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Artificial 3D Skeletal Muscle Tissues to Model and Investigate Disease Onset and Progression Mechanisms

Sheeza Mughal



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Doctoral Thesis

**Artificial 3D Skeletal Muscle Tissues to Model and Investigate Disease Onset
and Progression Mechanisms**

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Dr. Juan Manuel Fernández Costa



UNIVERSITAT_{DE}
BARCELONA

Tesi doctoral

Teixits musculars esquelètics artificials en 3D per modelar i investigar els mecanismes d'inici i progressió de malalties.

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Sheeza

Prologue

The work presented in the thesis aims to develop and use 3D artificial skeletal muscle tissues to model two idiopathic diseases of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and post-acute sequelae of SARS-CoV2 as well as the first human cell derived disease model of steroid myopathy.

The structure of the thesis has a general Introduction section followed by two main sections: 1) Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Long COVID-19 2) Steroid Myopathy. Each section is further divided into sub-chapters (Introduction, Materials and Methods, Results, Discussion, and Conclusion).

The two sections are followed by a general discussion and conclusion of the thesis. The appendices include scripts for the codes used and published works.

Abstract

This thesis investigates the innovation and application of three-dimensional (3D) *in vitro* skeletal muscle tissue models to study and model muscle pathophysiology, particularly in idiopathic and corticosteroid-induced myopathies. Based on the historical evolution of cell culture techniques, this work highlights the limitations of conventional two-dimensional systems in reproducing native muscle structure, bioenergetics, and functional features. In the first section of this work, 3D human skeletal muscle constructs were developed using human muscle progenitor cells and exposed these tissues to sera from patients with Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and long COVID (LC-19). Structural, functional and transcriptomic analyses post-treatment revealed disease-specific, time-dependent disturbances in mitochondrial function, protein homeostasis, and inflammatory signaling. The disease pathology in these models demonstrated mitochondrial stress, IL-6–mediated catabolism, and metabolic reprogramming toward glycolysis, with ME/CFS samples showing particularly severe contractile deficits.

In the second part, a steroid myopathy model was produced by exposing 3D muscle tissues to dexamethasone. Taurine supplementation effectively restored contractile function by promoting protein synthesis and suppressing proteolysis by regulating the AKT/mTOR pathway modulation, even in the continued presence of dexamethasone. Together, these models provide powerful platforms for investigating disease mechanisms and testing therapeutic interventions, offering new mechanistic insights into muscle dysfunction. These insights are particularly important in conditions with unknown etiologies. Future prospects include developing an *in vitro* construct together with immune and neural components, increasing patient cohorts, and improving these models for clinical translation, particularly for testing patient stratification.

Summary in Catalan

Aquesta tesi investiga la innovació i l'aplicació de models tridimensionals (3D) de teixit muscular esquelètic in vitro per estudiar i modelar la fisiopatologia muscular, especialment en miopaties idiopàtiques i induïdes per corticosteroides. A partir de l'evolució històrica de les tècniques de cultiu cel·lular, aquest treball posa de manifest les limitacions dels sistemes convencionals bidimensionals per reproduir l'estructura, la bioenergètica i les característiques funcionals del múscul nadiu. En la primera part del treball, es van desenvolupar construccions musculars esquelètiques humanes 3D utilitzant cèl·lules progenitores musculars humanes, i es van exposar aquests teixits a sèrum de pacients amb encefalomièlitis miàlgica/síndrome de fatiga crònica (EM/SFC) i COVID persistent (LC-19). Les anàlisis estructurals, funcionals i transcriptòmiques posteriors al tractament van revelar alteracions específiques de cada malaltia i dependents del temps en la funció mitocondrial, la homeòstasi proteica i la senyalització inflamatòria. La fisiopatologia observada en aquests models va mostrar estrès mitocondrial, catabolisme mediat per IL-6 i reprogramació metabòlica cap a la glucòlisi, amb dèficits contràctils especialment severes en les mostres d'EM/SFC.

En la segona part, es va desenvolupar un model de miopatia esteroïdal exposant els teixits musculars 3D a dexametasona. La suplementació amb taurina va restaurar eficaçment la funció contràctil promovent la síntesi proteica i suprimint la proteòlisi mitjançant la modulació de la via AKT/mTOR, fins i tot en presència continuada de dexametasona. Aquests models proporcionen plataformes potents per investigar mecanismes patològics i provar intervencions terapèutiques, oferint noves perspectives mecanístiques sobre la disfunció muscular. Aquests coneixements són especialment rellevants en condicions amb etiologies desconegudes. Les perspectives futures inclouen el desenvolupament de construccions in vitro amb components immunitaris i neuronals, l'augment de les cohorts de pacients i la millora d'aquests models per a la seva traducció clínica, especialment en l'estratificació de pacients.

Paraules clau: Musculatura esquelètica, funció mitocondrial, senyalització inflamatòria, miopatia, funció contràctil.

List of Abbreviations

AAV: Adenoassociated Virus
ActRIIA and ActRIIB: Activin Type II Receptors A and B
ASOs: Antisense Oligonucleotides
ATP: Adenosine Triphosphate
BMPs: Bone Morphogenetic Proteins
ChR2: Channelrhodopsin-2
CMCMA: Methacrylated Carboxymethylcellulose
DEGs: Differentially Expressed Genes
DETs: Differentially Expressed Transposons
DHPR: Dihydropyridine Receptors
DMD: Duchenne Muscular Dystrophy
EAM: Experimental Autoimmune Myositis
ECC: Excitation-Contraction Coupling
ECM: Extracellular Matrix
ERK1/2: Extracellular Signal-Regulated Kinase 1/2
ETC: Electron Transport Chain
FAK: Focal Adhesion Kinase
FMN: Flavin Mononucleotide
FoxO: Forkhead Box O
GelMA: Gelatin Methacryloyl
GRMD: Golden Retriever Muscular Dystrophy
Grb2: Growth Factor Receptor-Bound Protein 2
IGF-1: Insulin-Like Growth Factor 1
LINES: Long Interspersed Nuclear Elements
LHON: Leber's Hereditary Optic Neuropathy
LTRs: Long Terminal Repeats
MCU: Mitochondrial Calcium Uniporter
MEK1/2: Mitogen-Activated Protein Kinase

Kinase 1/2

MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes
mPTP: Mitochondrial Permeability Transition Pore
mTOR: Mammalian Target of Rapamycin
MuRF1: Muscle RING Finger 1
NCX: Sodium-Calcium Exchanger
NHPs: Non-Human Primates
NOX: NADPH Oxidase
OXPHOS: Oxidative Phosphorylation
PDK1: Phosphoinositide-Dependent Kinase 1
PI3K: Phosphoinositide 3-Kinase
PIP2: Phosphatidylinositol-4,5-Bisphosphate
PIP3: Phosphatidylinositol-3,4,5-Trisphosphate
ROS: Reactive Oxygen Species
RSK: Ribosomal S6 Kinase
RYRs: Ryanodine Receptors
SERCA: Sarcoplasmic Reticulum Ca²⁺-ATPase
SINES: Short Interspersed Nuclear Elements
SMAD2/3: SMAD Family Member 2/3
SOD: Superoxide Dismutase
SOS: Son of Sevenless
SR: Sarcoplasmic Reticulum
SRF: Serum Response Factor
TCA: Tricarboxylic Acid Cycle
TGFβ: Transforming Growth Factor Beta
TSC1/2: Tuberous Sclerosis Complex 1/2
UCPs: Uncoupling Proteins
UPS: Ubiquitin-Proteasome System
YAP: Yes-Associated Protein

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Chapter 1. General Introduction

Preamble

Throughout history, the understanding of human body has transcended across many levels. In olden times, inferences on human anatomy were commonly driven from observations based on the study of animals which continued for the next ten centuries until the Renaissance. The first documented human dissection was carried out by Alexandrian scientists as early as 3rd century B.C. From 15th century, the studies on human anatomy largely progressed through grave-robbing by Leonardo Da Vinci for his art and later by Andreas Vesalius for his comprehensive opus on human anatomy “The Fabric of the Human Body”, who was also not shy to include anecdotes on personal experience of grave-robbing in his work. Expressing hope that *De Fabrica* will help “the painter, the sculptor and the molder”, Vesalius’s findings considerably helped understand human anatomy through detailed illustrations in addition to public dissections in anatomical theaters. He thoroughly mapped the human body from the skeleton to the brain’s “waterways and plumbing systems” that helped coordinate feelings and movements in the body, the many layers of muscles and the intricate network of human blood vasculature.

“I have done my best to this single end, to aid as many as possible in a very recondite as well as laborious matter, and truly and completely to describe the structure of the human body which is formed not of ten or twelve parts—as it may seem to the spectator—but of some thousands of different parts”- Andreas Vesalius.

From here on, research into organ systems, tissues and the ultracellular structure originated. The human body, as we know it, is a complex interwoven scaffold of multiple specialized organ systems with a synchronized function. One of these specialized organ systems is the musculoskeletal system which comprises bones, cartilage, tendons, muscles and connective tissue. This system supports and keeps tissues and organs in place.



Second Muscle Figure, after Jan Steven van Calcar, 1543. J. Paul Getty Museum, USA

1.1. Skeletal Muscles

Skeletal muscles are pivotal, not only for the structure and function of the human body, but also for regulating overall homeostasis. They anchor to tendons providing the force and flexibility necessary for maintaining posture, voluntary movements among other physiological processes. Their ability to execute coordinated contraction and relaxation dynamics enable controlled movements of limbs and other parts of the human body. Fueled by ATP hydrolysis, the actin and myosin filaments slide against one another allowing for this movement. From menial tasks such as grasping objects, to walking and running, skeletal muscles provide the biomechanical force and neuromuscular integration for moving bones at the joints. Muscle groups such as erector spinae and abdominal types help to counteract against gravity, ensuring an upright posture and functional integrity (1). By converting chemical energy to mechanical work, muscles release heat as a byproduct thereby helping to regulate body temperature. Furthermore, during extreme cold, shivering (involuntary contractions) helps to regulate core body temperature by thermogenesis (2). Alongside functional contributions, skeletal muscles are also a major site for glucose metabolism and storage. Insulin-stimulated glucose uptake into skeletal muscle helps maintain blood glucose levels and provides energy for muscle activity (3). Moreover, skeletal muscles serve as reservoirs for amino acids, which can be mobilized during periods of fasting or stress to support other vital bodily functions. In lower extremities, skeletal muscle contractions provide support to the circulatory system by facilitating venous return to the heart against gravity thereby protecting against venous pooling and ensuring an efficient blood supply (4). Alongside these crucial contributions to body health and stability, skeletal muscles are endocrine organs. They secrete myokines during contractions which are signaling molecules effecting key regulatory processes like inflammation, metabolism, regeneration and inter-organ communication (paracrine signaling). A skeletal muscle is, therefore, a multi-faceted organ contributing towards a myriad of physiological functions crucial to human form and function.

1.1.1. Anatomy of a skeletal muscle tissue

Skeletal muscle is one of the two types of striated muscles, the other being cardiac muscles. While the latter type is self-stimulating and non-fatiguing type of muscle, the former represents a class of innervated striated muscles that perform high energy voluntary contractions and experience fatigue. A skeletal muscle tissue is characterized by systematically organized bundles of myofibers (5). Enclosed and defined by a tubular sheath known as the sarcolemma, each myofiber comprises of the basic contractile unit of a muscle cell known as the sarcomere. The sarcomere, in turn, is composed of two types of myofilaments: thick filaments with myosin and thin filaments containing actin. Bundles of myofibers constitute a fascicle encased by a connective tissue called the perimysium and a collection of fascicles group together to form a muscle tissue enveloped by the epimysium (6). The striated appearance of a skeletal muscle is due to the arrangement of actin and myosin filaments. At each end of the sarcomere there is a thin, dark line called the Z-disk. In the center of the sarcomere there is a group of thick filaments called the A band. The thin filaments are laterally arranged at the Z-disk and the M line intersects the H-bands. Myosin is not only a structural protein but also an enzyme involved in ATP hydrolysis. Furthermore, it is also associated with other myosin binding proteins (types C and H) that contribute to accurate positioning of myosin and consequently play a fundamental role in regulating force generation by the actin-myosin complex (7). Titin, the largest protein in the human body, runs through the length of the sarcomere ensuring that force generation is homogenous across the length of the sarcomere (8). Proteins located at the M-line are responsible for connecting myosin with titin, allowing for an efficient and coordinated contraction of the sarcomere (Figure 1.1).

The extracellular matrix (ECM) of the skeletal muscle is non-contractile and apart from efficient vascularization and neural networks, it also contains fibroblasts and macrophages (9). Although non-contractile, the ECM is flexible enough to adjust to the contraction/relaxation cycles of the muscle. It also helps in transmitting a homogenous force through the muscle and from the muscle to the joint or tendon.

Furthermore, it is also important for ensuring focal adhesion for cellular interaction. ECM remodeling has been implicated in a lot of muscle pathologies and stress phenotypes such as diabetes, as well as both chronic and acute muscle/ nerve injury (10,11).

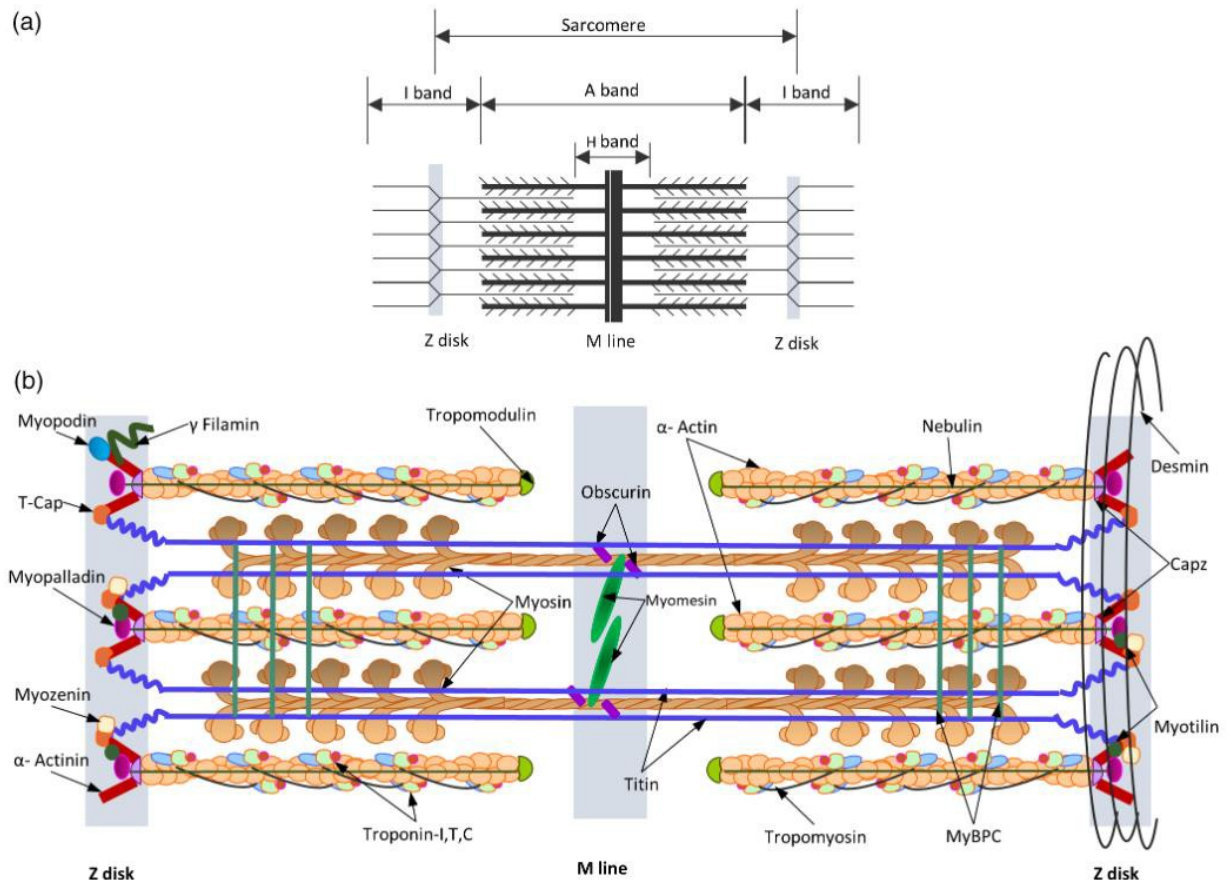


Figure 1.1 Contractile Machinery of a Skeletal Muscle. a) Cross-sectional diagram of a striated skeletal muscle, which shows the thin and thick filaments in a sarcomere (the functional muscle cell unit). b) Schematic of a sarcomere with the relevant proteins. Myosin binding heads crawl along the length of the actin which contracts the entire sarcomere. Titin and nebulin serve to maintain the thick and think filaments. Obscurin lines the M-line, while myopodin, myopalladin, myozerin are situated in the Z-disk; all proteins working in tandem to preserve the structure of the filaments. Desmin forms links between individual myofibrils. Adapted from (12).

1.1.2. Mechanism of contractile performance and force generation

Skeletal muscle contractile assembly is composed of a well-structured arrangement of multiple proteins that help regulate contraction as shown in Figure 1.1. Muscle contraction is initiated by the excitation-contraction coupling process. An action potential traveling along a motor neuron reaches the neuromuscular junction,

triggering the release of acetylcholine (13). This neurotransmitter binds to receptors on the muscle fiber membrane, leading to depolarization and the release of calcium ions from the sarcoplasmic reticulum. The Troponin (Tn)-Tropomyosin complex works in response to sarcomeric Ca^{2+} levels (14). The surge in intracellular calcium concentration facilitates the binding of calcium to troponin, causing conformational changes that move tropomyosin away from actin's myosin-binding sites. Consequently, myosin heads attach to actin, forming cross-bridges and generating force through ATP-dependent cycles. This process known as Excitation-Contraction Coupling or ECC occurs at the juncture of T-tubules and the sarcoplasmic reticula. The depolarization of dihydropyridine receptors (DHPR) which is a voltage-gated Ca^{2+} channel located at the T-tubule, by the action potential releases Ca^{2+} ions from the sarcoplasmic reticulum (15). This particular Ca^{2+} channel is considered to be an important voltage sensor of the skeletal muscle, responsible for opening of the ryanodine receptors (RYRs). An efficient regulation of Ca^{2+} ions is key to a healthy muscle contractile function through the cross-bridge cycle (16).

Contractile force generation is also dependent on the type and size of muscle fibers. There are four different types of muscle fibers: Type I or slow-twitch fibers, Type II fast-twitch fibers with subtypes IIA, B and X (17). The exact subtype of type II fibers is determined during tissue formation. Type I fibers have a low glycolytic activity and high mitochondrial content allowing them to obtain energy through oxidative metabolism. They are resistant to fatigue as they are capable of long contractions with low-force production. Furthermore, they are vascularized and have a high myoglobin content. On the contrary, type II fibers are more reliant on glycolytic oxidation to compensate for increased ATP hydrolysis and therefore, capable of producing greater force (18). The fiber types also depend on their calcium kinetics. Fast fibers have low cytosolic Ca^{2+} and an abundance of SERCA (Sarcoplasmic Reticulum Ca^{2+} -ATPase) pumps capable of performing rapid sarcomeric shortening (19). Skeletal muscles are capable of high adaptive plasticity depending on the degree of physical activity. More specifically, they can switch fiber types in response to changes in nerve activity or due to exercise (20,21). The switch to oxidative fast fiber type (IIA) is more significant compared to a switch to the slow fiber type (22).

This switching mechanism has been relevant in metabolic studies relevant to different disease models (23,24).

1.1.3. Muscle Atrophy and Hypertrophy

Compared to other tissues, skeletal and cardiac muscles have a dense packing of proteins and organelles. This implies that muscle fiber size and function are largely dependent on protein and organelle turnover. To this end, there needs to be a balance between the rate of biogenesis and degradation within the muscle fiber to determine its cellular volume. An increase in cell volume due to elevated cytosolic accumulation of proteins is known as hypertrophy. Alternatively, catabolic processes resulting in shrinkage or decrease of cell volume, due to an increased degradation of cytosolic components, is called atrophy (25). There is an extensive interconnected network of biological pathways that are regulated by both autonomous and non-autonomous signals to strike a balance between the two processes (26). It is also important to note that the skeletal muscle is very heterogeneous with respect to the fiber types and therefore their metabolic properties which means that there is often drastic variation in response to the same stimulus. For instance, during starvation, the soleus muscles being Type I will not be as sensitive to nutrient deficiency as compared to the Type II muscles (27). However, type I muscles will be more sensitive to wastage due to inactivity compared to Type II. Furthermore, while pathways like Insulin/IGF1 and TGF β /Activin/BMP regulate muscle mass by affecting both protein synthesis and degradation, the relationship between protein turnover and muscle growth or loss is more complex than the traditional view suggests (28,29). Muscle hypertrophy is not always driven solely by increased protein synthesis and reduced degradation, nor is atrophy simply an increase in degradation. Exercise, for instance, enhances protein synthesis but also activates degradative pathways such as the autophagy–lysosome and ubiquitin–proteasome systems (30). Similarly, during protein breakdown, amino acids released from these processes can activate mTOR (mammalian target of rapamycin), potentially promoting protein synthesis even in conditions of muscle atrophy (Figure 1.2).

1.1.3.1. Increase in cell volume- Hypertrophy

Two common pathways that are responsible for inducing muscle hypertrophy are the Insulin-IGF1-AKT-mTOR and the RAS-MAPK-ERK pathways. The Insulin-IGF1-AKT-mTOR pathway is a critical regulator of muscle hypertrophy, primarily by promoting protein synthesis and inhibiting protein degradation (Figure 1.2) (31). This pathway is initiated when insulin or insulin-like growth factor 1 (IGF-1) binds to their respective receptor tyrosine kinases, the insulin receptor (IR) and IGF1 receptor (IGF1R), on the muscle cell membrane (Figure 1.2). This binding activates the insulin receptor substrate (IRS) proteins, which subsequently recruit and activate phosphoinositide 3-kinase (PI3K). Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3), creating docking sites for phosphoinositide-dependent kinase 1 (PDK1) and AKT (also known as Protein Kinase B, PKB) (32).

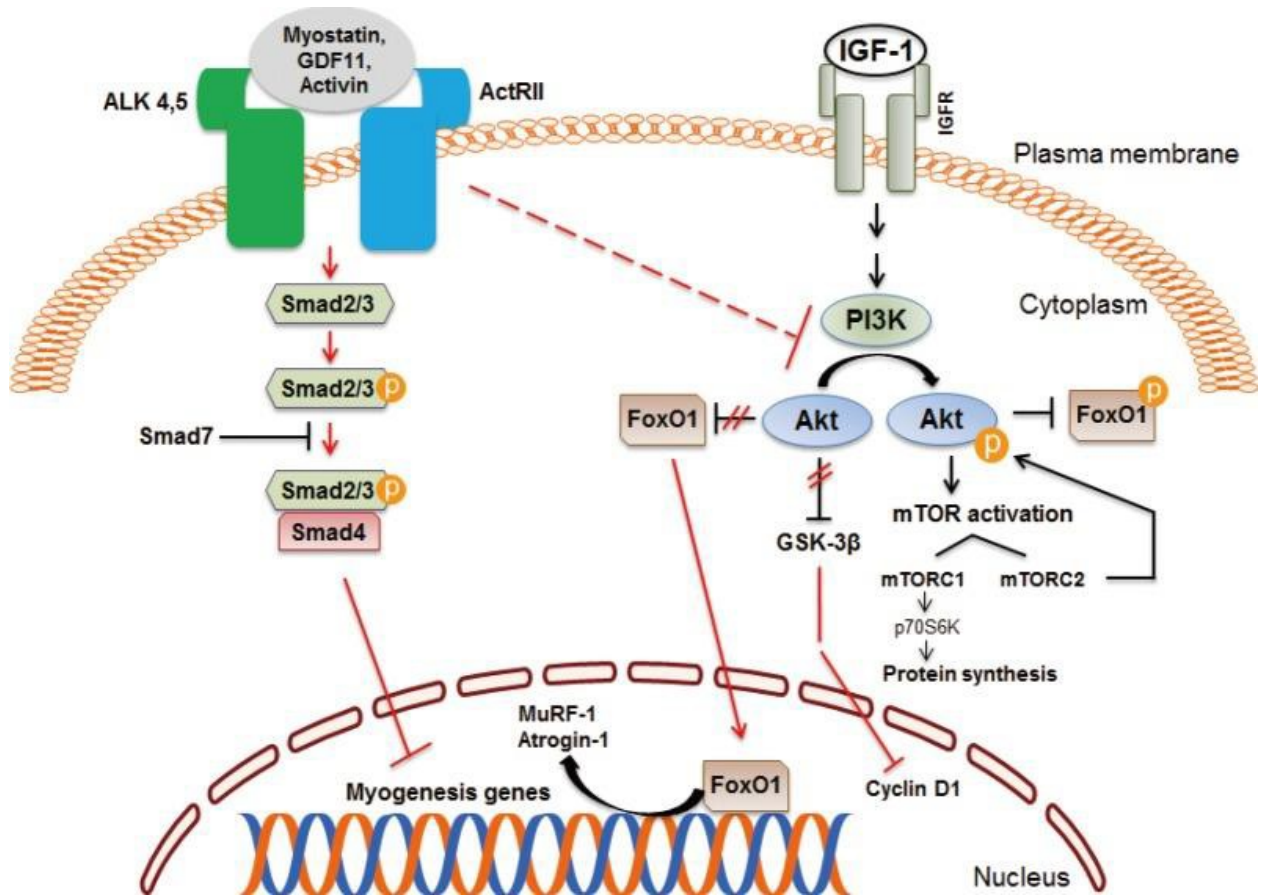


Figure 1.2. Signaling pathways pertaining to Insulin Growth Factor 1 (IGF-1) and Myostatin in the skeletal muscle. IGF-1/Akt pathway activates the mTOR, which triggers hypertrophy. Myostatin and related family members pass through the Activin receptor (ActRII) and the Smad2/3. The inhibition of mTOR further activates myostatin signaling. Adapted from (33)

Once AKT is phosphorylated by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), it becomes fully active and exerts its hypertrophic effects. A major downstream target of AKT is the tuberous sclerosis complex 1/2 (TSC1/2), a negative regulator of Rheb (Ras homolog enriched in brain). AKT inhibits TSC1/2, leading to the activation of Rheb, which in turn stimulates mTORC1 (mammalian target of rapamycin complex 1), the central hub of muscle growth regulation (34) (Figure 1.2).

mTORC1 phosphorylates two major effectors involved in protein synthesis namely:

1. Ribosomal protein S6 kinase 1 (S6K1) – Enhances translation by promoting ribosome biogenesis and increasing mRNA translation efficiency (35).
2. Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) – Releases eukaryotic translation initiation factor 4E (eIF4E), facilitating cap-dependent translation initiation (36).

In addition to promoting protein synthesis, AKT inhibits protein degradation by phosphorylating and inactivating Forkhead box O (FoxO) transcription factors, which are responsible for upregulating the expression of muscle atrophy-related genes, including those encoding components of the ubiquitin-proteasome system (UPS) and autophagy-lysosome system. By simultaneously stimulating protein synthesis and suppressing degradation, the Insulin-IGF1-AKT-mTOR pathway plays a central role in driving muscle hypertrophy (37).

The second pathway, RAS-MAPK-ERK, contributes to muscle hypertrophy primarily through its role in cell growth, gene expression, and protein synthesis (24) (Figure 1.3). This pathway is activated by mechanical stimuli, exercise, and growth factors such as IGF-1, which bind to receptor tyrosine kinases (RTKs), including IGF1R. Upon activation, RTKs initiate a cascade of phosphorylation events by recruiting adaptor proteins like Grb2 (growth factor receptor-bound protein 2) and SOS (son of sevenless), leading to the activation of the small GTPase RAS. Activated RAS interacts with and activates RAF (rapidly accelerated fibrosarcoma kinase), which then phosphorylates and activates MEK1/2 (mitogen-activated protein kinase kinase 1/2). MEK1/2, in turn, phosphorylates and activates ERK1/2 (extracellular signal-regulated kinase 1/2), the core effectors of this pathway (38) (Figure 1.3).

Once activated, ERK1/2 translocates to the nucleus and phosphorylates transcription factors such as c-Myc, ETS like protein-1 (ELK1), and Serum Response Factor (SRF), which drive the expression of genes involved in muscle growth and adaptation (39). ERK1/2 also phosphorylates ribosomal S6 kinase (RSK), promoting protein synthesis by enhancing the activity of translation machinery. Additionally,

ERK1/2 modulates mechanotransduction pathways by influencing focal adhesion kinase (FAK) and Yes-associated protein (YAP), both of which are key regulators of muscle adaptation to mechanical loading (40). This pathway is particularly important in exercise-induced muscle hypertrophy, where mechanical stress activates MAPK signaling, leading to enhanced muscle growth (41).

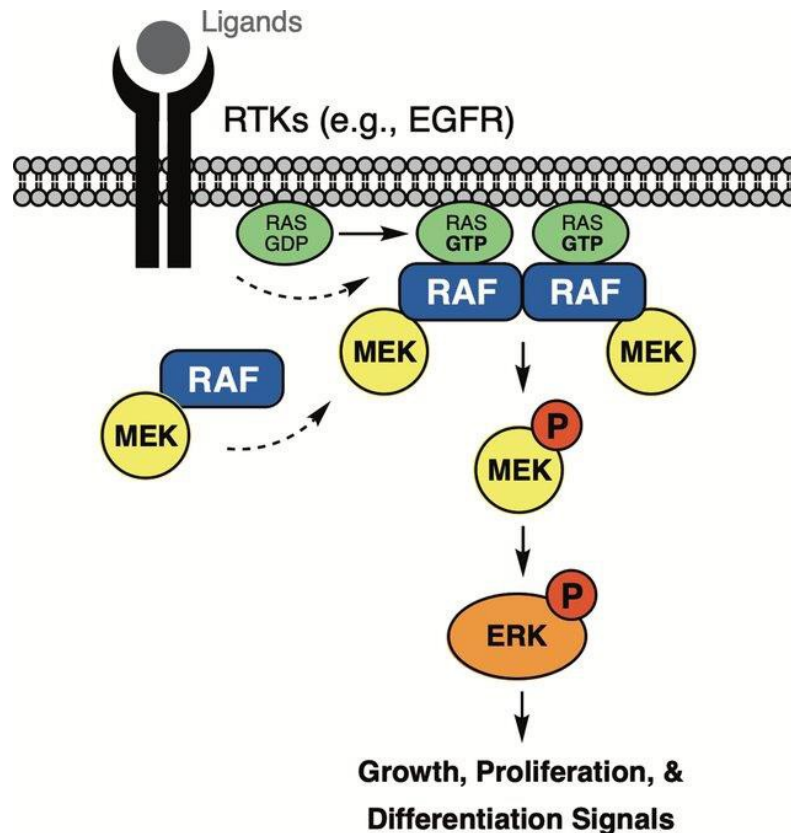


Figure 1.3. Signaling cascade of RTK/Ras/MAP Kinase pathway. The EGFR activation on the cell membrane in recruits guanine-nucleotide exchange factors, which lead to the loading of RAS with GTP. This RAS in turn recruits RAF-kinases to the cell membrane where they get activated. Their substrates are MEK1 and MEK2, which are phosphorylated once RAF-kinases are activated. Phosphorylated MEK1/2 then activate ERK, which leads to its downstream targets affecting growth and proliferation. Figure adapted from (42)

Unlike the Insulin-IGF1-AKT-mTOR pathway, which predominantly regulates protein synthesis and degradation, the RAS-MAPK-ERK pathway influences transcriptional reprogramming, cell cycle progression, and anabolic responses in muscle cells (43). Together, these two pathways synergistically promote muscle hypertrophy, ensuring both rapid protein translation and long-term structural adaptations.

In addition to these pathways, other factors are also involved in modulating muscle mass. One of these is the TGF β (Transforming Growth Factor Beta) superfamily, which includes myostatin, activins, and bone morphogenetic proteins (BMPs), and plays a crucial role in regulating muscle mass by balancing anabolic and catabolic processes. Myostatin and activins act as negative regulators of muscle growth by binding to activin type II receptors (ActRIIA and ActRIIB), leading to the activation of SMAD2/3 signaling (44). Once phosphorylated, SMAD2/3 forms a complex with SMAD4, translocates to the nucleus, and represses genes involved in muscle hypertrophy while promoting protein degradation through the ubiquitin-proteasome system. This inhibitory effect restricts muscle growth and contributes to atrophy.

In contrast, BMP signaling promotes muscle hypertrophy by activating SMAD1/5/8, which enhances the expression of pro-growth genes and counteracts myostatin-induced atrophy (45). BMPs also stimulate mTOR signaling, reinforcing anabolic processes. The balance between myostatin/activin-mediated catabolism and BMP-driven anabolism determines overall muscle mass, making this pathway a critical target for therapeutic interventions in muscle-wasting conditions.

1.1.3.2. Increased degradation- Atrophy

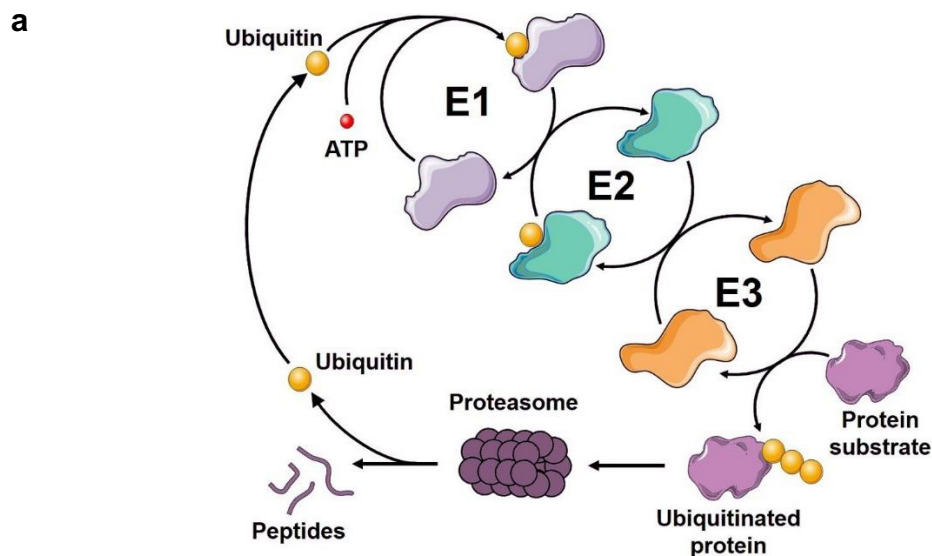
When the balance tilts towards increased protein degradation, atrophic pathways are activated. Atrogenes, which are genes responsible for inducing atrophic pathways, have been found at the heart of different catabolic or muscle wasting conditions (46). These pathways can be triggered due to a variety of stimuli such as infections, elevated inflammatory cytokines, oxidative stress, mitogens, and nutrient deficiency among others (47–49). A muscle undergoing atrophy is characterized by a progressive loss in muscle mass and protein content, decline in basal force of contraction and a decrease in overall fiber cross-sectional area. The two major contributing pathways to atrophy are the ubiquitin-proteasome system and the autophagy-lysosome system.

The Ubiquitin-Proteasome system is a key mechanism responsible for degrading proteins in muscle cells, particularly under conditions that lead to muscle atrophy (50) (Figure 1.4a). This system functions through a highly regulated sequence of steps, beginning with the tagging of proteins for degradation via ubiquitination. The process is initiated by ubiquitin-activating enzymes (E1), which use ATP to activate ubiquitin molecules. These activated ubiquitin molecules are then transferred to ubiquitin-conjugating enzymes (E2) and finally attached to specific target proteins by ubiquitin ligases (E3) (51). The selectivity of protein degradation in muscle cells is largely determined by E3 ligases, with muscle RING finger 1 (MuRF1) and atrogin-1 (MAFbx) playing central roles in breaking down key structural and regulatory proteins. Once polyubiquitination is completed, these tagged proteins are directed to the 26S proteasome, a complex that unfolds and degrades them into smaller peptides (52). The breakdown products are then further processed into amino acids, which can be recycled for new protein synthesis or used for energy production. Activation of the UPS in muscle is largely driven by stress-related signaling pathways, including those controlled by FoxO transcription factors, which increase the expression of atrophy-related genes. This pathway is particularly active in atrophying conditions like disuse, denervation, fasting, and disease-associated cachexia, where the rate of protein degradation exceeds synthesis, leading to a reduction in muscle mass (53).

The Autophagy-lysosome system (ALS) is another critical pathway involved in muscle atrophy, primarily responsible for breaking down larger protein complexes, organelles, and long-lived proteins that cannot be processed by the proteasome (54) (Figure 1.4b). This pathway is initiated when a double-membraned structure, known as a phagophore, forms and expands to engulf cytoplasmic components, eventually developing into an autophagosome. The regulation of this process involves a series of proteins encoded by autophagy-related (ATG) genes, which orchestrate membrane expansion and cargo selection. A major regulator of autophagy is the mTOR which suppresses autophagy under nutrient-rich conditions. When nutrients or energy are scarce, mTOR activity decreases, allowing activation of the ULK1 complex, which initiates autophagosome formation. Once the autophagosome is

fully formed, it merges with lysosomes, where enzymes, including cathepsins, degrade its contents into smaller molecules for reuse. In skeletal muscle, autophagy helps maintain cellular integrity by removing damaged mitochondria and other dysfunctional components. However, excessive autophagic activity, triggered by stress conditions such as prolonged fasting, denervation, or chronic disease, contributes to muscle loss by accelerating the degradation of essential cellular components (55).

These two systems are interconnected and work in tandem to regulate muscle protein degradation. While the proteasome primarily targets short-lived proteins and myofibrillar components, the lysosomal pathway is responsible for larger cellular structures (56). Under normal physiological conditions, muscle mass remains stable due to a balance between protein synthesis and degradation. However, in conditions such as aging, or chronic disease, the activation of these degradation pathways surpasses protein synthesis, leading to progressive muscle atrophy (57) (Figure 1.4).



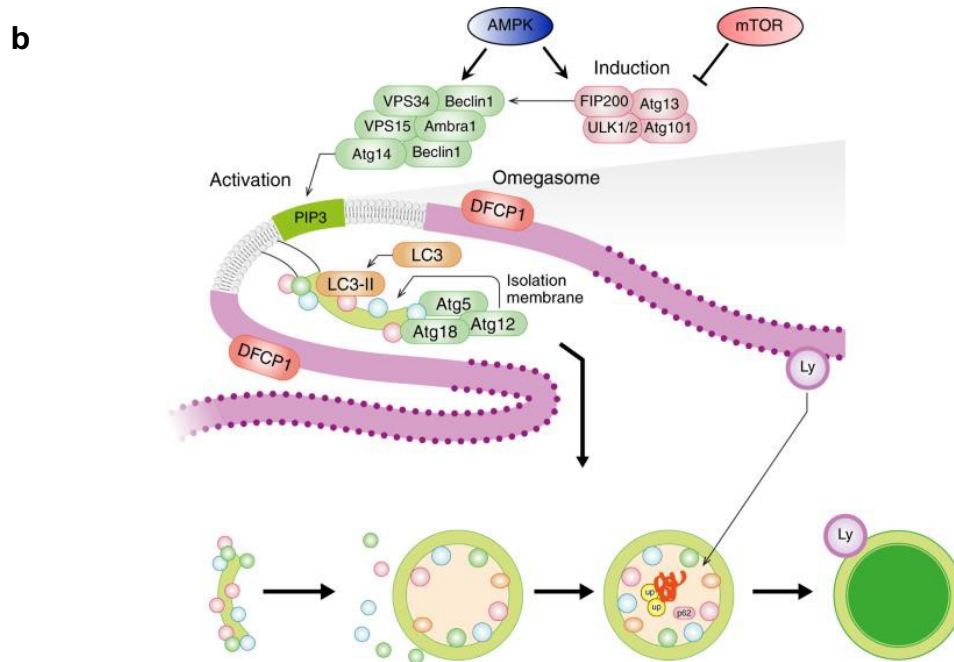


Figure 1.4. a) The Ubiquitin Proteasome System of protein clearance divided into distinct steps. E1 is the activating step where the ubiquitin molecule is phosphorylated, which is then captured by the conjugating enzymes in E2 step to form an intermediate. The final E3 step is where the ubiquitin molecule is recruited by a ligase to be conjugated to the substrate, hence conferring it with a tag. The tagged protein is sent to the proteasome where it is degraded and the ubiquitin is recycled. Figured adapted from (58) b) Schematic representation of autophagosome formation. ULK1/2-FIP200-Atg13-Atg101 complex assembles to induce Beclin1 complex to activate PIP3 in the membranes of organelles. These complexes can be induced by mTOR and AMPK. PIP3 then recruits LC3 conjugation system which commits the membranes to become autophagosomes. This then buds out and docks to the lysosome awaiting cargo. Adapted from (26).

1.1.4. Muscle Bioenergetics

1.1.4.1. Mitochondrial localization and cellular arrangements

A continuous supply of ATP is necessary to ensure sustained muscle contraction. The source of this ATP is predominantly the process of oxidative phosphorylation or OXPHOS taking place in mitochondria during high-intensity muscle activity (59). Different fiber types have different percentage of mitochondria occupying the muscle volume in either the intermyofibrillar or subsarcolemmal spaces. Depending on their location, the mitochondria may differ not only in function and network organization but also in structure. For example, in the subsarcolemmal space the mitochondria tend to cluster together in between sarcolemma and myofibrils and have dense cristae formation (60). The subsarcolemmal mitochondria are mostly globular in shape and extend their network through the intermitochondrial

junctions to further connect with the intermyofibrillar type, thus forming the mitochondrial reticulum. In contrast to subsarcolemmal mitochondria, the intermyofibrillar mitochondria are more complex in their arrangement and localization depending on the fiber types (61). In slow oxidative type fibers, they are mostly located in grid-like uniform networks around myosin filaments while in fast-glycolytic muscle fibers they arrange themselves perpendicularly to the line of contraction around the I-bands. The location and arrangement of intermyofibrillar mitochondria therefore contributes strongly to the fiber cross-sectional area. As a greater percentage of mitochondria around the Z-line will reduce cross sectional area of the sarcomeres in that particular space contributing to irregular spacing between subsequent myosin filaments, thereby impacting fiber contractility (62) (Figure 1.5).

Among the two mitochondrial types discussed, the subsarcolemmal mitochondria possess a high proton motive force for the electron transport chain (ETC) through complex IV by virtue of their position close to the capillaries. The proton motive force is one of the key contributors in determining membrane potential through the mitochondrial network. Other contributing processes include movement of ions such as Na^+ , Cl^- and K^+ , calcium homeostasis, membrane permeability determined by uncoupling proteins (UCPs), proton leaks (induced or otherwise) and the mitochondrial transport dynamics for inorganic phosphate through ATP/ADP interchange (63).

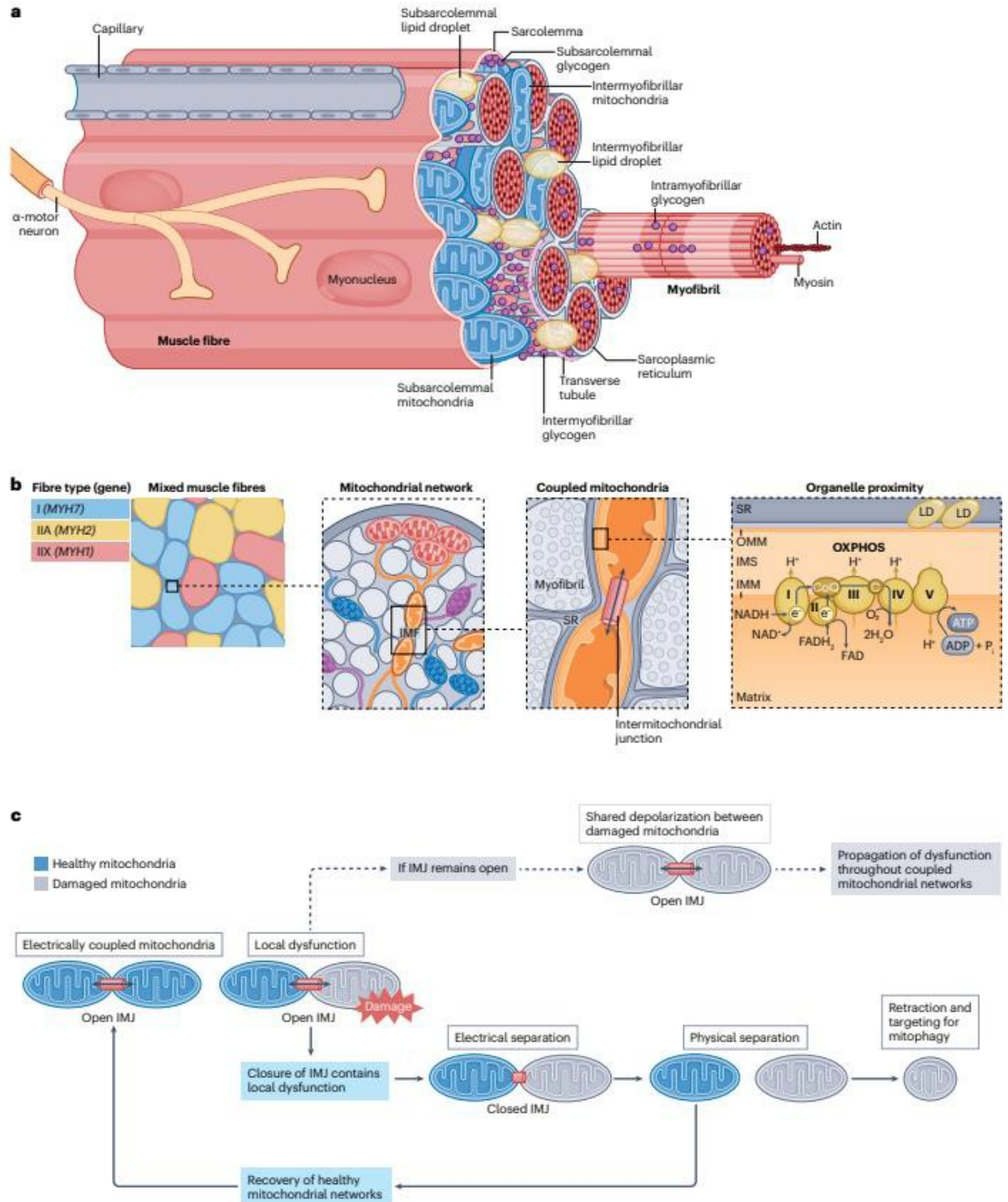


Figure 1.5. Skeletal muscle structure a) Cross-section of the skeletal muscle comprising of muscle bundles and connective tissues. Muscle bundles are composed of muscle fibers, which are coated with basal lamina attached with the sarcolemma. Stem cells of the muscle are contained within their own niches in the sarcolemma. Sodium/potassium pumps, transverse tubules, and myofibril proteins help to contract the fiber via excitation-contraction coupling. Glycogen granules occur within the myofibers and sarcolemma, and so are intramyocellular lipids. b) Three types of fibers are present within the skeletal muscle, namely I, IIA and IIX. These types differ in

their mitochondrial protein content and configuration, which impacts metabolism. Mitochondria within the muscle make an interconnected reticulum, which distributes energy from subsarcolemmal mitochondria to the intermyofibrillar mitochondria. c) Mitochondria within the skeletal muscle form junctions and networks to share energy. These junctions help the creation of smaller network, where the defective mitochondria are swiftly removed without any consequences to the overall energy generation. Adapted from (64).

