐	☐
8	get out of bed?	☐	☐	☐
9	move a chair?	☐	☐	☐
10	do the dusting/cleaning?	☐	☐	☐
11	do the shopping?	☐	☐	☐
12	tie the laces of your shoes?	☐	☐	☐
13	catch an object (e.g. a ball)?	☐	☐	☐
14	use dustpan and brush?	☐	☐	☐
15	empty dustbin?	☐	☐	☐
16	make up your bed?	☐	☐	☐
17	vacuum clean?	☐	☐	☐
18	serve coffee/tea on a tray?	☐	☐	☐
19	dance?	☐	☐	☐
20	stand up from squatting position?	☐	☐	☐
21	stand on one leg?	☐	☐	☐
22	walk uphill?	☐	☐	☐
23	walk 3 flights of stairs?	☐	☐	☐
24	carry and put down heavy object (10 kg)?	☐	☐	☐
25	run?	☐	☐	☐

Figure 88: The 25-item DM1- ACTIV^{C}© Questionnaire

The questionnaire demonstrated its validity and utility in the OPTIMISTIC EU FP7 Project.[493]

5. Modified Fatigue Impact Scale (MFIS)

Measuring fatigue may be complicated because of the subjectivity and multidimensionality of the symptom. Self-report instruments ask people to describe and rate their fatigue. Among many possible instruments,[494] the present investigators chose the Modified Fatigue Impact Scale (MFIS), a derived and reduced adaptation of the Fatigue Impact Scale (FIS)[464] that assesses the effects of fatigue in the time frame of the last month.

The MFIS consists of 21 items distributed in 3 subscales: physical, cognitive and psychosocial. The score is obtained by adding the individual score for each item, which presents 5 possible responses ranging from 0 = no problem to 4 = extreme problem (Likert scale). The final score ranges from 0 to 84 (maximum total score), with higher scores reflecting more severe fatigue. A score of 38 points is established as the cut-off point to define the presence of fatigue or not. Administration time is approximately 5-10 minutes for the full-length version. **Figure 89** and **Figure 90** disclose the instructions and items, respectively.

Modified Fatigue Impact Scale (MFIS)

Fatigue is a feeling of physical tiredness and lack of energy that many people experience from time to time. But people who have medical conditions like MS experience stronger feelings of fatigue more often and with greater impact than others.

Following is a list of statements that describe the effects of fatigue. Please read each statement carefully, the circle the one number that best indicates how often fatigue has affected you in this way during the past 4 weeks. (If you need help in marking your responses, tell the interviewer the number of the best response.) Please answer every question. If you are not sure which answer to select choose the one answer that comes closest to describing you. Ask the interviewer to explain any words or phrases that you do not understand.

Because of my fatigue during the past 4 weeks

Instructions for Scoring the MFIS
Items on the MFIS can be aggregated into three subscales (physical, cognitive, and psychosocial), as well as into a total MFIS score. All items are scaled so that higher scores indicate a greater impact of fatigue on a person's activities.

Physical Subscale
This scale can range from 0 to 36. It is computed by adding raw scores on the following items: 4+6+7+10+13+14+17+20+21. _____0

Cognitive Subscale
This scale can range from 0 to 40. It is computed by adding raw scores on the following items: 1+2+3+5+11+12+15+16+18+19. _____0

Psychosocial Subscale
This scale can range from 0 to 8. It is computed by adding raw scores on the following items: 8+9. _____0

Total MFIS Score
The total MFIS score can range from 0 to 84. It is computed by adding scores on the physical, cognitive, and psychosocial subscales. _____0

Figure 89: The 21-item Modified Fatigue Impact Scale (MFIS) Questionnaire. Instructions for patients and scoring.

		Never	Rarely	Sometimes	Often	Almost Always
1.	I have been less alert.	0	1	2	3	4
2.	I have had difficulty paying attention for long periods of time.	0	1	2	3	4
3.	I have been unable to think clearly.	0	1	2	3	4
4.	I have been clumsy and uncoordinated.	0	1	2	3	4
5.	I have been forgetful.	0	1	2	3	4
6.	I have had to pace myself in my physical activities.	0	1	2	3	4
7.	I have been less motivated to do anything that requires physical effort.	0	1	2	3	4
8.	I have been less motivated to participate in social activities.	0	1	2	3	4
9.	I have been limited in my ability to do things away from home.	0	1	2	3	4
10.	I have trouble maintaining physical effort for long periods.	0	1	2	3	4
11.	I have had difficulty making decisions.	0	1	2	3	4
12.	I have been less motivated to do anything that requires thinking	0	1	2	3	4
13.	My muscles have felt weak	0	1	2	3	4
14.	I have been physically uncomfortable.	0	1	2	3	4
15.	I have had trouble finishing tasks that require thinking.	0	1	2	3	4
16.	I have had difficulty organizing my thoughts when doing things at home or at work.	0	1	2	3	4
17.	I have been less able to complete tasks that require physical effort.	0	1	2	3	4
18.	My thinking has been slowed down.	0	1	2	3	4
19.	I have had trouble concentrating.	0	1	2	3	4
20.	I have limited my physical activities.	0	1	2	3	4
21.	I have needed to rest more often or for longer periods.	0	1	2	3	4

Figure 90: The 21-item Modified Fatigue Impact Scale (MFIS) Questionnaire

6. Test of the 90 symptoms (Symptom checklist 90 R)

The Symptom Checklist-90-Revised (SCL-90-R)[495] is a 90-item self-report symptom inventory developed in the mid-1970s[496,497] (from a symptom list from Johns Hopkins University HSCL, to measure psychological symptoms and psychological distress.

It is a tool developed to evaluate patterns of symptoms present in individuals and can be used both in community tasks and in clinical diagnosis. It evaluates the: somatizations, obsessions and compulsions, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation and psychoticism.

The SCL-90-R assesses psychological distress in terms of nine primary symptom dimensions and three summary scores termed global scores (**Table 117**, adapted from Derogatis *et al.*[498]).

Table 117: Dimensional structure of SCL-90-R questionnaire

Measures	Concepts	Number of items	Item positions in the SCL-90-R
Symptom dimensions	Somatization (SOM)	12	1, 4, 12, 27, 40, 42, 48, 49, 52, 53, 56, 58
	Obsessive-Compulsive (OBS)	10	3, 9, 10, 28, 38, 45, 46, 51, 55, 65
	Interpersonal Sensitivity (INT)	9	6, 21, 34, 36, 37, 41, 61, 69, 73
	Depression (DEP)	13	5, 14, 15, 20, 22, 26, 29, 30, 31, 32, 54, 71, 79
	Anxiety (ANX)	10	2, 17, 23, 33, 39, 57, 72, 78, 80, 86
	Hostility (HOS)	6	11, 24, 63, 67, 74, 81
	Phobic Anxiety (PHOB)	7	13, 25, 47, 50, 70, 75, 82
	Paranoid Ideation (PAR)	6	8, 18, 43, 68, 76, 83
	Psychoticism (PSY)	10	7, 16, 35, 62, 77, 84, 85, 87, 88, 90
	Additional items	7	19, 44, 59, 60, 64, 66, 89
Global measures	Global Severity Index (GSI)		
	Positive Symptom Distress Index (PSDI)		
	Positive Symptom Total (PST)		

Each of the 90 items that make it up is answered on the basis of physiological distress in the last 7 days, scored on a five-point scale (0-4, Likert scale), wherein (0 = «not at all»; 1 = «a little bit»; 2 = «moderately»; 3 = «quite a bit»; 4 = «extremely»). For each dimension, the score is the mean from its constitutive items. Followed by a mathematical analysis, a person with a T score above 65 is considered at risk, whereas a pathological situation is scored above 80.[499] The exact items comprised in SCL-90-R are listed below (patients were requested to score each one according to the Likert scale from 0 to 4, as previously explained):