The intermyofibrillar mitochondria possess a higher percentage of ATP synthase (complex V) (65). The membrane potential generated by the subsarcolemmal mitochondria is subsequently harnessed by the intermyofibrillar mitochondria to generate ATP for sustained contractions. The mitochondrial reticulum therefore plays an effective role as the cellular electrical grid.

1.1.4.2. Oxidative Phosphorylation

Oxidative phosphorylation takes place in the inner mitochondrial membrane through a series of four complexes/enzymes, among which three are the proton pumps. This process is responsible for the extraction of ATP from nutrient substrates such as carbohydrates and fats. In glycolysis and subsequent citric acid cycle through substrate level phosphorylation, 4 ATP molecules are generated alongside 10 NADH and 2 FADH₂ (66). The last two electron carriers are fundamental to OXPHOS as their electrons are transferred to molecular oxygen while generating 34 ATP molecules through various redox reactions.

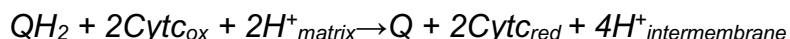
The first point of electron entry in the electron transport chain is through complex 1 (NADH dehydrogenase or NADH: Ubiquinone oxidoreductase). NADH carries 2 electrons from the Krebs's cycle and donates them to flavin mononucleotide (FMN) producing FMNH₂. These electrons then pass through the Fe-S (iron-sulfur) clusters and are finally transferred to coenzyme Q or ubiquinone forming ubiquinol (67). As a result of this transfer, a total of 4 H⁺ ions are transferred to the mitochondrial intermembrane space.

Complex II (Succinate dehydrogenase) plays a similar role as complex I but instead of NADH as an electron donor it accepts FADH₂ which is reduced primarily by electrons donated from succinate which is a Krebs's cycle intermediate. The electrons donated by FADH₂ pass through the Fe-S clusters and eventually reduced

ubiquinone to ubiquinol(68). This process, however, does not pump any H^+ ions to the intermembrane space.

Cytochrome *c* oxidoreductase generates a Q cycle which transfers the electrons from ubiquinol/complex III (QH_2) to cytochrome *c* and pumps a total of 4 H^+ ions to the intermembrane space per 2 electrons (2 for each QH_2 oxidized). Of the two electrons brought by a ubiquinol, one moves on to complex IV through cytochrome *c* while the second is transferred to cytochrome *b* generating semiquinone which is ultimately converted to ubiquinol by cytochrome *b* using the electron brought by a second ubiquinol molecule to the complex (69).

Overall reaction:



Complex IV cytochrome *c* oxidase uses the electrons from cytochrome *c* to reduce molecular O_2 , forming 2 molecules of H_2O and releasing 2 protons to the intermembrane space. This complex contains two main catalytic subunits. Subunit one contains two heme groups (a , a_3) and a copper center (CuB) which helps in reducing oxygen. The oxygen molecule forms a peroxide bridge with cytochrome a_3 before reduction by CuB (70). Subunit II contains a CuA center (two copper atoms) responsible for accepting the electrons from cytochrome *c* and then transferring them to heme a_3 in subunit I.

Complex V, ATP Synthase (F_0F_1 ATPase) has two main subunits namely F_0 membrane-bound, responsible for forming the proton channel and F_1 facing the matrix, responsible for the ATP synthesis. The protons from the proton gradient established by the previous complexes are brought back into the matrix through the F_0 subunit. Their movement rotates the γ (gamma) subunit, inducing conformational change in the F_1 subunit allowing it to generate ATP (71). Each full rotation yields 3 ATP molecules.

1.1.4.3. Sites of Reactive Oxygen Generation during OXPHOS

Reactive oxygen species (ROS) are produced as byproducts of OXPHOS when electrons from the ETC leak out and react with molecular oxygen (O_2). This mostly happens at Complexes I and III, where electrons leave ubiquinone (CoQ) or iron-sulfur (Fe-S) clusters before finishing their transfer to downstream carriers. Superoxide anion (O_2^-), a primary ROS, is created when these lost electrons decrease oxygen. It can then be further transformed into hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\cdot$), both of which are extremely reactive and have the potential to harm biological components. A certain amount of ROS generation is unavoidable even if the ETC is made to effectively transport electrons to oxygen at Complex IV while reducing electron leakage (72). The ETC is designed to minimize electron leakage and to ensure their efficient transfer across the chain. Regardless, a certain degree of ROS generation is unavoidable. Superoxide dismutase (SOD), catalase, and glutathione peroxidase are among the mitochondrial antioxidant defense systems that normally neutralize ROS to stop cellular damage. However, excessive ROS production, which is frequently brought on by hypoxia, high metabolic demand, or mitochondrial dysfunction, can result in oxidative stress, which damages proteins, lipids, and DNA and contributes to aging and a number of illnesses, including cancer, cardiovascular disease, and dementia (73).

1.1.4.4. Calcium homeostasis and ROS- Cause or Effect?

By oxidizing proteins involved in the control of calcium (Ca^{2+}) transport, excessive ROS upset calcium (Ca^{2+}) homeostasis. This causes the intracellular Ca^{2+} to become dysregulated. Ca^{2+} ions leak from the sarcoplasmic reticulum (SR) into the cytoplasm as a result of ROS-induced oxidation of RyRs, leading to fatigue, prolonged muscular contraction, and possibly Ca^{2+} -dependent death (74). Similarly, ROS may induce changes in SERCA, inhibiting return of Ca^{2+} to the SR and resulting in Ca^{2+} sequestering, impacting muscle relaxation. ROS may also impact the voltage gate Ca^{2+} channels which further increases Ca^{2+} influx to the cytoplasm, further accentuating mitochondrial Ca^{2+} accumulation and enhancing ROS generation as a result. Generation of ROS and mitochondrial Ca^{2+} overload then continues in a self-perpetual cycle of oxidative stress and calcium dysregulation (75).

ROS production can itself be significantly influenced by calcium homeostasis. Through the mitochondrial calcium uniporter (MCU), mitochondria acquire Ca^{2+} when cytosolic Ca^{2+} levels rise. Excessive Ca^{2+} intake causes mitochondrial overload, although moderate Ca^{2+} levels can increase ATP synthesis by stimulating tricarboxylic acid cycle (TCA) enzymes. The ETC is disrupted by high Ca^{2+} concentrations, which leads to electron leakage at Complexes I and III. These electrons subsequently combine with molecular oxygen to create superoxide (O_2^-), an important ROS. Furthermore, high levels of mitochondrial Ca^{2+} can cause the inner mitochondrial membrane's non-specific channel, the mitochondrial permeability transition pore (mPTP), to open, which can lead to membrane depolarization, cytochrome c release, ATP depletion, and additional ROS generation. Neurodegenerative disorders, apoptosis, and muscular weariness are all associated with chronic mPTP opening (76). Furthermore, one of the main non-mitochondrial sources of ROS, NADPH oxidase (NOX), is activated by increased Ca^{2+} levels. Superoxide radicals are produced when NOX enzymes catalyze the transfer of electrons from NADPH to oxygen. This pathway increases the production of ROS and contributes to oxidative stress in metabolic illnesses, ischemia-reperfusion injury, and muscle problems (77) (Figure 1.6).

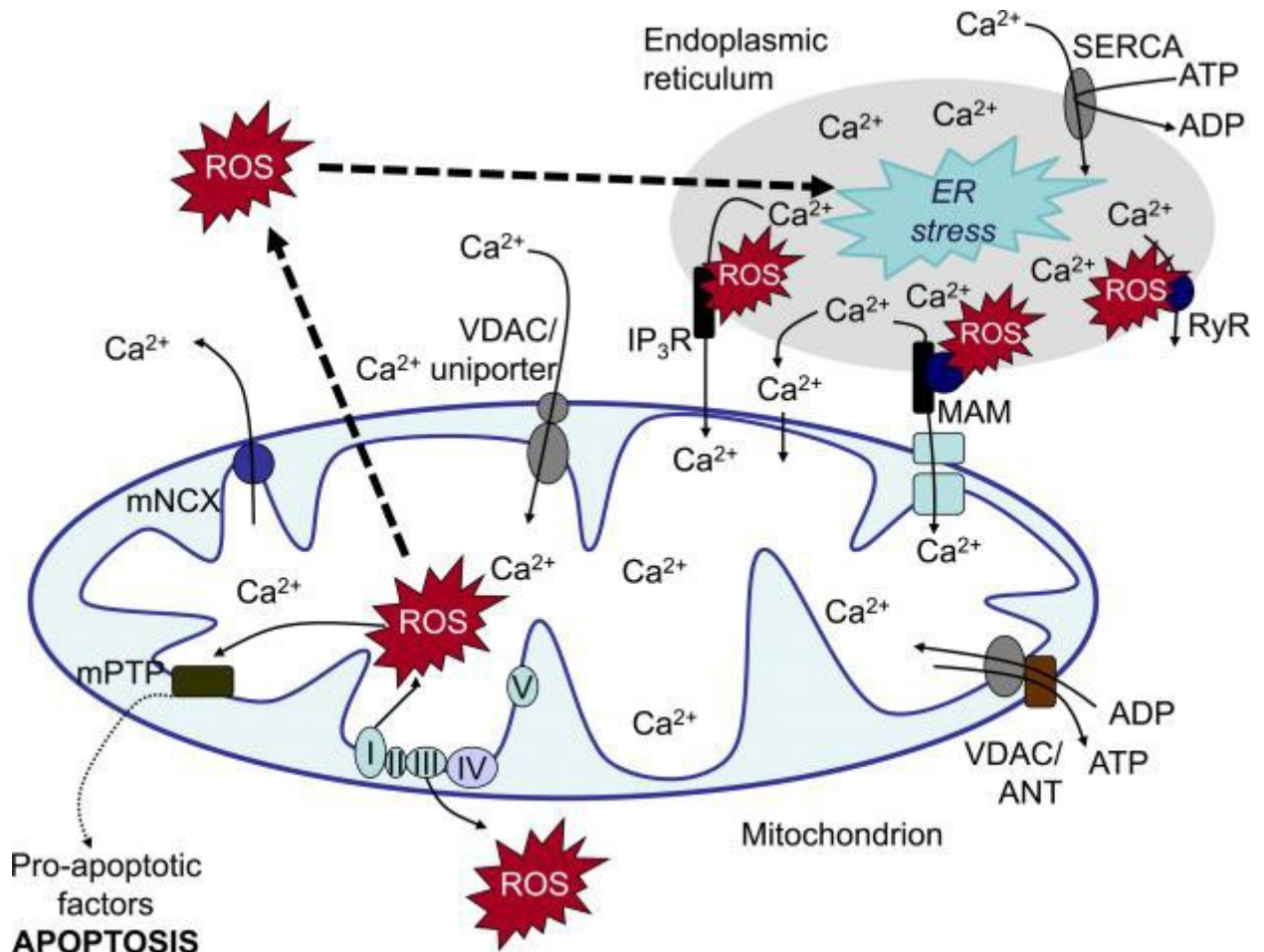


Figure 1.6. Schematic of the ER-mitochondria cross-talk. ER stores calcium, which it release through the calcium release channels in the cisternae. These channels are also present in the mitochondrial associated membranes (MAMs). Calcium can enter into the mitochondria through either the calcium uniporter or voltage dependent channels. ROS generation can occur if the calcium levels are too high, which can further promote the release of ER calcium channels. These two factors can then trigger the mPTP, which then can cause apoptosis. Adapted from (78)

1.2. Skeletal Muscle related Diseases

1.2.1. Metabolic Myopathies

Muscle weakness, cramping, and exhaustion, especially during activity, are symptoms of a class of illnesses known as metabolic myopathies, which are brought on by abnormalities in the enzymes involved in muscle metabolism. McArdle disease, a glycogen storage condition brought on by a lack of myophosphorylase, an enzyme that converts glycogen to glucose during exercise, is one of the most

prevalent metabolic myopathies (79). Patients are unable to efficiently generate energy.

Another subset of metabolic diseases are mitochondrial myopathies, which are caused by mutations in nuclear or mitochondrial DNA that impair mitochondrial function. Due to mitochondria's inability to generate enough ATP, these conditions frequently result in muscular weakness, exercise intolerance, and systemic symptoms like cardiomyopathy and neuropathy (80). These deficiencies may also result in overproduction of ROS. To counteract mitochondria's inability to produce sufficient energy, metabolic myopathies are usually managed with dietary changes, supplements, and, occasionally, particular pharmaceutical treatments.

1.2.2. Muscular Dystrophies

A class of hereditary diseases known as muscular dystrophies is distinguished by gradual muscle weakening and deterioration. There are more than 70 different types of muscular dystrophies. One of the most well-known type is Duchenne Muscular Dystrophy (DMD), an X-linked recessive condition in which the dystrophin protein is either absent or malfunctioning due to mutations in the dystrophin gene. Muscle cell death results from the loss of dystrophin, a vital component of the muscle cell membrane, which compromises the structural integrity of the muscle fibers and makes them more vulnerable to mechanical stress (81). Early childhood muscle weakness that begins in the lower limbs and progresses to the upper limbs and trunk is the hallmark of DMD, which usually affects boys. Patients usually develop cardiomyopathy, respiratory failure, and loss of ambulation as the illness worsens. Other types of muscular dystrophies include Becher muscular dystrophy, myotonic dystrophy, facioscapulohumeral (FSHD), congenital (CMD), Limb-girdle (LGMD) and Emery-Dreifuss dystrophy (EDMD) (82). All these types have muscle weaknesses and degeneration at their core but differ on the basis of severity, age and the types of muscles affected.

1.2.3. Steroid Myopathies

Muscle weakening and wasting brought on by long-term steroid (corticosteroid) use is known as steroid myopathy. Prednisone is a common glucocorticoid prescribed to treat many inflammatory and autoimmune conditions such as rheumatoid arthritis, lupus, and asthma. Long-term usage of these medications, however, can result in a variety of muscle-related adverse effects, which mostly impact the proximal muscles, or those nearest to the torso, including the shoulder and hip flexors (83). Although the precise process by which steroids cause muscle weakness is not entirely understood, it is believed to involve a number of processes, such as changes in muscle cell signaling, decreased protein synthesis, and muscle protein breakdown. Muscle fiber atrophy results from prolonged exposure to elevated corticosteroid levels, especially in type II fast-twitch fibers, which are more vulnerable to degeneration by glucocorticoids (84).

1.3. Models to study muscular disorders and diseases

Disease modelling has adopted different strategies and model organisms to understand not only the pathomechanism of disease progression but also to understand patient-specific nuances in some diseases. Moreover, the developed disease models are further used to conduct some pharmacokinetic and pharmacodynamic studies in drug testing. Common models include animals, *in vitro* culture techniques like monolayers and 3D microphysiological systems.

1.3.1. Animal Models

Animals are frequently used in biomedical research for studying multiple muscular disorders. These models help understand pathophysiology, causes, and therapeutic approaches. Researchers can track the onset and progression of muscle degeneration, test new treatments, and investigate underlying biochemical processes in a controlled, repeatable method by simulating human diseases in animals (85). Before moving on to human clinical trials, researchers can test new medicines and get a better understanding of disease mechanisms using animal models of muscle illnesses. This is particularly significant for complex diseases that often have multiple etiologies and are difficult to reproduce in conventional

monolayer cell culture systems that lack the complexities observed in an *in vivo* environment (86).

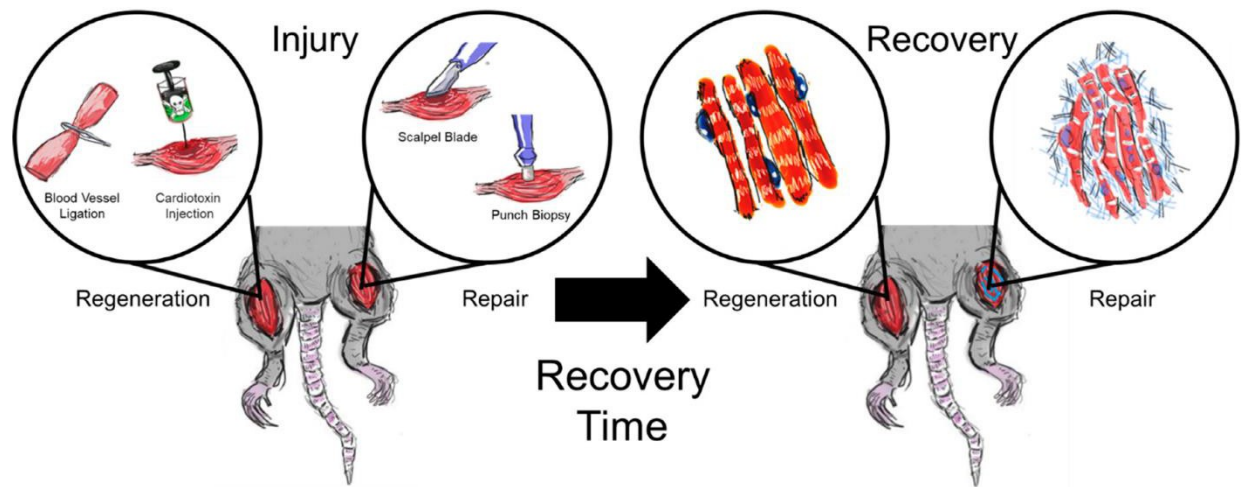


Figure 1.7. Two models to investigate muscle repair and regeneration. Models based on inducing injury regenerate post-recovery. Models based on repair may lead to intermediate myofibers which are laced with fibrotic tissue. Adapted from (87)

In vivo animal models more closely mimic the metabolic, physiological and anatomical complexity of entire organ systems and the intricacies of their interactions (Figure 1.7). In the context of living things, these models make it easier to research immune responses, tissue regeneration, systemic impacts, and therapeutic efficacy. Mice are among the most widely used animal models, and because of their genetic resemblance to humans, short lifetime, and simplicity of genetic manipulation, they have emerged as the gold standard for studying muscle disorders in both molecular and functional contexts. Numerous transgenic, knockout, and knock-in mice models have been employed to investigate a range of muscle disorders, such as inflammatory myopathies, mitochondrial myopathies, and muscular dystrophy (88).

One of the most popular models for researching DMD, a hereditary condition marked by muscle weakening and degeneration brought on by a deficiency of dystrophin, is the *mdx* mouse. The dystrophin gene is altered or deleted in the *mdx* mouse, simulating the genetic abnormality present in DMD patients in humans (89). Researchers have been able to investigate the gradual muscular weakness, fibrosis, and inflammation linked to DMD because of the *mdx* mouse model, which has

yielded important insights into the disease's etiology. The development of treatments for DMD, including gene therapy, exon skipping, and anti-inflammatory medications, has been greatly expedited by this model. Antisense oligonucleotides (ASOs) have been investigated as a treatment to bypass aberrant exons in the dystrophin gene and restore partial dystrophin production in muscle fibers in recent research using the *mdx* mouse (90). This method has demonstrated potential in lowering fibrosis and enhancing muscular function.

Bigger and more complex animals like dogs offer a superior model for researching skeletal muscle illnesses that entail cardiomyopathy or impact bigger muscle groups, even if mice are helpful for genetic manipulation. A mutation in the dystrophin gene causes Golden Retriever Muscular Dystrophy (GRMD), a naturally occurring form of DMD in dogs that shares clinical characteristics with humans (91). Because of their larger, complex muscles and more human-like immune systems, the GRMD dog is an excellent model for extrapolating preclinical findings to human patients, which makes them particularly helpful for evaluating gene treatments and cell-based therapies. Muscle cell transplantation techniques, in which stem cells from healthy tissue are infused into the injured muscle to repair damaged fibers and restore function, have been tested on GRMD dogs (92). Subsequent human clinical trials have been informed by the findings from animal models (93).

Mutations in nuclear genes producing mitochondrial proteins or mitochondrial DNA (mtDNA) are frequently the cause of mitochondrial disorders, including Leber's Hereditary Optic Neuropathy (LHON) and MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes) (94). To investigate the etiology of these disorders and assess possible therapies targeted at reestablishing mitochondrial function, mice with mutations in nuclear mitochondrial genes or mtDNA are used as model animals (95). The role of mitochondrial malfunction in muscle weakness, fatigue/lethargy, and exercise intolerance is studied using transgenic mice that exhibit abnormalities in the mtDNA linked to MELAS. These models aid in the assessment of possible mitochondrial-targeted treatments, including antioxidant supplements and coenzyme Q10 (96).

Rats are frequently utilized in the study of muscular disorders because of their bigger size and more complicated skeletal muscle structure than mice, especially when more thorough muscle biopsies or functional assessments are needed. Rats are nevertheless very helpful in studying the mechanical characteristics and pathophysiological development of muscle disorders, despite not being as genetically tractable as mice. In order to explore muscular dystrophies in a larger organism and provide a more realistic simulation of human muscle function, the dystrophin-deficient rat model was created. These rats experience cardiomyopathy, weakness, and muscular atrophy that resemble human symptoms (97). Gene therapies like adeno-associated virus(AAV)-mediated gene therapy, which attempts to restore dystrophin expression and stop muscle degeneration, have been tested in the dystrophin-deficient rat model (98). These rats are also a valuable resource for researching medication delivery methods for systemic treatment of dystrophies and successful gene therapy. Autoimmune illnesses like polymyositis and dermatomyositis, which cause inflammation of the muscles as a result of immune system malfunction, are being studied in rat models (99). Immunizing animals with skeletal muscle antigens causes inflammation and injury to the muscles in the experimental autoimmune myositis (EAM) rat model. The effectiveness of immunosuppressive medications and biological therapies that target particular immune pathways implicated in the pathophysiology of myositis has been assessed using the EAM rat model (100).

Because of their quick development, early-stage transparency, and genetic resemblance to humans, zebrafish are being employed more and more in studies of muscular diseases. Because of these characteristics, zebrafish are a perfect model for cellular and molecular research on muscle growth, regeneration, and disorders. Similar to the *mdx* animal model, zebrafish with dystrophin gene mutations show muscular weakening and degeneration, qualifying it as a model for researching the pathophysiology of different muscular dystrophies (101). Because zebrafish embryos are transparent, scientists can see firsthand how muscle injury develops and how the body responds to it in real time. Small molecule drugs that could improve muscle regeneration or lessen fibrosis in DMD have been screened for

using zebrafish models. In these models, researchers have investigated ASOs and other possible therapeutic interventions, with encouraging outcomes in terms of enhancing muscle function (102). Non-human primates (NHPs) are beneficial in some areas of muscular illness research, particularly when studying disorders that require a more human-like immune system and muscle function, despite their less widespread use due to ethical concerns and expensive expenses. Early research on gene therapy and stem cell-based treatments for muscular dystrophy has made use of NHP models (103). Compared to smaller animals, they provide a better model of how humans will react to these therapies because of their larger size and more intricate structure.

These animal models are not only expensive but also have low predictive accuracy and a high percentage of drug development failures. Furthermore, misleading results may result in adverse impacts on humans (104). Therefore, alternatives to animal models are frequently preferred.

1.3.2. 2-D *in vitro* models

As discussed at length in previous sections, skeletal muscle is a metabolically complex tissue capable of exhibiting high plasticity. Traditionally, this tissue has been studied extensively using biopsies from healthy donors and patients. As an alternative to biopsies and animal models, cell culture techniques using primary and immortalized cell lines such as murine C2C12 and L6 myoblasts from rat have been used to study cell biology and development of mature skeletal muscle fibers (105,106).

Mechanistic investigations to understand different muscular diseases such as mitochondrial myopathies, dystrophies and metabolic disorders have frequently employed different cell types such as induced pluripotent stem cells (iPSCs), primary myoblasts, immortalized muscle precursor cells, and satellite cells. Each of these cell types has both advantages and disadvantages.

Induced Pluripotent Stem Cells (iPSCs): iPSCs are somatic cells by origin that have been reprogrammed to differentiate into different lineages including skeletal

muscles. This makes them invaluable for modelling and understanding genetic disorders (107). Dystrophies such as Duchenne Muscular Dystrophy (DMD) and Limb Girdle Dystrophy (LGMD) have been modelled using iMyoblasts expressing (PAX3+/MYOD1+) (108). These cells were found to retain disease-specific mutations that enabled testing different drugs. iPSCs have also been used to study epigenetic dysregulation in Facioscapulohumeral Muscular Dystrophy (FSHD) in which disease pathology has been correlated with disruption in the expression of DUX4 gene (109). For mitochondrial pathologies, iPSCs show positive and negative implications. They may have reduced mtDNA and variable heteroplasmy. The former implies that the iPSCs may contain lesser number of mtDNA copies than the parent somatic cells, this may result in a shift from dependence on mitochondrial energy production to glycolysis. Variable heteroplasmy, on the other hand refers to a combination of normal and mutated mtDNA in the same cell (110). This may happen due to a sharp drop in mtDNA during reprogramming which ultimately leads to a random distribution of mtDNA in the cells. Consequently, some iPSCs made from the same cell type may have healthy mtDNA while others may acquire mutated mtDNA copies. Additionally, the iPSCs may harbour new mtDNA mutations or may have deleted the ones originally present. Therefore, the derived iPSC lines may not exactly mirror the original mtDNA profile and potentially lead to misleading results. For this reason, monitoring mtDNA in iPSCs has been an important part of the quality control process (111). Isogenic iPSCs, that have the same genetic background and differ only in the presence of a specific mutation, are preferred to study gene defects (112).

Primary Myoblasts: Commonly isolated from biopsies of patients and healthy donors, primary myoblasts closely mimic the original muscle physiology but offer several challenges such as a short lifespan and senescence as well as variable purity and differentiation depending largely on the methods of isolation (113).

Immortalized myoblasts: These are muscle precursor cells that have been genetically modified to bypass cellular ageing, allowing the lines to proliferate and

differentiate more than the primary myoblasts. These immortalized lines are frequently used to model diseases and study drug delivery (114).

Satellite cells: These are the internal stem cells within a muscle responsible for regeneration. mtDNA deletions, which are a common cause of mitochondrial myopathies and muscle aging, are present in satellite cells from patients with mitochondrial DNA deletion syndromes (115). However, when these satellite cells are activated to proliferate and repair muscle, the proportion of cells carrying mtDNA deletions decreases significantly.

While these cultures are easy to maintain and can be used for high throughput drug screening, they tend to produce immature myotubes which are not physiologically relevant (116). This is because of the nature of the vessel in which the cells are grown in, which is typically either glass or plastic. These stiff substrates can lead to a spontaneous detachment of myotubes and reduce gene-expression (117). While a coating of ECM proteins on these substrates can help alleviate the problem to some extent, the true microenvironment of a muscle is still not replicated in the truest sense. This is where investigators started exploring different materials to allow 3D cell culturing (118).

Since a 3D culture uses scaffold systems to give it the third dimensional conformation differently from a standard 2D cell culture, it is considered as a bridge between 2D cell culture and real tissue. They can be cultured in concentrated, gel-like materials as well as in suspension. The application and use-case determine the medium to be used (119). Furthermore, donor tissue cells can be cultivated in conditions akin to those from which they were isolated. This indicates that, in addition to maintaining the cells' shape and polarity, cell-cell connections are also maintained, resulting in superior cell signalling compared to their 2D counterparts. Moreover, gene expression and metabolic activity are comparable when the cells develop under *in vivo* like circumstances (119).

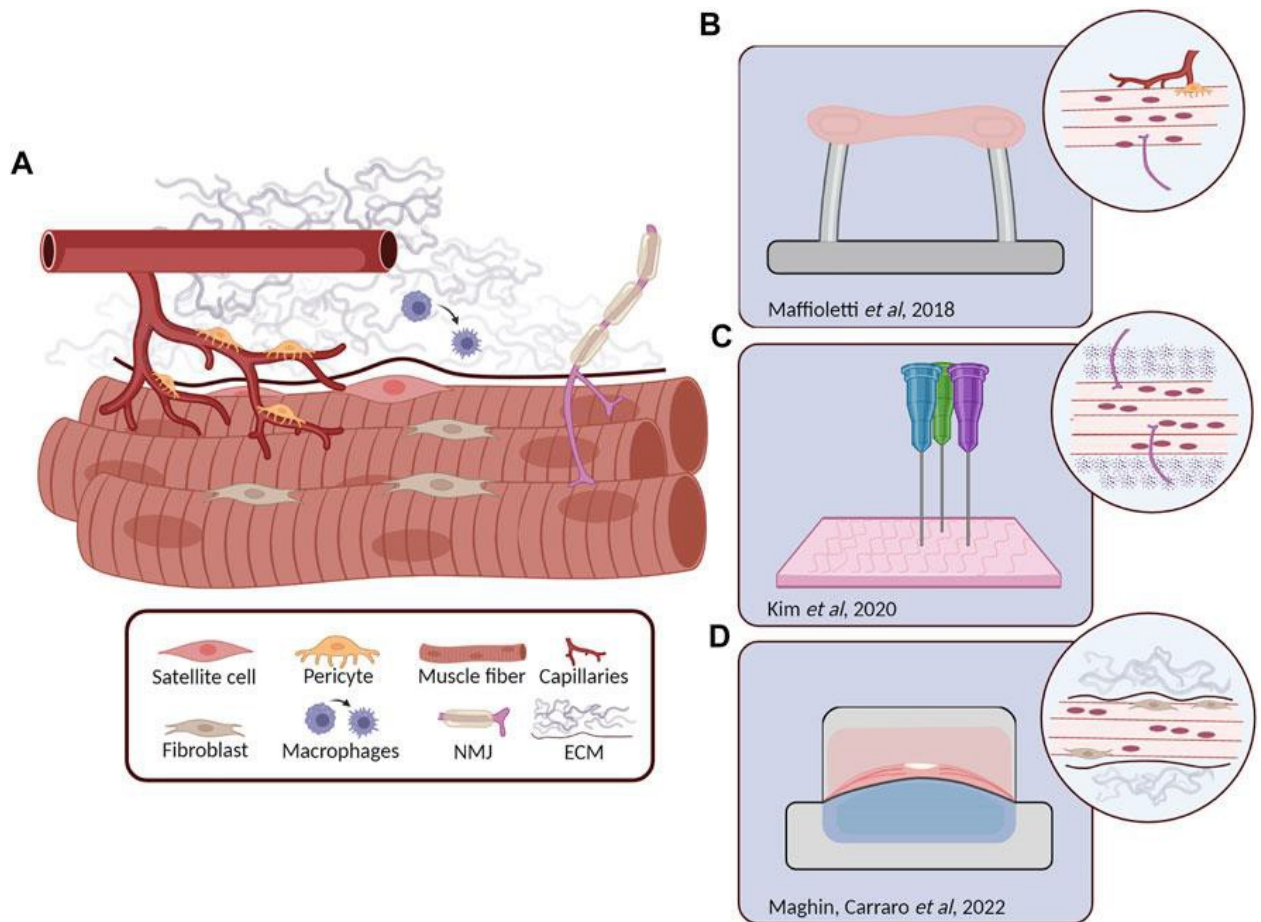


Figure 1.8. 3D tissue models of the skeletal muscle. A) Different types of cells present in a mature skeletal muscle. ECM proteins surround myofibers, while satellite cells are contained within the basal lamina. Motor neurons form the neuromuscular junction and the pericytes originate from the vasculature. Macrophages are recruited to the site of muscle injury. B) Pillar-based system, with induced pluripotent cells wrapped around embedded in a fibrin hydrogel. C) Bioprinting of innervated myofibers with an aligned pattern. D) Bioreactor to generate 3D model of the skeletal muscle after long-term dynamic culture, with the cells self-aligning within the ECM. Adapted from (120–123)

1.3.3. 3-D Biomimetic Muscle Models

3D muscle models overcome the various pitfalls associated with 2D cultures. They can allow for longer culture times, improve gene expression, increase the size and maturation of myotubes and also enable the measurement of contractile function (124,125). The last feature enables such cultures to go beyond the conventional measurements of gene and protein expression typical of 2D cultures and towards a more *in vivo* muscle physiology. This is why a bioengineered 3D muscle model should replicate not just the structure but the function of a native muscle and should

model the phenotype of muscle-associated diseases. Different approaches and materials have been adopted to achieve the structural complexity of skeletal muscles as shown in Figure 1.8.

1.3.3.1. Replication of Environmental Cues

To mimic said environment, it is crucial to incorporate biochemical and mechanical signals through a specific ECM, which can also provide good cell-adhesion in three dimensions and maintain the structural and functional integrity. Some biomaterials are used more commonly than others, and these include collagen, laminin and fibrin which are all intrinsic ECM proteins. Bian and colleagues have shown that these ECM scaffolds not only provide support but also alignment cues which in turn enhances myotube differentiation (126). On the other hand, there are also scaffold-free approaches, which allow the cultured cells to secrete their own ECM. A pioneering study by Strohman et al in the 1990s established that using a plastic wrap as a substrate in conjunction with fibroblasts was enough for the cells to exude ECM and hence self-assemble into muscle fibers (127). This has now evolved into much more cutting-edge micropatterning of surfaces, and by using fibrin gel (128). Contemporary literature presents three main techniques to culture myofibers: micropatterning, electrospinning and bioprinting. Muscle progenitor cells seeded on micro-patterned or micro-molded substrates have an improved aligned of sarcomeres and increased contractile capacity (129). This suggests a positive effect of topological signals on muscle form and function. Micro-contact printing has also been employed to develop 2D micropatterned cell-adhesive substrates achieving alignment of myotubes (130). Electrospinning using piezoelectric materials capable of being electrically polarized upon mechanical stimulation has also been employed to develop mature myofibers (131). 3D extrusion bioprinting is another technique commonly used to develop aligned muscle fibers in highly hierarchical scaffolds (132). Growth of myotubes on top or within these scaffolds provide the necessary topographical cues to ensure maturation. Similarly, E-jet 3D printing has been used to develop multi-layered scaffolds that mimic innate extracellular matrix with materials like poly-lactic-co-glycolic acid (PLGA) (133). Studies have optimized

different parameters like gaps within the scaffold to investigate cellular adhesion and proliferation (134). Molding or formation of tissue hydrogel constructs using soft lithography is another commonly adopted method which employs the uniaxial tension between two posts to grow mature myobundles perpendicular to the posts (135). Depending on the stiffness of these posts, the tissue constructs structural phenotype changes.

However, apart from the scaffold, engineering a biomimetic muscle *in vitro* requires other elements as well. Within the organism, a functional muscle receives stimulation from motor neurons but in the *in vitro* setting, this stimulation can be provided by exposing the muscle to an external electrical field. In this way, pulses of specific frequencies can be provided constantly in culture and can help the muscles to differentiate into different types of fibers. A 2006 study showed a case, where a two-week electrical stimulation turned the cells into slow and fast type isoforms (136). This was also replicated at the MHC level in a separate study (137). In more recent years, researchers have also incorporated sensory motor-neurons which can further regulate motor input by co-culturing human myoblasts with sensory neurons to yield intrafusal fibers (138,139).

The important aspect of mimicking skeletal muscles *in vitro* is to monitor contractile force, which can be done through chemical and physical stimuli (140). Electrical signals, as already detailed above, is one way in which this can be done, but there are also mechanical and optical stimulation strategies to this avail (141,142). Optical stimulation involves incorporating light-sensitive proteins in tissues through gene delivery, for instance channelrhodopsin-2 (ChR2) (143). Through this light-sensitive opsin, contractile activity can be controlled non-invasively, as the protein triggers membrane depolarization (142,144). Mechanical stimulation involves the generation of tensile stress on the tissue, which leads to the induction of mechano-transduction pathways. Muscular development is ensured, hence, by an increase in myotube number and diameter (141).

1.3.3.2. Replication of Mechanical Cues:

Mechanical cues are crucial for the proper and healthy development, maintenance and function of skeletal muscles. These stimulations regulate alignment, growth, maturation and the overall structural and functional phenotype of the skeletal muscle tissue (145). These cues also contribute to the development, integration and coordination of tendons, joints and bones. From embryonic development mechanical cues help shape and differentiate skeletal muscle tissues (146). In mature muscles, physical exercise or activity triggers an increase in muscle mass or muscle hypertrophy (147). On the contrary, inactivity has been found to cause muscle atrophy (148). From a molecular and mechanistic perspective, mechanosensitive pathways including YAP/TAZ, IGF-1-PI3K-Akt-mTOR, and mechanosensitive ion channels, convert the external mechanical cues to changes in cellular biochemical pathways that regulate protein synthesis (149).

Replicating these mechanical cues is, therefore, important for the formation and maturation of skeletal muscle tissues *in vitro*. Biomimetic mechanical environments drive muscle precursor cells to undergo differentiation (150). Skeletal muscle tissues have captured interest in studying strain application due to the structure-function correlation exhibited by the myofibers (151). Specific chamber systems have to be designed to maintain the structure and allow functional flexibility of skeletal muscles *in vitro* (152). These chambers have provisions to enclose tissues, media, sensors and any actuators. The different types of mechanical cues include:

Mechanical stretching: which involves applying static or repetitive stretch to muscle constructs in order to improve alignment, induce hypertrophy and to analyze or improve muscle performance (153). Specific types of stimuli include cyclic stretch, static stretch and multiaxial loading.

Electrical stimulation: Electric pulses of different frequencies for different exposure times can help accelerate myotube formation and maturation (154). Furthermore, it also helps to analyze changes in muscle contractile performance in response to different drugs or treatments.

Substrate stiffness: Topography and stiffness of the scaffold or matrix used to grow tissues influence cellular response to mechanical cues, ultimately affecting the growth, formation and organization of myotubes (155). Micro- and nano-patterned scaffolds can direct cell orientation and organization, further supporting the development of functional muscle tissue (156).

1.3.3.3. 3D Muscle Models

3D bioprinting can be used to fabricate muscle tissue constructs by encapsulating myoblasts within polymers like GelMA (Gelatin methacryloyl), or alginate (157). The size of the nozzle and extrusion pressure can be modulated, and in the process the cells align with and grow in the direction of printing (158). Within the hydrogel, they can then differentiate into mature myotubes. To assist in this alignment, cell aligners, like Au nanowires could also be added within the encapsulation mix and a field applied later (159). Recent work has also utilized composite polymers, for instance the combination of GelMA with methacrylated carboxymethylcellulose (CMCMA) to improve long-term culture and to prevent degradation of the hydrogel structure (160). Another approach is to develop hydrogels in a similar fashion but around fixed posts to generate tissues (161). One study showed that molds of polydimethylsiloxane (PDMS) with an anchored cantilever design could effectively be used to cast hydrogels (162). The extruded encapsulated mix thence grows around the post or pillars and can be used for force measurement after electrical stimulation. Another way to fabricate muscle tissues is to create microporous cryogels, whose pore-size can be regulated (163). This can help improve not just the myofiber alignment but also fusion and maturation as found by a 2019 study where gelatin-cellulose cryogels were used with C2C12 myoblasts (164).

The first functional *in vitro* human skeletal muscles were developed by the Bursac group using primary myoblasts. The said constructs were developed by forming cell-laden Matrigel®-fibrin hydrogels within a PDMS platforms within a Cerex® nylon frame. The nylon was used to provide anchor points during electrical

stimulation. This construct was used to study changes in the population of PAX7+ satellite cells due to different chemical, mechanical and structural cues. Other models have used Fibrin-Geltrex® hydrogels using Velcro as artificial tendons to produce uniaxial tension. The innate heterogeneity in the pathomechanism of different muscular diseases emphasizes the need for a personalized approach to diagnostic and therapeutic interventions. 3D *in vitro* skeletal muscle models present an important platform with immense bench to bedside translational potential. Additionally, these constructs can be used to study temporal progression of diseases using not only the functional phenotypes but also multi-omics characterizations in a patient-specific manner.

1.4. Brief overview of diseases in focus

This thesis focuses on investigating two types of diseases: Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and long COVID-19 which are idiopathic conditions with unknown etiology and steroid myopathy which is a corticosteroid induced muscle wasting as a consequence of excessive use of steroid-based medication or due to underlying adrenal tumors.

1.4.1. Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome

Systemic Exertion Intolerance Disease (SEID) also known as Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome is a heterogenous, multisystemic, chronic condition which induces physical and cognitive impairment. This condition affects approximately 17 to 24 million people worldwide (165). Despite this prevalence and a significant impact on patients' lives, the underlying pathomechanism of the disease remains poorly understood.

The first evidence of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome dates to the beginning of 20th Century when multiple outbreaks of unknown etiologies were recorded across different regions in 1934. In the beginning, these outbreaks were incorrectly attributed to poliomyelitis because of similar but not identical symptoms including but not limited to neuromuscular discomfort and prolonged

fatigue post viral infection (166). However, after poliovirus was isolated and studied, researchers had to reconsider this classification as the pathomechanism, and progression of poliomyelitis was significantly different from the clinical presentation of this illness. The earliest recorded cases were from Los Angeles (1934), Iceland (1948) and London (1955) (167). These cases presented the same symptom profile but did not test positive for any viral or other infections. The scientists and clinicians then suspected that perhaps this illness was the long- term sequelae of a previous infection that significantly impacted the immunity and neuromuscular functions of patients.

Until the middle of 20th century the recurrent pattern of these global outbreaks was recognized and registered by epidemiologists and clinicians. The clinical definition of the illness, however, remained poor (168). The first term to define the range of symptoms experienced by these patients was “Benign Myalgic Encephalomyelitis”. It included extreme fatigue or malaise, severe myalgia, lymphadenopathy and pharyngitis. Despite evidence of significant morbidity, the prefix *Benign* was used to emphasize that the disease did not directly cause mortality. It was added to emphasize that the illness, although severely debilitating, was not fatal and therefore did not demand a similar level of urgency as other well-characterized neurological diseases (169). However, during this time, the researchers observed altered muscle metabolism, immune response and neurological function. The lack of consistent laboratory results and variability between cohorts hampered formal recognition of the condition.

Despite extensive clinical evidence, the qualification of ME/CFS or SEID as a distinct identifiable disease or a nosological entity was frequently challenged. In the 1970s, there was a spike in cases and the patients were mostly female (168). This prompted the medical authorities at the time to suggest that the condition was psychosomatic in nature. Implying that the physical discomfort was due to an underlying psychological stress, anxiety or trauma. Then ensued a series of recommendations to rename the condition as “Myalgia nervosa”- discrediting a well-evidenced physiological definition for a psychological one. This re-classification was

derived from prevalent biases in medicine during history where conditions that predominantly affected women were dismissed as hysteria or somatization disorders. Due to limited funding, institutional support and broader recognition, detailed scientific research on ME/CFS suffered while the psychiatric explanations dominated diagnoses and subsequent treatment interventions. Patients were prescribed Cognitive Behavioral therapy (CBT) or Graded Exercise Therapy (GET), which later, not only proved to be controversial but actually threatened patient safety by inducing severe PESE.

Growing clinical evidence, however, repeatedly and strongly refuted the psychosomatic hypothesis. Longitudinal research proved that the condition induced quantifiable immunological and neurological impairments. These impairments included chronic inflammation, autonomic dysfunction and immune system imbalance (170). In the 1980s, progress in virology and immunology further solidified that ME/CFS was not a psychiatric condition but in fact had deeper biological and mechanistic underpinnings. These researchers also recorded that a noteworthy percentage of these patients presented with a history of previous viral infections such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), or enteroviruses, which led to the hypothesis that an underlying or a reactivated viral infection could be a possible contributing factor (170). Furthermore, studies identifying altered energy metabolism, mitochondrial abnormalities, and changes in cytokine profiles also began surfacing. All this accumulating scientific evidence further consolidated the physiological foundation of this disease (171).

Consequently, the prefix Benign was considered inappropriate due to the extreme functional impairment and discomfort reported by the patients. It was not until the late 20th century that the nomenclature evolved to “Myalgic Encephalomyelitis/Chronic Fatigue Syndrome” an umbrella term which represented both the neurological features (ME) and the principal symptom of chronic, fatigue (CFS) (172). This dual naming system allowed to include the historical diagnostic criteria and general clinical picture, accommodating different scientific explanations and diagnostic frameworks. From there on, development of diagnostic criteria such

as the 1988 Holmes Criteria, the 1994 Fukuda Criteria, and later the Canadian Consensus Criteria (2003) and the International Consensus Criteria (2011) enabled better organized outlines to define and diagnose the condition (173–175) (Figure 1.9).

The complex pathophysiology of ME/CFS is still being elucidated by current research, with recent findings demonstrating the involvement of neurological disorders, metabolic abnormalities, and immunological dysfunction (176–178). Both positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have revealed abnormalities in brain activity, particularly in areas linked to executive processes and sensations of pain (179,180). Studies have also linked ME/CFS to altered gut microbiome composition, small fiber neuropathy, and decreased cerebral blood flow, suggesting a complicated etiology that includes both central and peripheral diseases (181,182). Although there are still challenges in achieving widespread clinical recognition and consistent diagnostic standards, the evolution of ME/CFS nomenclature and classification demonstrates the progress made in understanding its scientific underpinnings. Despite the ongoing biases and therefore difficulty in acquiring broader clinical recognition and standardized diagnostic guidelines, contemporary research continues to probe deeper into the mechanism of disease manifestation and physiological progression (183,184).

ME/CFS DIAGNOSTIC CRITERIA

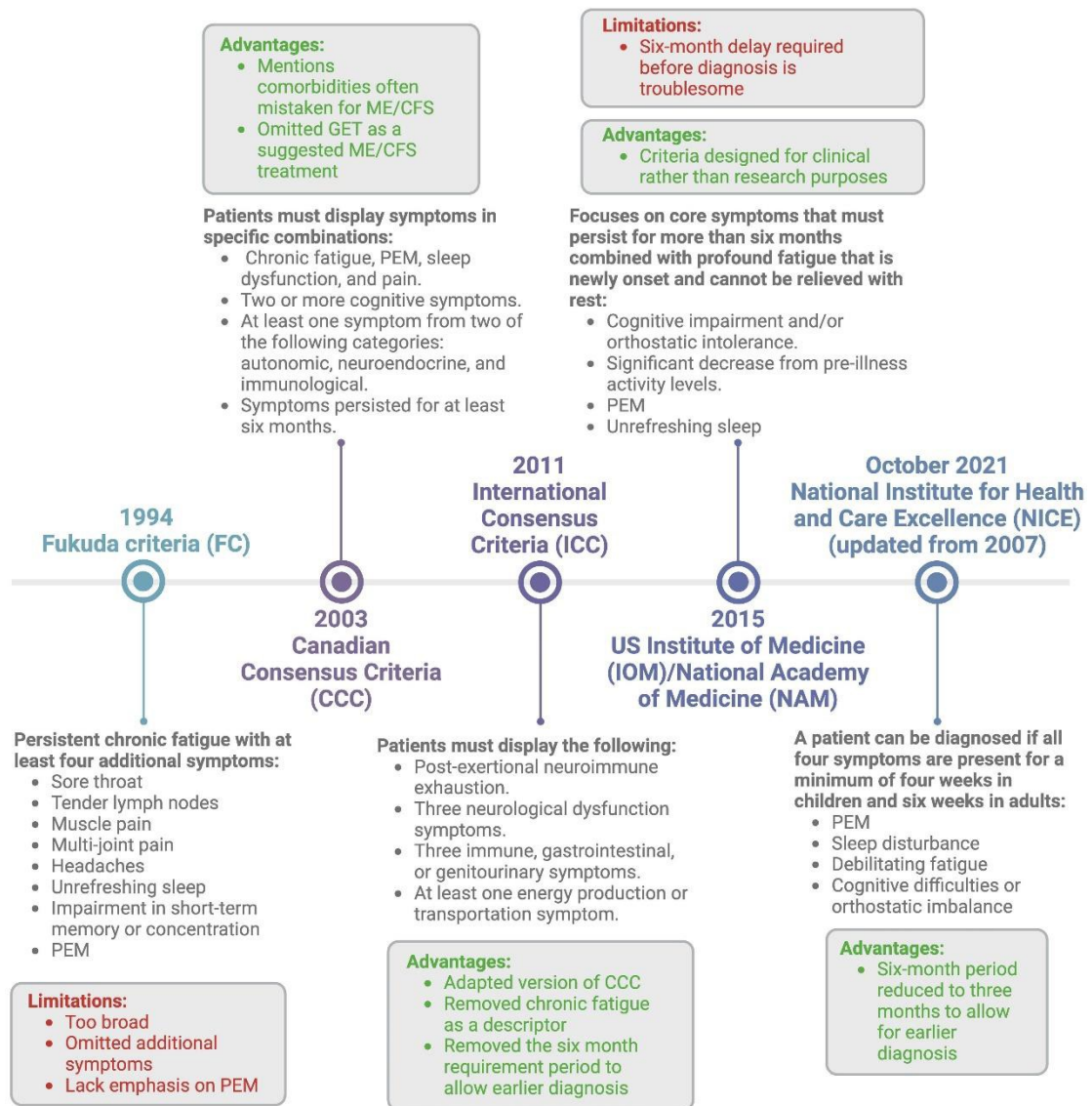


Figure 1.9. An overview of the various ME/CFS diagnostic criteria developed in the last 3 decades. Adapted from (165)

Multiple theories exist in contemporary literature suggesting various explanations; The theory of Energy Envelope, proposed by Jason et al., (1999), suggests that ME/CFS patients have a decreased threshold of energy production (185). When this limited energy capacity threshold is exceeded, symptoms may worsen. The theory implicates impaired mitochondrial function in CFS patients which compromises their capability for aerobic respiration. The alternative, anaerobic respiration, is less efficient, not only yielding less ATP per glucose molecule but also

leading to an accumulation of lactic acid, resulting in a decrease in blood and tissue pH, potentially impairing muscle function and inducing fatigue (186). The Push-Crash Cycle explanation suggests that CFS patients experience a burst of adrenaline and energy causing them to be overexerted and eventually crash (187). Studies have also implicated hyperactivation of inflammatory processes and cytokine storms to explain PESE/PEM (188–193). Dysfunctional mitochondrial oxidative phosphorylation and metabolic disturbances are till date the most widely agreed upon explanations for peripheral fatigue (194–196). Investigating metabolic alterations can help in identifying misallocation of energy or metabolic plasticity to manage against stress which eventually leads to fatigue. Furthermore, studies also point to the epigenetic reprogramming in ME/CFS patients that dysregulate expression of Transposable elements or transposons that in turn effect the expression of multiple protein encoding genes and by extension alter key biological processes.

1.4.2. Long COVID-19 or post-acute sequelae of COVID-19

Coronavirus disease (COVID-19) is a severe contagious respiratory disease caused by the SARS-CoV-2 virus. It spreads through the respiratory system by contacting contaminated surfaces and by coming into close contact with infected people. In approximately 10% of these patients, symptoms continue to persist for longer than three months (Figure 1.10). The symptoms are similar to those experienced by ME/CFS patients such as impaired exercise tolerance, extreme fatigue and post exertional malaise or post exertional symptom exacerbation after both physical and mental exertion (197). This has been termed as the post-acute sequelae of COVID-19 or long COVID-19 because they cannot be explained by other diseases or comorbidities. Contemporary scientific hypotheses suggest mitochondrial dysfunction, local hypoxia, disturbed immune system response, local and systemic inflammation (198). There is, however, no consensus due to a large variability between patients and lack of proper assessment and diagnostic tools. Furthermore, mechanistic hypotheses to investigate long COVID-19 are still in their

infancy and require rigorous extensive research to conclusively understand the pathophysiology (199,200).

Other possible mechanisms of progression manifest through the neuropsychiatric, cardiovascular, respiratory system, digestive, reproductive, musculoskeletal and immune systems. There is also evidence that the additional morbidity or vulnerability brought on by long COVID-19 is responsible for proportionally increasing the rate of reinfection (201). While vaccination provides sufficient protective barrier, the risk of developing long COVID-19 persists.

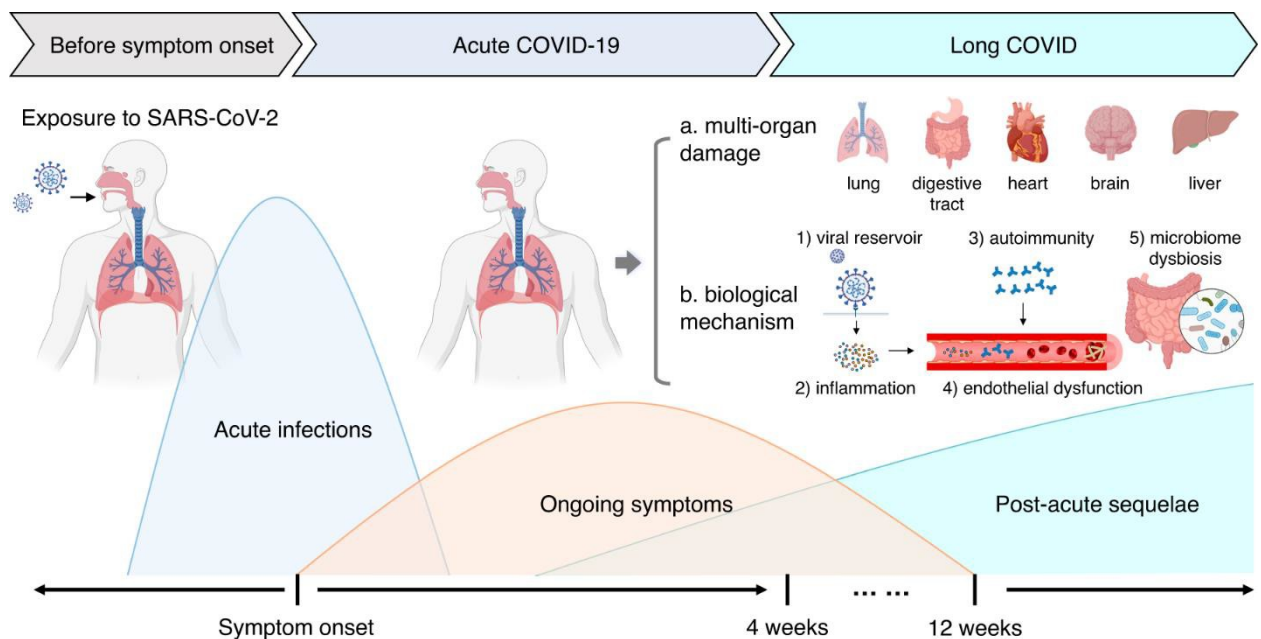


Figure 1.10. A timeline of long COVID onset. Post acute COVID phase, the time period between 4-12 weeks and continuing further is classified as Long-COVID. In the inset are the organ systems affected and the pathomechanisms. Figure adapted from (198)

1.4.2.1. Neuropsychiatric System

The long COVID-19 patients commonly present with the clinical symptoms involving the neuropsychiatric system such as cognitive disorder, dizziness, brain fog, memory loss, sensory and motor aberration, paresthesia, sensitivity to light, sound and scents, loss of sense of taste and smell (202). Among these symptoms, cognitive impairments were the most prevalent symptoms in patients suffering with long COVID-19, approximately 22% of the patients reported impaired cognition,

thinking, recollection of memory and perception. Furthermore, 25% of patients report experiencing brain fog which includes impairments in speech fluency, attention deficit, and executive function disorders (203,204). While these symptoms do not threaten a high morbidity, they do impede normal day-to-day activities. Furthermore, in children these neuropsychiatric effects induce a high risk of developing seizures, epilepsy and other psychotic problems. Different explanations have been suggested to understand the onset and persistence of these neurological symptoms. One hypothesis suggests the continued presence of SARS-CoV-2 virus in the patients' nervous system (205). Research investigating this hypothesis has evidenced direct neuronal invasion which induced symptoms like Guillain-Barre syndrome, anosmia and encephalitis (206). Animal testing and autopsy data also showed persistence of viral particles in the brain months after the first infection (207,208). Another explanation is neuroinflammation which is evidenced by MRI studies showing structural changes in the brain such as reduction of grey matter in patients (209). Furthermore, increased levels of inflammatory cytokines such as CCL11 have been associated with decreased neurogenesis, activation of glial cells, neurodegeneration and cognitive decline (210). Problems in the endothelial-vascular systems have also been reported indicating reduced density of the capillary network and increase in endothelial markers evidencing persisting vascular damage in brain and other organs months after the preliminary infection (211).

1.4.2.2. Musculoskeletal System

Muscle weakness and peripheral fatigue are two of the cardinal symptoms of long COVID-19 and reported as core symptoms by 92% of patients (212). These symptoms may be because of several interconnected mechanisms. Skeletal muscles also express the AEC2 and TMPRSS2 receptors, targeting them for SARS-COV2 attack. The autopsy results, however, found low or negative viral load in skeletal muscle tissues of COVID-19 patients with known musculoskeletal symptoms (213). A more compelling explanation is the role of systemic inflammation that may induce muscle wasting mechanisms such as elevated levels of pro-inflammatory cytokines (INF- γ , TNF- α , IL-10 and IL-6). There is also evidence

of circulating micro-clots in muscle biopsies and vascular inflammation which may also contribute to nervous system sensitization, peripheral fatigue and muscular pain in patients (214) (Figure 1.11). Furthermore, local hypoxia is also a widely accepted explanation for severe COVID-19 patients (215). Hypoxia inducing factors (HIFs) may trigger not only signpost anerobic metabolic pathways but may also trigger muscle wasting and impaired regeneration. An increased abundance of glycolytic fibers, shift to anaerobic respiration and calcium overload due to H^+ / Na^{2+} imbalance that adversely impacts muscle contractile performance and mitochondrial function leading to low ATP levels and elevated ROS.

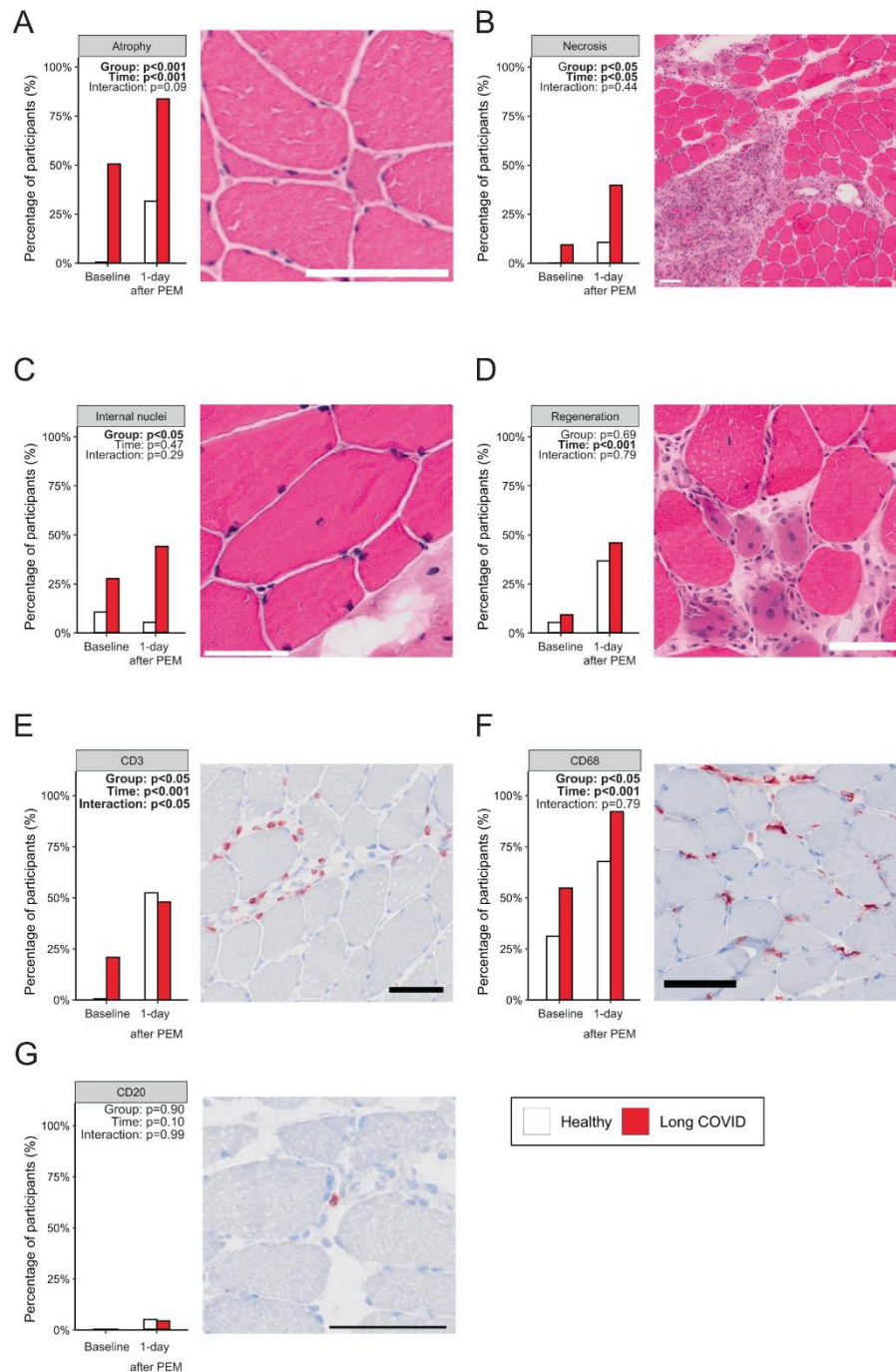


Figure 1.11. Features in skeletal muscles of long COVID patients. A) atrophic fibers in patients with long COVID and post exercise. B) Necrotic fibers appear in patients with long COVID post-exercise. C) fiber repair was apparent in long COVID patients by the presence of internal nuclei D) Regenerative fibers were more abundant in the post-exercise group. E) Long COVID cohort had higher CD3+ T-cell infiltration. F) Long COVID cohort had higher CD68+ macrophages. G) CD20+ B-cells population was not abundant post-exercise in the skeletal muscle. Adapted from (190)

1.4.2.3. Immune Microenvironment

Changes in the immune microenvironment are also recognized as key contributors to the disease. During the first three months, elevated levels of TNF alpha and INF- γ induced protein 10 have been observed in patients that ultimately developed long COVID-19 (216). Additionally, IL-6 levels have also been found to be upregulated in patients with long COVID-19. Approximately eight months after infection, long COVID-19 patients have a characteristic immune profile with activated innate immunity, increased expression of interferons IFN- β and IFN- λ 1, as well as reduced expression of naïve B and T lymphocytes. Correlating the chronic immune profile with the early phase profile offers a potential niche to understand the role of immune dysregulation in inducing disease symptoms in muscles and other organs.

1.4.3. Steroid Myopathy

Corticosteroid-induced myopathy or steroid myopathy is an endocrine-related muscle disorder. It is triggered by an excess of systemic circulating corticosteroids which could be endogenous due to adrenal tumors, excessive ectopic adrenocorticotrophic hormone (ACTH) production or triggered by the overconsumption of exogenous corticosteroids during steroid treatment against other diseases such as respiratory inflammation (217). Corticosteroids trigger muscle atrophy by hyperactivating the ubiquitin-proteasome pathway. The 9 α -fluorinated steroids, such as dexamethasone and triamcinolone are more likely to induce a myopathy compared to other steroids. Several weeks of steroid therapy may initiate muscle weakness, but chronic use of high doses of steroid therapy may result in more acute steroid myopathy which may threaten paralysis or respiratory failure in patients (218). Patients experience proximal muscle weakness which is particularly prominent lower extremities. Contrary to other muscular myopathies, in patients with steroid myopathy, standard diagnostic biomarkers such as creatine kinase, aspartate aminotransferase, lactate dehydrogenase and aldolase levels are often normal in the early disease stages (219). These markers might only be elevated in critically ill patients, making it difficult to get a timely diagnosis. Corticosteroid-induced myopathy or steroid myopathy is an endocrine-related muscle disorder. It is triggered by an excess of systemic circulating corticosteroids

which could be endogenous due to adrenal tumors, excessive ectopic adrenocorticotrophic hormone (ACTH) production or overconsumption of exogenous corticosteroids during steroid treatment against other diseases such as respiratory inflammation (217). Corticosteroids trigger muscle atrophy by hyperactivating the ubiquitin-proteasome pathway. The 9 α -fluorinated steroids, such as dexamethasone and triamcinolone are more likely to induce a myopathy compared to other steroids. Several weeks of steroid therapy may initiate muscle weakness, but chronic use of high doses of steroid therapy may result in more acute steroid myopathy which may threaten paralysis or respiratory failure in patients (218). Patients experience proximal muscle weakness which is particularly prominent lower extremities. Contrary to other muscular myopathies, in patients with steroid myopathy, standard diagnostic biomarkers such as creatine kinase, aspartate aminotransferase, lactate dehydrogenase and aldolase levels are often normal in the early disease stages (219). These markers might only be elevated in critically ill patients, making it difficult to get a timely diagnosis.

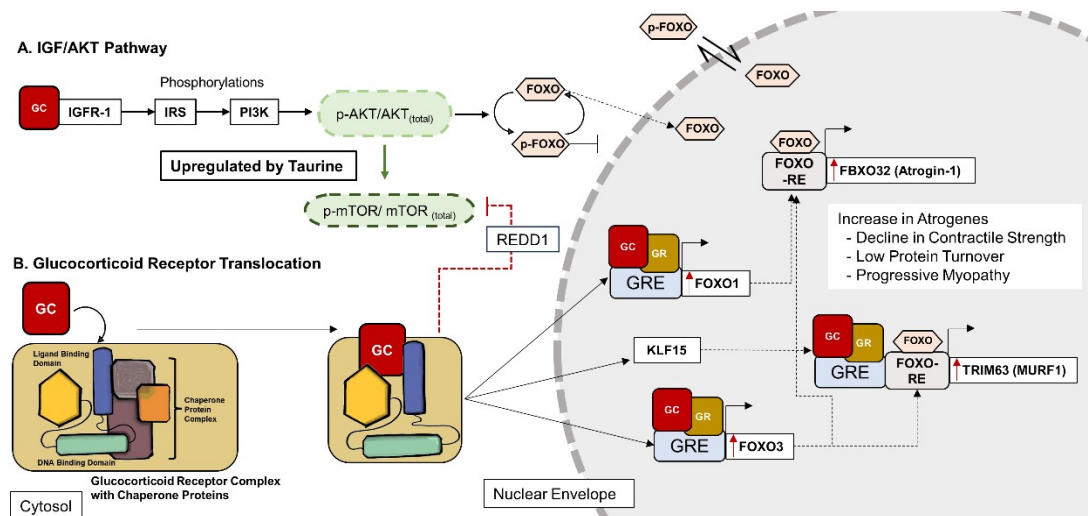


Figure 1.12: Pathways implicated in glucocorticoid exposure and the mechanism of action of taurine. GC, glucocorticoid; GR, glucocorticoid receptor complex; RE, responsive element.

Mechanistically, corticosteroid induced myopathy can be triggered due to a dysregulation of catabolic, anabolic or both pathways. Corticosteroids may upregulate catabolic processes such as the ubiquitin-proteasome system, calpains

and cathepsins to induce proteolysis of myofibrillar proteins. These processes cause actin to dissociate from myosin and induce myocyte apoptosis. Dysregulation of anabolic mechanisms is triggered by an inhibition of amino acid transport and IGF-I production. Differentiation of satellite cells into myofibers is also upset and overall protein synthesis and myogenesis is, therefore, disrupted. Furthermore, corticosteroids with high mineralocorticoid activity also lower the levels of sodium and potassium in the serum, thereby contributing to muscle weakness. Patients express an increased intolerance to exercise due to weakness and pain.

At the molecular level, decreased production of IGF-I and increased myostatin have been observed in muscular atrophy caused by glucocorticoids. IGF-I usually counteracts the catabolic mechanisms induced by corticosteroids by inhibiting a major positive regulator of atrogenes, the FOXO (Forkhead box O) transcription factor. This inhibition is achieved by activating PI3K/Akt/mTOR pathway. *mTOR* inhibition by corticosteroids has also been observed which results in a decreased phosphorylation of 4E-BP1 and S6K1. Supplementation with exogenous IGF-I may help in overriding the effect of glucocorticoids and suppress muscle cell atrophy.

Chapter 2: General Objectives

Skeletal muscle tissues are highly adaptable organs capable of exhibiting not only contractile flexibility but also metabolic plasticity. These are among the most important features that make skeletal muscle tissues central to investigate metabolic and idiopathic conditions such as Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome and Long COVID-19. As mentioned before, these diseases have unknown etiologies and mechanisms of progression, but both have peripheral fatigue and post-exertional malaise at their core. Furthermore, both conditions have predominantly patient-specific features and tend to differ from patient to patient.

Steroid myopathy, on the other hand, is comparatively better characterized in terms of its pathophysiology and mechanism of progression. As mentioned before, it usually develops as a side effect of excessive systemic corticosteroids and progressively causes extreme muscle atrophy. Therefore, in the initial stages the disease remains unnoticed and undiagnosed.

This thesis hypothesizes that well-differentiated artificial 3D skeletal muscle tissues made by encapsulating immortalized muscle progenitor cells or primary myoblasts in a hydrogel of Matrigel and Fibrin are potent tools to investigate idiopathic and unknown disease conditions such as ME/CFS and Long COVID-19 in a patient-specific manner. The thesis also hypothesizes that these artificial tissues can be used not only to model diseases such as steroid myopathy but also to investigate the molecular pathways involved in inducing atrophy and simultaneously testing therapeutic interventions in a much quicker and efficient way.

The objectives of this research are therefore:

- I. To fabricate and characterize *in vitro* skeletal muscle disease models (acute and chronic) for investigating muscle pathology in ME/CFS and Long-COVID-19 using patient sera.
- II. To develop and characterize the first artificial skeletal muscle model of human steroid myopathy and to test amino acid supplementation as a means to reverse muscle atrophy.

**Section A: Myalgic Encephalomyelitis/Chronic
Fatigue Syndrome and Long COVID-19**

Chapter 1A: Introduction

1A.1. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

1A.1.1. Current State of the Art

Clinical evidence has substantially established skeletal muscle involvement in both Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome (ME/CFS) and post-acute sequelae of SARS-CoV-2. Investigation techniques include force measurements using hand grip and quadricep strength, cyclic ergometry to test for VO₂ max, imaging and histopathology using patient biopsies. Cyclic ergometry and exercise testing in patients resulted in an impaired exercise capacity, high fatiguability, rapid exhaustion, metabolic reprogramming favoring anerobic respiration and mitochondrial dysfunction. Contemporary literature also suggests vascular dysregulation and hypoperfusion as causative mechanisms of nervous and muscular ambiguities observed in both disease conditions. In their study, Kedor et al., (2022) correlated diminished muscle strength with biomarkers of hypoperfusion in both ME/CFS and long COVID-19 patients (220). Furthermore, they also found correlation between markers of inflammation for long COVID-19 patients. Other studies highlight endothelial dysfunction, inflammation induced changes in the capillary wall, impaired deformability or flexibility of red blood cells, and enhanced vasoconstriction to obstruct efficient circulation (214,221). Additionally, this obstructed microvasculature has been held responsible for diastolic dysfunction and low stroke volume (222,223). Skeletal muscle biopsies have evidenced muscle atrophy, changes in endothelial basement membrane, necrosis and inflammation (190,224–226). Muscle biopsy studies of long COVID-19 patients presenting with fatigue and reduced exercise capacity at least six months after the preliminary acute infection did not show any capillary obstructions and micro-clots that were present in patients in the early stages of long COVID-19 onset. Furthermore, there was no evidence of viral reactivation or persistence of viral capsid and only mild to no inflammation (214,227). In the same studies for the patient cohort exposed to exercise, key parameters like the capillary-fiber ratio, intra and extracellular lactate concentrations were found to be unaffected. There was, however, an increase in glycolytic fibers, mitochondrial dysfunction and necrosis in the muscles for the cohort

exposed to physical exertion. Findings from these studies rule out the role of capillary disturbance in inducing muscle pathologies but necrosis and mitochondrial dysfunction continue to be the commonly reported explanations and observations in the patient groups.

Biopsy studies, metabolomics and transcriptomics analyses indicate diminished mitochondrial performance. Electron microscopy has been frequently used to study the ultrastructure of mitochondria, including cristae formation and the inner mitochondrial membrane. Studies indicate that subsarcolemmal mitochondria appeared to be more affected compared to the interfibrillary mitochondria. Deep transcriptomics between ME/CFS and healthy controls considering sex-based differences also reveal key differences such as downregulation of beta-fatty acid oxidation and mitochondrial dysfunction in patient groups. It further postulates that ME/CFS could possibly be a centrally mediated disorder (228). Biopsy based studies evidencing disturbances in muscle pathophysiology during disease conditions are tabulated in Table 1.

Table 1: Research suggesting muscle pathophysiology in Long COVID-19 (PCS) and ME/CFS patients.

Study	Participants	Time Since Infection	Methods	Main Findings
Appelmans et al.	25 long COVID-19 patients with PEM (based on DSQ-PEM)	1–2 years	Light microscopy (pre/post exercise), <i>In vitro</i> mitochondrial function	Decreased exercise capacity and <i>in vitro</i> signs of mitochondrial dysfunction. Exercise led to increased amyloid deposits outside capillaries, focal fiber atrophy, more glycolytic fibers, moderate leukocyte infiltration, and focal necrosis. Post-exercise muscle damage

					increased. Some regeneration and mild pre-existing damage observed.
Bizjak et al.	13 COVID-19 patients, ME/CFS patients (CCC criteria)	long COVID-19 15	Long COVID-19: 15 months ME/CFS: 23 months	Light and electron microscopy, Ergometry, <i>in vitro</i> mitochondrial function	Both groups had reduced exercise tolerance and mitochondrial dysfunction. Long COVID-19 patients showed no visible mitochondrial structural damage. ME/CFS patients exhibited altered mitochondrial morphology, including cristae damage and subsarcolemmal clustering, suggesting more advanced mitochondrial involvement.
Aschman et al.	11 COVID-19 and long COVID-19-ME/CFS (CCC) patients	long COVID-19 and long COVID-19-ME/CFS	6–12 months	Light and electron microscopy, MRI, Muscle proteomics	Muscle weakness with type-2b fiber atrophy, increased macrophages, and inflammation. Capillary density decreased; basement membranes thickened. Gene expression related to mitochondrial functions were downregulated. No signs of active myositis were observed.

Hejbøl et al.	16 COVID-19 patients	long	5–14 months	Light and electron microscopy, Electromyography	Muscle weakness and myopathic EMG patterns. Muscle fiber atrophy and signs of regeneration observed. Mitochondrial damage included loss of cytochrome c oxidase activity, abnormal cristae, and subsarcolemmal accumulation. Inflammatory markers such as T lymphocytes and HLA-I expression detected. Structural capillary changes noted.
Agergaard et al.	8 COVID-19 patients	long	Median 9.8 months	Light and electron microscopy, Electromyography (quantitative and single fiber)	Reduced muscle strength with EMG confirming myopathic changes. Microscopy revealed damage at the neuromuscular junction, including abnormalities in terminal motor nerves and the motor endplate.
Walitt et al.	8–17 ME/CFS patients		Median 33 ± 15 months	RNA sequencing, Mitochondrial genome analysis, Mitochondrial function testing	In males, gene expression showed downregulated mitochondrial and glucose metabolism and upregulated fatty

				acid β -oxidation. In females, both mitochondrial and fatty acid metabolism genes were downregulated. No difference in mitochondrial function of blood immune cells, indicating potential tissue-specific dysfunction.
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To explain alterations in mitochondrial function, researchers have proposed several contributing mechanisms like inflammation, persisting viral infection, autoantibodies, ionic imbalance and calcium overload. The process causing calcium overload has been explained by increased hypoperfusion during exercise causing overload of sodium proportionately calcium overload. This hypothesis suggests that impaired function of the in transporters and hypoperfusion may be potential explanations for the necrosis observed in muscles and the mitochondrial dysfunction as well. Increased accumulation of sodium ions in muscles has been evidenced by a Magnetic Resonance Imaging (MRI) study. The study also showed that sodium accumulation and elevation were found with or without exercise in different types of muscles at varying degrees. This overload of sodium and alternatively calcium has been considered to explain impaired energy metabolism, exercise intolerance experienced by the patients. Furthermore, muscle cramps and fasciculations have also been explained using this hypothesis.

Electron microscopy has revealed that mitochondrial damage in ME/CFS patients is localized specifically to the subsarcolemmal region, with interfibrillary mitochondria remaining intact. This distinct pattern suggests a localized pathogenic mechanism, likely related to calcium overload in areas near the sodium-calcium exchanger (NCX). Under conditions of elevated intracellular sodium, NCX may

operate in reverse, importing excess calcium and triggering mitochondrial injury. In contrast, long COVID-19 patients in the same study did not exhibit this damage pattern. However, separate findings in long COVID-19, though without clear ME/CFS diagnostic confirmation, have demonstrated mitochondrial abnormalities such as reduced cytochrome c oxidase activity, subsarcolemmal mitochondrial accumulation, and structural cristae defects. These alterations may stem from post-infectious capillary or microvascular dysfunction, indicating a potentially self-perpetuating cycle of impaired mitochondrial function and disrupted vascular perfusion. While initial vascular damage may improve over time, the persistence or escalation of mitochondrial dysfunction could contribute to the transition from long COVID-19 to long COVID-19 -ME/CFS, especially in the presence of additional vascular risk factors. Studies assessing mitochondrial function in peripheral blood immune cells of ME/CFS patients have yielded inconclusive data, suggesting that the pathology is perhaps tissue specific.

A fully developed mitochondrial dysfunction is a self-sustaining condition driven by ROS, particularly by inhibiting ion transporters. Furthermore, metabolic reprogramming and switch to anaerobic respiration evidenced through an increased proportion of glycolytic fibers due to mitochondrial dysfunction and disrupted perfusion also leads to excessive proton generation and leakage, causing sodium overload. This implies that even small physical exertions may lead to calcium accumulation causing calcium-induced necrosis and mitochondrial dysfunction.

As mentioned before, a significant proportion of patients with long COVID who experience exertional intolerance and prolonged post-exertional malaise eventually meet the diagnostic criteria for ME/CFS, typically presenting more severe and persistent symptoms. While many long COVID patients show gradual improvement within 1–2 years, those who develop ME/CFS often experience chronic or worsening illness. Does this imply that ME/CFS is ultimately the more severe and prolonged fate of long COVID patients, or are there actually different specific covert pathomechanisms that are activated to enable this transition and present with the same clinical profile? Evidence suggests a time-dependent shift in vascular

involvement. In the early phase following SARS-CoV-2 infection, impaired capillary microcirculation is largely due to inflammatory damage to capillary walls and pathological changes in blood components. In the later stages, however, vascular impairment appears more functional and dynamic in nature, involving endothelial dysfunction, ROS generated from mitochondrial dysfunction, and potentially autoantibodies targeting vasoregulatory receptors. As stated before, histological studies no longer detect early capillary damage in chronic long COVID cases meeting ME/CFS criteria. Additionally, mitochondrial pathology appears more pronounced in ME/CFS. While structural mitochondrial damage is absent in long COVID patients, ME/CFS muscle biopsies consistently show morphologically altered and damaged mitochondria, particularly in the subsarcolemmal region, pointing to a progressive mitochondrial involvement unique to the chronic disease state. This work aims to answer 2 main questions:

- 1- What are the mechanistic differences in mitochondrial dysfunction, altered muscle metabolism, and microvascular impairment between long COVID and ME/CFS patients?
- 2- Do immune-related factors, such as patient-derived IgG antibodies, contribute to mitochondrial dysfunction and muscle pathology in long COVID and ME/CFS?

1A.1.2. Objectives

This section of the thesis fabricates artificial 3D skeletal muscle tissues from immortalized human satellite cells and primary myoblasts to study the effect of systemic factors from ME/CFS and long COVID-19 patients on the structure and function of muscle form and function. It then investigates the transcriptome of the samples by comparing to healthy controls and studying the expression of transposable elements. It then probes deeper into the mitochondrial structure and functions for both acute and chronic exposures. Furthermore, an oxygen sensing nanoprobe was inoculated in the tissue to measure real time oxygen consumption

dynamics alongside changes in metabolomics of tissues treated with sera from patients before and after cryotherapy using Raman micro spectroscopy.

The objectives are as follows:

1. To fabricate and characterize *in vitro* skeletal muscle disease models (acute and chronic) of ME/CFS and Long-COVID-19 using patient sera.
2. To investigate the expression and effect of transposable elements in inducing disease conditions through bioinformatics tools.
3. To test if the above characterized ME/CFS disease conditions can be induced by patient derived IgG antibodies.
4. To develop *in vitro* skeletal muscle disease models using primary myoblasts inoculated with oxygen sensing nanoprobe to monitor real time oxygen consumption dynamics upon patient serum exposure.
5. To evaluate metabolic changes in the primary myoblasts derived artificial skeletal muscle tissues using Raman spectroscopy.

Chapter 2A: Materials and Methods

2A.1. Study Population

This pilot cohort study was conducted involving 4 individuals with ME/CFS (mean age: 49.1 ± 3.1 years), 5 long COVID patients (mean age: 48.3 ± 2.4 years) with ongoing post-exertional fatigue for at least 12 months and 4 age-, sex and BMI-matched non-fatigued sedentary controls (mean age: 46.7 ± 1.8 years) recruited consecutively between September 2021 and December 2022 from the largest outpatient tertiary referral center in Spain (ME/CFS Clinical Unit, Vall d'Hebron University Hospital, Barcelona, Spain) and Hospital Clinic, Barcelona, Spain. After receiving verbal and written information on the study protocol, all subjects signed informed consent before participation. The study protocol was reviewed and approved by the IRB of Vall d'Hebron University Hospital (reference number: PR/AG 201/2016). All procedures were conducted in accordance with the ethical standards of the board and the 1964 Declaration of Helsinki for human research including its later amendments.

2A.2. Eligibility Criteria

ME/CFS patients were potentially eligible if they were female, aged ≥ 18 years and had a confirmed diagnosis for ME/CFS by a specialist physician based on international consensus criteria (2011 ICC), the recommended diagnostic criteria for ME/CFS research purposes (229). Long COVID patients were eligible for enrollment if they were female, aged ≥ 18 years and, following a confirmed diagnosis of acute COVID-19 infection based on a positive SARS-CoV-2 RT-PCR test on nasal swab during the COVID-19 pandemic, were still suffering from persistent fatigue, unexplained post-exertional malaise and other core symptoms/signs ≥ 3 months after the acute COVID-19 infection, and met the same clinical case criteria (2011 ICC) for ME/CFS (229). No-fatigued sedentary donors were eligible if they had neither experienced any post-exertional fatigue/malaise and post-acute infection illnesses-associated symptoms/signs nor had been in recent contact with anyone who was infected with SARS-CoV-2 (COVID-19) within 90 days prior to the study, no significant neurological, cardiac, endocrine, or neuroimmune disorders, no alcohol or drug dependence, and did not use daily prescribed medications. All no-

fatigued sedentary subjects were recruited through word-of-mouth from the local community and did not meet the case criteria for either ME/CFS or long COVID at the time of enrollment.

All participants were of Caucasian descent, from the same geographical area, and had a sedentary lifestyle at the time of the study. They were subject to stringent exclusion criteria, as previously described (230). The major exclusion criteria were a relevant previous or current diagnosis of autoimmune disorder, multiple sclerosis, psychosis, major depression disorder, heart disease, hematological disorders, infectious diseases, sleep apnea or metabolic disorders; pregnancy or breast-feeding; smoking habit; strong hormone-related drugs; and preexisting fatigue-associated symptoms or evidence of multiorgan failure that did not meet the case criteria for ME/CFS and long COVID used in this study.

2A.3. Blood Collection and Processing

After a 12-hour overnight fasting, 6 ml of peripheral whole blood were collected from each participant between 8:00 a.m. and 10:00 a.m. by a trained phlebotomy nurse (venipuncture from an antecubital vein with a 19-gauge needle) into SST-tubes for serum isolation (BD Vacutainer, Becton Dickinson, Sarstedt, Barcelona, Spain) in the USIC outpatient clinical unit, Vall d'Hebron University Hospital, and Hospital Clinic, Barcelona Spain. One tube was transported and delivered to the local core laboratory within 2 h of collection for routine blood analyses, following standard and recommended procedures. The other blood tube was immediately centrifuged at 2,500 rpm for 15 min at 4°C (Thermo Scientific, Waltham, MA, USA), after which serum specimens was collected and stored in aliquots at –80 °C until further assays. An aliquot of the serum samples was shipped on dry ice to IBEC laboratory for further studies in the 3D “*in vitro*” human skeletal muscle model. No serum sample was thawed more than twice.

2A.4. Study Design

To understand the pathomechanism and develop an *in vitro* model of ME/CFS and LC-19 disease manifestation in a skeletal muscle we adopted a sequential experimentation design. This type of study design helps in understanding idiopathic diseases, where each experiment is planned based on the inferences obtained from a previous experiment. In summary, we made 3D *in vitro* skeletal muscle tissues from immortalized human muscle progenitor cells. After 6 days of differentiation, we treated the mature tissues with 5% patient or control sera for 48, 96 and 144 hours and then proceeded with subsequent analyses.

2A.5. Fabrication of PDMS Platforms

The 3D polydimethylsiloxane (PDMS) platforms were fabricated as published before (231). Briefly, Master molds were 3D printed using SolusProto (Reify3D, CA, USA) with a Direct Light Projection 3-D printer (Solus DLP 3D Printer, Reify3D) at low resolution (80 x 45 mm with a pixel distance of 45 microns). The design was converted to a Standard tri-angle Language (STL) format for printing. After removing all traces of uncured resin by thorough washing and cleaning with ResinAway, the thermo-cured 3D master molds were activated with ozone plasma for 30 s and silanized in a vacuum desiccator with a few drops of trichloro (1H,1H,2H,2H-perfluorooctyl)-silane (PFOTS, Sigma-Aldrich). These 3D printed molds were used to prepare negative molds with PDMS (5:1), which were then silanized after thorough washing. Post silanization, the negative molds were used to fabricate the final casting molds with PDMS (10:1). After fabrication, all the casting molds were thoroughly washed and dried before using for cell encapsulation (217).

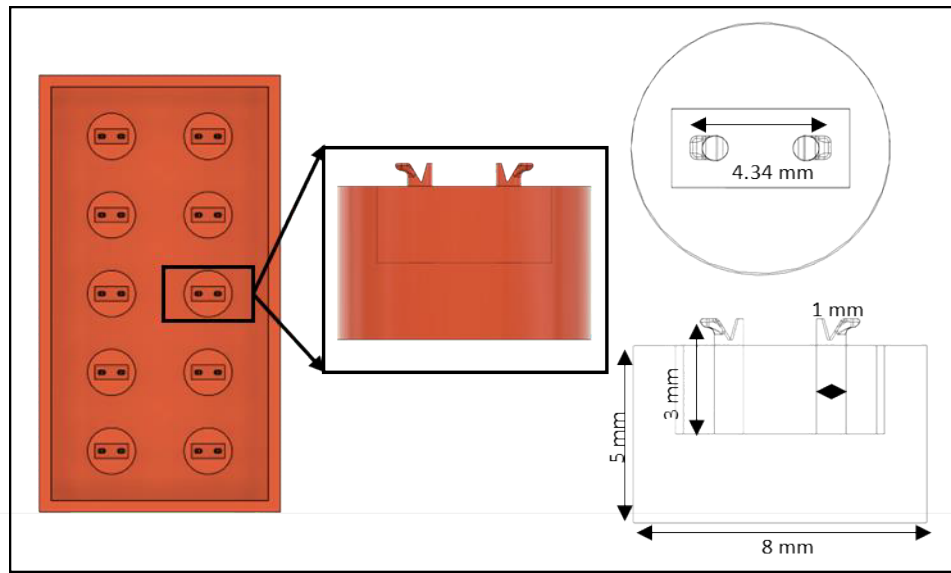


Figure 1: Design of the Master Mold and final PDMS platforms.

Surface of the final casting platforms was treated with filtered 5% Pluronic F-127 (Sigma-Aldrich) in PBS for at least 2 h at 4°C to facilitate hydrogel detachment. Pluronic was removed by tapping the chip on a filter paper without grazing the bottom of the well. The platforms were stored at 4°C until tissue fabrication.

2A.6. Cell Culture

Human immortalized muscle progenitor cells were obtained from the Institut NeuroMyoGene, Lyon, France (232). Specifically, cell line control 2-E4 (CNT2-E4) was used with a doubling time of 3.55 days and 99.40% of CD56+ cells. Primary myoblasts were obtained from the Morten group, Nuffield Department of Women's and Reproductive Health, John Radcliff Hospital, Oxford, United Kingdom.

The cells were cultured in skeletal muscle basal growth medium (SMC-GM, C23060, PromoCell) with supplemental mix (C23060, PromoCell), 1% penicillin-streptomycin (10,000 U mL⁻¹, 15140-122, Thermo Fisher Scientific) and 10% fetal bovine serum (10270-106, Thermo Fisher Scientific). For differentiation, the cells were cultured in Dulbecco's modified Eagle medium (DMEM), high glucose, GlutaMAX (Gibco, Thermo Fisher Scientific), 1% v/v penicillin-streptomycin-glutamine (P/S-G, 100×

Gibco, Thermo Fisher Scientific) and 1% v/v insulin-transferrin-selenium-ethanolamine supplement (ITS-X, 100×, Gibco, Thermo Fisher Scientific).

2A.7. Tissue Fabrication

An established protocol in our group was adopted to fabricate functional skeletal muscle tissues (231). Briefly, for encapsulation, the human muscle precursor cells were trypsinized and resuspended in skeletal muscle growth medium. The cells were encapsulated at a density of 2.5×10^7 cells mL^{-1} in 30% v/v Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning), 2 U mL^{-1} thrombin from human plasma (Sigma-Aldrich) and 4 mg mL^{-1} fibrinogen from human plasma (Sigma-Aldrich). During hydrogel casting, care was taken to avoid bubbles and cold plasticware was used to prevent polymerization of Matrigel. The mixture was spread as homogenously as possible between the pillars without grazing the surface. After hydrogel introduction all tissues were incubated at 37°C for 30 min to allow for matrix compaction before adding skeletal muscle growth medium supplemented with 1 mg mL^{-1} of 6-amino-caproic acid (ACA, SigmaAldrich). The hence formed tissues were allowed to grow for 2 days after which differentiation was initiated for another 6 days by replacing growth medium with differentiation medium (DM) supplemented with 1 mg mL^{-1} ACA (DM/ACA). Subsequently, half of the DM/ACA was replenished every 2 days to maintain tissue survival.

2A.8. Sera Treatment

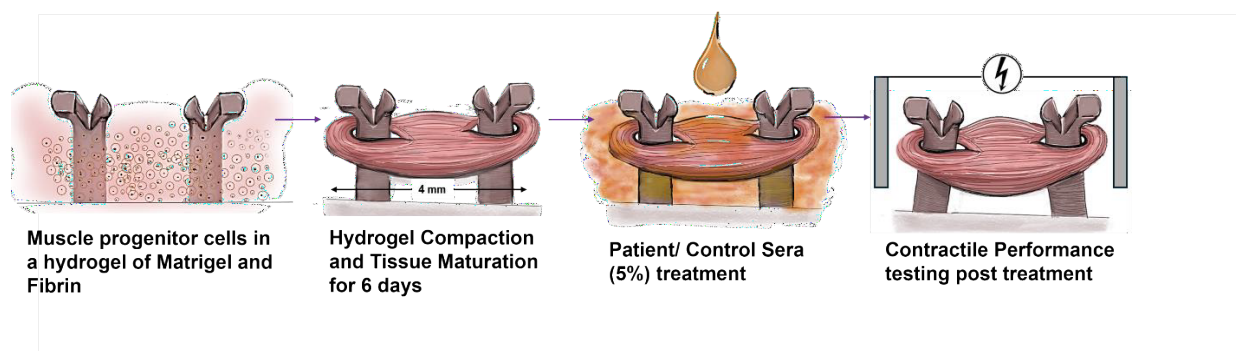


Figure 2: General schematic of tissue fabrication and treatment

Muscle constructs with visible structural abnormalities, such as failure to display hydrogel compaction were excluded. Experiments were performed in triplicate on different days and/or in separate batches to ensure the reproducibility of our findings. To design treatment experiments, serum samples were randomly assigned to the control or treatment groups. Investigators performing data analysis were blinded to the study hypotheses. After 6 days of differentiation, tissues were treated with 5% (v/v) of serum supplemented in DM/ACA for 48, 96 and 144 hrs. The serum was diluted from the stock in DM/ACA and added to the respective cultures.

2A.9. IgG Treatment

Purified IgGs were kindly gifted by Dr. Bhupesh Prusty from the University of Latvia, Līga. IgGs were isolated from 5 Healthy and 5 ME/CFS patients and a recommended dosage of 1µg/mL was used to treat cell monolayers and artificial muscles. The IgGs were supplemented in the differentiation medium for treatment which continued for 48 hours, after which the cell monolayers were fixed for immunostaining while the tissues were progressed for contractility analyses, calcium transition analyses and then fixed for immunostaining post cryosectioning. Details of these procedures are provided in the following sections.