1. Headaches
2. Nervousness or shakiness inside
3. Unwanted thoughts, words, or ideas that won't leave your mind
4. Faintness or dizziness
5. Loss of sexual interest or pleasure
6. Feeling critical of others
7. The idea that someone else can control your thoughts
8. Feeling others are to blame for most of your troubles
9. Trouble remembering things
10. Worried about sloppiness or carelessness
11. Feeling easily annoyed or irritated
12. Pains in heart or chest
13. Feeling afraid in open spaces or on the streets
14. Feeling low in energy or slowed down
15. Thoughts of ending your life
16. Hearing words that others do not hear
17. Trembling
18. Feeling that most people cannot be trusted
19. Poor appetite
20. Crying easily
21. Feeling shy or uneasy with the opposite sex
22. Feeling of being trapped or caught
23. Suddenly scared for no reason
24. Temper outbursts that you could not control
25. Feeling afraid to go out of your house alone
26. Blaming yourself for things
27. Pains in lower back
28. Feeling blocked in getting things done
29. Feeling lonely
30. Feeling blue
31. Worrying too much about things
32. Feeling no interest in things
33. Feeling fearful
34. Your feelings being easily hurt
35. Other people being aware of your private thoughts
36. Feeling others do not understand you or are unsympathetic
37. Feeling that people are unfriendly or dislike you
38. Having to do things very slowly to insure correctness
39. Heart pounding or racing
40. Nausea or upset stomach
41. Feeling inferior to others
42. Soreness of your muscles
43. Feeling that you are watched or talked about by others
44. Trouble falling asleep
45. Having to check and double-check what you do
46. Difficulty making decisions
47. Feeling afraid to travel on buses, subways, or trains
48. Trouble getting your breath
49. Hot or cold spells
50. Having to avoid certain things, places, or activities because they frighten you
51. Your mind going blank
52. Numbness or tingling in parts of your body

53. A lump in your throat
54. Feeling hopeless about the future
55. Trouble concentrating
56. Feeling weak in parts of your body
57. Feeling tense or keyed up
58. Heavy feelings in your arms or legs
59. Thoughts of death or dying
60. Overeating
61. Feeling uneasy when people are watching or talking about you
62. Having thoughts that are not your own
63. Having urges to beat, injure, or harm someone
64. Awakening in the early morning
65. Having to repeat the same actions such as touching, counting, washing
66. Sleep that is restless or disturbed
67. Having urges to break or smash things
68. Having ideas or beliefs that others do not share
69. Feeling very self-conscious with others
70. Feeling uneasy in crowds, such as shopping or at a movie
71. Feeling everything is an effort
72. Spells of terror or panic
73. Feeling uncomfortable about eating or drinking in public
74. Getting into frequent arguments
75. Feeling nervous when you are left alone
76. Others not giving you proper credit for your achievements
77. Feeling lonely even when you are with people
78. Feeling so restless you couldn't sit still
79. Feelings of worthlessness
80. Feeling that familiar things are strange or unreal
81. Shouting or throwing things
82. Feeling afraid you will faint in public
83. Feeling that people will take advantage of you if you let them
84. Having thoughts about sex that bother you a lot
85. The idea that you should be punished for your sins
86. Feeling pushed to get things done
87. The idea that something serious is wrong with your body
88. Never feeling close to another person
89. Feelings of guilt
90. The idea that something is wrong with your mind

7. Global Physical Activity Questionnaire (GPAQ)

The Global Physical Activity Questionnaire (GPAQ) was developed under the auspices of the World Health Organization (WHO) in 2002 as part of the WHO STEPwise Approach to Chronic Disease Risk Factor Surveillance (STEPS).[500]

To quantify levels of physical activity in the adult population, WHO developed a Global Physical Activity Questionnaire (GPAQ)[501], which helps countries monitor insufficient physical activity as one of the main factors risk of suffering from non-communicable diseases (see **Figure 91**, extracted from reference [501]). The GPAQ has been integrated into the WHO "progressive" approach, which is applied to the surveillance of the main risk factors for non-communicable diseases.

GPAQ comprises 16 questions grouped to capture physical activity undertaken in different behavioural domains, these are work, transport and discretionary (also known as leisure or recreation)[500] subdivided into sedentary, vigorous intensity and moderate intensity, based on a typical week.