2A.10. Electric Pulse Stimulation and Force Analyses

Post treatment, the bioengineered skeletal muscle tissues were placed in a 12-well plate with custom-built graphite electrodes connected to a pulse generator (Multifunction Generator WF1974, NF Corporation). An XL S1 cell incubator maintained at 37°C and 5% CO₂ was used to acquire brightfield images of the plate on top of a Zeiss Axio Observer Z1/7 microscope. The tissues were electrically stimulated to induce both twitch and tetanic contractions. Square monophasic waves of 1 to 50 Hz were used to generate an electrical field strength of 1 V mm⁻¹ and applied for 10 s. The duty cycle was adjusted as needed to ensure all pulses were 1 ms long. Post analyses the cells were fixed and stored.

Videos were recorded for both pillars at 10X and 39 frames per second (fps) during stimulation. Contractile parameters were calculated using pillar displacement from origin during deflection. Force, Power and Contraction Velocity were calculated using the following formulae:

$$F = \frac{6\pi ED^4}{64a^2(3L - a)} \cdot d$$

$$F = k \cdot d$$

$$V(max) = \frac{Displacement(max)}{TTP + T(delay)}$$

$$P = F \cdot V$$

where: **F** is force exerted by the tissue; **E** is the Young's modulus of the PDMS (calculated to be 1.6 ± 0.1 MPa.); **D** is diameter of the post; **a** is location of the tissue on the pillar, **L** is the height of the post; and **d** is displacement of the pillar. Spring constant **k** was calculated using the above parameters to be 3.54 N m^{-1} . **V_(max)** is the maximum Contractile Velocity (233), **TTP** is the time in seconds to reach peak displacement, **T_(delay)** is the delay time in seconds between each stimulation and **P** is power in μW calculated as shown in Figure 1M.

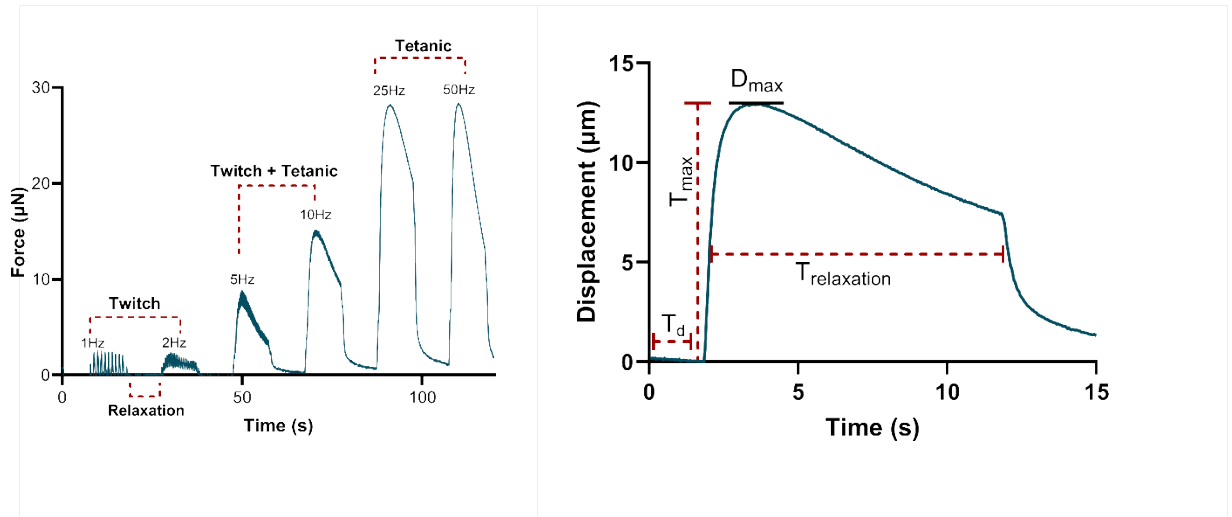


Figure 3: Parameters from Tetanic Contractions to measure Contractile Velocity.

2A.11. RNA Extraction, mRNA Library Preparation and Sequencing

Post treatment tissues were flash frozen in liquid nitrogen. Sterilized pestles were used to homogenize the tissues in QIAzol reagent (QIAGEN), and the total RNA was extracted using the miRNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. Total RNA content was assessed using Nanodrop ND-1000 full spectrum spectrophotometer. Technical replicates were pooled, and the total RNA concentration was adjusted to $100 \text{ ng } \mu\text{L}^{-1}$, approximately $1.3 \mu\text{g}$ of total RNA per biological replicate or serum. The quantity and quality of the total RNA sample were assessed using the Qubit RNA BR Assay kit (Thermo Fisher Scientific) and the RNA 6000 Nano Bioanalyzer 2100 Assay (Agilent).

RNA-Seq libraries were prepared by Centro Nacional de Análisis Genómico (CNAG) using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus (Illumina), following the manufacturer's recommendations, starting with $0.5 \mu\text{g}$ of total RNA as the input material. The final library was validated on an Agilent 2100 Bioanalyzer using the DNA 7500 assay.

The libraries were sequenced on the NovaSeq 6000 (Illumina) with a read length of $2 \times 51 \text{ bp}$ following the manufacturer's protocol for dual indexing. Image analysis, base calling, and quality scoring of the run were performed using the manufacturer's software Real Time Analysis (RTA v3.4.4), followed by the generation of FASTQ sequence files

2A.12. RNA-Seq Data Processing and Analysis

RNA-seq reads were mapped against the human reference genome (GRCh38) with *STAR* 2.7.8a (234) (ref 1) using ENCODE parameters. Genes were quantified with *RSEM* 1.3.0 (235) using the gencode 44 annotation. Differential

expression was performed with *limma* (236) using the *voom* transformation of the counts (237). For the comparisons LC-19 vs CNT and CFS vs CNT the differentially expressed genes between untreated- CNT were removed. Multidimensional scaling analyses was performed with the *limma* function *plotMDS*. Functional enrichment for the up and down-regulated genes was performed separately with *g:Profiler* (238). Gene set enrichment analysis was done with the pre-ranked list of genes by the *limma* *t moderated* statistic, using *FGSEA* 1.12.0 (239).

2A.13. Transposon Overlap Analyses

To analyze the overlap of different transposons on genes we used the pipeline shown in Figure 4. Briefly, two main files were obtained i) all genes annotated GTF file and ii) LOCATION file for Transposable Elements in our dataset. Using the differential expression data, we filtered the up and downregulated transposons according to a set criterion ($FC > \pm 1$, and $\text{adj } p\text{-value} < 0.05$). We then removed the detailed names of Transposons to enable convenient intersection between the data files by keeping the same nomenclature. We first found chromosomal locations of our filtered list of Differentially Expressed Transposons (DETs) by intersecting with the LOCATION file. Once we had the chromosome locations for our filtered DETs, we checked if they overlapped with any genes by intersecting with the GTF file. This allowed us to check for total or direct overlaps and genes nearest to the transposons. For this study we considered direct overlapped genes. We then annotated the output file. Extracted the gene IDs, removed versions from ENSEMBL IDs and checked for differentially expressed genes in our list for both up and downregulated datasets. After getting the final list of genes we conducted Gene Ontology Enrichment Analysis using *g:Profiler* or DAVID.

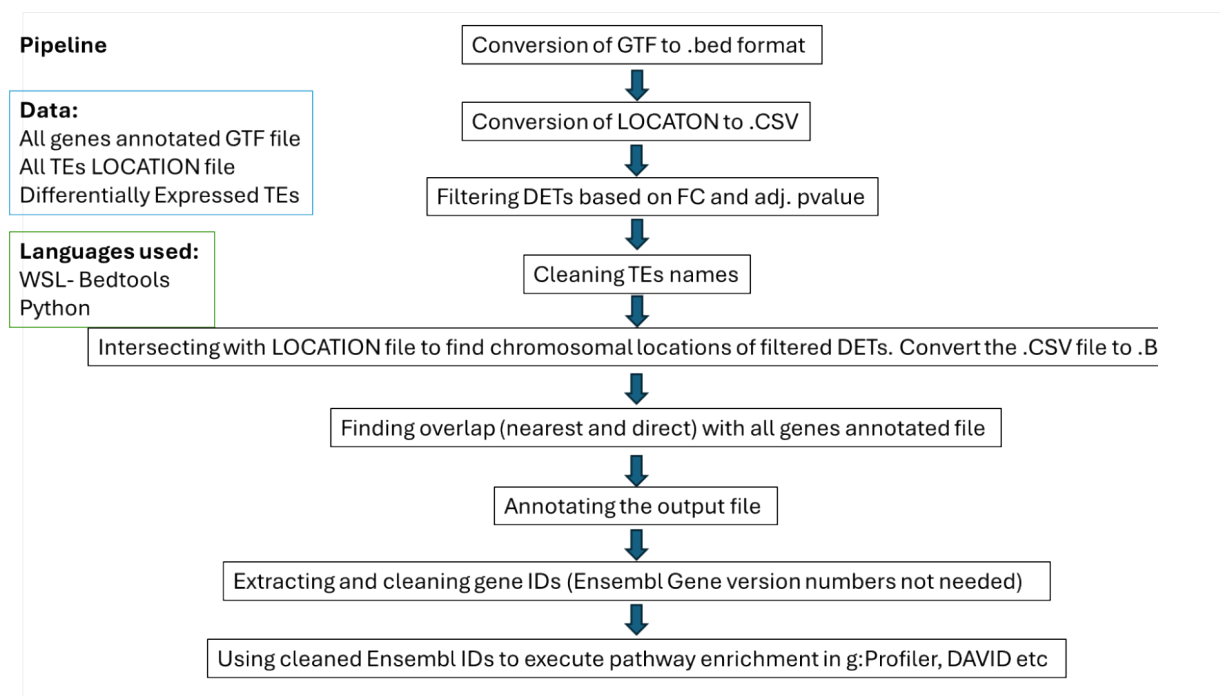


Figure 4: Pipeline for studying transposon expression, annotation and genetic overlap.

Refer to Appendix 1. Transposable Elements overlap and Annotation Scripts for detailed WSL-Bedtools and Python codes.

2A.14. qRT-PCR Validation

qRT-PCR was performed as described before (217). Briefly, 1 µg of RNA was digested with DNaseI (Invitrogen) and retrotranscribed with SuperScriptII (Invitrogen) using random hexanucleotides. For each biological replicate, qRT-PCR reactions from 10 ng of cDNA were carried out in triplicate using TaqMan probes (Applied Biosystems). GAPDH, RPLP0 and B2M were used as the endogenous control after comparing their stable expression across different treatment regimens. Thermal cycling was performed using the QuantStudio 5 RT-PCR System (Applied Biosystems). Relative expression to the endogenous gene and the control group was obtained by the $2^{-\Delta\Delta C_t}$ method. Pairs of samples were compared using a two-tailed unpaired t-test ($\alpha=0.05$), applying Welch's correction when necessary.

2A.15. Cryosectioning and Immunofluorescence

After treatments or EPS, the tissues were fixed in 10% formalin (approximately 4% formaldehyde) (Sigma-Aldrich). For transversal sections, tissues were incubated in 30% sucrose solution for 48h to preserve cellular structure against crystallization. The tissues were then embedded in Optimal Cutting Temperature (OCT) compound (PolyFreeze, Sigma-Aldrich) in plastic Cryomolds® (VWR, PA, USA) using chilled isopentane. Transverse sections (15 µm) were obtained by sectioning with a Leica CM1900 cryostat. The sections were transferred to SuperFrost Plus Adhesion slides (Fisher Scientific) and stored at -20°C.

A PAP pen (ImmEdge, Vector laboratories, CA, USA) was used to encircle tissue sections to minimize waste of antibodies. Sections were rehydrated using PBS and permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 15 minutes. Blocking was performed using 0.3% Triton X-100 with 3% Donkey serum in PBS for 30-40 min at room temperature. The tissue sections were then incubated with primary antibodies (see Table S1 for details) in blocking buffer at 4°C overnight.

After overnight incubation, the sections were washed three times with PBS-T and incubated with fluorophore-tagged secondary antibodies and phalloidin (Table S1) in blocking buffer for 60 min at room temperature. Post secondary antibody labelling, three PBS-T washes were done, and sections were mounted using VECTASHIELD Plus Mounting Medium with DAPI (Palex). Sections were secured with coverslips and sealed using transparent enamel.

2A.16. Imaging and Quantitative Morphological Analyses

Sections were analyzed using brightfield and confocal imaging with ZEISS Axio Observer Z1/7 and ZEISS LSM800 microscopes, respectively. 20X and higher magnifications were used to procure images from the confocal microscope. After acquisition, images were processed and analyzed using Fiji/ImageJ. To calculate myotube diameter, SAA or phalloidin and DAPI channels were used. Images were binarized and the free hand tool was used to mark the boundary for obtaining Ferret's diameter ($n \geq 50$ per image, at least three images were procured per tissue). Additionally, Cellpose was also used to quantify Feret's Diameter for myotube.

Briefly SAA and F-actin channels were exported, and the diameter was adjusted using the overlays generated from Cellpose (Diameter, 80). This generates PNG cell masks or overlays which have to be renamed. Using the LabelsToRois in ImageJ, Fiji, individual myotube diameters were quantified and obtained in a summary file. Cellpose was operated using Anaconda Powershell.

Mitochondrial Network Analysis in 2-D monolayer cultures was performed using MiNA (Mitochondrial Network Analyzer) (StuartLab) plugin in Fiji/ ImageJ. Care was taken to ensure same scale parameters for quantifying images obtained from different magnifications. For each condition, three technical replicates were imaged. The quantification was then performed using at least three images from each condition relative to the number of nuclei. To evaluate mitochondrial morphology, Mitochondrial Morphometry macro was used in Fiji/ImageJ (S4).

2A.17. Raman Spectroscopy

Tissue sections were put on aluminum-coated microscopic slides and air-dried. They were then washed carefully and thoroughly with ddH₂O to remove OCT and air-dried. Care was taken to not remove the sample from the aluminum slide. Raman spectra were acquired using an HR Evolution confocal Raman microscope (Horiba Jobin–Yvon, UK Ltd) equipped with a 532 nm neodymium–yttrium aluminum garnet laser. The laser power on the samples was 5-10 mW, attenuated using neutral density filters. An air objective (50X, NA = 0.65) was used to focus on tubular structures. Raman scattering from the focal point was detected by a charge-coupled device (CCD) cooled at -70 °C. Raman Spectra were measured from 320 to 3400 cm⁻¹ with 300 grooves per mm grating. The acquisition parameters were 5 s per spectrum. An average of 5–10 spectra per myotube per section per tissue were acquired for each treatment group. After data acquisition, quality control was performed to remove spectra with low signal-to-noise ratios.

2A.18. MitoStress Test - Seahorse Analysis

Immortalized human muscle progenitor cells were seeded at a density of 4000 cells per well in Seahorse XF HS Mini cell culture plates. Cells were allowed to proliferate until the formation of a uniform monolayer with little to no empty spaces. Once confluent, the growth medium was replaced with differentiation medium for 6 days after which 5% serum treated was initiated for 48 hours. Once the 48 h treatment was completed, the media was removed, and cells were washed with PBS thrice. The standard assay media was prepared by supplementing Seahorse XF DMEM Media with 10 mM Glucose, 1 mM pyruvate and 2 mM L-Glutamine. Subsequent analyses and treatment steps were followed from the Seahorse XF Cell Mito Stress Test Kit user guide. The concentrations for Oligomycin (1 μ M and 2 μ M) and FCCP (1.5, 1.8 and 2 μ M) were optimized on untreated cells. Final concentrations of the treatments were 1.5 μ M of Oligomycin, 1.5 μ M of FCCP and 0.5 μ M of Rotenone/Antimycin A. Post experiment, the cells were lysed in RIPA Buffer for protein quantification and normalization.

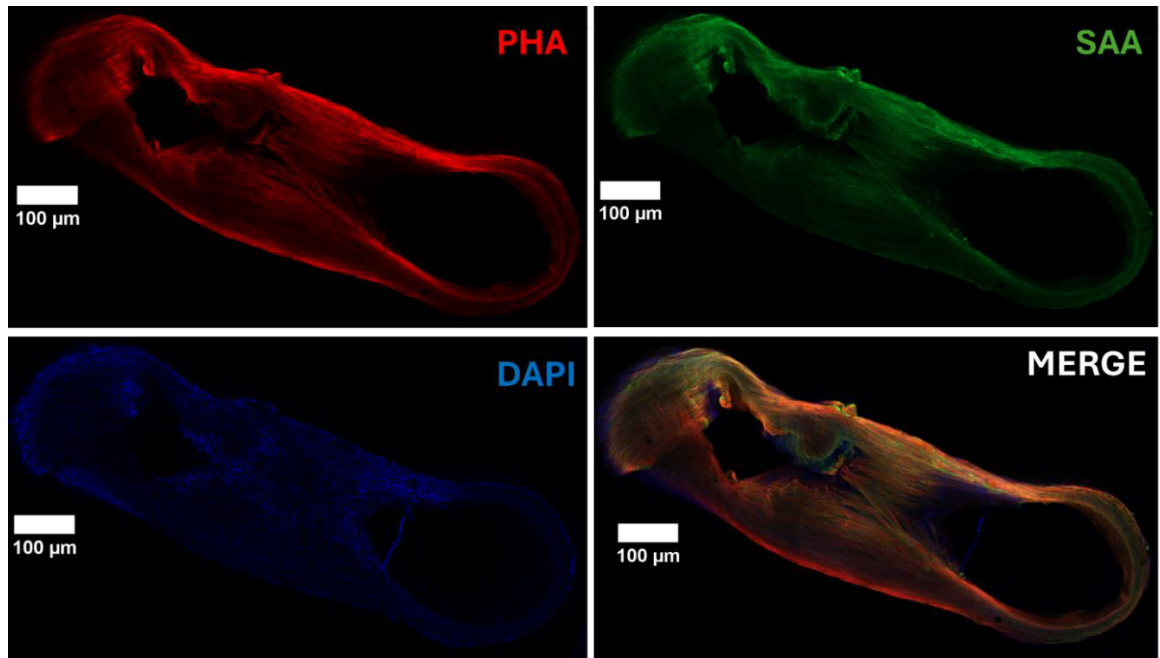
2A.19. Statistical Analyses

Statistical analyses were performed using Prism 9.5 software (GraphPad). All data were tested for normality by using the Shapiro–Wilk test ($P \geq 0.05$). The comparisons between more than two groups were performed by one-way ANOVA. For two groups, unpaired two-tailed parametric Student's T-test with Welch's correction was applied, whereas for non-normal data sets, unpaired two-tailed non-parametric Student's t-test with Mann–Whitney U test was performed. In the case of ANOVA, if a significant trend was observed, Tukey's post hoc tests with 95% confidence interval were applied ($P < 0.05$).

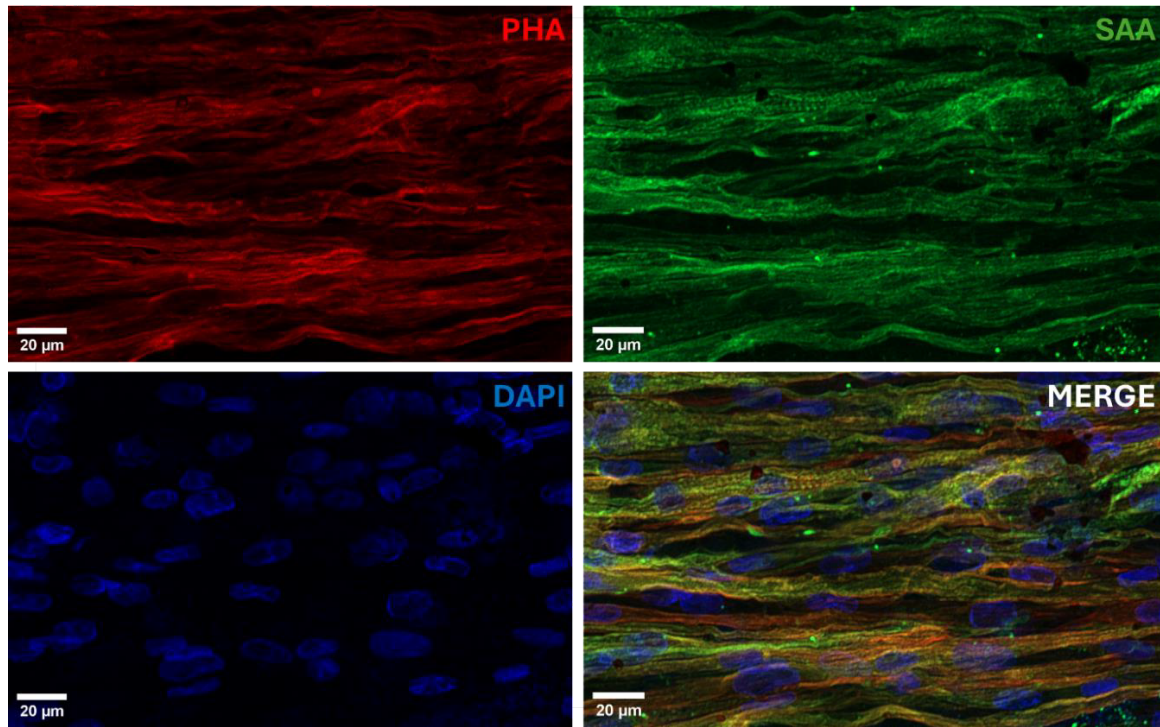
Chapter 3A: Results

3A.1. Comparative Analysis of Muscle Contractile Performance.

Patients diagnosed with ME/CFS and Long COVID-19 frequently experience low power output, impaired contractile force and extreme fatigue (177,240). Among the multiple theories existing in contemporary literature, the role of systemic factors in inducing multi-organ complications is also widely believed. To investigate the impact on muscle function, we fabricated 3-D *in vitro* skeletal muscle tissues from healthy immortalized human myogenic progenitor cells as discussed before (231) and exposed them to patient and control sera for a short duration of 48 hours. Prior to treatment, all tissues were well-differentiated with long, multinucleated myotubes aligned perpendicular to the two pillars as shown in Figure 1. Patients diagnosed with ME/CFS and Long COVID-19 frequently experience low power output, impaired contractile force and extreme fatigue (177,240). Among the multiple theories existing in contemporary literature, the role of systemic factors in inducing multi-organ complications is also widely believed. To investigate the impact on muscle function, we fabricated 3-D *in vitro* skeletal muscle tissues from healthy immortalized human myogenic progenitor cells as discussed before (231) and exposed them to patient and control sera for a short duration of 48 hours. Prior to treatment, all tissues were well-differentiated with long, multinucleated myotubes aligned perpendicular to the two pillars (Figure 1).



A



B

Figure 1: Confocal images of Immunostained well-differentiated 3D *in vitro* skeletal muscle tissues A) Whole mount tissues stained with F-actin (red), α - sarcomeric actinin- SAA (green), and DAPI (blue) at 5X Magnification, Scale bar= 50 μ m B) Longitudinal sections stained with F-actin (red), α - sarcomeric actinin- SAA (green), and DAPI (blue) at 10X Magnification, Scale bar= 100 and 50 μ m

The contraction regime included progressively increasing stimulation frequencies to induce both twitch and tetanic contractions. The voltage was kept constant at 10 VPP (peak to peak voltage) with 10 seconds of relaxation after each 10 second stimulation period.

Post 48-hour treatment, the tissues exposed to patient sera, henceforth named as diseased tissues, had a weaker force of contraction as evidenced by both twitch and tetanic spectra (Figure 2A, B). Furthermore, the maximum contractile force observed at all frequencies was also significantly lower for the diseased groups compared to the controls (Figure 2C). Although statistically insignificant, the diseased CFS tissues had a much lower contractile force compared to diseased LC-19 tissues. Time in Peak (TIP) helps in assessing the ability of a tissue to maintain peak performance during stimulation. Shorter TIP values may indicate a diminished ability of the muscle tissue to sustain its peak force due to factors such as impaired energy metabolism, contractile performance or gradual atrophy. The TIP for diseased treatment groups is significantly shorter compared to the healthy controls (Figure 2D). At a higher frequency of 50 Hz, these tissues had a significant fatiguability as indicated by a much shorter time to reach 50 % relaxation post stimulation (Figure 2E). Quantifying the power of contraction helps to interpret the ability of a muscle to execute work (Figure 2F). All the contractile parameters observed for 1 Hz (Twitch), 25 Hz (Twitch-Tetanic) and 50 Hz (Tetanic) frequencies were significantly diminished for all the diseased tissues, albeit more for CFS samples (Figure 2G-I).

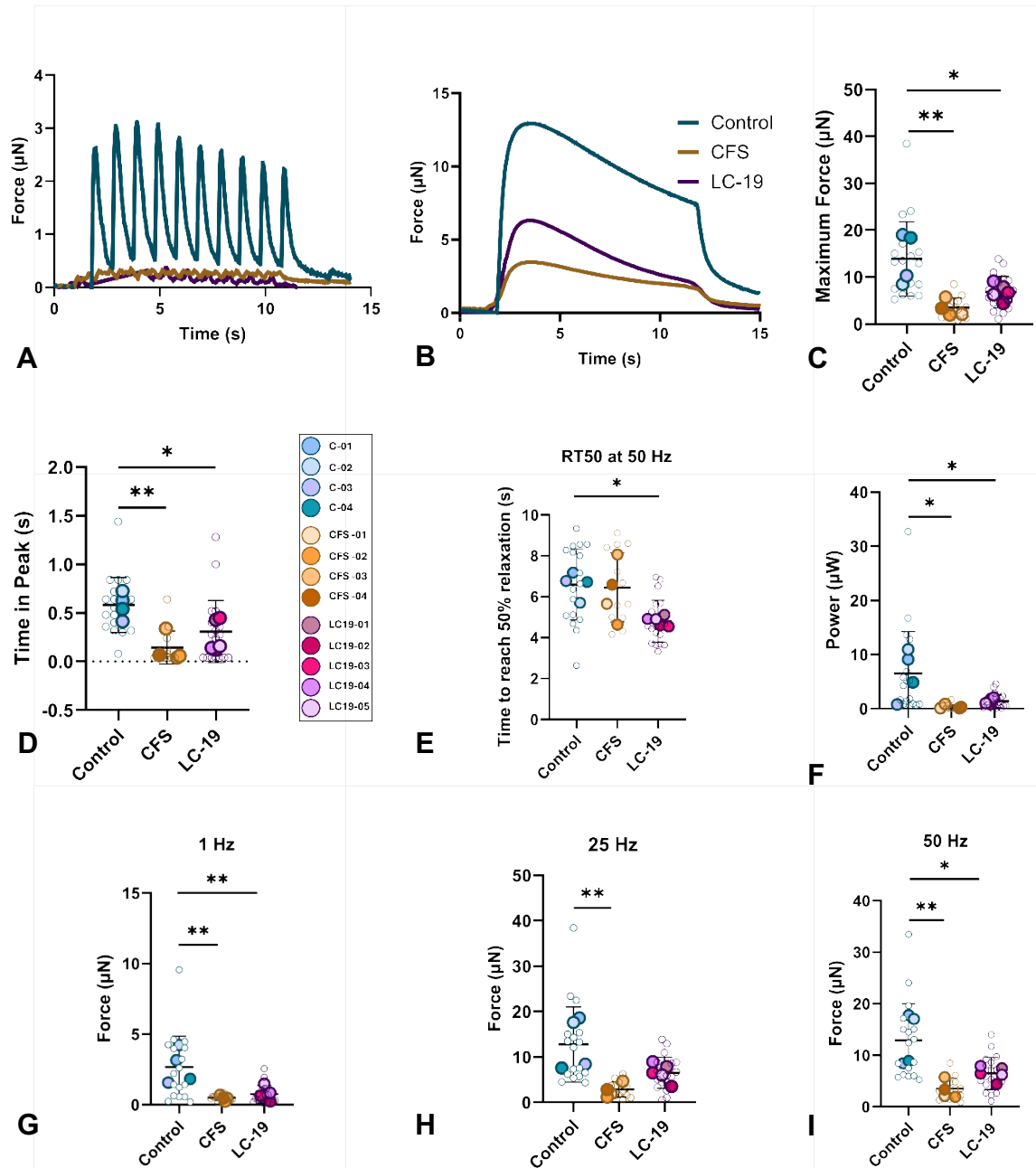


Figure 2: Comparative Contractile Dynamics post sera treatment for 48h. A) Twitch spectrum during 1 Hz stimulation. B) Tetanic spectrum during 50 Hz stimulation. C) Maximum Contractile Force of tissues post treatment. D) Retention time during maximum contraction, Time in Peak (TIP). E) Time taken by the tissues to reach 50% relaxation post contraction (RT50). F) Power of Contraction G) Maximum Force during 1 Hz stimulation. H) Maximum force during 25 Hz stimulation. I) Maximum Force at 50 Hz. Statistical Analyses: Biological replicates: CFS and Control, n= 4; LC-19 n=5. Technical

Replicates: n= 3-7. Data show the mean $\pm$ s.d. Statistical analysis: one-way ANOVA with Tukey's post hoc test * $P\leq 0.05$; ** $P\leq 0.01$

A functional muscle is capable to sustain an increase in its force of contraction proportionally with the stimulus until a certain limit is reached, beyond which depletion of the energy reserves or changes in structural integrity (such as damage) induce impairment (241). For diseased LC-19 tissues, this limit was reached at a lower stimulating frequency compared to the control and CFS samples. At 25 Hz, a majority of LC-19 tissues appeared to show peak performance following which they could no longer maintain their maximum force against the stimulus at higher frequencies contrary to the trend observed for the other two treatment groups (Figure 3).

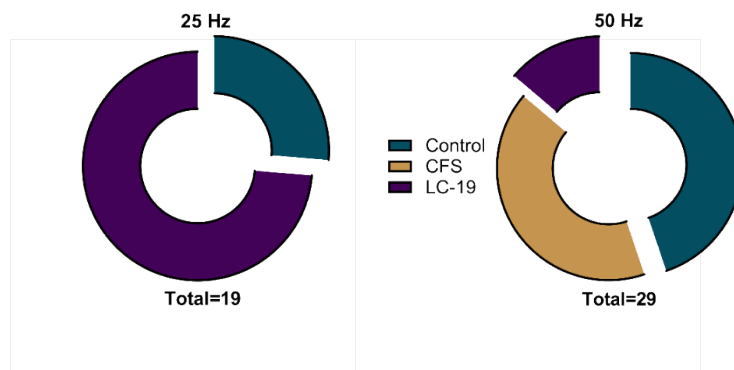


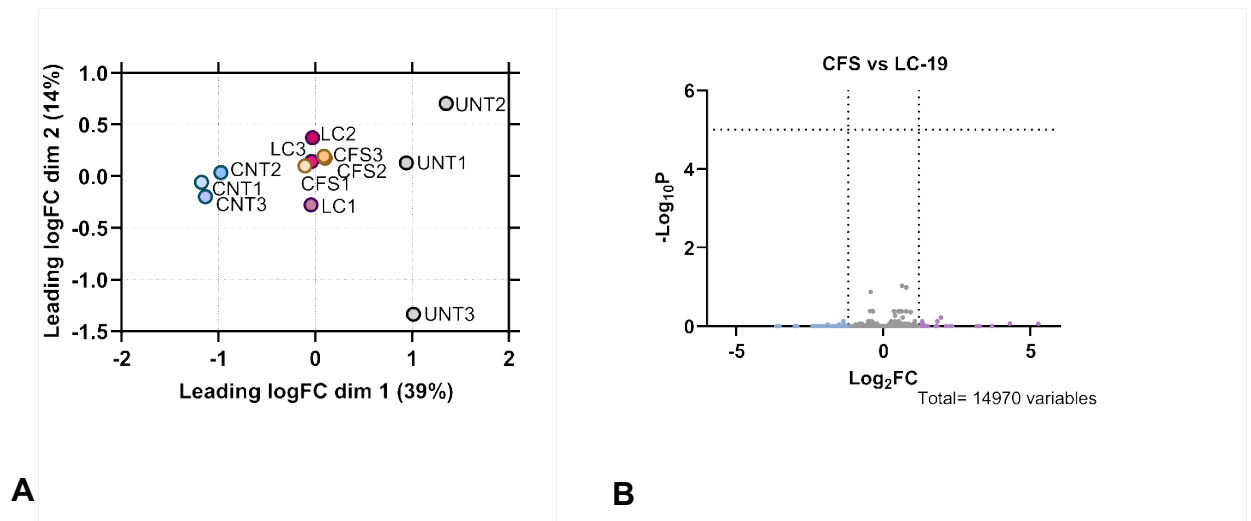
Figure 3: Sample distribution- Maximum Contractile force A) Tissues with maximum force at 25. B) Tissues with Maximum force at 50 Hz. Biological replicates: CFS and Control, n= 4; LC-19 n=5. Technical Replicates: n= 3-7.

These findings indicate that patient sera not only lowered the overall ability of the muscle to execute work but post peak performance, the tissues experienced high fatiguability and inability to reach the same performance again, particularly for

diseased LC-19 tissues. The model, at short exposure, therefore, replicates muscle weakness and fatigue frequently experienced by patients. It also signposts the applicability of the Push-Crash Cycle theory on diseased tissues.

3A.2. Comparative Transcriptomics Analyses

To investigate the underlying mechanistic changes in diseased tissues that caused contractile impairment, transcriptomic analyses of the short exposure tissue samples was performed using total RNA Sequencing. Interestingly, the Multidimensional Scaling (MDS) analyses indicated that both ME/CFS and LC-19 treatments clustered together with no significant *Differentially Expressed Genes* (DEGs) (Figure 4A). There were, however, several DEGs when ME/CFS and LC-19 tissues were compared to Controls (Figure 4B, C). These findings highlight: a) the similarity between the two disease conditions, and b) they are mechanistically and functionally different from the controls used in this study. We further performed Gene Ontology (GO) Enrichment Analyses, to identify dysregulated pathways. Genes of interest were filtered based on *Fold Change* and *adjusted p-values* for both up and downregulated genes ($FC > \pm 1$, and $\text{adj } p\text{-value} < 0.05$). Expression of genes with maximum fold change identified by total RNA Sequencing was then validated by qRT-PCR



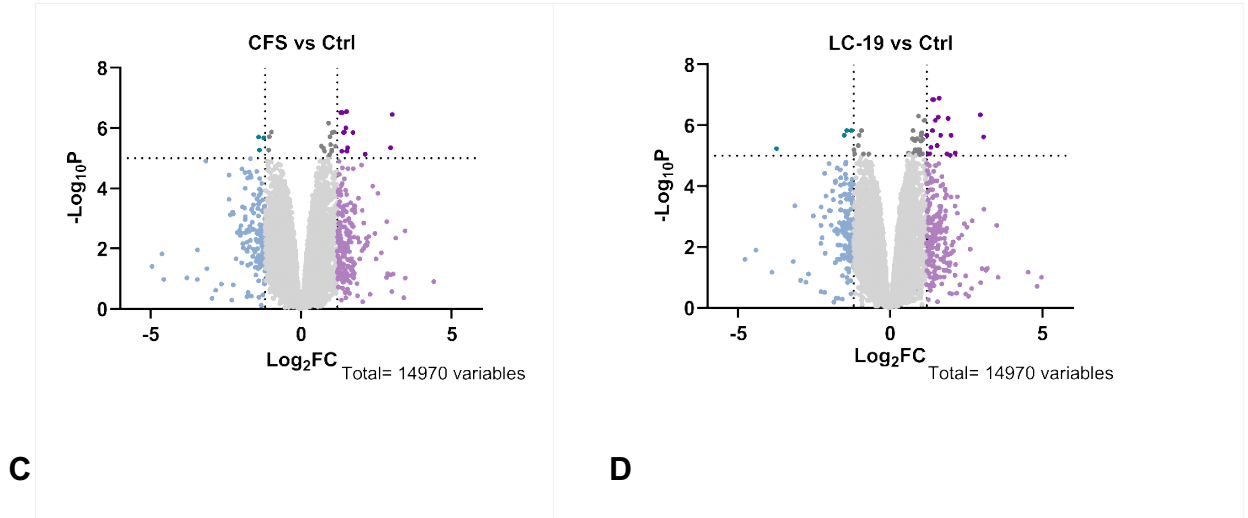
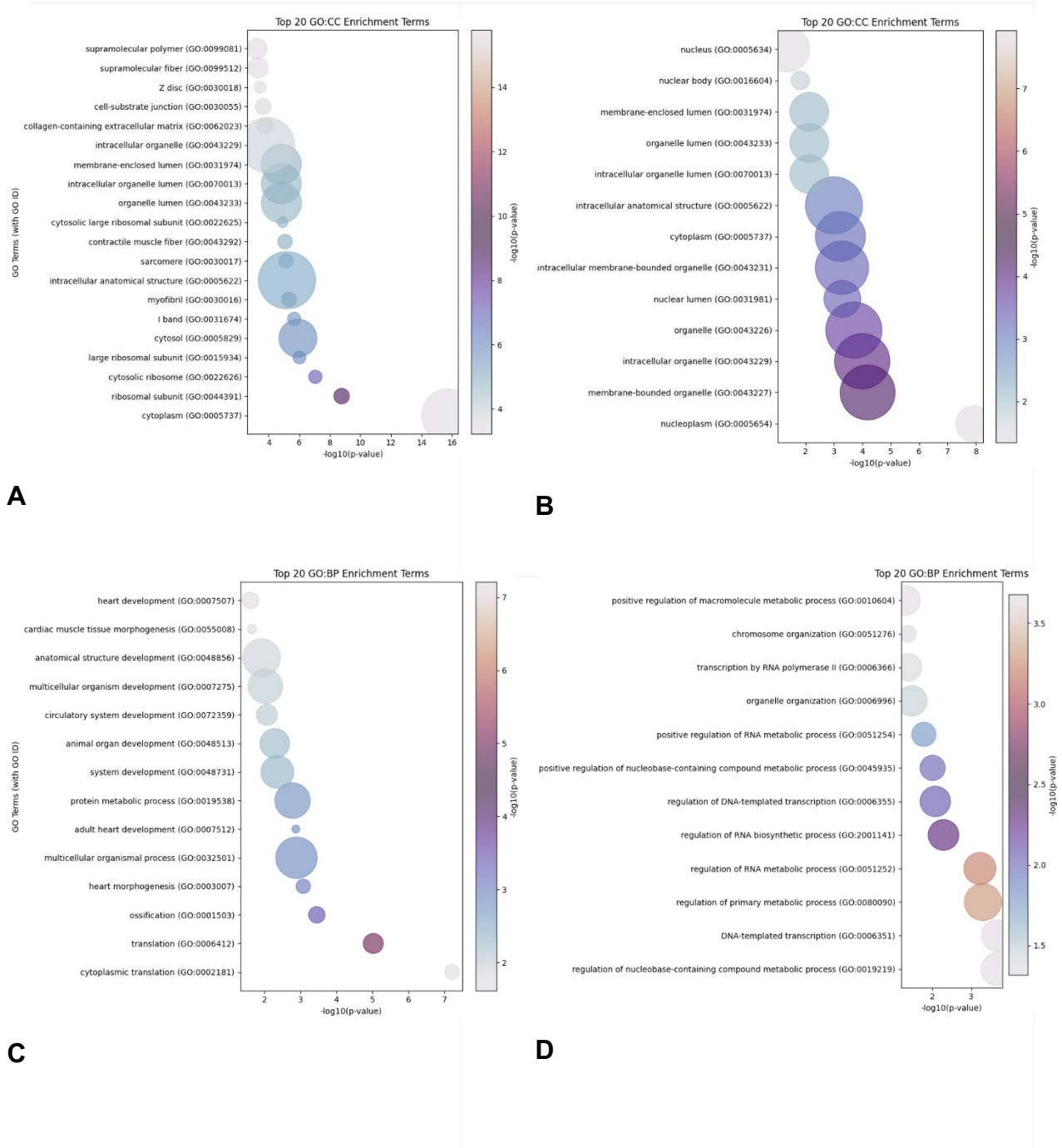


Figure 4: RNA-Seq -MDS and Differential Expression Analyses A) A Multidimensional Scaling Map for samples. B) Volcano plot of Differentially Expressed genes (DEGs) between ME/CFS and Control. C) Volcano plot of Differentially Expressed genes (DEGs) between LC-19 and Control. D) Volcano plot of Differentially Expressed genes (DEGs) between ME/CFS and LC-19.

3A.2.1. Group 1: ME/CFS versus Healthy Control samples

The findings from Gene Ontology enrichment analyses (Figure 6) indicate key changes in muscle structure and contractile function along with cellular metabolism, particularly protein synthesis and translation. The fold enrichment score is based on the total number of differentially expressed genes that are participating in a particular cellular pathway and whether they are over or underrepresented compared to the expected number of genes in a specific GO term.

Section A: ME/CFS and Long COVID-19: Results



Section A: ME/CFS and Long COVID-19: Results

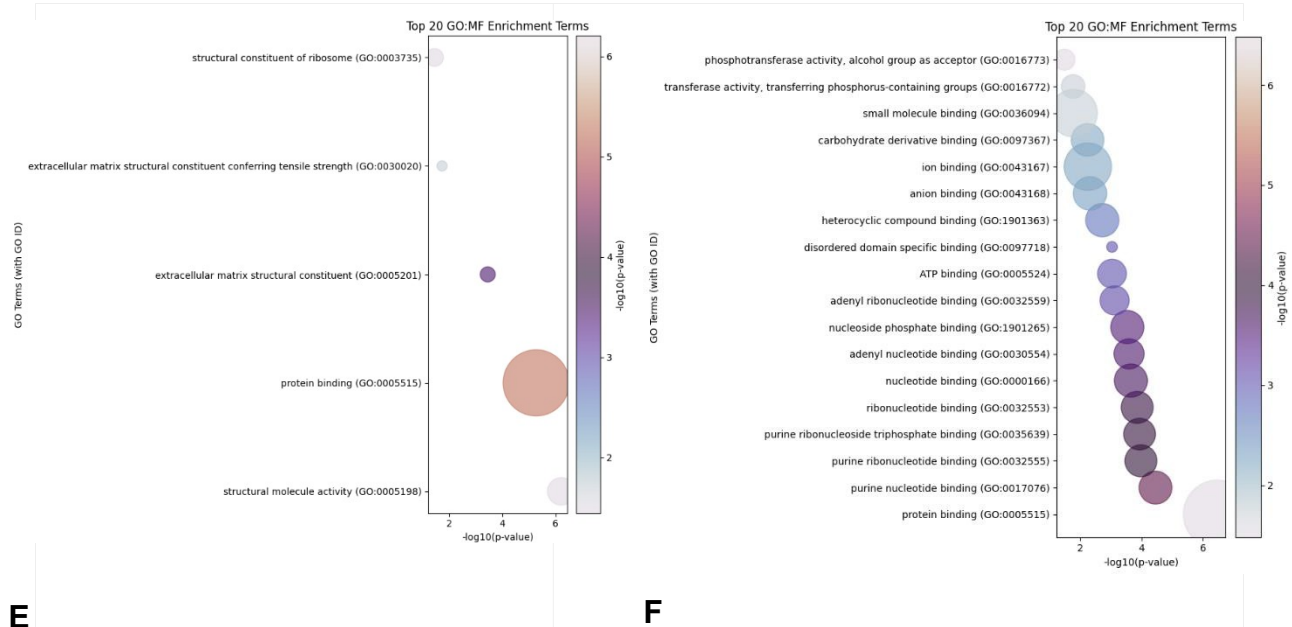


Figure 5: CFS vs CNT Gene Ontology Enrichment using gProfiler A) GO: Cellular Component for Upregulated DEGs B) GO: Cellular Component for Downregulated DEGs C) GO: Bioprocesses for Upregulated DEGs D) GO: Bioprocesses for Downregulated DEGs E) GO: Molecular Function for Upregulated DEGs F) GO: Molecular Function for Downregulated DEGs

FE (Fold Enrichment):

$$\frac{\frac{\text{Observed Genes in Term}}{\text{Total Genes in Dataset}}}{\frac{\text{Expected Genes in Term}}{\text{Total genes in Background}}}$$

Where:

FE > 1= More genes than expected

FE < 1= Less genes than expected

The Fold Enrichment Score helps to understand if the genes in the dataset are enriching a GO term which in turn determines if that term is enriched or depleted. This means that even if the genes in the dataset are upregulated but there are fewer

genes in the data set than expected, the overall term is downregulated (242). For example, a set of genes that are upregulated in response to a particular stress, may indicate depletion in GO terms related to "cell growth", which could signpost that stress is inhibiting cell growth.

Based on GO, although ME/CFS samples exhibit upregulation of several ECM-related genes, overall, the enrichment analysis points to a depletion of ECM-associated pathways (Figure 6A and Table 1). Rather than a general improvement in ECM integrity, this disparity might be the result of a compensatory reaction. Weakened cell-matrix interactions are further suggested by the downregulation of focal adhesion and cell-substrate junction components (GO:0030055 and GO:0005925), which may be a contributing factor in the musculoskeletal abnormalities and post-exertional malaise (PEM) seen in ME/CFS patients.

Table 1: Extracellular Matrix and Cell-ECM interactions

GO Term	Description	FE	p-value	Interpretation
GO:0062023	Collagen-Containing ECM	0.61	0.00065	Depleted
GO:0031012	Extracellular matrix	0.42	0.0084	Depleted
GO:0030055	Cell-substrate junction (links muscle cells to ECM)	0.58	0.00097	Depleted
GO:0005925	Focal adhesion (connected ECM to cytoskeleton)	0.53	0.0020	Depleted

Furthermore, according to the FE scores, the depletion of GO terms for sarcomere, myofibril, Z-disc and I band (Table 2) indicated a potential weakening of the contractile apparatus. Particularly the depletion of I-band and Z-disc signal diminished structural integrity and support necessary to accomplish muscle contraction.

Table 2: Structural Components of the Skeletal Muscle

GO Term	Description	FE	p-value	Interpretation
GO:0030016	Myofibril	0.65	0.00065	Depleted
GO:0030017	Sarcomere (basic contractile unit)	0.64	0.00088	Depleted
GO:0030018	Z disc (anchoring point for actin filaments)	0.55	0.0037	Depleted
GO:0031674	I band (region of sarcomere with thin filaments)	0.71	0.00055	Depleted
GO:0099512	Supramolecular fiber (includes actin, myosin)	0.35	0.0158	Depleted

This translates to weaker force production and an increased vulnerability to damage during exertion. Additionally, depletion of supramolecular fibers also indicates disrupted force transmission across the myotube. An enrichment in cytoplasmic related processes, particularly translation, indicates activation of a compensatory mechanism in which the tissue increases protein production in an attempt to counteract damage against stress (Table 3).

Table 3: Metabolic Changes

GO Term	Description	FE	p-value	Interpretation
GO:0002181	Cytoplasmic translation	1.13	5.11e-06	Enriched
GO:0006412	Translation (protein synthesis)	1.11	2.84e-07	Enriched

GO Cellular Components also indicated depletion of terms related to mitochondrial components suggesting possible impairments in mitochondrial function and ATP synthesis. Mitochondrial inner membrane components critical for OXPHOS were depleted, indicating energy deficits. Mitochondrial outer double membraned envelope, which ensures mitochondrial stability and metabolite transport, also indicates depletion (Table 4).

Table 4: Mitochondrial function

GO Term	Description	FE	p-Value	Interpretation
GO:0005743	Mitochondrial Inner Membrane	0.38	0.0158	Depleted
GO:0005740	Mitochondrial Envelope	0.28	0.0498	Depleted
GO:0019866	Organelle Inner Membrane	0.34	0.0236	Depleted
GO:0043231	Intracellular Membrane-Bounded Organelle	0.38	0.0010	Depleted

Supporting the enrichment of cytoplasmic compensatory processes (GO:0002181), ribosomal activity indicated an increase as well (Table 5). This and changes in the process of protein synthesis evidenced by the high FE scores of different ribosomal categories (such as large ribosomal subunit, cytosolic ribosome and ribosomal subunit (1.06-1.32)), suggest upregulation of compensatory processes. Enrichment of these processes is, however, not sufficient to restore muscle structure and function as evidenced by the impaired contractile force shown earlier in Figure 2.

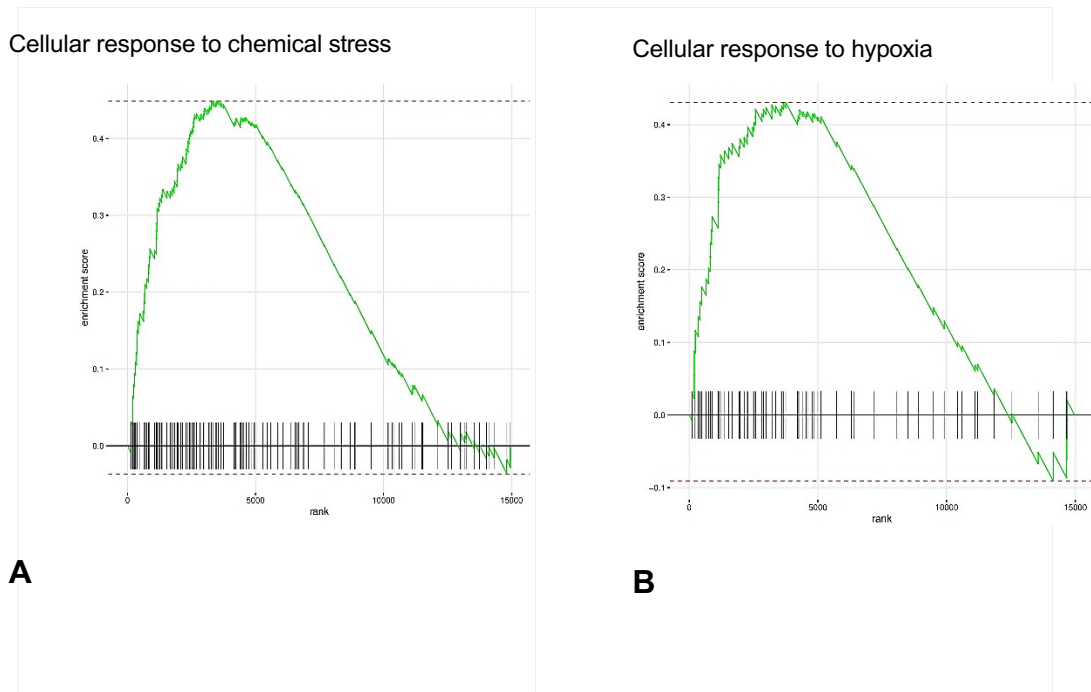
Table 5: Ribosomes and Translation

GO Term	Description	FE	p-Value	Interpretation
GO:0022626	Cytosolic Ribosome	1.32	5.26e-07	Enriched

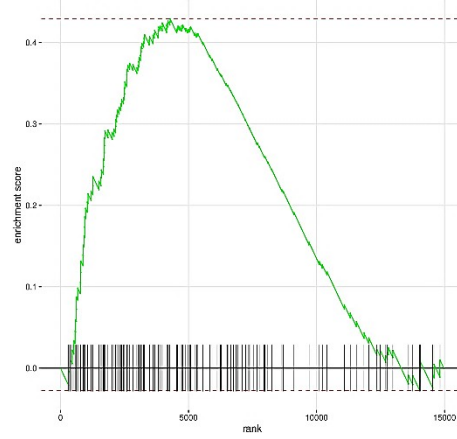
GO:0044391	Ribosomal Subunit	1.27	1.99e-07	Enriched
GO:0002181	Cytoplasmic Translation	1.13	5.11e-06	Enriched

Therefore, based on this discussion, for all significant but depleted GO terms shown in Figure 6, a low fold enrichment indicates that although the genes constituting them may be upregulated, but the overall pathway is either depleted or not functioning optimally against stress.

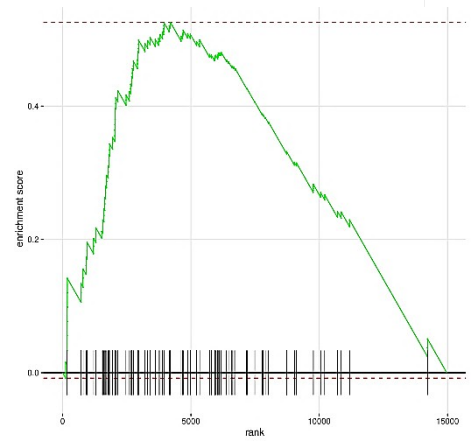
Gene Set- Enrichment Analysis (Figure 7) which takes into account the ranking of genes to identify whether there are more up or downregulated genes in the data set for a particular function or process also indicated an upregulation of cellular responses to different types of stress and a strong inclination of the system to support protein synthesis and muscle contraction.



Cellular response to starvation

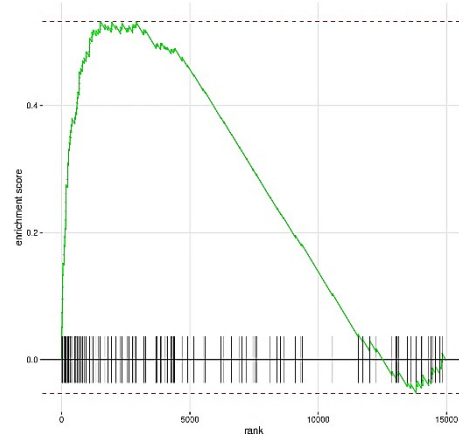


Mitochondrial translation elongation



C

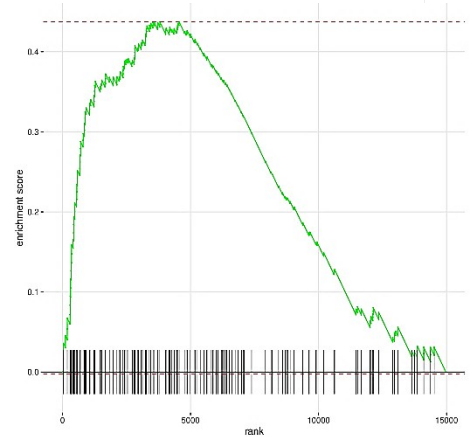
Muscle contraction



E

D

Protein localization



F

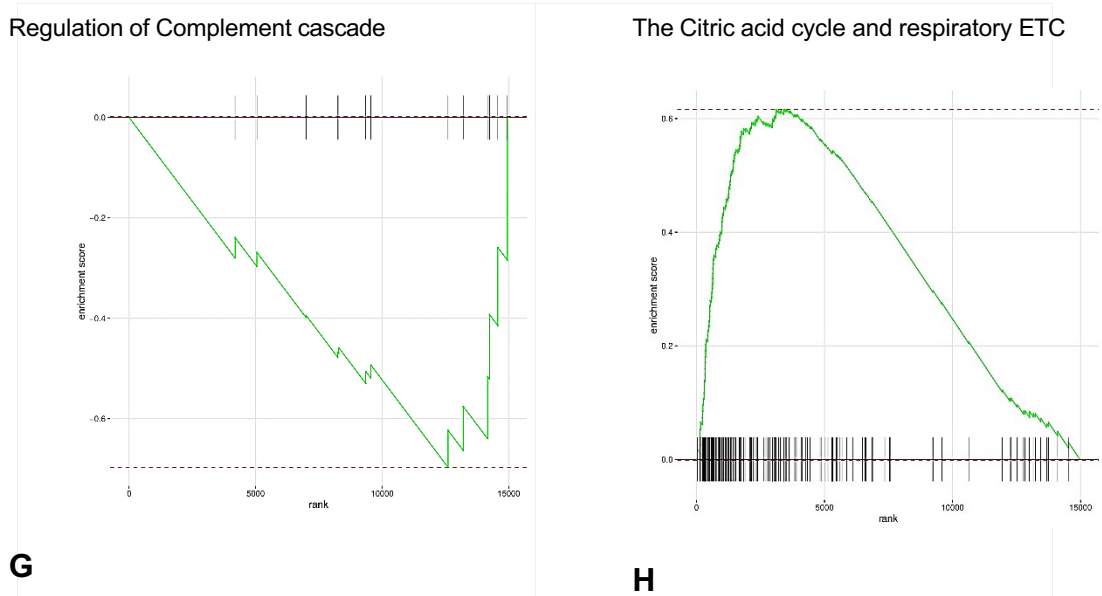


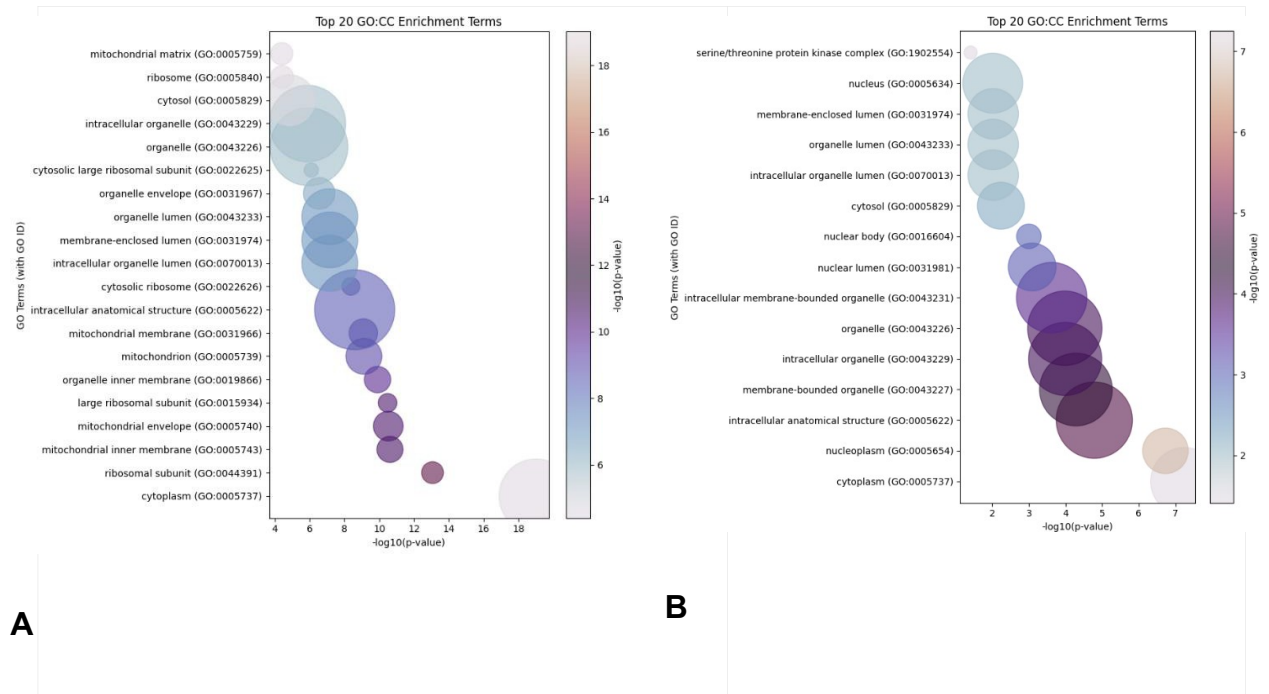
Figure 6: GSEA of CFS vs CNT A) Cellular response to chemical stress B) Cellular response to hypoxia C) Cellular response to starvation D) Mitochondrial translation elongation E) Muscle Contraction F) Protein localization G) Regulation of complement cascade H) TCA cycle and respiratory electron transport chain

Overall, from this data, post-viral skeletal muscle fatigue appears to arise from competing biological demands between tissue repair and energy production. Viral infection triggers enhanced protein synthesis through upregulated ribosomal components (GO:0002181, GO:0044391) and cytoplasmic translation processes (GO:0006412), driving an increase in repair-related biosynthesis. However, mitochondrial dysfunction creates an energy deficit, with depleted ATP synthesis (GO:0006754) and ETC activity (GO:0022900), reducing oxidative phosphorylation capacity. Paradoxically, mitochondrial structural components like the inner membrane (GO:0005743) show enrichment while envelope integrity declines (GO:0005740), indicating failed optimization of energy production. This imbalance forces muscle cells into a resource allocation crisis and triggers energy intensive repair processes (GO:0043603) to compete with contractile function, leading to incomplete protein folding and impaired calcium handling. Mild activation of hypertrophic signaling pathways (GO:0007507) and myofibril reorganization (GO:0030016) further strain the system, creating a self-perpetuating cycle of cellular

stress and metabolic exhaustion that perhaps manifests clinically as persistent fatigue.

3A.2.2. Group 2: LC-19 Vs CNT

Integrated GO analysis from the upregulated DEGs shows a coordinated biological response against long COVID-19 patient sera exposure (Figure 8). This response is indicated by an increase in protein synthesis, mitochondrial function and energy metabolism, in addition to broader changes in the structural organization of intracellular compartments.



Section A: ME/CFS and Long COVID-19: Results

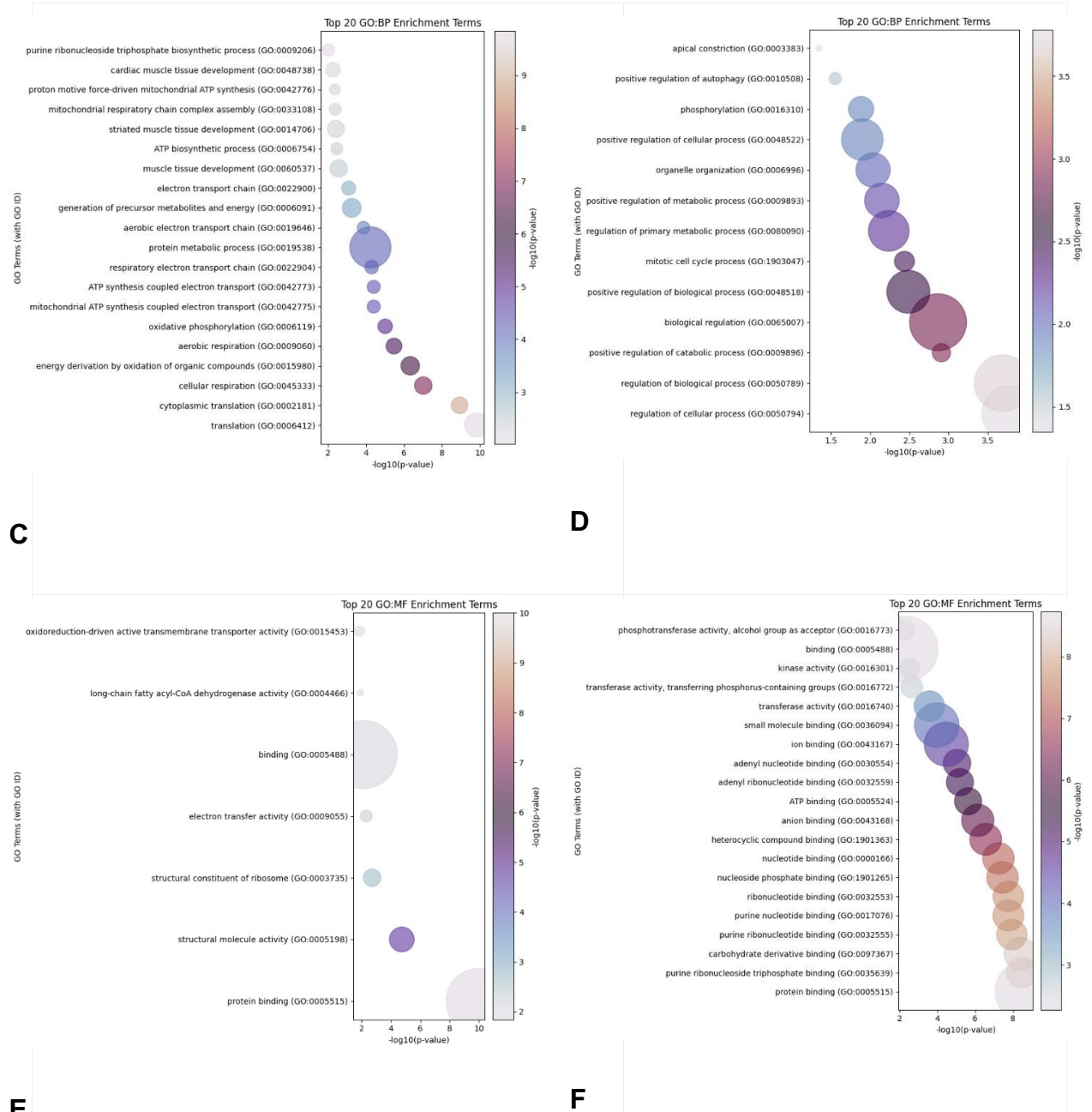


Figure 7: LC-19 vs CNT Gene Ontology Enrichment using gProfiler A) GO: Cellular Component for Upregulated DEGs B) GO: Cellular Component for Downregulated DEGs C) GO: Bioprocesses for Upregulated DEGs D) GO: Bioprocesses for Downregulated DEGs E) GO: Molecular Function for Upregulated DEGs F) GO: Molecular Function for Downregulated DEGs

Trends in GO: Cellular Components (CC) indicate enrichment of ribosomal structures and activity as shown in Table 6 (for example ribosomal subunit-

GO:0044391, cytosolic ribosome- GO:0022626). This observation is further supported by the enrichment observed for ribonucleoprotein complexes, and polysomes (responsible for facilitating mRNA translation) suggesting enhanced translation.

Table 6: *Protein Synthesis and Ribosomal Activity*

GO Term	Description	F E	p- Value	Interpretation
GO:0006412	Translation	0.66	7.55E-09	Enriched – Increased protein synthesis, possibly due to inflammation or repair processes.
GO:0002181	Cytoplasmic translation	0.87	7.97E-07	Enriched – Elevated cytoplasmic protein production linked to stress response.
GO:00044391	Ribosomal subunit	1.58	6.16E-10	Enriched – Strong upregulation of ribosomal components.
GO:00022626	Cytosolic ribosome	1.48	3.40E-08	Enriched – Indicates high ribosome activity in cytosol.
GO:00015934	Large ribosomal subunit	1.47	2.51E-08	Enriched – Upregulated synthesis machinery components.
GO:00005840	Ribosome	1.41	3.04E-09	Enriched – Elevated translation overall.
GO:00043604	Amide biosynthetic process	0.96	5.54E-07	Enriched – Active protein/peptide biosynthesis.
GO:00043603	Cellular amide metabolic process	0.79	4.83E-06	Enriched – High protein turnover/metabolism post-viral insult.

These observations align proportionately with the GO: Bioprocess (BP) enrichment trend which shows significant enrichment in processes such as translation (GO:0006412), cytoplasmic translation (GO:0002181), and peptide metabolic processes (GO:0006518). The data represents an increased demand of structural and functional protein in response to systemic stress factors suggesting compensatory mechanisms (Figure 8).

Furthermore, a substantial enrichment of mitochondrial components was also observed (mitochondrial respirasome- GO:0005746, mitochondrial matrix, mitochondrial inner membrane- GO:0005743 and other mitochondrial ribosomal and respiratory complexes) as shown in Table 7-8. In GO: BP, however, mitochondrial function appeared to be low performing (cellular respiration-GO:0045333, oxidative phosphorylation- GO:0006119, aerobic respiration- GO:0009060, and ATP biosynthetic process- GO:0006754).

Table 7: *Mitochondrial Function*

GO Term	Description	FE	p-Value	Interpretation
GO:0005746	Mitochondrial respirasome	1.29	4.41E-07	Enriched – Increased mitochondrial respiratory supercomplex activity, reflecting a response to energy demands.
GO:0098803	Respiratory chain complex	1.22	1.30E-06	Enriched – Indicates enhanced or possibly dysregulated electron transport chain activity.
GO:0005743	Mitochondrial inner membrane	1.13	1.01E-08	Enriched – Upregulation of the mitochondrial inner membrane structure, likely due to mitochondrial remodeling.
GO:0031966	Organelle inner membrane	1.03	4.24E-08	Enriched – Indicates mitochondrial remodeling in response to cellular stress or damage.
GO:0005740	Mitochondrial envelope	0.95	4.63E-08	Depleted – Suggests mitochondrial structural changes, likely related to post-infection remodeling.

Table 8: *ATP synthesis and Electron Transport Processes*

GO Term	Description	FE	p-Value	Interpretation
GO:0019646	Aerobic electron transport chain	0.85	0.00037	Depleted. May reflect reduced mitochondrial activity or decreased expression of ETC genes. Could point to metabolic inefficiency or stress.
GO:0022900	Electron transport chain	0.62	0.0073	Depleted. General ETC downregulation—aligns with lower oxidative phosphorylation and energy output.

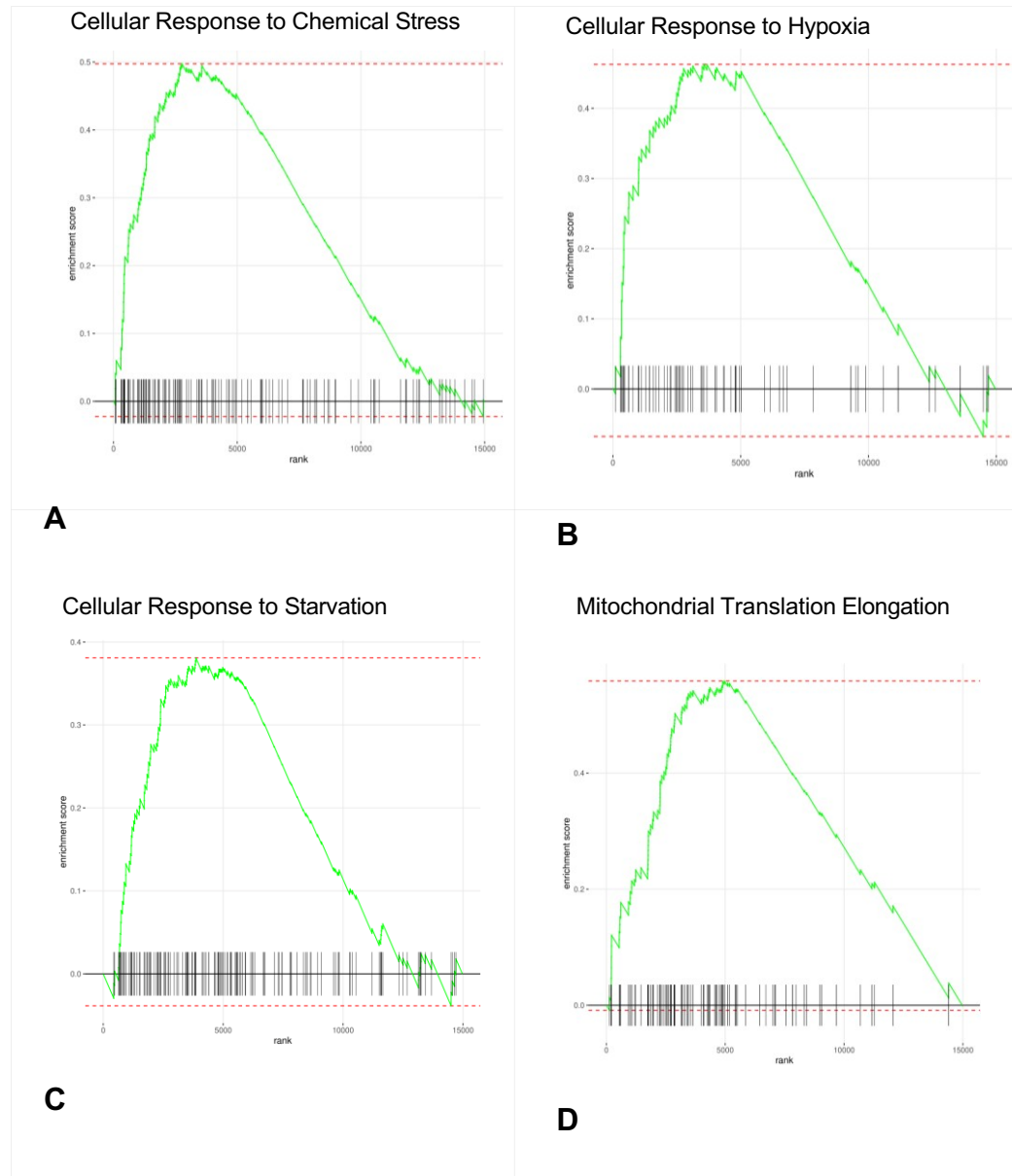
GO:0006754	ATP biosynthetic process	0.77	0.009	Depleted. Suggests reduced ATP synthesis via oxidative means—potential contributor to muscle fatigue or weakness.
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Additionally, the data also shows an enrichment in other metabolic processes which indicates an increased anabolic tendency in the cells which is also reflected in GO: CC such as enrichment in the upregulated genes for cytoplasm and the cytosol. Furthermore, muscle-specific GO terms (muscle hypertrophy in response to stress- GO:0014898, and striated muscle adaptation- GO:0014888) indicates activation of adaptive processes in the muscles, signaling hypertrophy and increased differentiation (Table 9).

Table 9: *Muscle Adaptation Processes against stress*

GO Term	Description	FE	p-Value	Interpretation
GO:0014898	Cardiac muscle hypertrophy in response to stress	1.26	0.0114	(Mildly Enriched) –Suggests activation of stress-responsive pathways common to both cardiac and skeletal muscle, possibly indicating hypertrophic signaling in response to overload or damage.
GO:0014888	Striated muscle adaptation	1.02	0.0193	(Marginally Enriched) – Suggests minor activation of muscle remodeling. May reflect attempts to adapt structurally to stress or altered workload, albeit in a subdued metabolic environment.

Gene Set- Enrichment Analysis (Figure 9) also indicated an upregulation of cellular responses to different types of stress and a strong inclination of the system to support protein synthesis and muscle contraction.



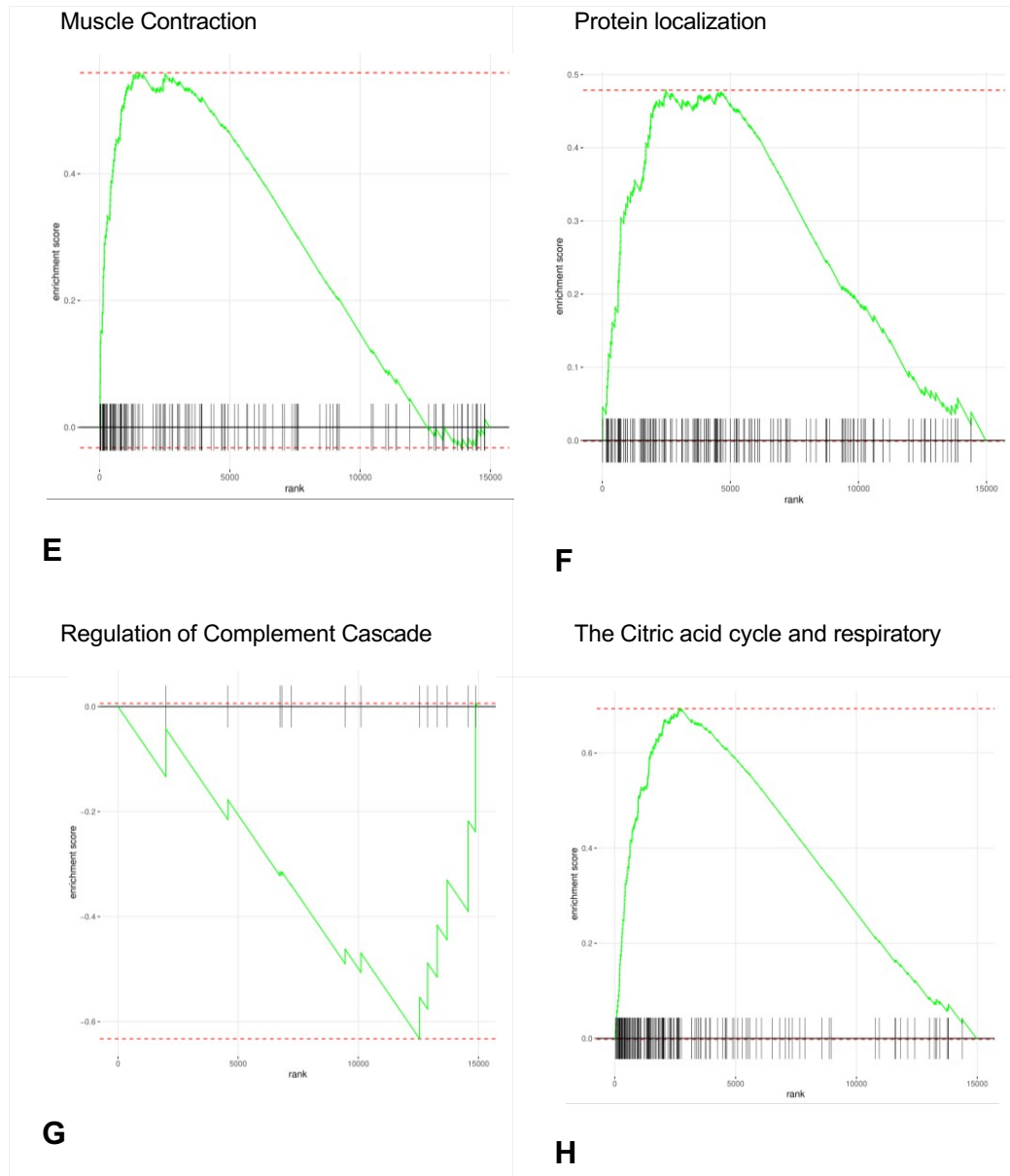


Figure 8: GSEA of LC-19 vs CNT A) Cellular response to chemical stress B) Cellular response to hypoxia C) Cellular response to starvation D) Mitochondrial translation elongation E) Muscle Contraction F) Protein localization G) Regulation of complement cascade H) TCA cycle and respiratory electron transport chain

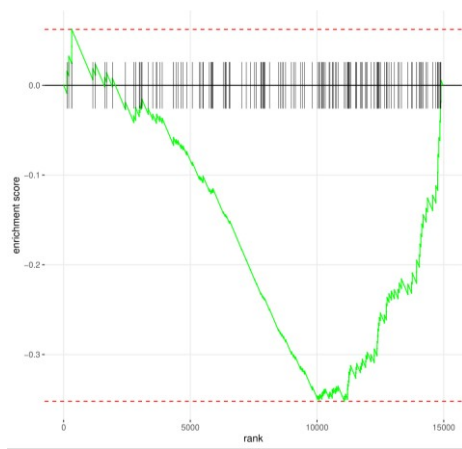
The overall analysis indicates a complicated interplay of pathophysiological processes observed in a post-viral skeletal muscle dysfunction. This interplay is particularly characterized by competing cellular priorities that result in persistent

fatigue. A hyperactive repair response appears to be at the center of this picture. This pattern is apparent by a substantial upregulation of ribosomal components (GO:0044391 FE=1.58, GO:0022626 FE=1.48) and elevated cytoplasmic translation (GO:0002181 FE=1.23). The consequent increase in protein synthesis then controls the synthesis of amides (GO:0043604 FE=0.55) and peptides (GO:0006518 FE=0.98) to repair damaged cellular components. According to the data, these repair processes are running parallel to mitochondrial dysfunction. OXPHOS appears to be impaired (GO:0006119 FE=0.8), and the activity of ETC is also reduced (GO:0022900 FE=0.62) with an expected decline in ATP production processes (GO:0006754 FE=0.77). Additionally, the muscle adaptation pathways appear to be activated but are perhaps operating sub-optimally. Hypertrophic signaling (GO:0014898 FE=1.26) and striated muscle remodeling (GO:0014888 FE=1.02) attempt to compensate against the stress but are inefficient due to lack of sufficient energy. Energy allocation is perhaps prioritized to ribosomal biogenesis, mitochondrial maintenance, and muscle adaptation (GO:0014888 FE=1.02). This imbalance due to energy constraints could lead to incomplete protein folding, impaired calcium handling and sustained muscle weakness. The GO analyses of this group presents a stressed system with upregulated compensatory systems (such as ribosomal activity) which are stressing the already strained metabolic machinery and inducing a self-sustaining cycle of dysfunction often observed in post viral syndromes.

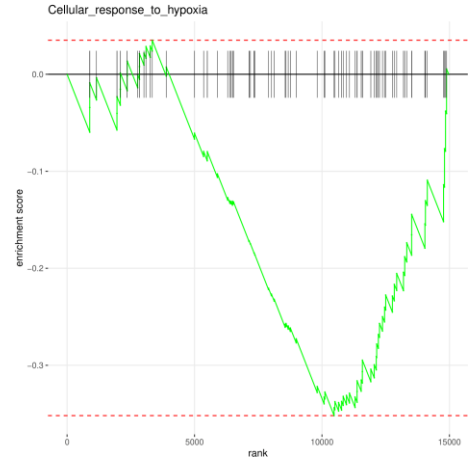
3A.2.3. Group 3: CFS vs LC-19

There were no DEGs between CFS vs LC-19 but Gene Set Enrichment Analysis (GSEA) for some pathways (Figure) indicated a tendency towards downregulated genes in ME/CFS samples compared to LC-19 for cellular responses towards stress, and hypoxia, while for cellular response to starvation, the genes were upregulated. Other processes like mitochondrial translation, protein localization, complement cascade and the TCA cycle were also downregulated in CFS compared to LC-19 samples.

Cellular response to chemical stress



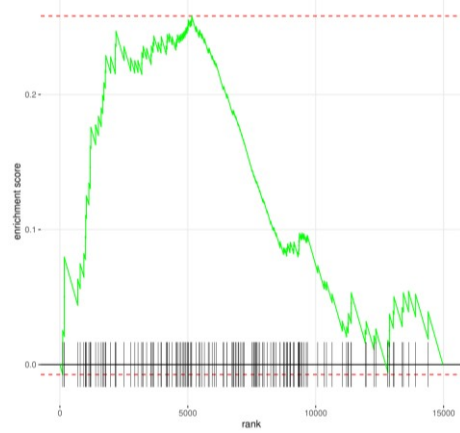
Cellular response to hypoxia



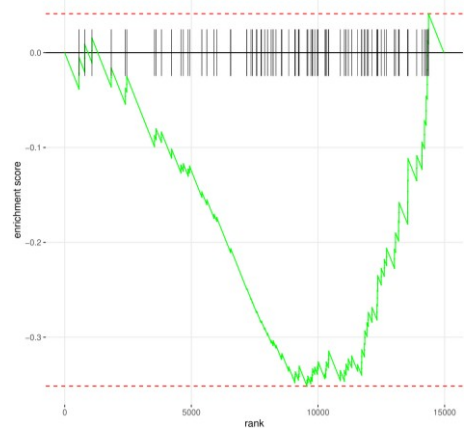
A

B

Cellular response to starvation



Mitochondrial translation elongation



C

D

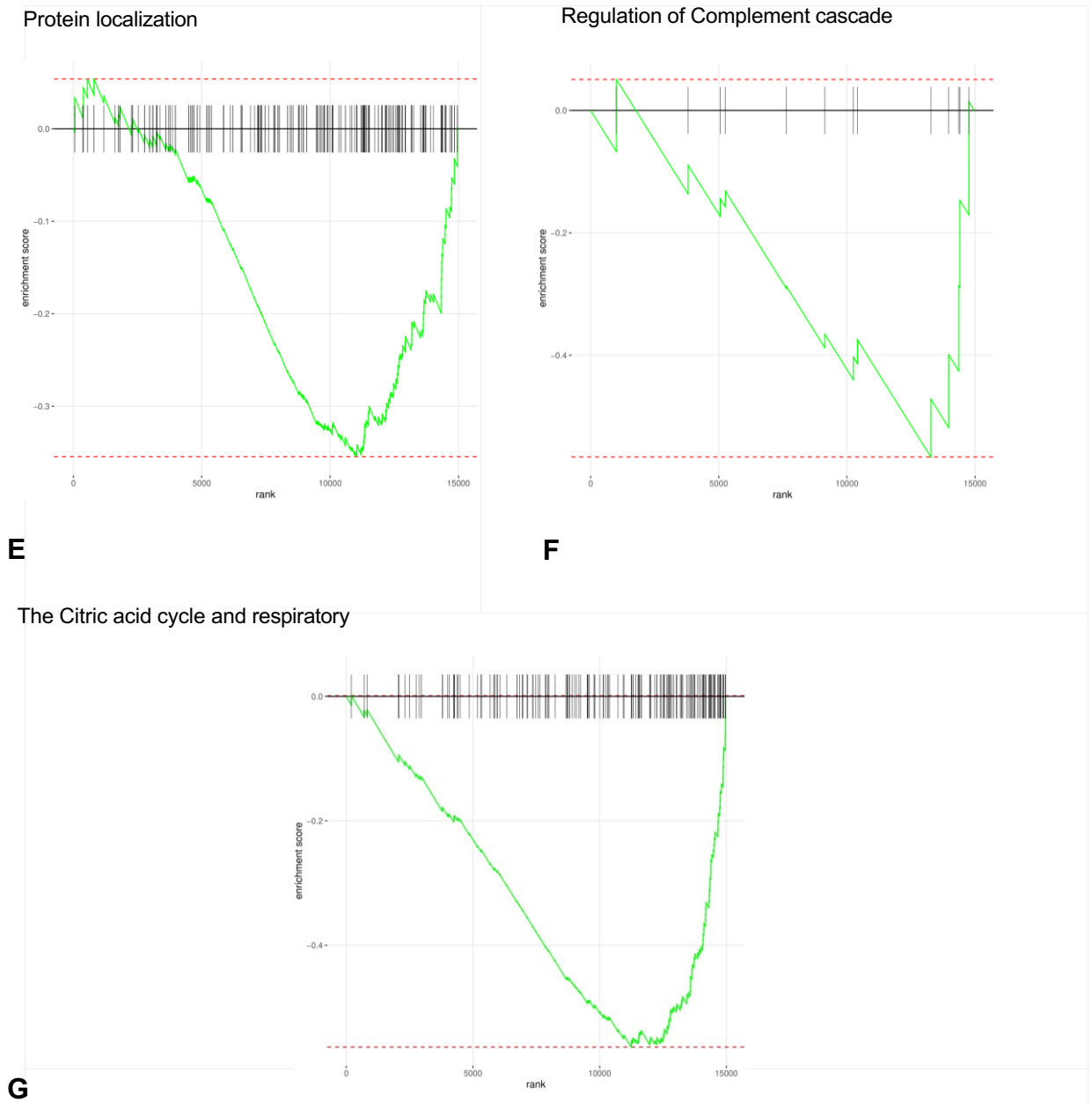


Figure 9: GSEA CFS vs LC-19 Cellular Response to A) Chemical Stress B) Hypoxia and C) Starvation D) Mitochondrial Translation E) Protein localization F) Regulation of Complement Cascade G) TCA cycle and Respiratory Electron Transport

3A.2.4. Summary- Comparative Transcriptomics

3A.2.4.1. Mitochondrial Complexes and Dynamics:

Gene expression data from RNA Sequencing indicated that transcript levels corresponding to Mitochondrial Respiratory Chain complexes were dysregulated in diseased groups, highlighting a compensatory response (243,244) (Figure 5M). The upregulation was comparatively more for the LC-19 samples compared to ME/CFS. The same trend was observed for mitochondria encoded genes and those encoded in the nuclear genome. Mitochondrial Dynamin Related Protein 1 (*DNM1L*) transcript levels were downregulated indicating a drop in mitochondrial fission (Figure 2M). This observation in conjunction with the upregulation of Mitofusin- 2 (*MFN2*) and *SMYD1* signposts towards adaptive changes in mitochondrial performance in favor of mitochondrial network fusion.

3A.2.4.2. TCA Cycle and Glycolysis

Upregulation in TCA cycle and Glycolytic gene expression for ME/CFS and LC-19 samples indicates cellular adaptation against increased energy demands.

3A.2.4.3. Mitochondrial Apoptosis

We also observed indication of mitochondrial apoptosis through the upregulated expression of *AIFM1* (Apoptosis-Inducing Factor, Mitochondria Associated 1) and *ENDO G* (Endonuclease G). *AIFM1* is involved in caspase-independent apoptosis and translocated from the mitochondria to the nucleus upon apoptotic stimulation. It has also been implicated in maintaining mitochondrial OXPHOS (245). Similar to *AIFM1*, *ENDO G* is also a pro-apoptotic mitochondrial protein. We also observed a downregulation in *HTRA2* (High Temperature Requirement A2) which suggested compromised mitochondrial protein quality control and accumulation of damaged or misfolded proteins (246).

Gene Ontology Analysis also revealed multiple other pathways that were altered indicating metabolic adaptations against stress in diseased tissues exposed to ME/CFS and LC-19 sera.

While there were no significant DEGs observed between ME/CFS and LC-19 diseased tissues, we were intrigued to compare trends between the two groups for

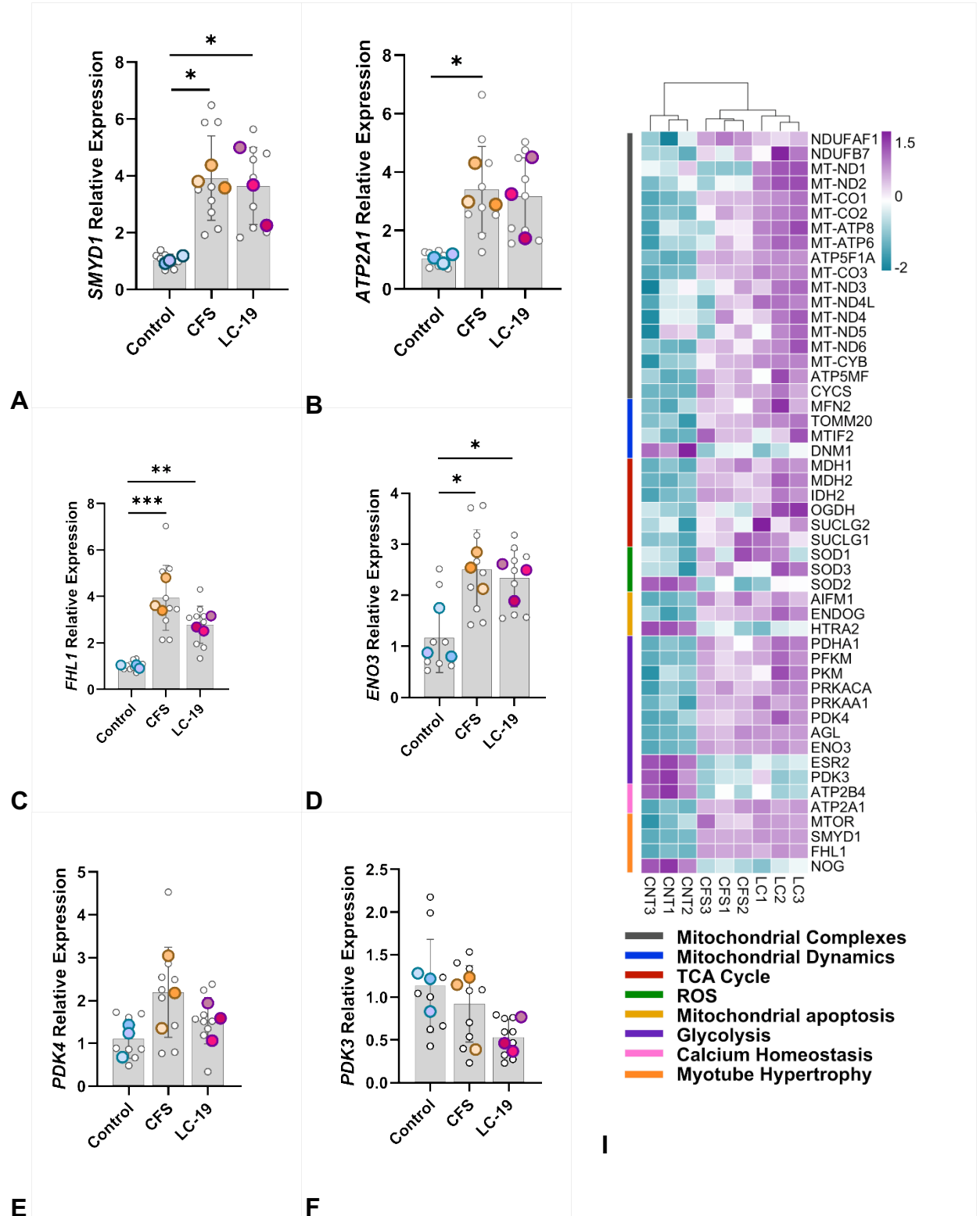
which we conducted the Gene Set Enrichment Analyses (Figure S3). For all the key compensatory pathways, ME/CFS tissues had a lower gene expression compared to the LC-19 diseased tissues except the genes involved in cellular response to starvation. This indicated increased compensatory capacity in tissues treated with LC-19 sera compared to those treated with ME/CFS. The relative expression for genes of interest has been validated through qRT-PCR (Figure 2 E-L).

3A.3. Validation of DEG expression

A lysine methyl transferase specific for striated muscles, SET and MYND Domain Containing 1 (*SMYD1*) plays an important role in muscle differentiation and mitochondrial bioenergetics including stabilization of respiratory complexes and cristae formation (247,248). It was found to be upregulated in ME/CFS and LC-19 tissues compared to the controls (Figure 10A, I). *SMYD1* gain-of-function has been previously associated with upregulation of mitochondrial respiration as a protective mechanism against injury (247). Furthermore, the levels of *ATP2A1* which codes for *SERCA1* Ca^{2+} -ATPase were also upregulated (Figure 10B, I). Predominantly present in type II fast twitch muscle fibers, this pump is a key regulator of relaxation dynamics of a striated muscle. Found in the sarcoplasmic reticulum (SR) of muscle cells it pumps the calcium ions from the cytoplasm into the SR, allowing muscle relaxation post contraction and restocking ions for the next contraction (249). Downregulation in *ATP2B4* (calcium ATPase isoform 4) observed in RNA-Seq, (responsible for removing intracellular calcium ions against the large gradients), indicates high calcium sequestering (Figure 10I). Coupled with an upregulation in *ATP2A1* levels, this indicates a disturbance in calcium homeostasis. Increase of calcium sequestering by *ATP2A1* in the sarcoplasmic reticulum has been related to a disturbance in mitochondrial function and an inducer of fatigue. Increased mitochondrial calcium can induce the production of ROS through the Electron Transport Chain, particularly the levels of superoxide radicals (250,251). Downregulation of *SOD2* (superoxide dismutase 2) was observed in diseased tissues, confirming the previously reported data (192). Coupled with an increase in

calcium sequestering, a decrease in *SOD2* levels indicates oxidative stress (Figure 10I).