GPAQ

Physical Activity			
Next I am going to ask you about the time you spend doing different types of physical activity in a typical week. Please answer these questions even if you do not consider yourself to be a physically active person. Think first about the time you spend doing work. Think of work as the things that you have to do such as paid or unpaid work, study/training, household chores, harvesting food/crops, fishing or hunting for food, seeking employment. *[Insert other examples if needed].* In answering the following questions 'vigorous-intensity activities' are activities that require hard physical effort and cause large increases in breathing or heart rate, 'moderate-intensity activities' are activities that require moderate physical effort and cause small increases in breathing or heart rate.			
Questions		**Response**	**Code**
Activity at work			
1	Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like *[carrying or lifting heavy loads, digging or construction work]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 4*	P1
2	In a typical week, on how many days do you do vigorous-intensity activities as part of your work?	Number of days ⌊__⌋	P2
3	How much time do you spend doing vigorous-intensity activities at work on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P3 (a-b)
4	Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking *[or carrying light loads]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 7*	P4
5	In a typical week, on how many days do you do moderate-intensity activities as part of your work?	Number of days ⌊__⌋	P5
6	How much time do you spend doing moderate-intensity activities at work on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P6 (a-b)
Travel to and from places			
The next questions exclude the physical activities at work that you have already mentioned. Now I would like to ask you about the usual way you travel to and from places. For example to work, for shopping, to market, to place of worship. [insert other examples if needed]			
7	Do you walk or use a bicycle (*pedal cycle*) for at least 10 minutes continuously to get to and from places?	Yes 1 No 2 *If No, go to P 10*	P7
8	In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?	Number of days ⌊__⌋	P8
9	How much time do you spend walking or bicycling for travel on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P9 (a-b)
Recreational activities			
The next questions exclude the work and transport activities that you have already mentioned. Now I would like to ask you about sports, fitness and recreational activities (leisure). [insert relevant terms].			
10	Do you do any vigorous-intensity sports, fitness or recreational (*leisure*) activities that cause large increases in breathing or heart rate like *[running or football,]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 13*	P10
11	In a typical week, on how many days do you do vigorous-intensity sports, fitness or recreational (*leisure*) activities?	Number of days ⌊__⌋	P11
12	How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P12 (a-b)
13	Do you do any moderate-intensity sports, fitness or recreational (*leisure*) activities that causes a small increase in breathing or heart rate such as brisk walking,(*cycling, swimming, volleyball*)for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P16*	P13
14	In a typical week, on how many days do you do moderate-intensity sports, fitness or recreational (*leisure*) activities?	Number of days ⌊__⌋	P14
15	How much time do you spend doing moderate-intensity sports, fitness or recreational (*leisure*) activities on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P15 (a-b)
Sedentary behaviour			
The following question is about sitting or reclining at work, at home, getting to and from places, or with friends including time spent [sitting at a desk, sitting with friends, travelling in car, bus, train, reading, playing cards or watching television], but do not include time spent sleeping. *[INSERT EXAMPLES] (USE SHOWCARD)*			
16	How much time do you usually spend sitting or reclining on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs min s	P16 (a-b)

Figure 91: The 16-item Global Physical Activity Questionnaire (GPAQ)

8. Food consumption frequency questionnaire (short CFCA)

Dietary intake assessment is complementary information that could be monitored along the present trial.

Behind the interest on measurements of the effect of nutritional pattern on health issues in the studied population, patients should not modify their diet, including the consumption of food containing chocolate or cocoa extracts, energy drinks or infusions containing caffeine or other stimulants during the trial in order to avoid interferences, overdose and mistakes in the interpretation of results.

Accordingly, patients were asked to keep a daily feeding log based on the semi-quantitative questionnaire on the frequency of food consumption (FFQ) validated in children.[502]

The Food Frequency Questionnaire consists of a list of foods and the frequency in which these patients' adherence to the food consumption regimen (see **Figure 92** in the next page).