Four-and-a-half LIM protein 1 (*FHL1*) has been implicated in inducing myotube hypertrophy (252). It was found to be overexpressed in the diseased tissues (Figure 10C, I). We also observed an upregulation in transcript levels of glycolytic enzymes such as *AGL* which codes for glycogen debranching enzyme and *ENO3* (beta Enolase) (Figure 10D, I). Both are responsible for regulating the glycolytic pathway and controlling the levels of glucose. Pyruvate dehydrogenase 4 (*PDK4*) is known to be an important metabolic regulator which increases fatty acid oxidation (Figure 10E, I). A shift towards fasting-type energy metabolism has been associated with an overexpression of *PDK4* (253). ME/CFS and LC-19 diseased tissues showed an increase in *PDK4* gene levels indicating metabolic plasticity under stress conditions warranting a high energy demand. The levels of *PDK3*, however, were downregulated favoring glucose oxidation (Figure 10F, I). Aberrant *PDK* levels have previously been associated with metabolic adaptation in ME/CFS patients (254). Downregulation of the *NOG* (noggin) gene has been associated with an increase in Bone Morphogenetic protein (BMP) signaling pathway implicated in inducing skeletal muscle hypertrophy which we observed in both LC-19 and ME/CFS treatments (Figure 10G, I) (255). Estrogen receptor type 2 or *ESR2* is known to regulate skeletal muscle growth, regeneration through activation of satellite cells, activating anabolic pathway, and metabolic homeostasis. Loss of *ESR2* expression negatively impacts all these processes (256–258) (Figure 10H, I).



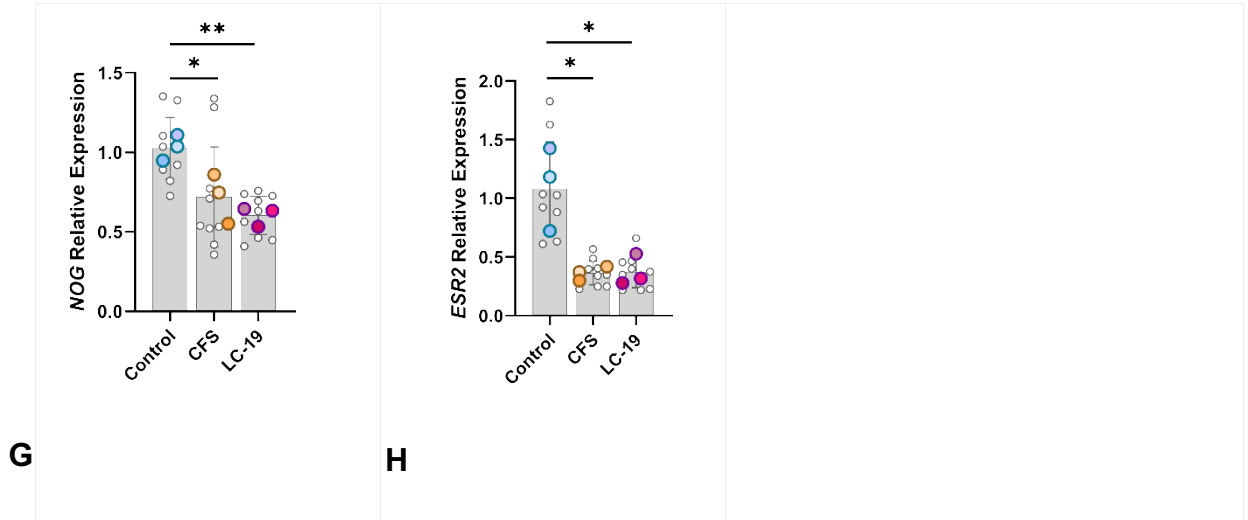


Figure 10: RNA-Seq Analyses of 48h tissue cohort. qRT-PCR Relative Gene Expressions of E) *SMYD1* F) *ATP2A1* G) *FHL1* H) *ENO3* I) *PDK4* J) *PDK3* K) *NOG* and L) *ESR2*. M) Heatmap of Differentially Expressed Genes. Statistical Analyses: Biological replicates: ME/CFS and Control, n= 3. Technical Replicates: n= 4. Data show the mean $\pm$ s.d. Statistical analysis: one-way ANOVA with Tukey's post hoc test * $P \leq 0.05$; ** $P \leq 0.01$.

3A.4. Expression Analyses of Transposable Elements

Multidimensional Scaling and Heatmap plots indicated a similar clustering of samples as observed for the gene data in the last section. There was a clear distinction between control and patient groups for all 3 comparisons (Figure 11).

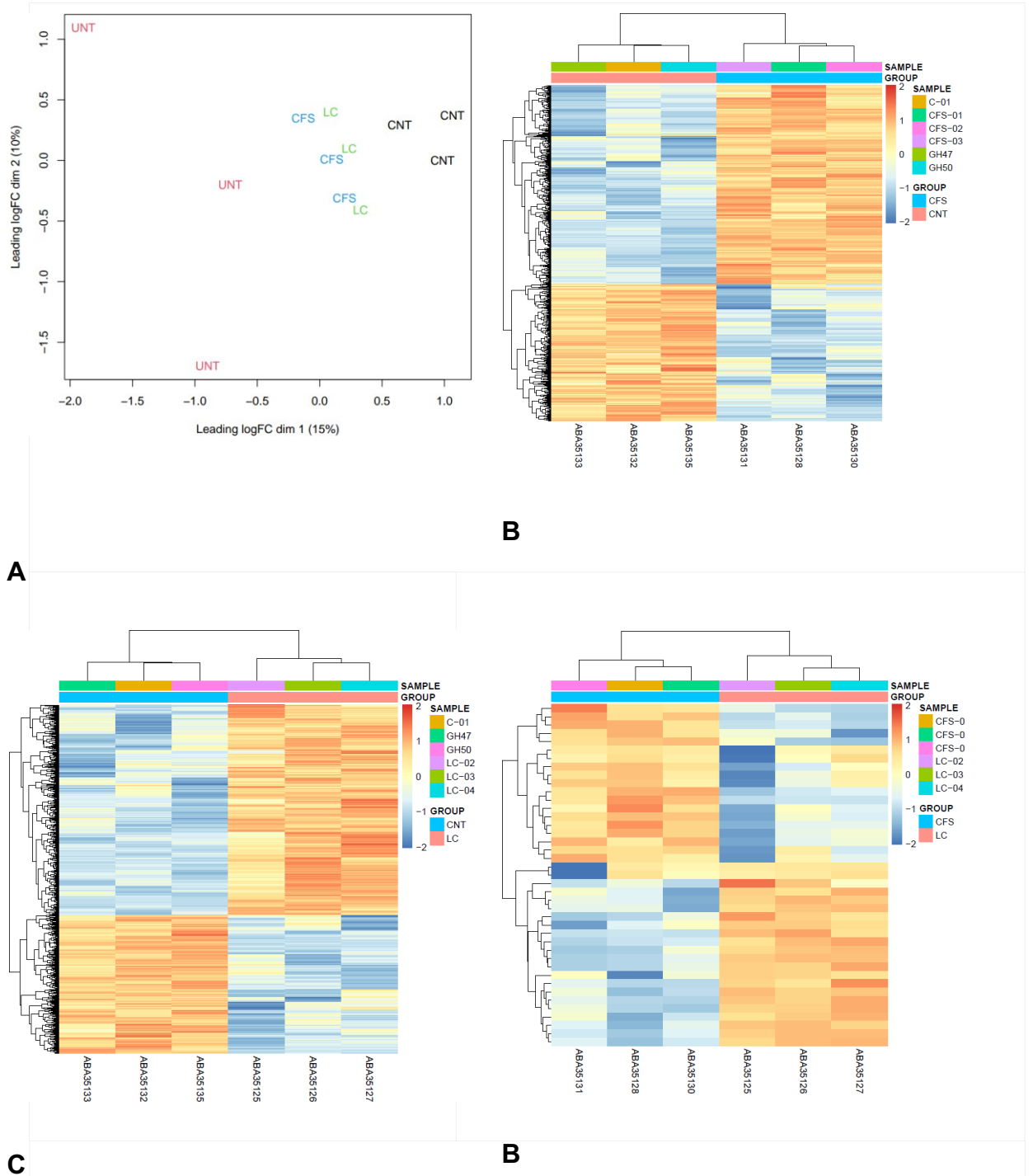
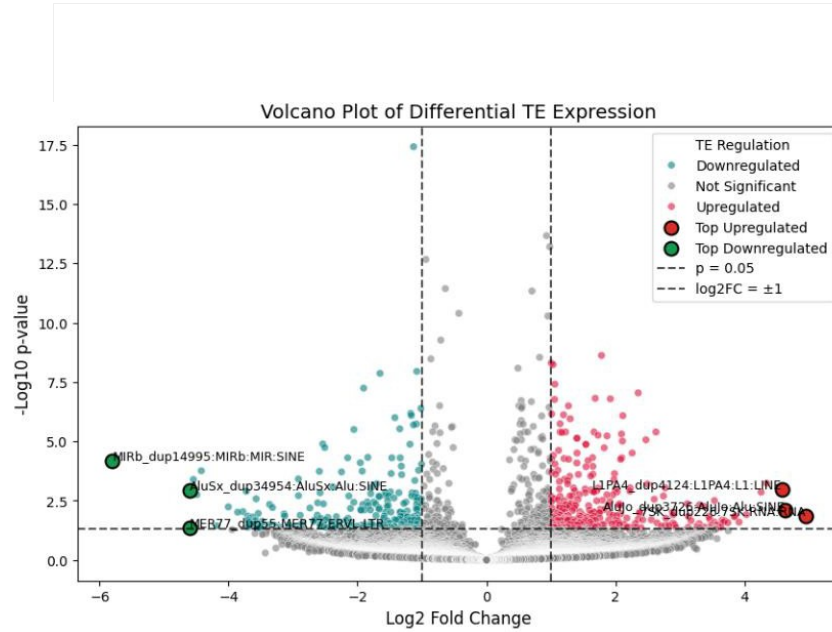
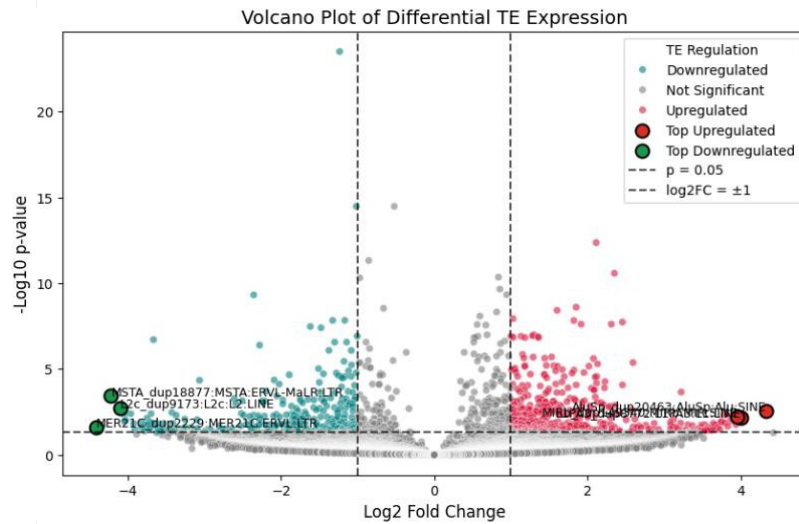


Figure 11: Transposon Expressional Analysis A) Multidimensional Scaling. Heatmaps B) CFS vs CNT C) LC-19 vs CNT D) CFS vs LC-19

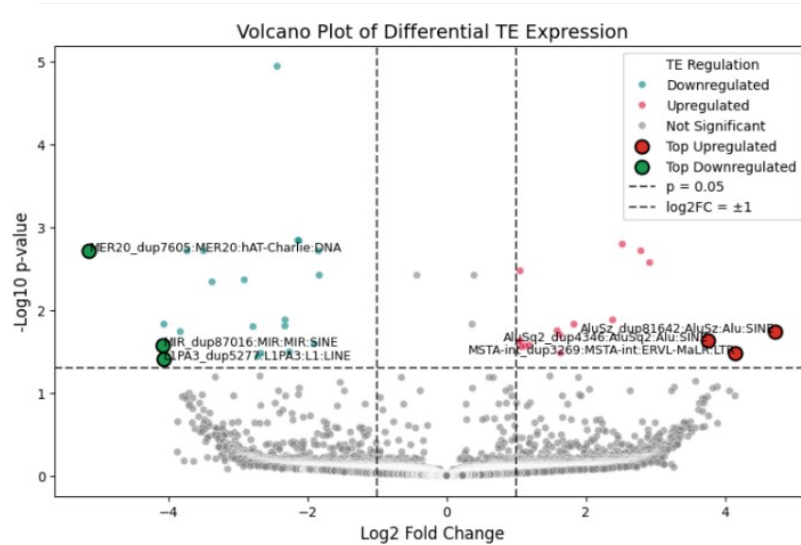
There were significant up and downregulated transposons between CFS vs CNT and between LC-19 vs CNT. Contrary to DEGs, we observed 44 differentially expressed transposons between CFS vs LC-19 as well (Figure 12).



A



B



C

Figure 12: TE-Analyses- Volcano plots Differentially Expressed genes A) CFS vs CNT B) LC-19 vs CNT and C) CFS vs LC-19

3A.4.1. Group 1: CFS vs CNT

3A.4.1.1. Classification of Transposons

Classification of transposons into LINEs (Long Interspersed Nuclear Elements), SINEs (Short Interspersed Nuclear Elements), LTRs (Long Terminal Repeats) and DNA transposons is based on their different mechanisms of movement across the genomes and the consequent effect on the genome. LINE transposons are the most abundant class followed by the rest. In upregulated transposons, SINEs were the second most abundant followed by the LTRs while in the downregulated, LTRs were the second most abundant followed by SINEs and DNA transposons (Figure 13 A-B).

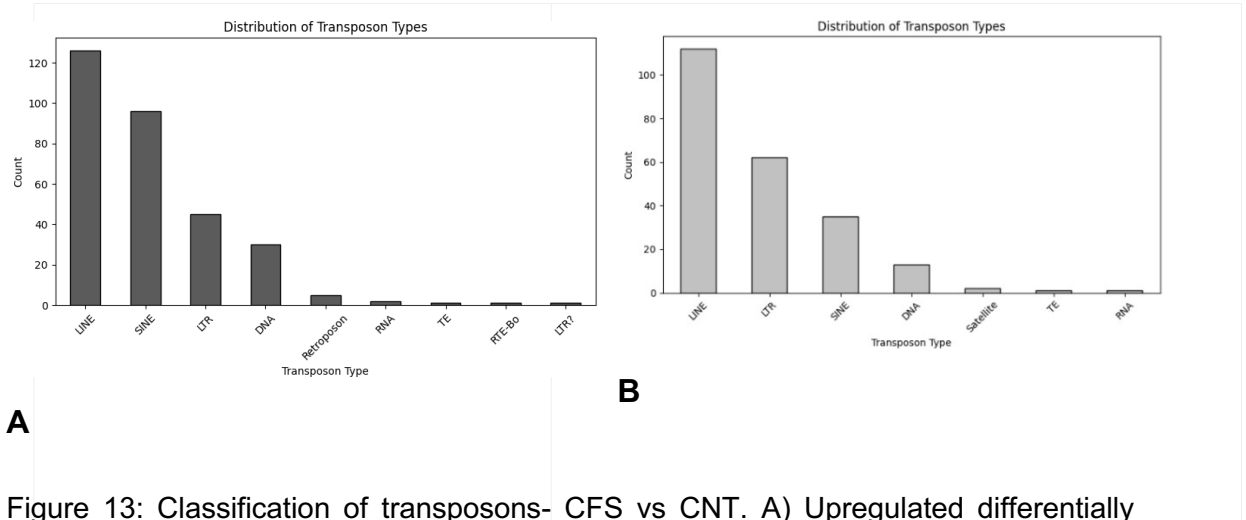
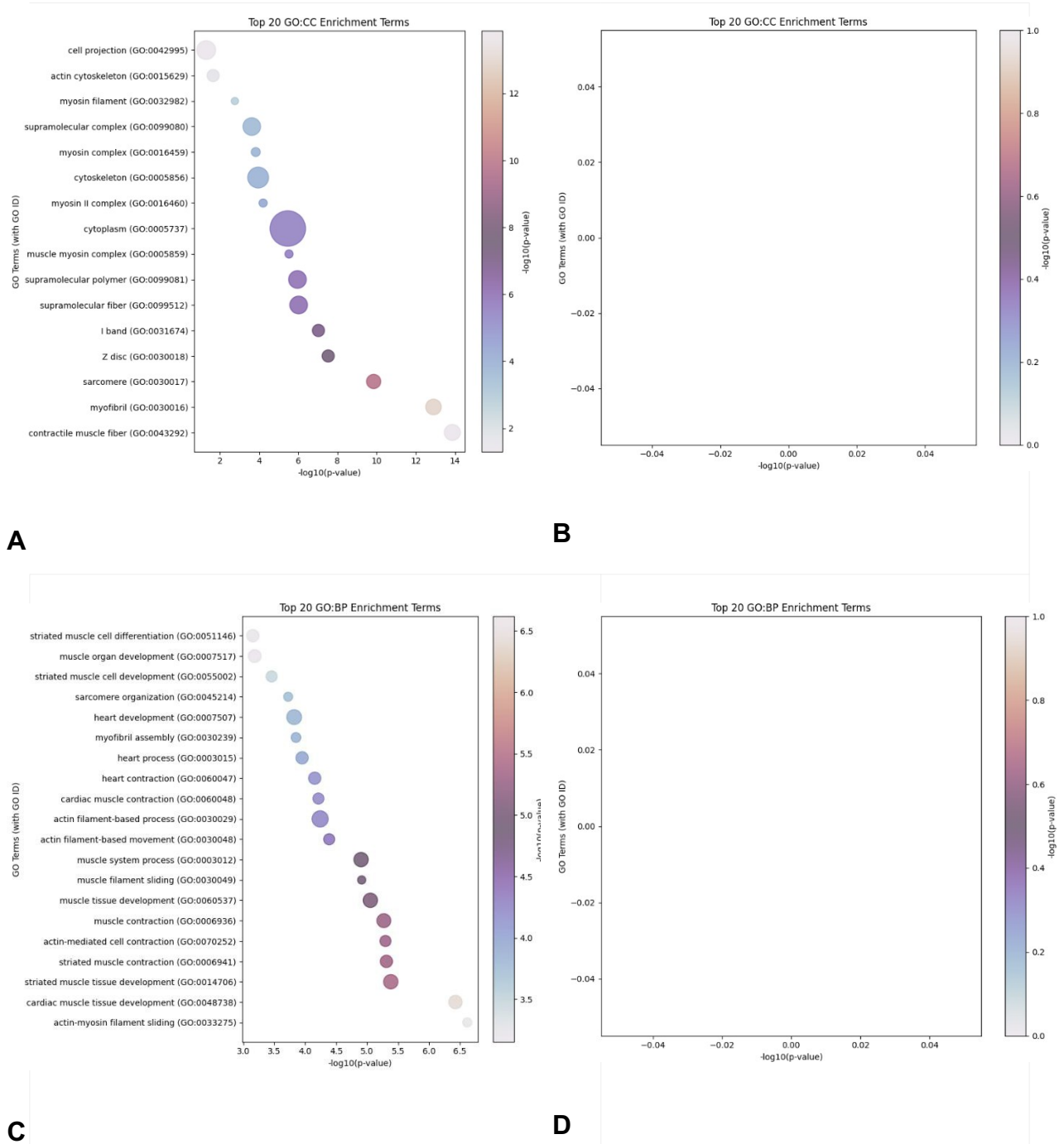


Figure 13: Classification of transposons- CFS vs CNT. A) Upregulated differentially expressed transposons B) Downregulated differentially expressed transposons.

3A.4.1.2. Gene Ontology Analysis of Overlapped Genes

Genomic mapping of transposons reveals their direct overlap with chromosomal loci critical for muscle function, implicating these mobile elements as cis-regulatory elements. Transposons (LINEs, LTRs, or SINEs) locations intersected with genes controlling contractile machinery, including those encoding actin-myosin components (e.g., MYH7, ACTC1) and sarcomere organizers (e.g., TTN, NEB). This colocalization correlates with hyperactivation of muscle-specific pathways such as actin-myosin filament sliding (GO:0033275, FE=1.87), sarcomere organization (GO:0045214, FE=1.41), and Z-disc assembly (GO:0030018, FE=1.41). Terms indicating structural changes were also prominent such as those implicating pathological hypercontractility (e.g., exaggerated actin-mediated contraction, GO:0070252) and myofibril overgrowth (GO:0030016, FE=1.86). Cardiac-specific genes (e.g., NKX2-5, GATA4) are preferentially targeted, aligning with enriched cardiac muscle development (GO:0048738) and contraction terms (GO:0060047). This data, overall, suggests mechanical stress in the skeletal muscle tissues.

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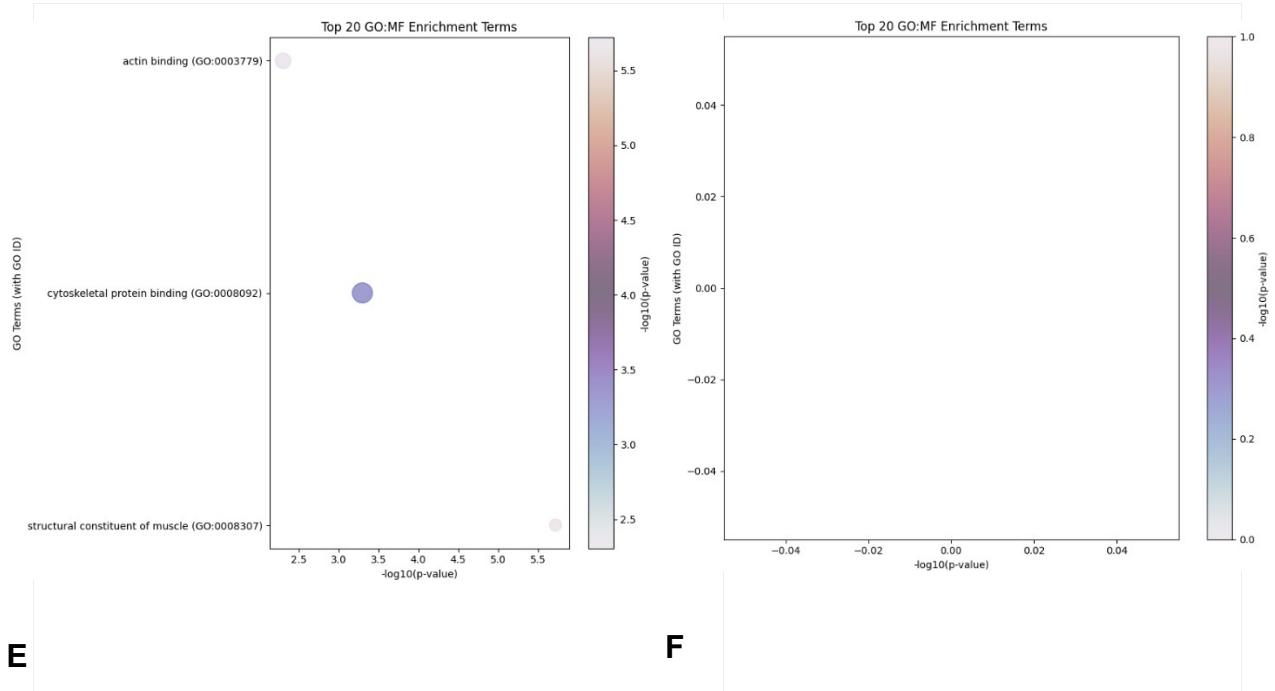


Figure 14: CFS vs CNT Gene Ontology Enrichment A) GO: Cellular Component for Upregulated DETs B) GO: Cellular Component for Downregulated DETs C) GO: Bioprocesses for Upregulated DETs D) GO: Bioprocesses for Downregulated DETs E) GO: Molecular Function for Upregulated DETs F) GO: Molecular Function for Downregulated DETs

In order to know which of the upregulated DEGs were directly overlapped with the upregulated transposons, a list of genes shown in Figure 15 and Table was obtained. Notably, myosin heavy chain genes (MYH6, MYH7) which are critical for cardiac and skeletal muscle contractility were directly overlapped by these transposons. This overlap also correlates with functional enrichments in actin-myosin filament sliding (GO:0033275) and sarcomere organization (GO:0045214) shown in Figure 14, suggesting a transposon-mediated effect by amplifying contractile machinery production to compensate for a lack of energy.

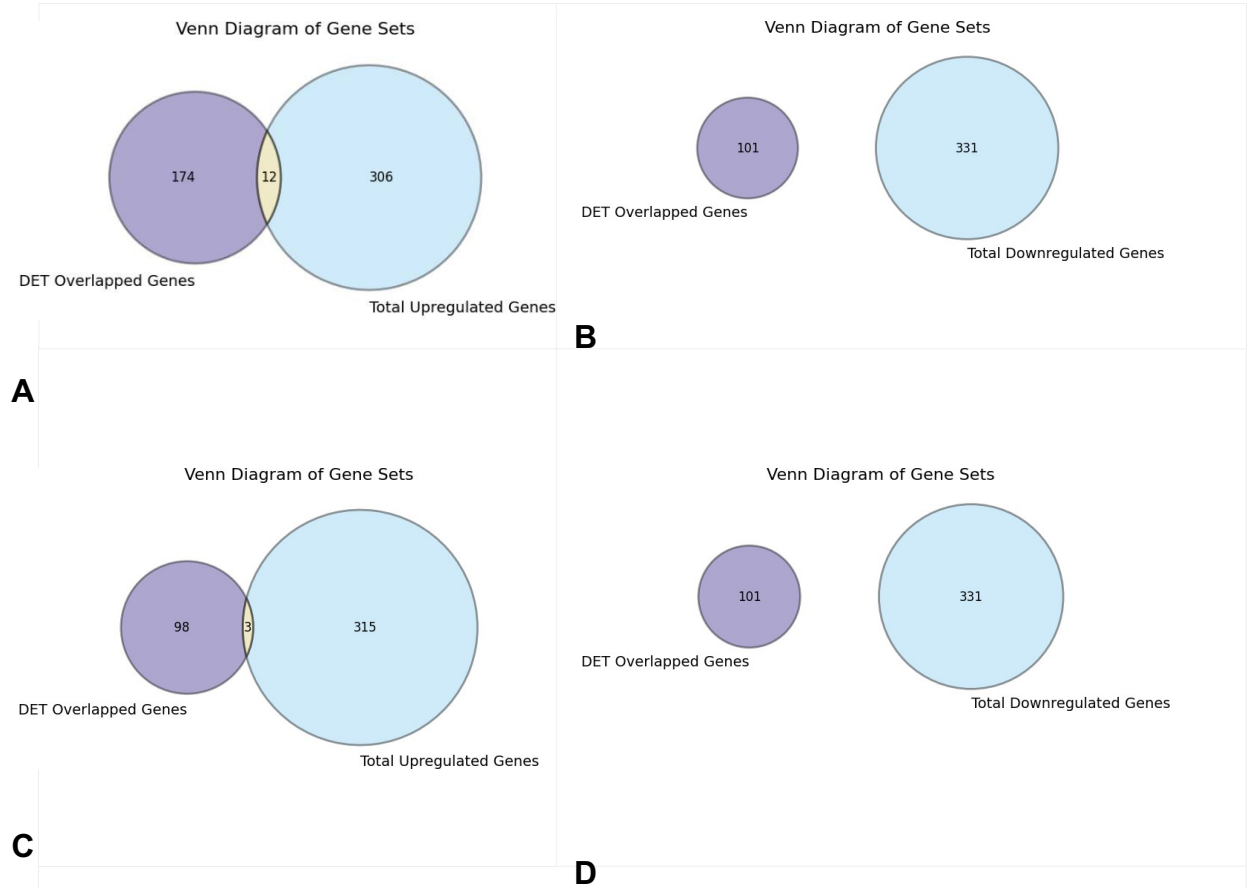


Figure 15: Venn Diagram CFS vs CNT A) Common Upregulated DEGs overlapped by Upregulated DETs. B) Common Downregulated DEGs overlapped by Upregulated DETs C) Common Upregulated Genes overlapped by downregulated DETs D) Common downregulated genes overlapped by downregulated DETs.

Table 10: *List of Upregulated DEGs overlapped with Upregulated DETs*

Ensembl ID	Gene Symbol	Gene Name / Description	Function
ENSG00000059728	MXD1	MAX Dimerization Protein 1	Transcriptional repressor; inhibits MYC activity; regulates cell proliferation.
ENSG00000144218	AFF3	AF4/FMR2 Family Member 3	Nuclear activator; involved in transcription elongation in lymphoid cells.

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ENSG000 0027513 1	PDE4D IPP2	PDE4DIP Pseudogene 2	Pseudogene; related to phosphodiesterase anchoring functions.
ENSG000 0025667 1	LIMS4	LIM Zinc Finger Domain Containing 4	May bind metal ions; limited functional data.
ENSG000 0019761 6	MYH6	Myosin Heavy Chain 6 (alpha-cardiac)	Key component in heart muscle contraction; associated with cardiomyopathy.
ENSG000 0024872 4	NPHP 3-AS1	NPHP3 Antisense RNA 1	Antisense RNA; function not well characterized.
ENSG000 0011516 5	CYTIP	Cytohesin 1 Interacting Protein	Regulates ARF signaling via cytohesin-1 interaction; influences immune signaling.
ENSG000 0016840 5	CMAH P	CMP-N-Acetylneuraminic Acid Hydroxylase Pseudogene	Pseudogene; human-inactive form of CMAH involved in sialic acid metabolism.
ENSG000 0012195 7	GPSM 2	G-Protein Signaling Modulator 2	Modulates G-protein signaling; involved in cell division and sensory system development.
ENSG000 0013811 9	MYOF	Myoferlin	Mediates membrane repair and muscle cell fusion.
ENSG000 0009205 4	MYH7	Myosin Heavy Chain 7 (beta-cardiac)	Cardiac and slow-twitch skeletal muscle myosin; associated with cardiomyopathies.
ENSG000 0006685 5	MTFR1	Mitochondrial Fission Regulator 1	Regulates mitochondrial morphology and energy metabolism.

Interestingly, none of the upregulated transposons overlapped with any of the downregulated genes as shown in Figure 15 while the downregulated transposons only overlapped with only 2 upregulated genes and a pseudogene (Table 11)

Table 11: List of Upregulated DEGs overlapped with Downregulated DETs

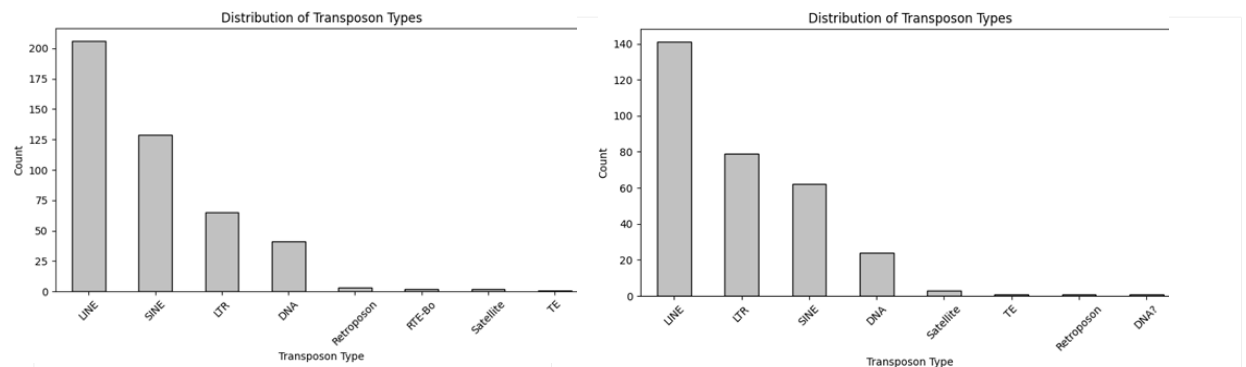
Ensemb IID	Gene Symb ol	Gene Name / Description	Function
ENSG00 000104 415	CCN 4	Cellular Communication Network Factor 4	Involved in cell adhesion, migration, and proliferation; associated with WNT signaling pathways.
ENSG00 000214 050	FBXO 16	F-Box Protein 16	Component of the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complex; involved in phosphorylation- dependent ubiquitination.
ENSG00 000275 131	PDE4 DIPP 2	PDE4DIP Pseudogene 2	Classified as a pseudogene related to PDE4DIP; lacks protein-coding potential.

Overall, the transposon expression and overlap patterns indicate positive adaptation attempts against stress as well by preferentially driving the expression of upregulated DEGs.

3A.4.2. Group 2: LC-19 Vs CNT

3A.4.2.1. Classification of Transposons

In upregulated transposons, SINEs were the second more abundant followed by the LTRs while in the downregulated, LTRs were the second most abundant followed by SINEs and DNA transposons (Figure 16).



A

B

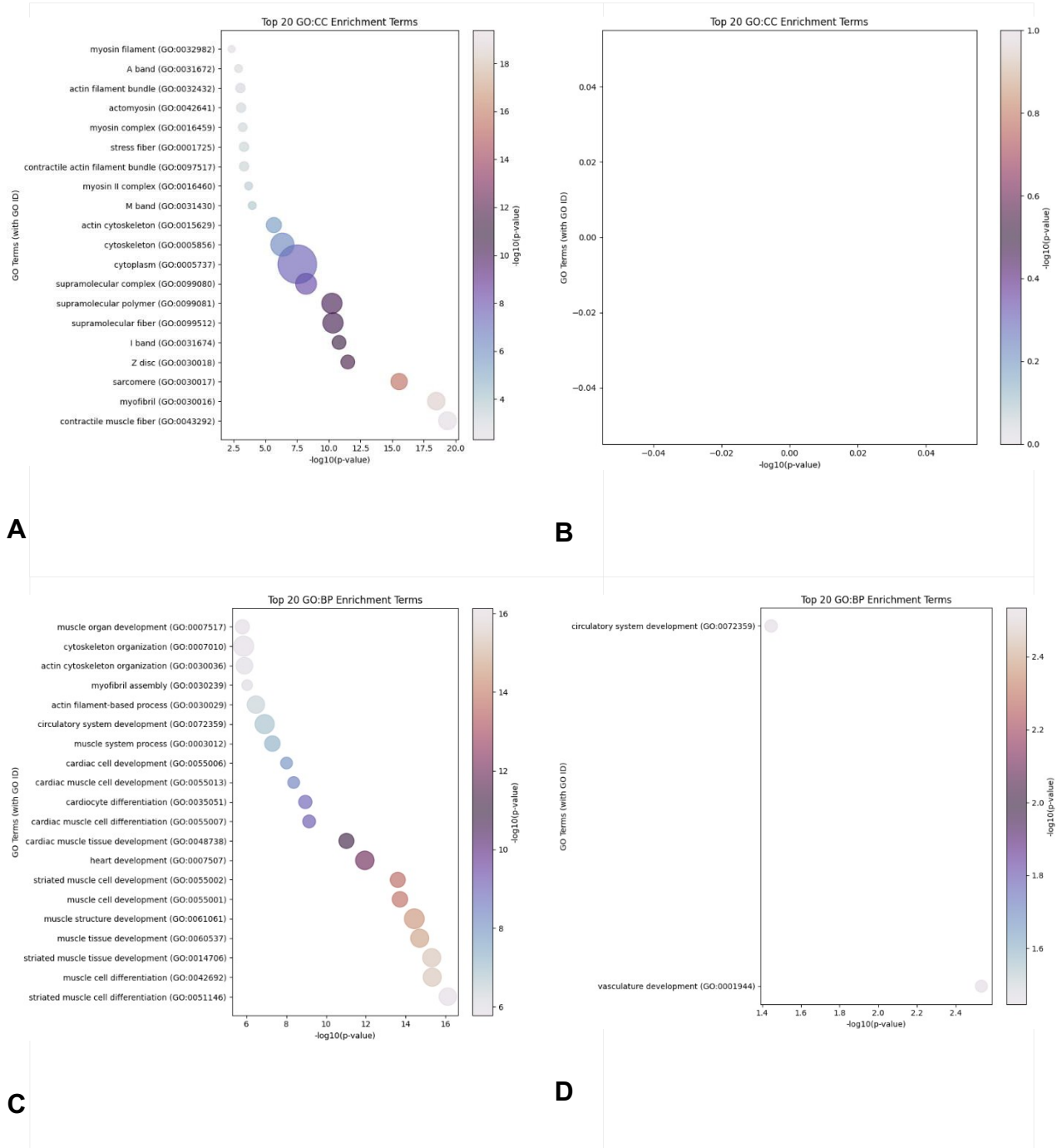
Figure 16: Classification of Transposons- LC-19 vs CNT A) Upregulated DETs B) Downregulated DETs.

3A.4.2.2. Gene Ontology Analysis of Overlapped Genes

Transposon overlapping tendency similar to that observed for the previous group was observed in this case as well (Figure 17). The overall trend appears dominated by muscle-specific processes, with strong enrichments in sarcomere organization (GO:0045214), striated muscle differentiation (GO:0051146), and myofibril assembly (GO:0030239). These terms reflect transposon-driven overexpression of structural genes like MYH6/MYH7 (myosin heavy chains) and TTN (titin), which contribute to the performance of actin/myosin in driving efficient muscle contraction. This trend indicates an attempt to upregulate the expression of contractile machinery (e.g., striated muscle contraction GO:0006941). The pattern was similar to that observed for DEGs in the same group as discussed in section 3A.2.2.

At the molecular level, there is a priority observed for structural and binding roles, including actin binding (GO:0003779) and structural constituent of muscle (GO:0008307). These terms highlight Transposon-mediated dysregulation of cytoskeletal proteins (e.g., α -actinin, tropomyosin). Cellular component enrichments further localize dysfunction to contractile machinery, including contractile fiber (GO:0043292), myofibril (GO:0030016), and sarcomere (GO:0030017) which are all structures that are central to muscle contraction. Notably, cardiac muscle hypertrophy (GO:0003300) emerges as a downstream consequence, linking transposon activity to pathological remodeling observed in hypertrophic conditions.

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Section A: ME/CFS and Long COVID-19: Results

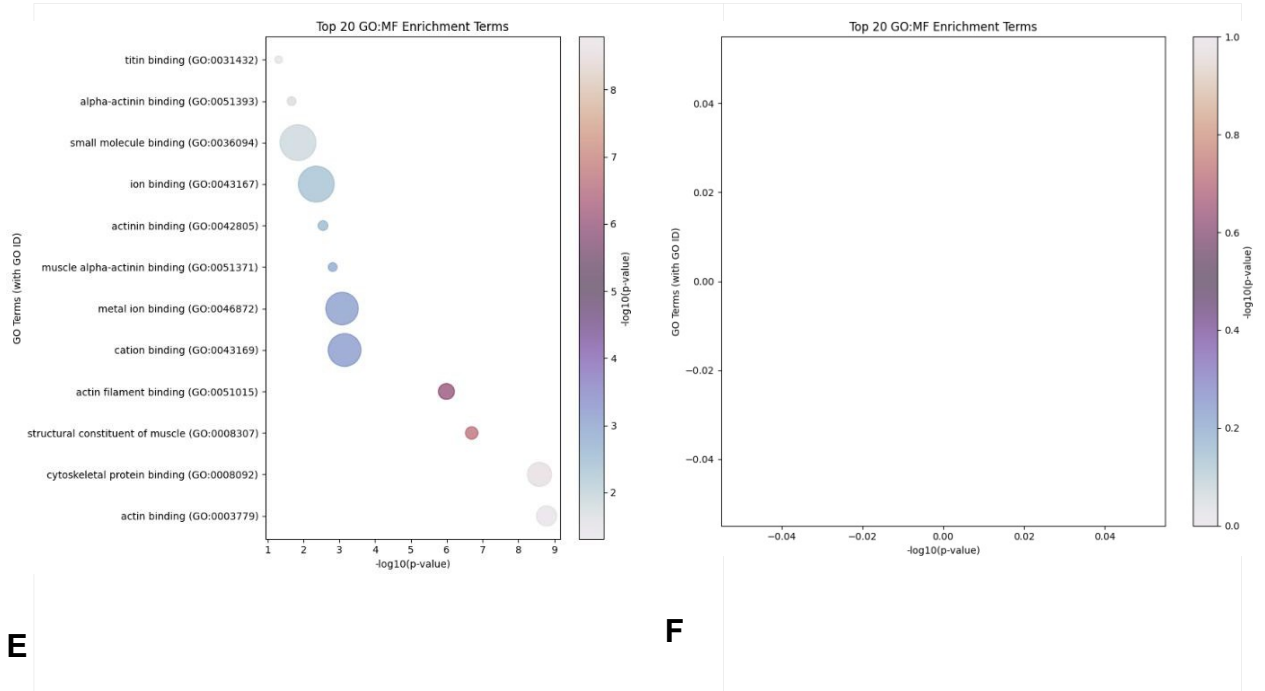
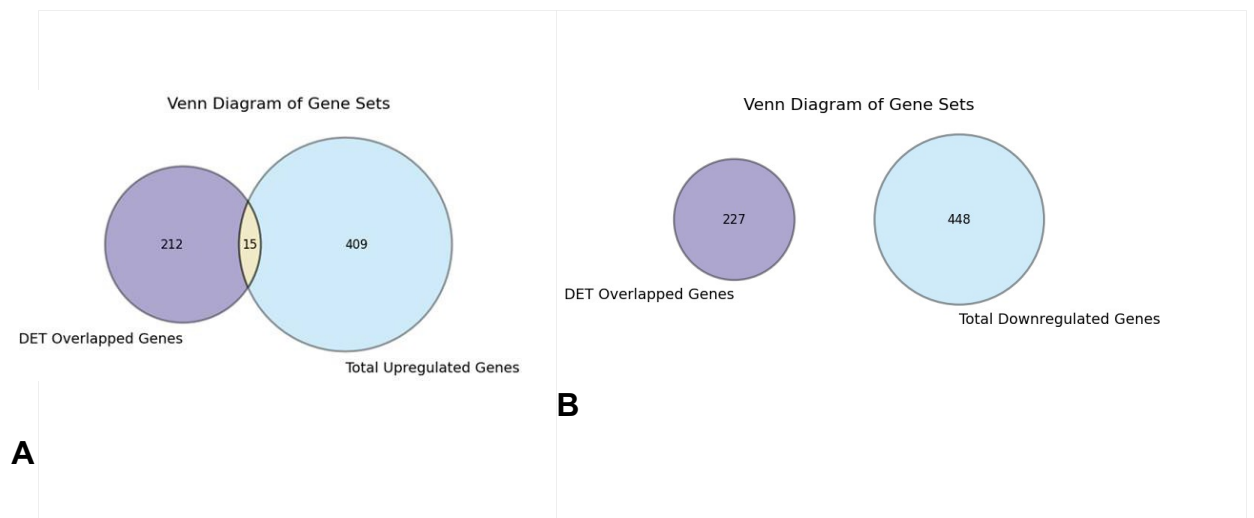


Figure 17: LC-19 vs CNT Gene Ontology Enrichment A) GO: Cellular Component for Upregulated DETs B) GO: Cellular Component for Downregulated DETs C) GO: Bioprocesses for Upregulated DETs D) GO: Bioprocesses for Downregulated DETs E) GO: Molecular Function for Upregulated DETs F) GO: Molecular Function for Downregulated DETs

We then checked which of the differentially expressed upregulated genes were overlapped directly with upregulated transposons and obtained 15 genes tabulated in Table. Furthermore, all the upregulated genes overlapped by downregulated transposons were pseudogenes and lcrRNA (Figure 18).



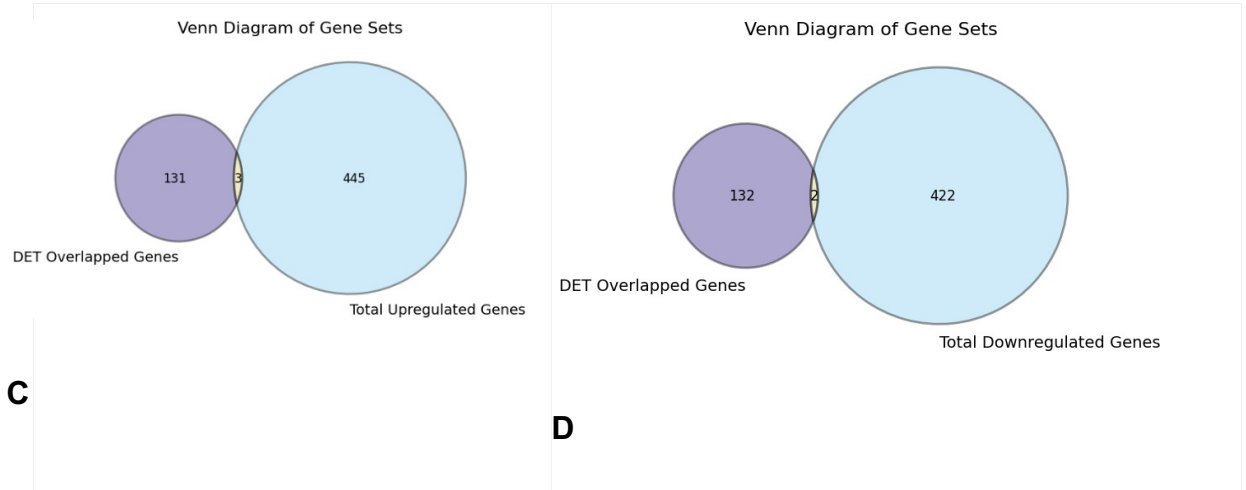


Figure 18: Venn Diagram LC-19 vs CNT A) Common Upregulated DEGs overlapped by Upregulated DETs. B) Common Downregulated DEGs overlapped by Upregulated DETs C) Common Upregulated Genes overlapped by downregulated DETs D) Common downregulated genes overlapped by downregulated DETs.

Table 12: List of Upregulated DEGs overlapped with Upregulated DETs

Ensembl ID	Gene Symbol	Gene Name / Description	Function
ENSG00000248092	NNT-AS1	Nicotinamide Nucleotide Transhydrogenase Antisense RNA 1	Long non-coding RNA; function not well characterized.
ENSG00000059728	MXD1	MAX Dimerization Protein 1	Transcriptional repressor; inhibits MYC activity; regulates cell proliferation.
ENSG00000275131	PDE4DIPP2	PDE4DIP Pseudogene 2	Pseudogene; related to phosphodiesterase anchoring functions.
ENSG00000256671	LIMS4	LIM Zinc Finger Domain Containing 4	May bind metal ions; limited functional data.

ENSG00000248724	NPHP3-AS1	NPHP3 Antisense RNA 1	Antisense RNA; function not well characterized.
ENSG00000159216	RUNX1	RUNX Family Transcription Factor 1	Essential for hematopoiesis; mutations linked to leukemia.
ENSG00000115165	CYTIP	Cytohesin 1 Interacting Protein	Regulates ARF signaling via cytohesin-1 interaction; influences immune signaling.
ENSG00000104415	CCN4	Cellular Communication Network Factor 4	Involved in cell adhesion, migration, and proliferation; associated with WNT signaling pathways.
ENSG00000141337	ARSG	Arylsulfatase G	Lysosomal enzyme; involved in degradation of sulfated glycosaminoglycans.
ENSG00000168405	CMAHP	CMP-N-Acetylneuraminic Acid Hydroxylase Pseudogene	Pseudogene; human-inactive form of CMAH involved in sialic acid metabolism.
ENSG00000175701	MTLN	Mitoregulin	Regulates mitochondrial function and energy metabolism.
ENSG00000133424	LARGE1	LARGE Xylosyl- and Glucuronyltransferase 1	Glycosyltransferase; modifies α -dystroglycan; crucial for muscle function.
ENSG00000092054	MYH7	Myosin Heavy Chain 7 (beta-cardiac)	Cardiac and slow-twitch skeletal muscle myosin; associated with cardiomyopathies.
ENSG00000270872	LINC00116	Long Intergenic Non-Protein Coding RNA 116	Long non-coding RNA; function not well characterized.
ENSG00000232931	LINC00342	Long Intergenic Non-Protein Coding RNA 342	Long non-coding RNA; potential biomarker for Huntington's disease.

3A.4.3. Group 3: CFS vs LC-19

3A.4.3.1. Classification of Transposons

In this group, we observed an abundance of SINEs in the downregulated transposons while for the upregulated transposons the trend remained the same as observed for previous groups

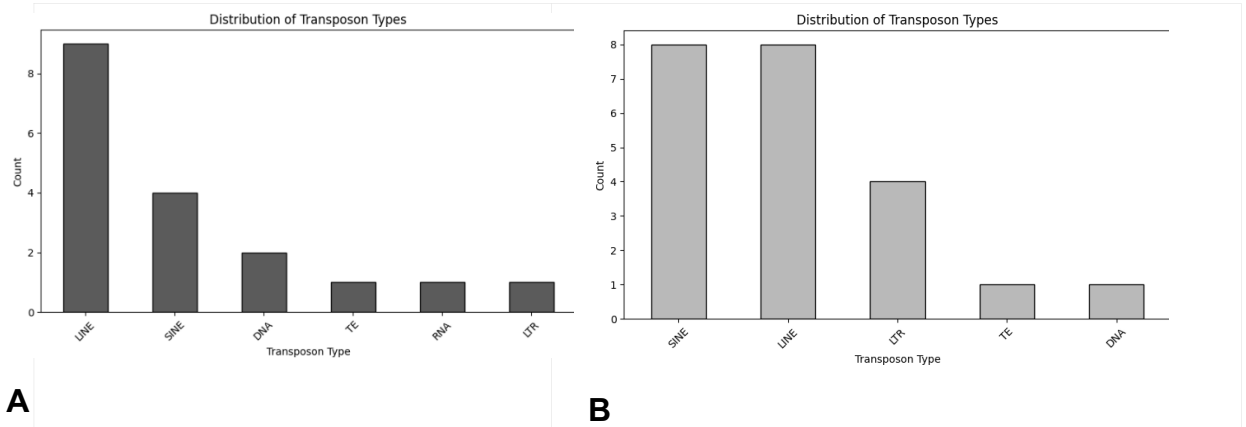


Figure 19: Classification of Transposons- CFS vs LC-19 A) Upregulated DETs B) Downregulated DETs.

3A.4.3.2. Gene Ontology of Overlapped Genes

As there were no differentially expressed genes in this group, intersection and subsequent pathway enrichment was not possible. List of genes that were overlapped by upregulated transposons have been tabulated in table below.

Table 13: Genes overlapped by upregulated DETs. (No DEGs in this group)

Ensemb IID	Gene Symbol	Gene Name / Description	Function
ENSG0 000022 7232	WASH7P	WAS Protein Family Homolog 7 Pseudogene	Pseudogene; related to the Wiskott-Aldrich syndrome protein family.
ENSG0 000026 8043	NBPF12	Neuroblastoma Breakpoint Family Member 12	Member of a gene family primarily located on chromosome 1; function not well characterized.
ENSG0 000026 6028	SRGAP2	SLIT-ROBO Rho GTPase Activating Protein 2	Involved in neuronal development; plays a role in neuronal morphogenesis and migration.

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ENSG0000170525	PFKFB3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3	Regulates glycolysis by synthesizing fructose-2,6-bisphosphate; involved in metabolic pathways.
ENSG0000067225	PKM	Pyruvate Kinase, Muscle	Catalyzes the final step in glycolysis; plays a role in energy production.
ENSG0000233024	NPIPA9	Nuclear Pore Complex Interacting Protein Family, Member A9	Predicted to be involved in nuclear pore complex interactions; function not well characterized.
ENSG0000254681	PKD1P5	Polycystic Kidney Disease 1 (Autosomal Dominant) Pseudogene 5	Pseudogene related to PKD1; lacks protein-coding potential.
ENSG0000185864	NPIP4	Nuclear Pore Complex Interacting Protein Family Member B4	Predicted to be involved in nuclear pore complex interactions; function not well characterized.
ENSG0000183569	SERHL2	Serine Hydrolase Like 2	Probable serine hydrolase; specific function not well characterized.
ENSG0000291035	GTF2H2C	General Transcription Factor IIH Subunit 2 Family Member C	Component of the transcription factor IIH complex; involved in nucleotide excision repair and transcription initiation.
ENSG0000202058	RN7SKP80	RNA, 7SK Small Nuclear Pseudogene 80	Pseudogene; related to 7SK small nuclear RNA.
ENSG0000183474	GTF2H2C	General Transcription Factor IIH Subunit 2 Family Member C	Component of the transcription factor IIH complex; involved in nucleotide excision repair and transcription initiation.
ENSG0000122642	FKBP9	FKBP Prolyl Isomerase 9	Peptidyl-prolyl cis-trans isomerase; involved in protein folding and trafficking.
ENSG0000104419	NDRG1	N-Myc Downstream Regulated 1	Involved in stress responses, hormone responses, cell growth, and differentiation.
ENSG0000102144	PGK1	Phosphoglycerate Kinase 1	Catalyzes an important step in glycolysis; involved in energy production.

3A.5. Structural and Mitochondrial Performance Analyses

To further investigate our transcriptomic findings, we undertook structural analyses of myotubes from our short exposure sample cohort and the mitochondria therein. Quantification of myotube diameter was performed by calculating Feret's diameter for individual, transversely cut tubes. Quantification of myotube diameters indicated an increased tendency of ME/CFS and LC-19 myotubes towards hypertrophy compared to the controls (Figure 20, 22A). Hypertrophy has been evidenced to be a compensatory response against stress which include self-repair processes (259).

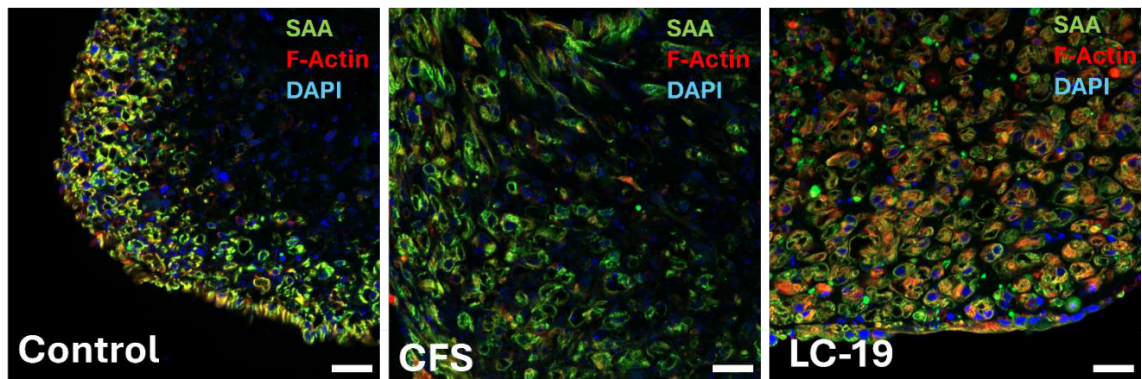


Figure 20: Structural and Functional Phenotypic Validation. Representative confocal images of transverse cross sections stained with an antibody against the mature muscle marker sarcomeric actinin (SAA), phalloidin (F-actin) and DAPI (nuclei). Scale bar: 40 μ m.

Moreover, quantification of mitochondrial networks indicated a trend (albeit non significant) towards mitochondrial fusion evidenced by increased mitochondrial branching and mean branch length (Figures 22 B, C). Mitochondria had a high aspect ratio and appeared to be hyperbranched in the cytoplasmic space across the length of a myotube as well as close to the nuclei (Figure 3E).

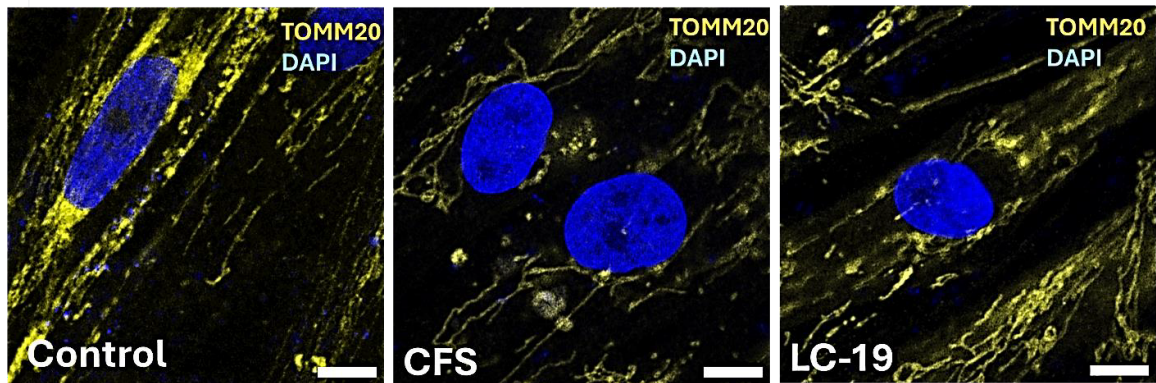


Figure 21: Representative confocal images of mitochondrial networks stained with inner mitochondrial membrane marker TOMM20 and DAPI. Scale bar= 10 μm

Fusion has been considered to be a positive response related to mitochondrial health, but evidence suggests that above control levels, excess fusion equates to an increased stress response (260–262). This coupled with an upregulation in pathways of mitochondrial protein elongation, OXPHOS and energy metabolism observed for ME/CFS and LC-19 in RNA Sequencing (Figure 7, 8) confirmed the mitochondrial compensatory response against stress.

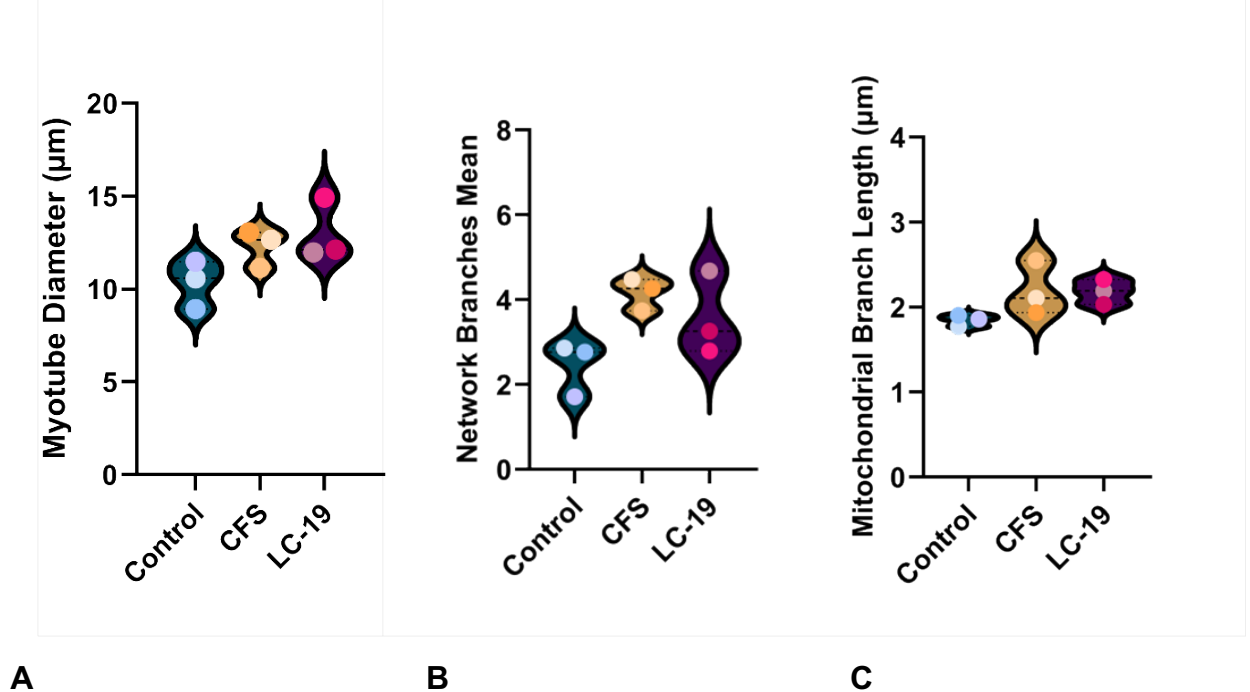


Figure 22: Morphological Quantification A) Quantification of myotube diameters $n=3$ B) Average network branches $n=3$ and C) Quantification of Mitochondrial Branch length.

Mitochondrial functional capacity was evaluated by the MitoStress Test and an increase in overall oxygen consumption by the diseased cells compared to the controls was observed (Figure 3F). The Extracellular Acidification Rate (ECAR) gives an overview of the glycolytic process in the cells as a response to treatments (Figure 3G). The ME/CFS samples had the highest ECAR of all the treatment groups.

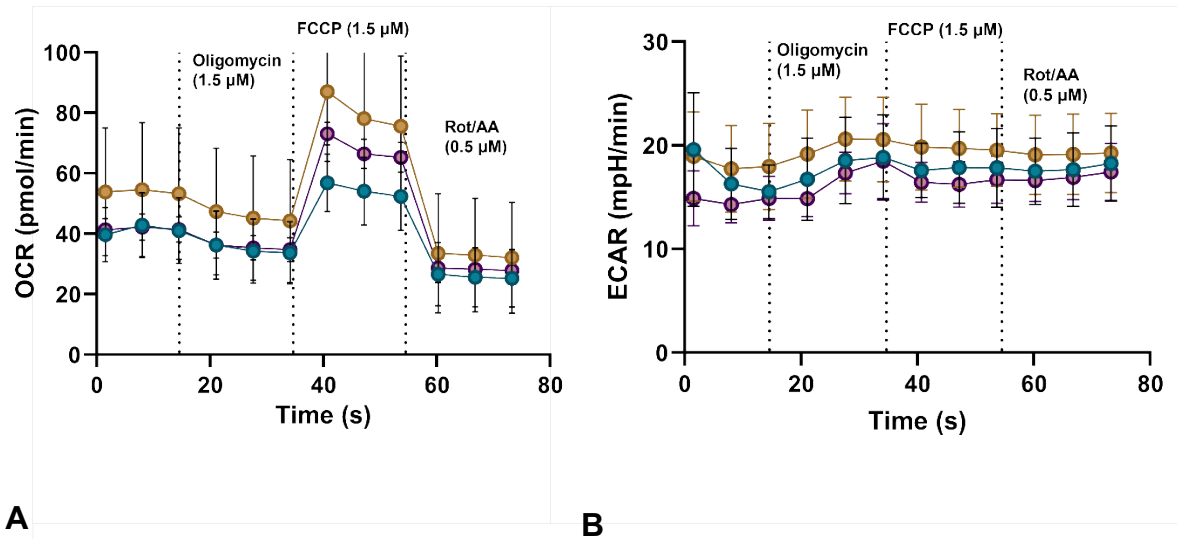
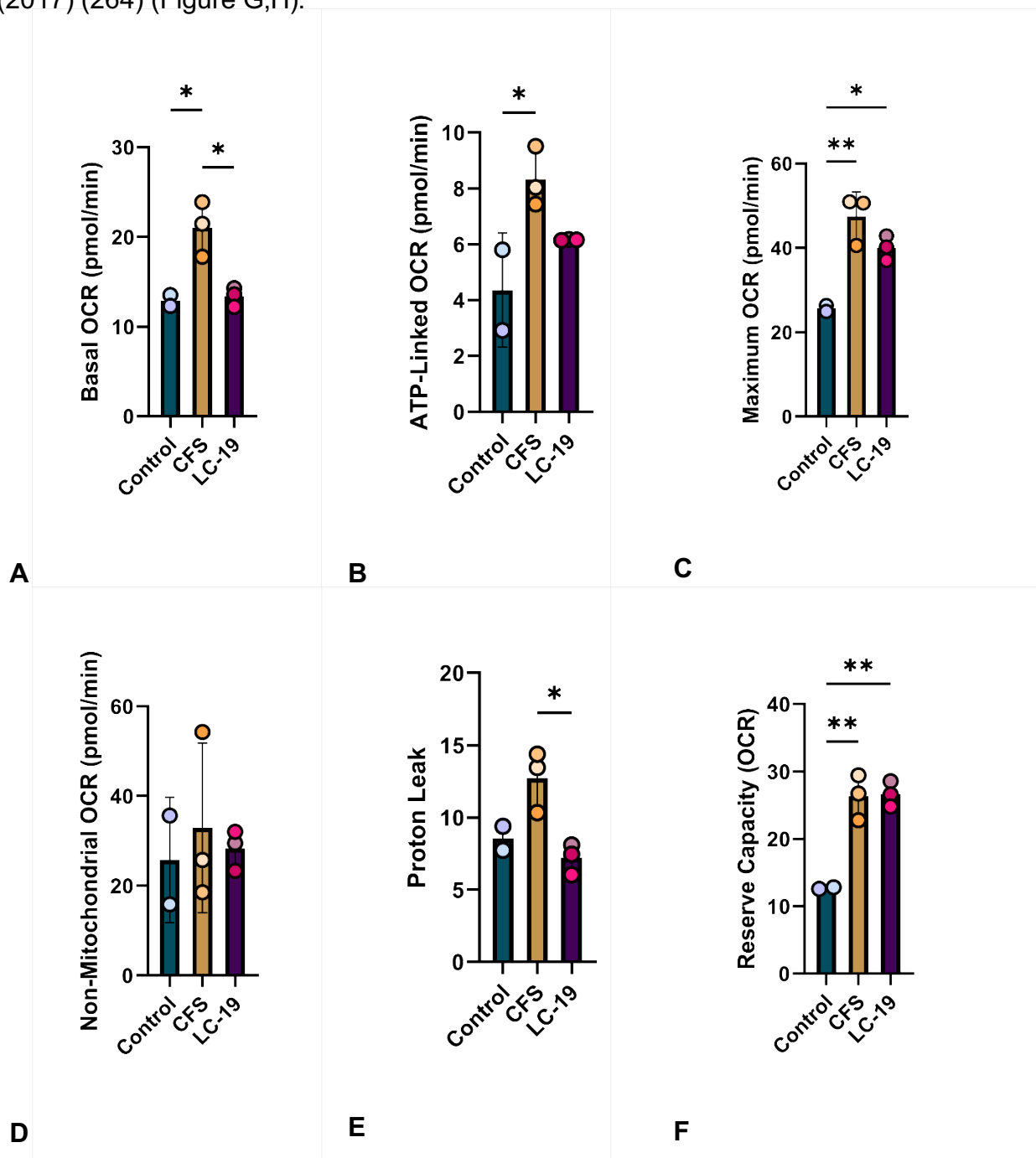
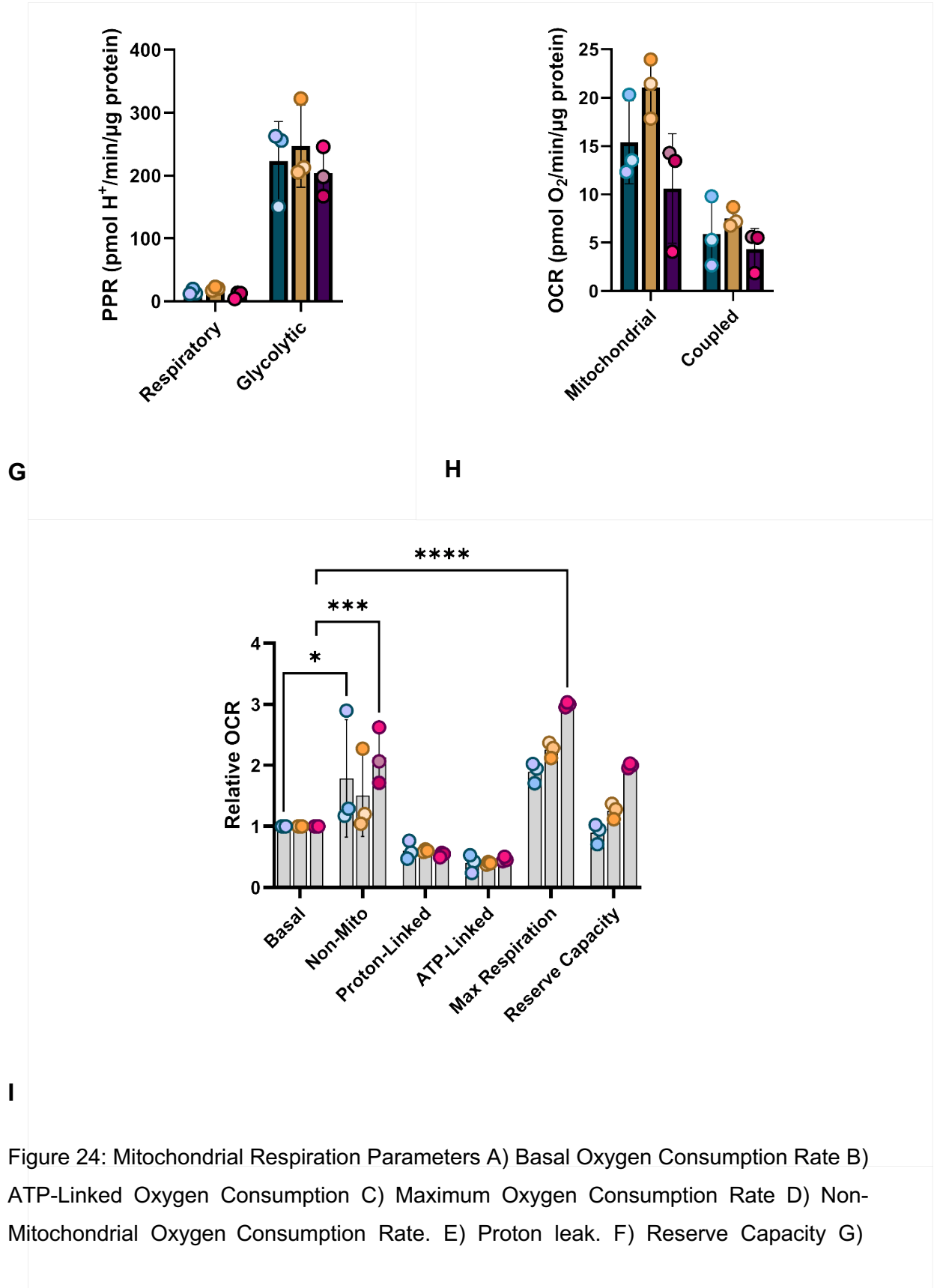


Figure 23: Mitochondrial Function- Mito Stress Test A) Profile for Oxygen Consumption Rate (OCR) from the MitoStress Test. B) Extracellular Acidification Rate (ECAR) profile.

Using the OCR data, performance of OXPHOS was assessed using different parameters as shown in Figure 24. The basal respiration for ME/CFS was substantially higher as were the ATP-linked and Maximum respirations, followed by LC-19 compared to the controls (Figure 24 A- F). An elevated non-mitochondrial respiration in diseased samples was particularly interesting (Figure 24D) as it is attributed to oxygen consumed by non-mitochondrial cellular enzymes and processes, predominantly those that originate from pro-oxidant and pro-inflammatory enzymes such as cyclo-oxygenases, cytochrome P450s or NADPH

oxidases (263). To investigate this further the Proton Production Rate (PPR) and the rate of coupled oxygen consumption (oxygen consumption coupled to ATP production) were calculated according to the protocol outlined by Mookerjee et al., (2017) (264) (Figure G,H).

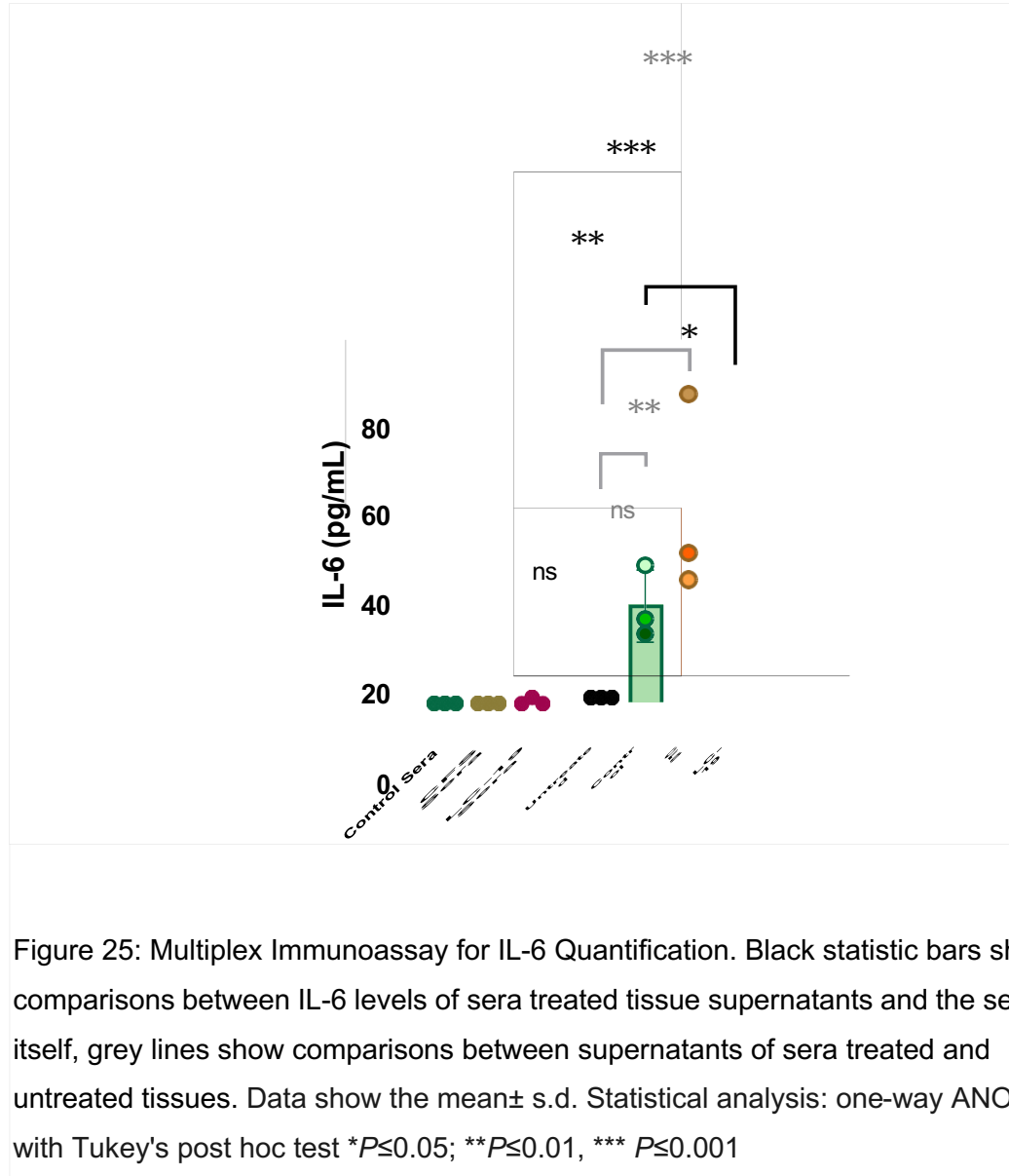




Comparative Mitochondrial and Coupled Proton Production Rate (PPR) and H) OCR I) Relative OCR normalized to basal respiration. Statistical Analyses: Biological replicates: CFS and Control, n= 3. Technical Replicates: n= 4. Data show the mean $\pm$ s.d. Statistical analysis: one-way ANOVA with Tukey's post hoc test * $P \leq 0.05$; ** $P \leq 0.01$.

At 48 hours of serum treatment, there is an increase in total oxygen consumption, of which the coupled OCR is the highest in tissues treated with ME/CFS (Figure 24H) samples followed by the controls and LC-19. A lower PPR in LC-19 samples (Figure 24G), elevated non-mitochondrial respiration further confirms that the major percentage of oxygen consumed is being utilized by compensatory processes against pro-inflammatory processes or to maintain calcium homeostasis. To check the dynamics within each treatment we normalized the rates of all mitochondrial processes to basal respirations and confirmed that in LC-19 samples, nonmitochondrial respiration is consuming most of the oxygen (Figure 24I). However, for ME/CFS samples all processes appear to be upregulated signaling not only a heavy energy burden but also perhaps an attempt to maintain calcium homeostasis for maintaining muscle contractile force.

3A.6. Secretome Analysis for comparative cytokine levels



Quantification of IL-6, IL-1 β and TNF- α was performed in sera supplemented DM, supernatants of untreated tissues and those treated with patient and control sera. The supernatants were collected post 48 hours exposure and before any EPS was performed. While the levels of IL-1 β and TNF- α remained under detection limits, IL-6 was upregulated only in tissue supernatants of sera-treated samples (Figure 25). This data indicates that IL-6 was predominantly secreted by the muscles post sera treatment and was not originally in the sera.

3A.7. Extended Exposure

At 48 hours, diseased tissues presented with a high compensatory response against patient sera. In order to check if this metabolic burden progressed to visible damage phenotypes, exposure to patient sera was increased up to 144 hours. Subsequently, functional response and structural changes (if any) were evaluated at 96- and 144-hour time points by electric pulse stimulation and immunostaining. For this experiment, we used tissues obtained from the same batch of encapsulation to avoid variability in handling and exposed them to LC-19 and control sera for 48, 96 and 144 hours. As can be seen in Figure 26, tissue survival significantly decreased on exposure to patient sera. In the beginning, gradual atrophy or broken tubes were observed at the periphery of the tissues following which the tissues started to lose strength and eventually broke. Overall, tissue survival decreased sharply with time for both diseased and control groups, but the decline was two-fold higher for the former than the latter.

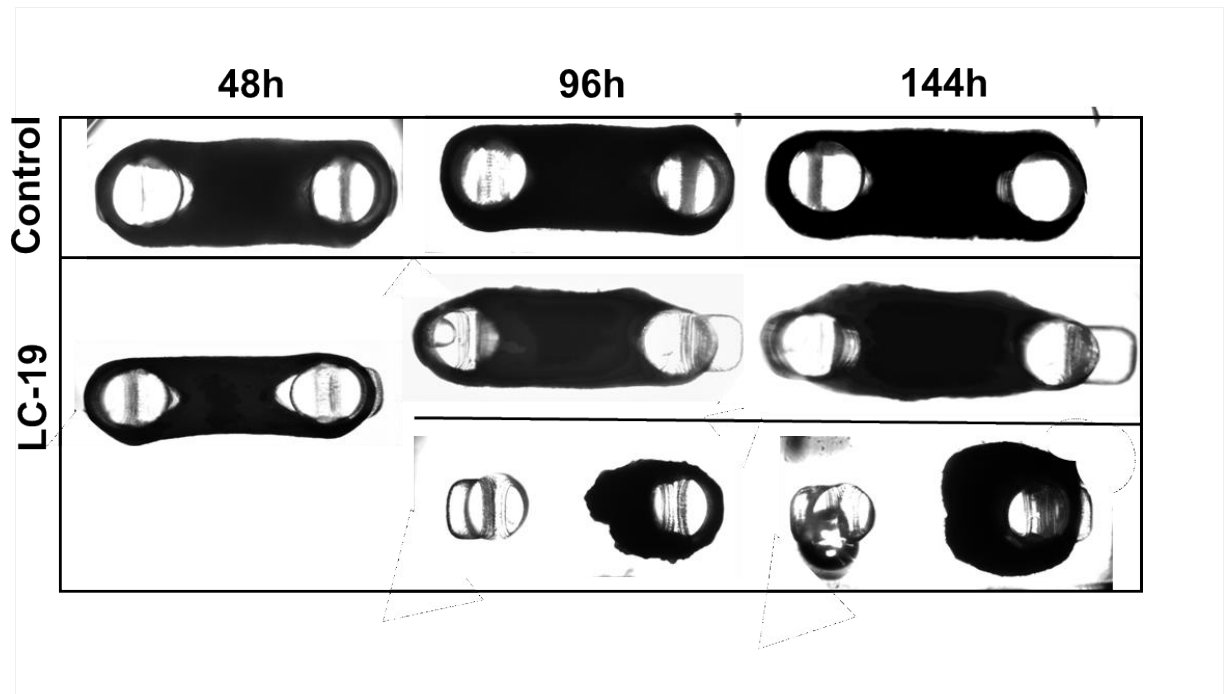


Figure 26: Structural Changes over Long Exposure through Brightfield.

Tissues exposed to patient sera had a weak contractile performance compared to the controls (Figure 27).

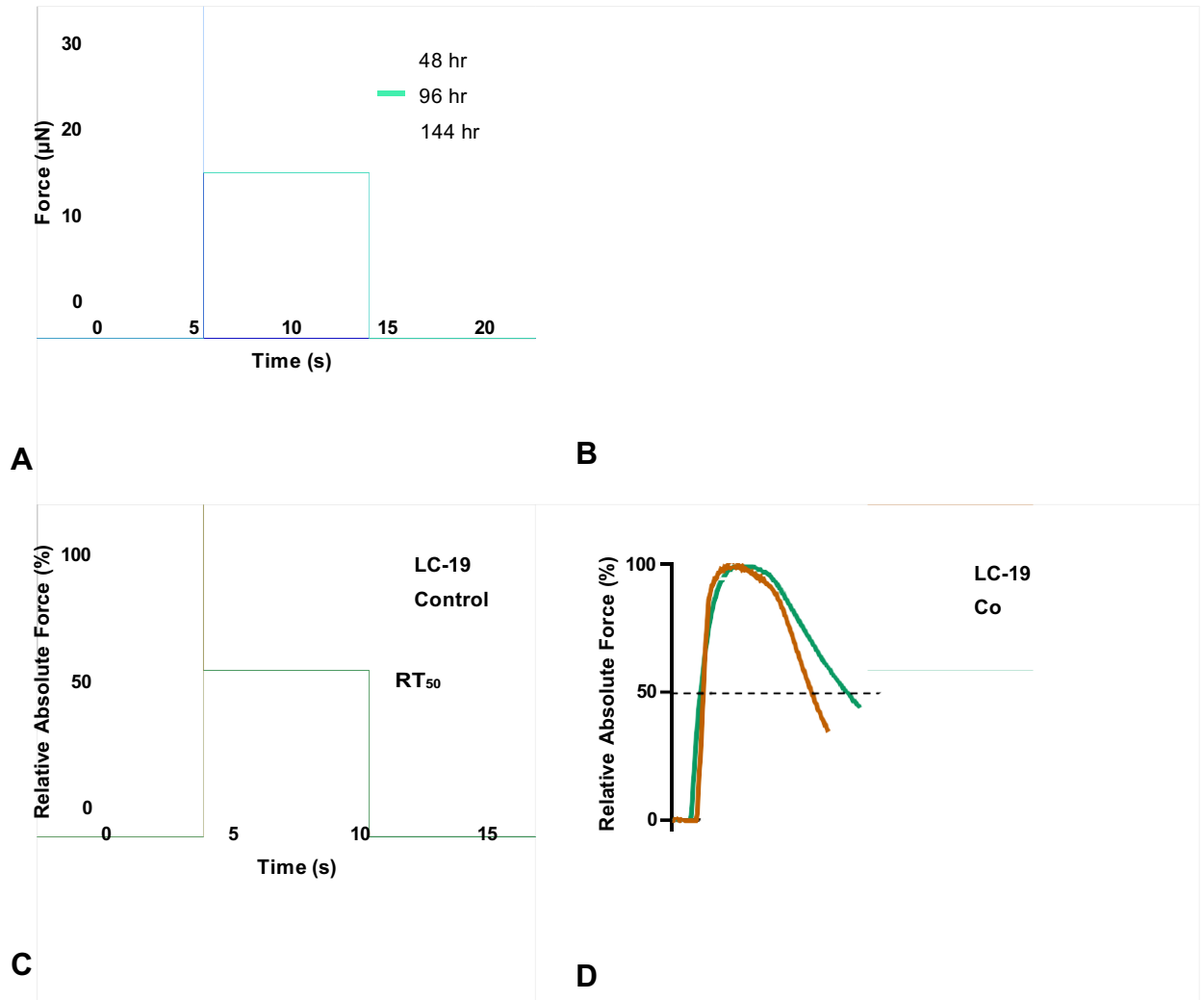


Figure 27: Comparative Contractile Performance LC-19 vs CNT at longer exposures A) Maximum Force of Contraction at 50 Hz of CNT tissues B) Maximum Force of Contraction of LC-19 at 50 Hz C) Time to reach 50% relaxation after 96 hours D) Time to reach 50% relaxation after 144 hours.

Furthermore, patient tissues not only had a weaker force of contractile compared to the controls at each time point but also had a shorter RT_{50} (Figure 27). Furthermore, quantification of myotube diameters showed progressive atrophy, with significantly decreased diameters over time (Figure 28)

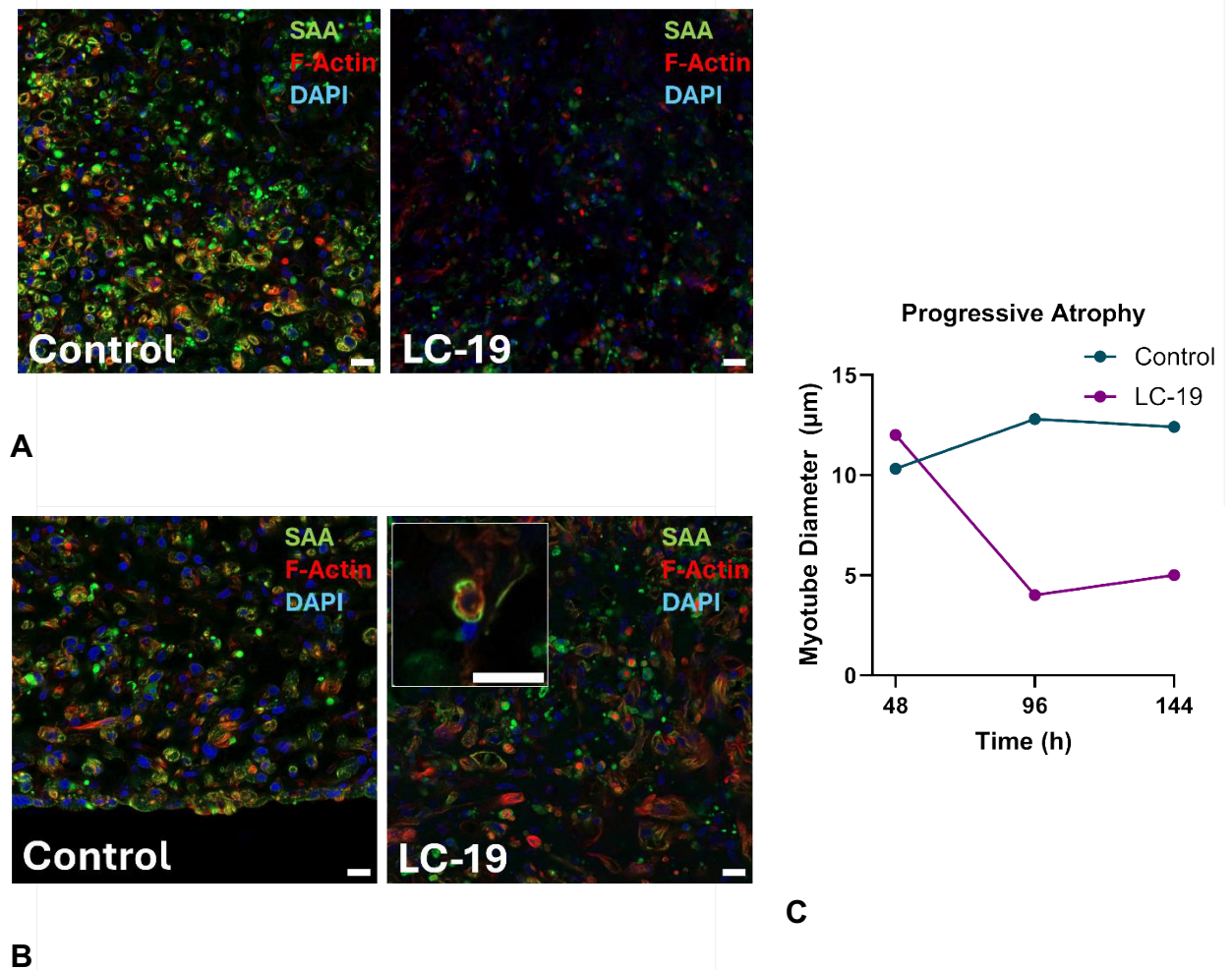
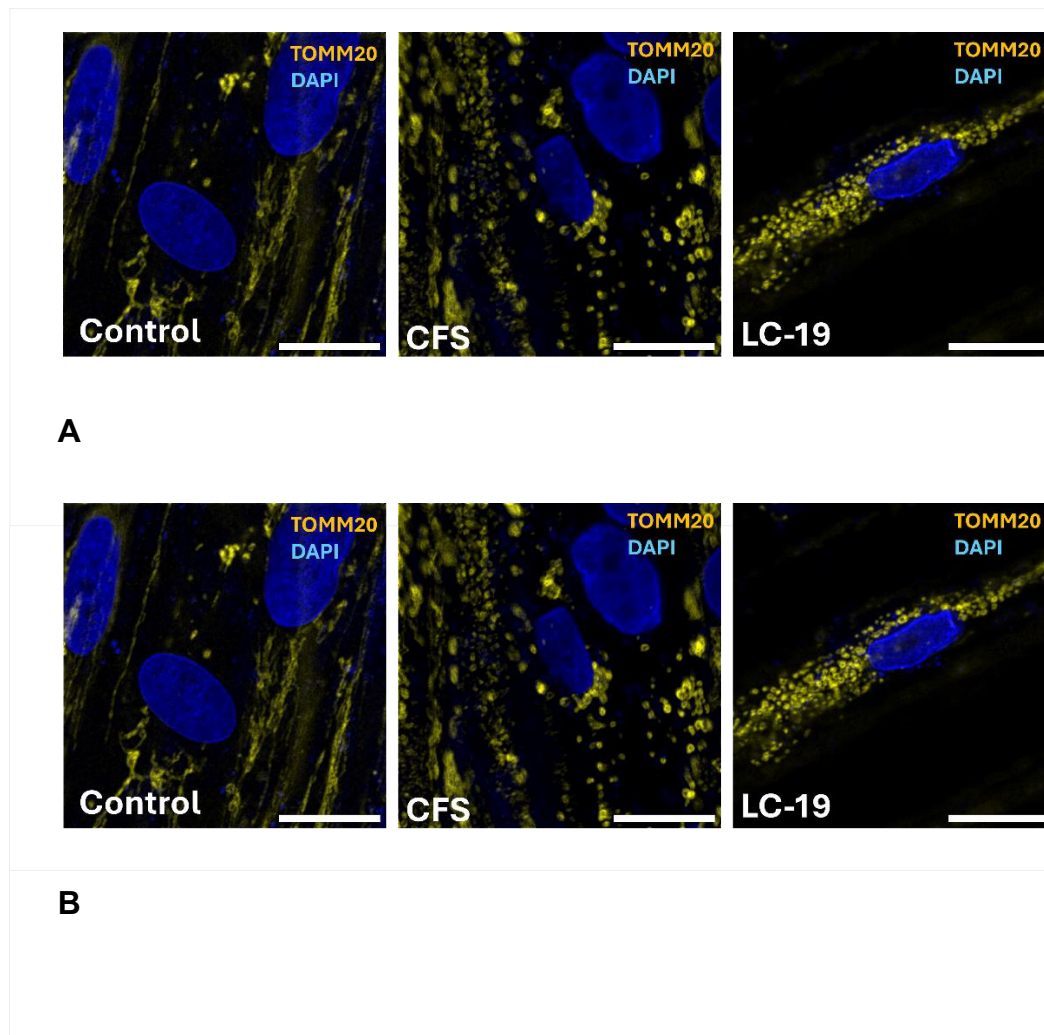
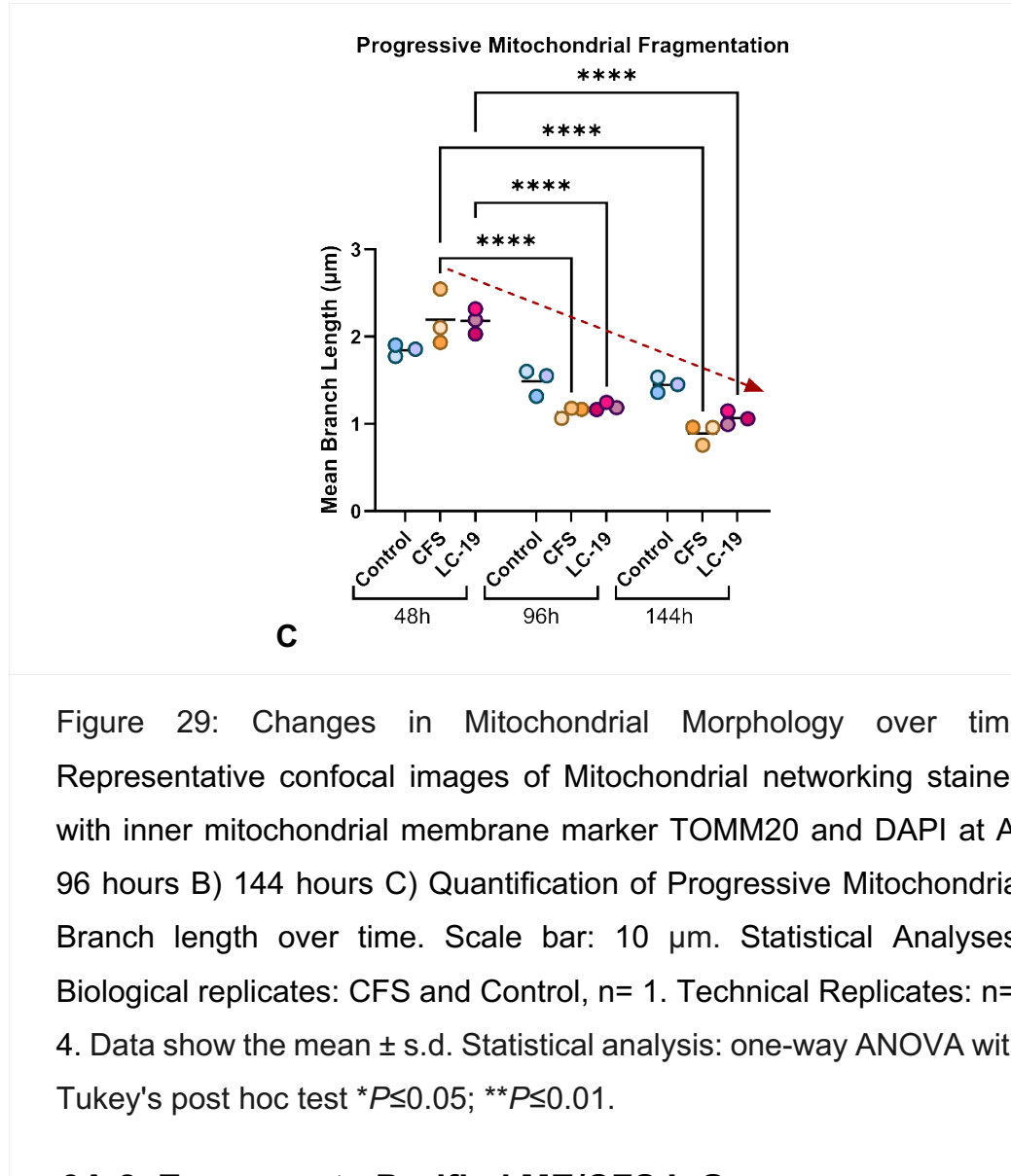


Figure 28: Changes in Myotube Diameter over time LC-19 vs CNT. Representative confocal images of transverse cross sections stained with an antibody against the mature muscle marker sarcomeric actinin (SAA), phalloidin (F-actin) and DAPI (nuclei) at A) 96 hours B) 144 hours C) Quantification of progressive atrophy. Scale bar: 20 μm (10 μm inset)

Quantification of mitochondrial morphology and networks for each time point indicated a decline in mitochondrial branching and mean branch length. The mitochondria not only assumed the familiar globular geometry observed during fragmentation but also toroidal conformations indicating changes in mitochondrial membrane potential at 144 hours of patient serum exposure (Figure 28). The toroidal conformations are typically observed with FCCP administration at high dosages due

to depolarization of the mitochondrial membrane, resulting in a drop in mitochondrial membrane potential and uncoupling of mitochondrial oxidative phosphorylation and ATP synthesis (265). These findings confirmed mitochondrial stress induced by systemic stress factors coupled with myotube atrophy at longer exposures. Our results of prolonged exposure to patient sera further warrant investigation due to small sample size but signpost at progressive deterioration of muscle structure, function and mitochondrial energy production, mimicking the conditions observed in patients.