ANEXO I. Cuestionario de Frecuencia de Consumo Alimentario - CFCA		
LISTADO DE ALIMENTOS	**¿CUÁNTAS VECES COME?**	
	A LA SEMANA	**AL MES**
Leche		
Yogur		
Chocolate: tableta, bombones, "Kit Kat", "Mars"…		
Cereales inflados de desayuno ("Corn-Flakes", "Kellog's")		
Galletas tipo "maría"		
Galletas con chocolate, crema…		
Magdalenas, bizcocho…		
Ensaimada, donut, croissant…		
	A LA SEMANA	**AL MES**
Ensalada: lechuga, tomate, escarola…		
Judías verdes, acelgas o espinacas		
Verduras de guarnición: berenjena, champiñones		
Patatas al horno, fritas o hervidas		
Legumbres: lentejas, garbanzos, judías…		
Arroz blanco, paella		
Pasta: fideos, macarrones, espaguetis…		
Sopas y cremas		
	A LA SEMANA	**AL MES**
Huevos		
Pollo o pavo		
Ternera, cerdo, cordero (bistec, empanada,…)		
Carne picada, longaniza, hamburguesa		
Pescado blanco: merluza, mero,…		
Pescado azul: sardinas, atún, salmón,…		
Marisco: mejillones, gambas, langostinos, calamares,…		
Croquetas, empanadillas, pizza		
Pan (en bocadillo, con las comidas,…)		
	A LA SEMANA	**AL MES**
Jamón salado, dulce, embutidos		
Queso blanco o fresco (Burgos,…) o bajo en calorías		
Otros quesos: curados o semicurado, cremosos		
	A LA SEMANA	**AL MES**
Frutas cítricas: naranja, mandarina,…		
Otras frutas: manzana, pera, melocotón, plátano…		
Frutas en conserva (en almíbar…)		
Zumos de fruta natural		
Zumos de fruta comercial		
Frutos secos: cacahuetes, avellanas, almendras,…		
Postres lácteos: natillas, flan, requesón		
Pasteles de crema o chocolate		
Bolsas de aperitivos («chips", "chetos", "fritos"…)		
Golosinas: gominolas, caramelos…		
Helados		
	A LA SEMANA	**AL MES**
Bebidas azucaradas ("coca-cola", "Fanta"…)		
Bebidas bajas en calorías (coca-cola light…)		
Vino, sangría		
Cerveza		
Cerveza sin alcohol		
Bebidas destiladas: whisky, ginebra, coñac,…		

Figure 92: The CFCA questionnaire

9. Neuropsychological evaluation: composed of two tests:

California Computerized Assessment Package (CalCap)[503]

In cognitive psychology, the concept reaction time (RT) is related to the amount of time that a subject needs to process information, from the presentation of a stimulus to the participant's response to the stimulus. Many factors affect reaction time, such as number, type and intensity of stimuli, age, gender, diseases, fatigue, and vision, among many others. Therefore, the measure of reaction time must be standardized in order to achieve reliable results.

The California Computerized Assessment Package (CalCap) is a computerized test developed by Eric N. Miller in 1990[504] for the purpose of detecting early signs of cognitive domains, to assess the existence of decline, through computerized and standardized evaluations of the speed of processing (reaction time), language skills, rapid visual scanning, form discrimination, recognition memory, and divided attention.

According to authors, the complete procedure takes approximately 20-25 minutes for administration and scoring. The software is copyright protected. Further information is available on the CalCap website[505] and in the CalCap user manual.[504]

WAIS-IV (Wechsler Intelligence Scale for Adults-IV)

In 1955, the psychologist David Wechsler disclosed the first version of the Wechsler Adult Intelligence Scale (WAIS). Since then, the test has been considered the gold standard to measure intelligence and cognitive abilities in adults and older adolescents, from 16 to 90 years old. The rationale behind the test according to Dr. Wechsler is that intelligence is made up of specific interrelated elements that can be isolated, defined, and measured. The test has been extensively revised, and now is in its fourth version[506,507].

The WAIS-IV is an individually administered test that assesses different aspects of intellectual abilities, divided into ten core subtests and five supplemental subtests that comprise four indexes (see **Table 118**): the Verbal Comprehension Index (VCI), Perceptual Reasoning Index (PRI), Working Memory Index (WMI), and Processing Speed Index (PSI).

Table 118: Dimensional structure of WAIS-IV questionnaire

Measures	Index score	Scores	Core subsets	Supplemental subsets
Full scale IQ (FSQI)	General Ability Index (GAI)	Verbal Comprehension Index (VCI)	Similarities Vocabulary Information	Comprehension
		Perceptual Reasoning Index (PRI)	Block design Matrix reasoning Visual puzzles	Picture completion Figure weights*
	Cognitive Proficiency Index (CPI)	Working Memory Index (WMI)	Digit span Arithmetic	Letter-number sequencing*
		Processing Speed Index (PSI)	Symbol search Coding	Cancellation*

*(16-69 years old only)

The test WAIS-IV provides four scores (Verbal Comprehension, Perceptual Reasoning, Working Memory and Processing Speed). Through these scores, the index scores General Ability Index (GAI) and Cognitive Proficiency Index (CPI) may be determined, which constitute the total combined performance score named Full Scale Intelligence Quotient (FSIQ)[508].

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