3A.8. Exposure to Purified ME/CFS IgGs

3A.8.1. Force of Contraction

Post-treatment with purified IgGs from the patient (ME/CFS), the contractile strength of the tissues remained unaffected. Normalizing with cross-sectional area of the whole hydrogel/ tissue and the effective cross-sectional area, tissue strength remained unaffected compared to the controls (Figure 29).

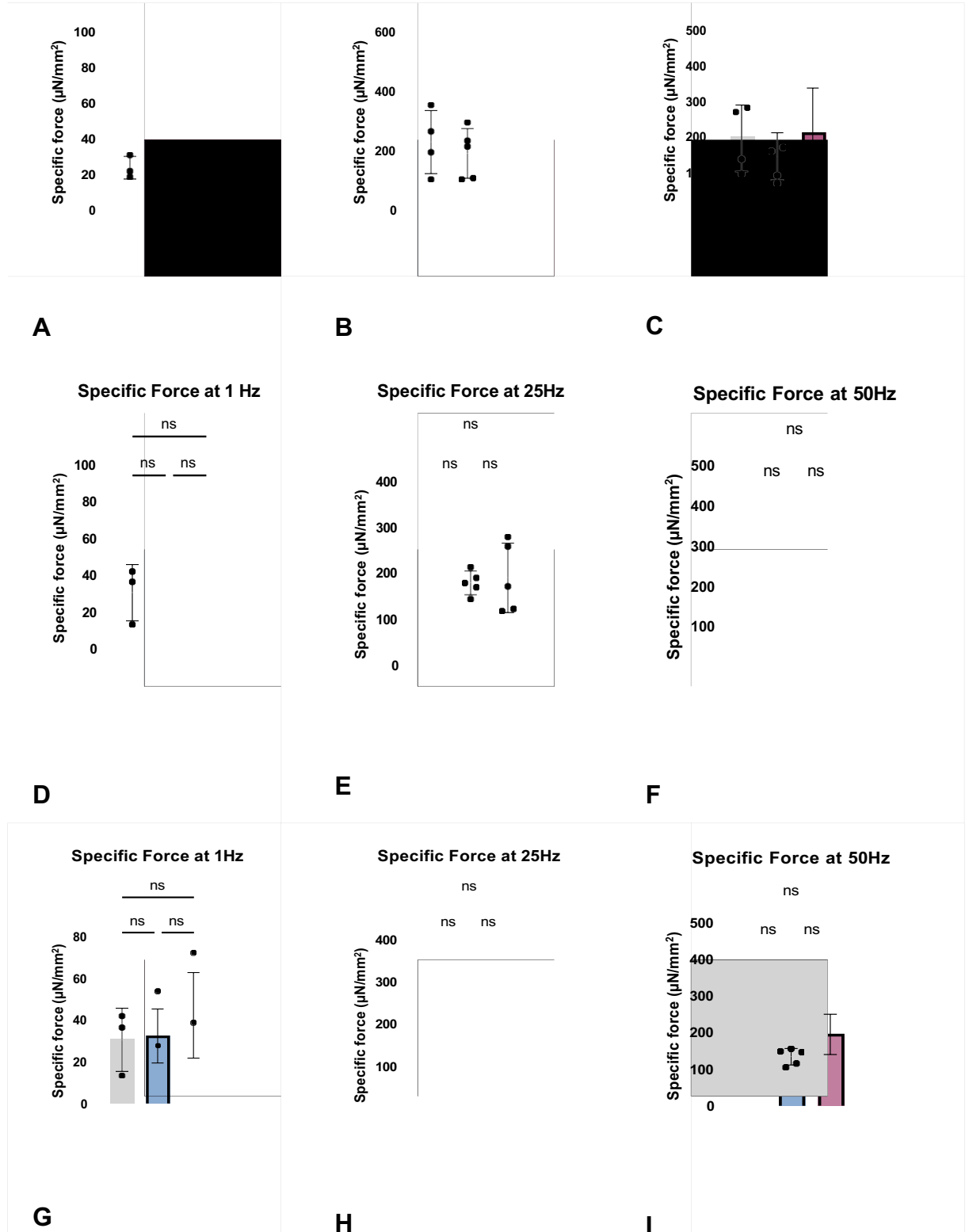
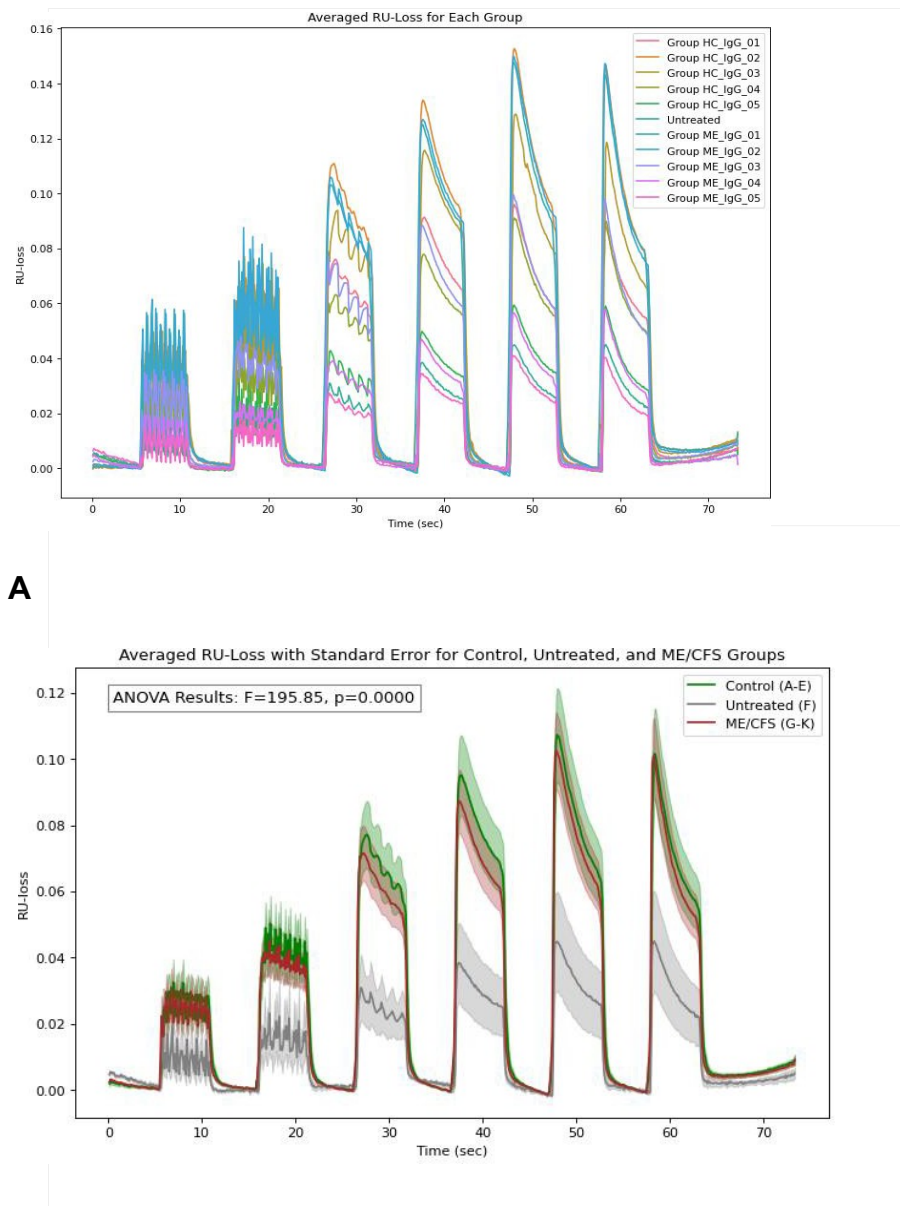


Figure 30: Contractile Dynamics post IgG-Treatment A-C) Raw Force values at 1 Hz, 25 Hz and 50 Hz. D-F) Specific force normalized with hydrogel cross-sectional area at

1 Hz, 25Hz and 50 Hz. G-I) Specific force normalized with effective cross-sectional area at 1 Hz, 25Hz and 50 Hz. Biological replicates n= 5, technical replicates n= 5-6

3A.8.2. Calcium transition dynamics

Calcium transition between analyses within the tissues also indicated no difference between the patient and the control tissues as shown in Figure 30A. There were some patient IgGs that had a lower calcium transition during contraction compared to the rest but average of all patient samples did not differ from the controls (Figure 30B).

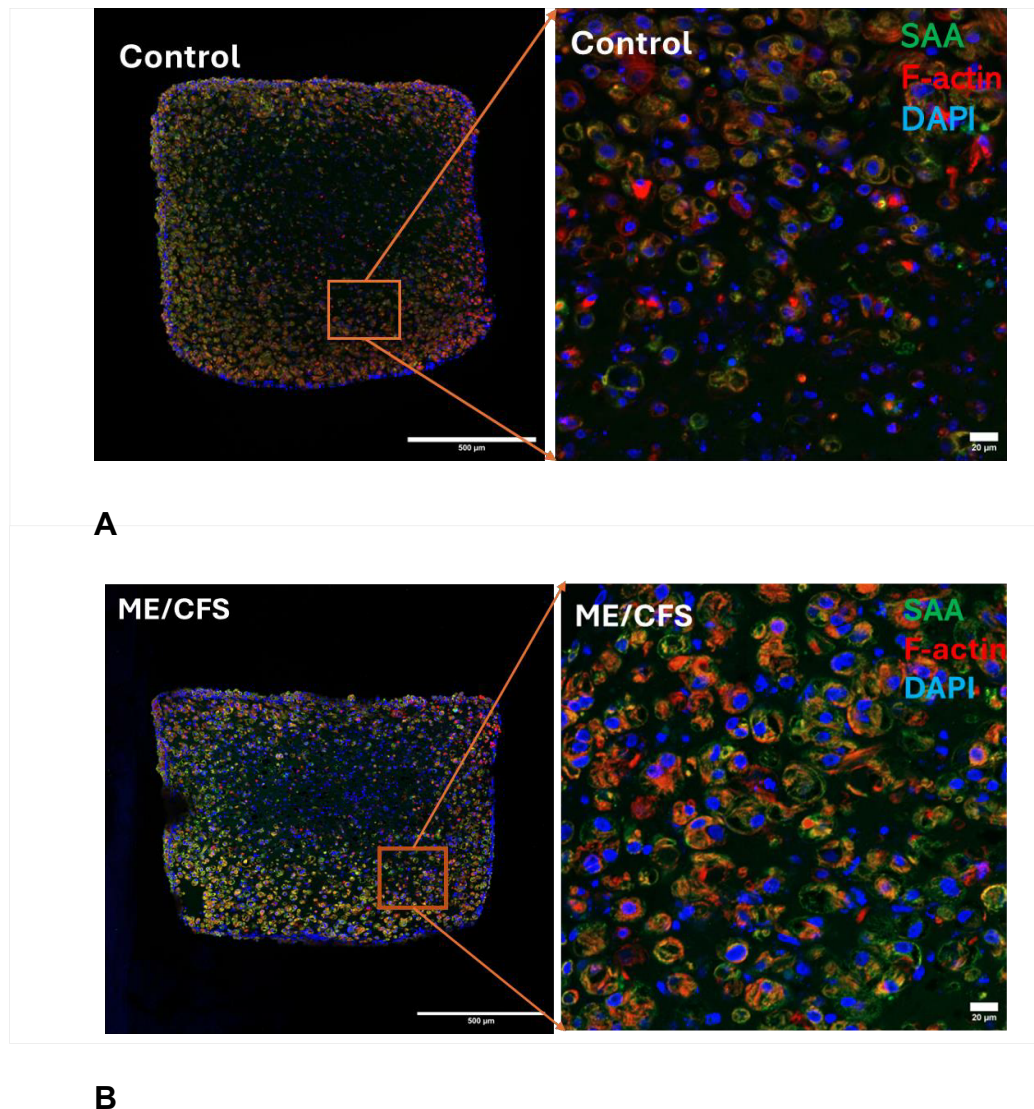


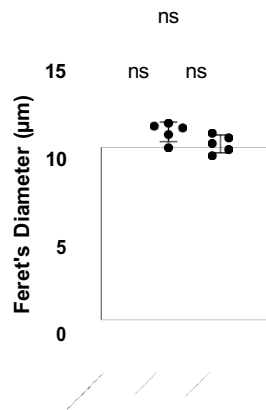
B

Figure 31: Changes in Calcium Dynamics A) All Replicates B) Averages

3A.8.3. Structural Analysis

Quantification of the myotube diameters for all treatments indicated no hypertrophy or atrophy in the tissues treated with patient IgGs for both 95 and 144 hour exposure durations (Figure 31).



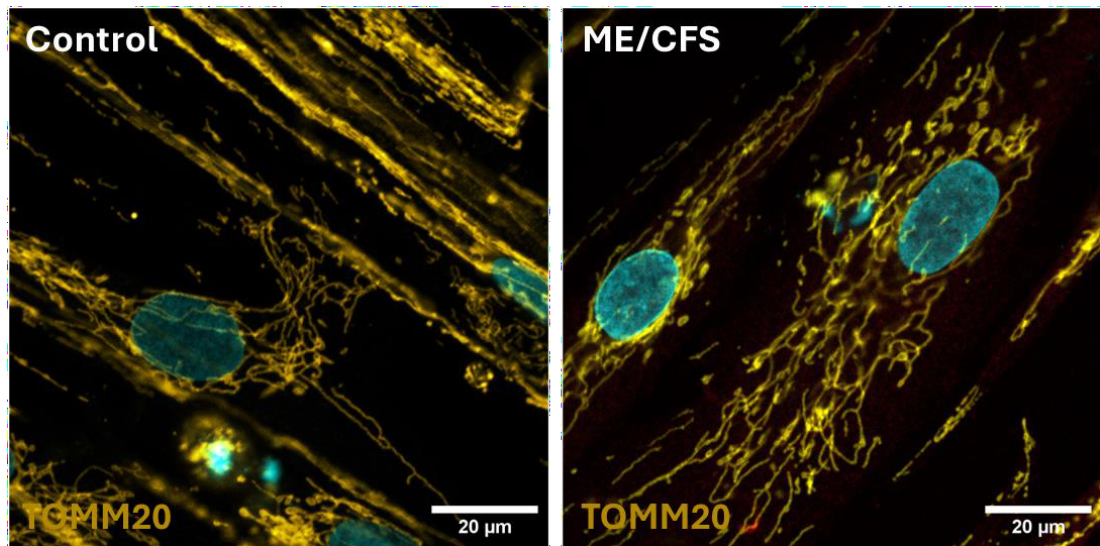


C

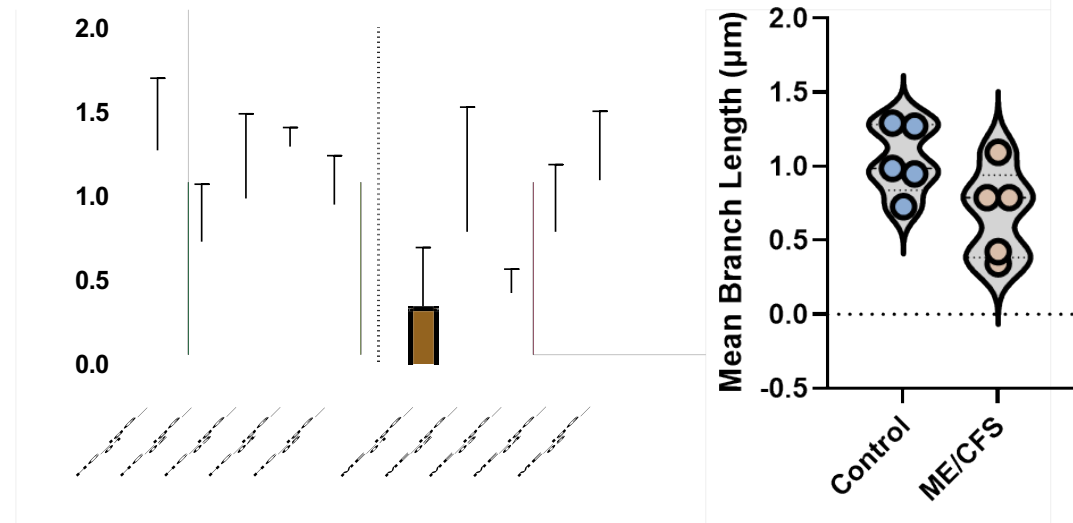
Figure 32: Changes in Myotube Diameter on IgG exposure A) Representative confocal images of Control transverse cross sections stained with an antibody against the mature muscle marker sarcomeric actinin (SAA), phalloidin (F-actin) and DAPI (nuclei) at 48 hours B) Representative confocal images of ME/CFSI transverse cross sections stained with an antibody against the mature muscle marker sarcomeric actinin (SAA), phalloidin (F-actin) and DAPI (nuclei) at 48 hours. Biological replicates n= 5, technical replicates n=5-6

3A.8.4. Mitochondrial Morphometry

Mitochondrial morphology of treated monolayers indicated a decreasing trend of mitochondrial fusion which for more prominent for some patient IgGs compared to others as shown in Figure (32).



A



B

C

Figure 33: Changes in Mitochondrial Morphology. A) Representative confocal images of Mitochondrial networking stained with inner mitochondrial membrane marker TOMM20 and DAPI for Control and ME/CFS. B) Quantification of Mean Branch Length for individual IgGs C) Quantification of Mean branch length for each treatment group. Scale bar: 20 µm. Statistical Analyses: Biological replicates: CFS and Control, n= 5 each. Technical Replicates: n= 4. Data show the mean ± s.d. Statistical analysis: one-way ANOVA with Tukey's post hoc test * $P \leq 0.05$; ** $P \leq 0.01$.

Oxygen sensing probe integration in primary myoblasts derived 3D *in vitro* skeletal muscle tissues and Raman spectroscopy to evaluate the effects of cryotherapy (if any)

Brief Overview:

This section seeks to evaluate the Raman signature for ME/CFS patients before and after they underwent cryotherapy using 3D *in vitro* skeletal muscles. Using this technique will allow understanding the response of patients to various therapeutic interventions by adopting different techniques and different combination of techniques. Brief exposure of the whole body to negative temperatures has been shown previously to improve clinical symptoms such autonomic functions and cardiovascular performance for ME/CFS patients (266). We were curious to test if sera from these patients effected the metabolic fingerprint in 3D *in vitro* skeletal muscles and therefore performed a pilot study to evaluate differences (if any) using Raman spectroscopy. We also assessed the real time oxygen consumption of tissues with continued serum exposure by integrating an oxygen sensing nanoprobe in the tissues. It is important to note that patient and sera cohort for these tests are different than the ones previously employed, and the 3D skeletal muscle tissues were fabricated using primary myoblasts.

Results:

Prior to analyzing treated samples, an average baseline spectrum was obtained for the untreated tissue sections. This was done keeping in mind the sucrose treatment during tissue fixation before cryosectioning. Sucrose helps in maintaining the structural integrity of the tissues during fixation and subsequent sectioning. Taking this into account helped to rule out any artifact. Both the spectra (with and without sucrose treatment) aligned and superimposed.

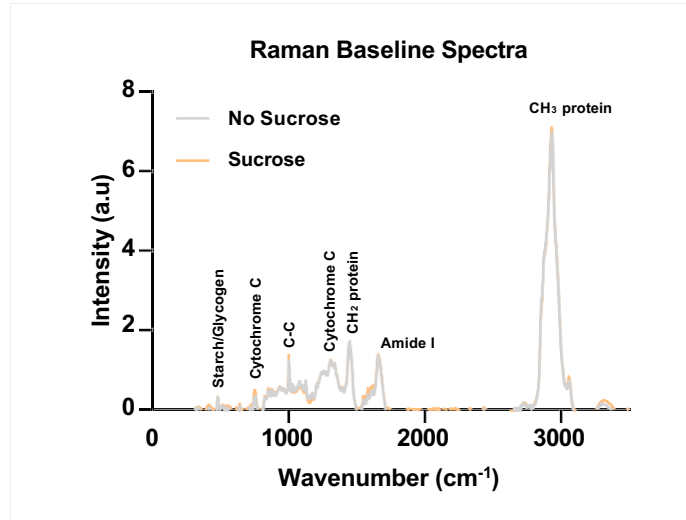


Figure 34: Basal Raman Spectra for Untreated Tissues

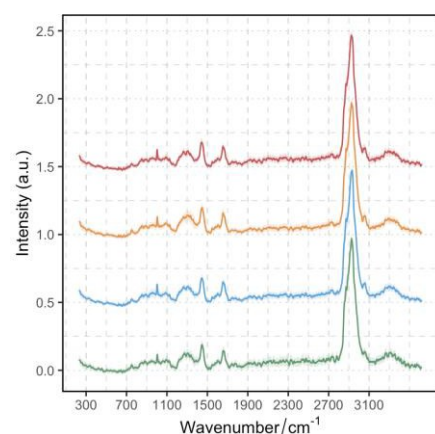
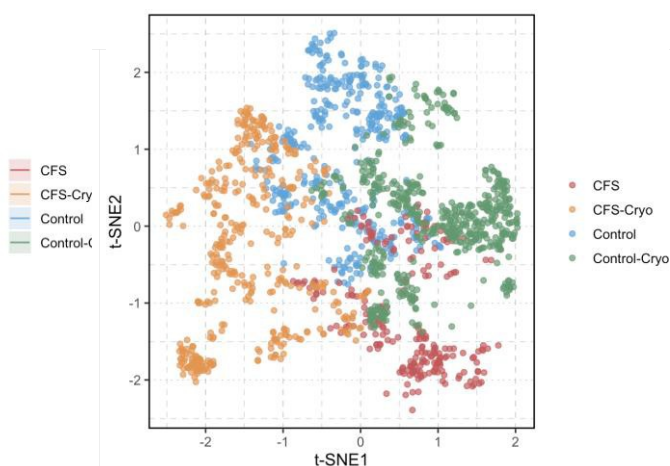
The treatment groups and their obtained spectra are tabulated in Table. Briefly, 4 types of sera were used in this study; 1) CFS_Cryo which are CFS patients that have undergone one session of whole-body cryotherapy. 2) CFS_Basal which is the sera obtained from the same CFS patients before any cryotherapy. 3) Control-Cryo are sera from Healthy donors that underwent one session of whole body cryotherapy 4) Control_Basal are sera from healthy donors without any cryotherapy.

Table 14: *Raman Spectra obtained per condition*

	Group	Sera	Spectra per sample	Total Spectra Acquired
1	CFS_Cryo	CFS_Cryo_S1	250	408
		CFS_Cryo_S2	158	
2	CFS_Basal	CFS_Basal_S1	118	247
		CFS_Basal_S2	129	
3	Control_Cryo	Control_Cryo_S1	361	533

		Control_Cryo_S2	172	
4	Control_Basa	Control_Basal_S1	157	401
		Control_Basal_S2	244	

An average spectrum was calculated from all the acquired spectra for each treatment condition (Figure 34 A) and then unsupervised tSNE visualization was undertaken to check if the data represented any distinct clusters between the groups (Figure 34 B). Raman spectroscopy data is high-dimensional as each spectrum is a collection of multiple data points across several Raman shifts and constituting thousands of measurements. It also not only provides spatially resolved measurements, but the acquired data itself carries information about molecular vibrations from the point of interest. These vibrations are in turn affected by different factors such as (structural features and chemical attributes). Therefore, performing an unsupervised tSNE on ungrouped data was essential to see if there was any distinct clustering. This analysis works by conducting several pairwise comparison tests for each data point and then groups the spectra.

**A****B**

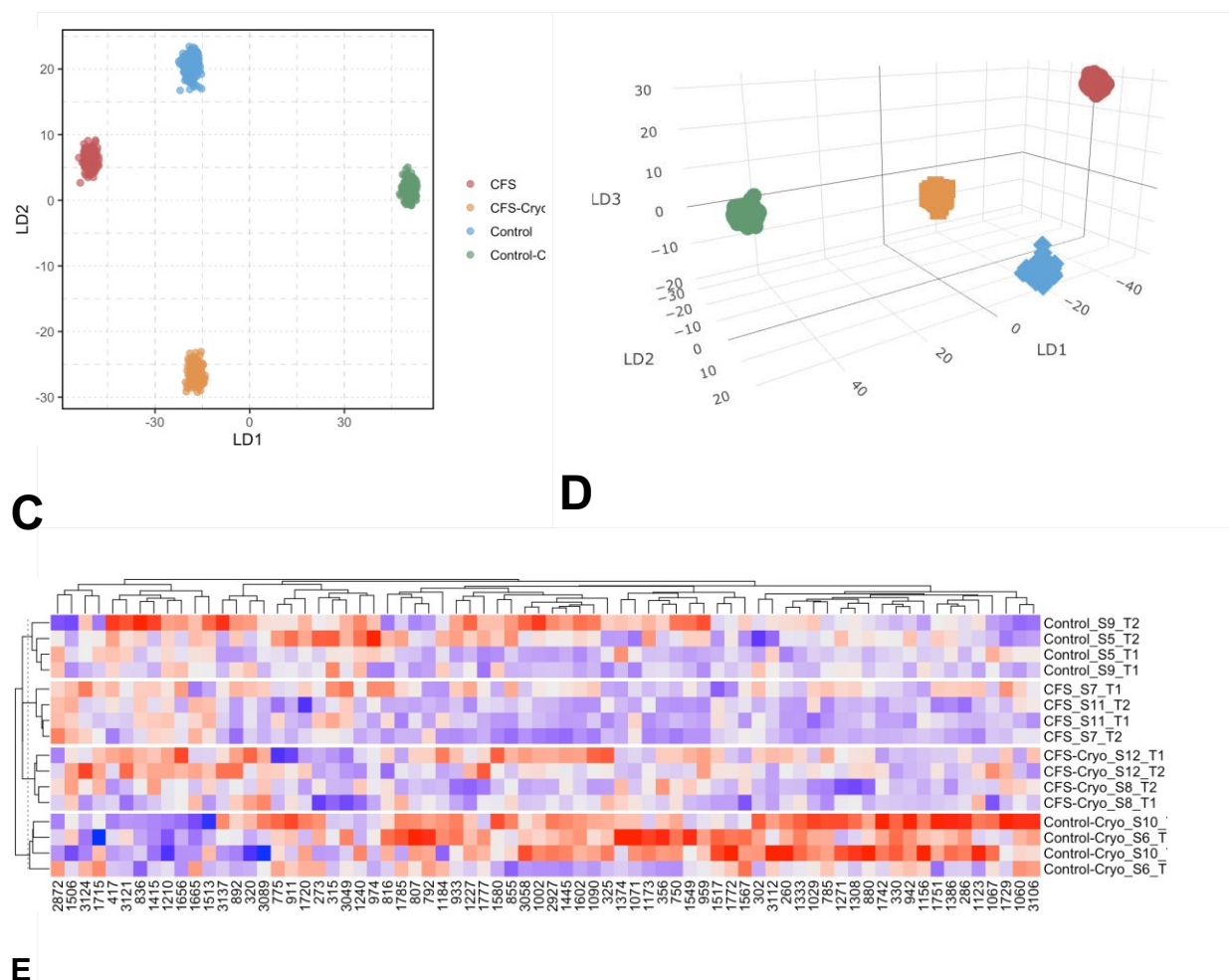


Figure 35: Raman Spectroscopy Analyses A) Average Spectra for each condition B) tSNE plot for ungrouped data C) LDA plot for grouped data

We further conducted a Linear Discriminate Analysis (LDA) for grouped data to analyse the variance and classification of each group. All four treatment groups are distinctly clustered from one other as observed in Figure 34 C-D. This trend can also be observed from the heatmap of individual peaks extracted from the spectrum (Figure 34 E).

We then quantified and compared a few key metabolites pertaining to mitochondrial function, protein and lipid metabolism (Figure 35). The CFS basal groups indicated consistent reduction in several key markers such as cytochromes, amino acids and unsaturated fatty acids. Cryotherapy appears to have generally

improved the Raman signal. An improvement in signals for cytochromes, lactate and glucose suggests an improvement in mitochondrial function.

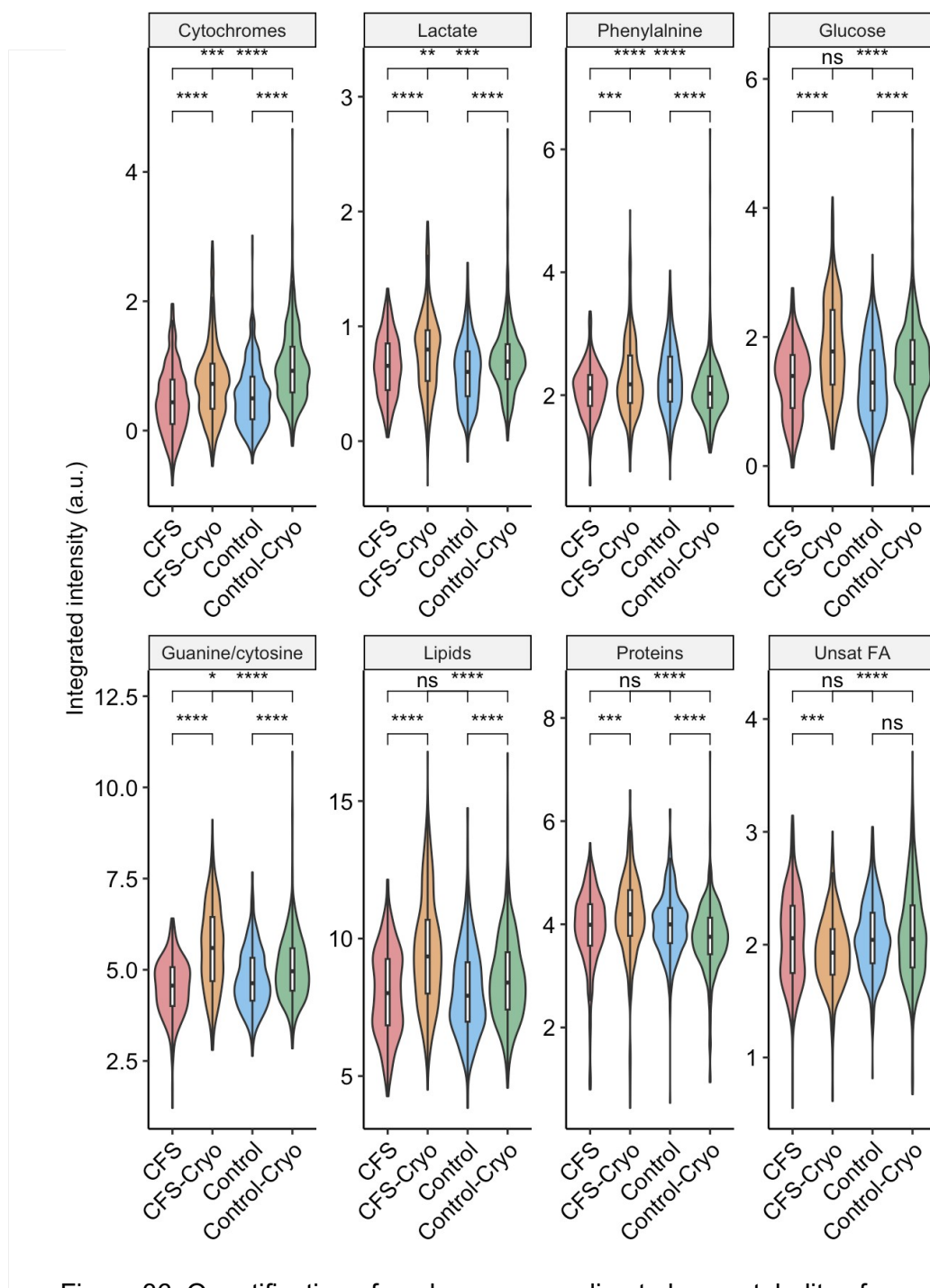
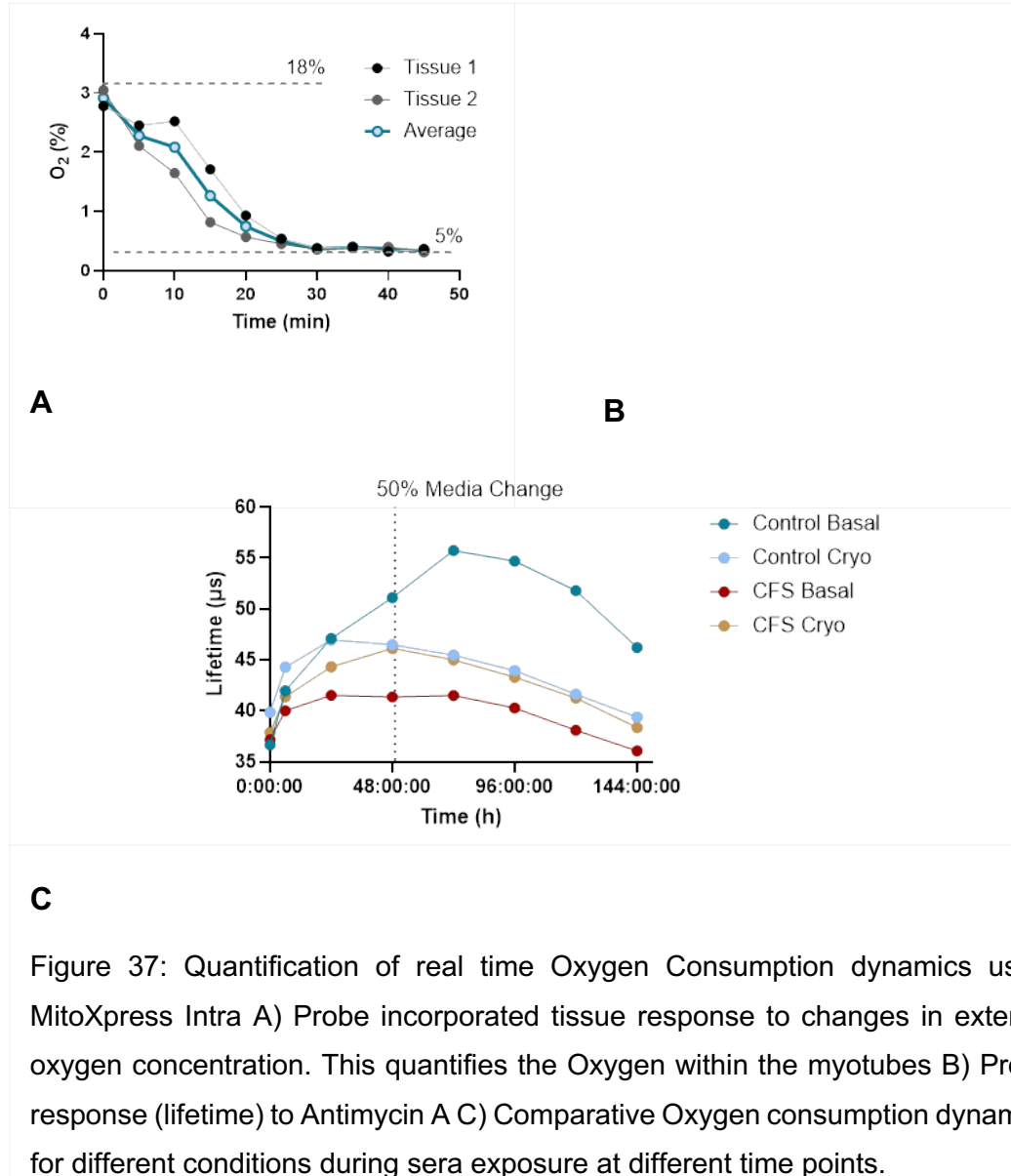


Figure 36: Quantification of peaks corresponding to key metabolites from Raman spectra for all four treatment conditions.



For this model, MitoXpress intra was incorporated within each tissue during encapsulation and the signal was validated by decreasing the external oxygen concentration and my introducing a mitochondrial toxin Antimycin A (2 mM) in the standard 18% oxygen environment (Figure 36 A-B). Lifetime or phosphorescence of

the probe is inversely proportional to the concentration of free oxygen in the environment. Higher lifetime indicates higher metabolic activity of the tissue. Interestingly, tissues treated with CFS sera indicated the lowest basal metabolic activity or oxygen consumption. This experiment was repeated twice with different tissues and the same trend was observed. For the sera obtained from patients who underwent cryotherapy, there was an improvement in the metabolic response. The same trend between the four treatment groups was observed across all time points (Figure 36 C).

4A. Discussion and Conclusion

4A.1. Discussion

This section employed 3D *in vitro* skeletal muscle tissues developed using muscle progenitor cells to investigate two idiopathic conditions of ME/CFS and LC-19. Both these conditions are known to share pathophysiological characteristics, especially fatigue profiles yet exhibit distinct organ-specific impacts. Both conditions involve multisystem dysregulation, including immune dysfunction, autonomic nervous system (ANS) abnormalities, and metabolic disturbances, but diverge in their acute inflammatory signatures, organ damage patterns and compensatory processes. While ME/CFS can be triggered by diverse infections such as Epstein Barr Virus, enteroviruses and other viruses, long COVID-19 is specifically triggered by SARS-CoV-2 infection. The clinical overlap observed in the metabolic signature of patients such as elevated lactate, fluctuations in citric acid cycle intermediates suggests systemic energy crisis rather than isolated muscle defects. Furthermore, changes in amino acid contents, pro-inflammatory components and circulating antibodies may further impact muscle function. Taking advantage of the significant leaps in 3D organ and tissue culture technologies, the impact of systemic factors on the functional performance and structural integrity of skeletal muscles was evaluated. This not only allowed to evaluate disease manifestation and progression as close as possible to the *in vivo* environment but also without causing any distress to the patients.

The study design included evaluation of contractile dynamics post serum exposure, deep comparative analysis of transcriptional profiles, impact on structural integrity, changes in mitochondrial network formation, performance of OXPHOS, multi-plex analyses of cytokines in the secretome of tissues post-treatment and treatment with purified IgG antibodies. Furthermore, changes in functional and structural phenotypes were also studied during long exposure to sera. Additionally, Raman spectroscopy was used to test changes in metabolic signature for patients who underwent one session of whole-body cryotherapy and an intracellular oxygen sensing probe to evaluate changes in oxygen consumption dynamics during serum exposure. Prior to treatment, however, cellular viability against the sera at 0.5% and

5% (v/v) concentrations was checked and no changes in cellular viability for both 2D and 3D cultures was observed. The multi-dimensionality of this experimental design allowed to thoroughly investigate and correlate the pathophysiological landscape in these idiopathic conditions.

The data from this study revealed interesting early and evolving mechanisms of skeletal muscle dysfunction induced by ME/CFS and LC-19 patient sera. The marked reduction in contractile force observed after 48 hours of exposure, especially in ME/CFS-treated tissues, appears to be the result of converging disruptions in mitochondrial bioenergetics, protein homeostasis, and inflammatory signaling. These molecular disturbances appeared to occur in a time-dependent manner, where early compensatory adaptations give way to maladaptive remodeling and catabolic stress. While both conditions indicated muscle weakness, there were distinct metabolic signatures in each.

Post 48-hour exposure to patient sera, the maximum contractile strength of ME/CFS and LC-19 treated tissues was severely compromised compared to the controls. A distinguishing feature of the LC-19 tissues was that they reached peak performance at lower frequencies (25 Hz) of the stimulation regimen and could not sustain the same strength at a subsequent higher frequency (50 Hz). This observation coupled with a shorter RT50 time at 50 Hz confirmed fatigue. Previous studies have also shown a lower peak power output in LC-19 patients observed through an exercise test on a cycle ergometer. The same was validated in this study using 3-D *in vitro* skeletal muscle tissues (190,267). The contractile performance of a skeletal muscle can be diminished due to structural damage, lack of creatine reserves, local hypoxia or insufficient production of ATP due to disturbances in mitochondrial function (268). The process of OXPHOS produces the maximum amount of ATP by reducing oxygen to sustain cellular bioenergetics (269). In skeletal muscles, a large proportion of this energy is expended to form Actin-Myosin cross-bridges necessary for contraction (270). The remainder is used for protein synthesis, glycolysis, ionic balance maintenance and calcium homeostasis (271). A disturbance or burden on any of these processes may result in an energy imbalance.

The transcriptional landscape observed via GO analysis provides further mechanistic context. Both ME/CFS and LC-19 samples demonstrated upregulation of genes associated with protein translation and synthesis, yet the functional outcomes diverged. In ME/CFS, this increased translational activity was not matched by enrichment of muscle contractile or mitochondrial genes. Instead, there was a depletion of transcripts linked to sarcomeric structure and mitochondrial machinery. This suggests a misallocation of translational resources, where protein synthesis could be redirected toward stress-responsive proteins such as, chaperones or acute-phase proteins, at the expense of functional myofibrillar components. The proteomic consequence of such misdirection may be a buildup of misfolded or non-functional proteins, contributing further to cellular stress and muscle fatigue (272,273). This aligns with existing proteomic data showing increased ubiquitination and proteasomal targeting in ME/CFS muscle, indicative of increased protein turnover and degradation under chronic stress (274–276). The depletion of contractile protein transcripts in ME/CFS samples supports histological evidence of fiber atrophy and necrosis in patient biopsies (183). This contrasts with LC-19's enriched muscle remodeling pathways, suggesting divergent repair capacities. Notably, *SMYD1* and *FHL1* upregulation in both conditions highlights attempts to stabilize sarcomeric proteins, but persistent oxidative damage in ME/CFS likely impairs functional assembly. The slight hypertrophy observed may reflect abortive repair (ME/CFS) or incomplete regeneration (LC-19), akin to "pseudo-hypertrophy" in neuromuscular disorders (277,278). Furthermore, the increase in *ATP2A1* indicates impaired calcium homeostasis in both disease conditions (279,280). Elevated *ATP2A1* levels may initially help in stabilizing calcium levels but at the expense of ATP, thereby significantly contributing to energy deficits.

From the perspective of ATP generation, an interesting insight from the Mito Stress test was the paradoxical elevation of oxygen consumption rate (OCR) and ATP-linked respiration in ME/CFS-treated tissues, which would typically imply heightened mitochondrial activity. However, rather than reflecting efficient bioenergetic output, this profile likely represents a state of mitochondrial overactivation or stress-induced hypermetabolism. Such a phenotype aligns with

prior reports describing “mitochondrial burnout” in ME/CFS, where prolonged energetic demands exceed mitochondrial capacity, leading to inefficiencies and increased susceptibility to oxidative damage (194). The initial tendency towards hyperfusion of mitochondrial networks observed across both ME/CFS and LC-19 conditions likely represents an early compensatory mechanism to preserve ATP production via enhanced network connectivity (281). However, this response appears to deteriorate with time, as progressive mitochondrial fragmentation sets in beyond 48 hours, coinciding with declining myotube diameter and reduced contractile force.

This fragmentation is not only structural but also functionally consequential. It is well-evidenced that the loss of mitochondrial cristae integrity diminishes ETC efficiency, and that fragmented mitochondria are associated with impaired calcium buffering, critical for excitation-contraction coupling (282–284). The observed progression from hyperfusion to fragmentation may reflect a tipping point where mitochondrial quality control mechanisms, such as mitophagy, may become overwhelmed, allowing dysfunctional organelles to accumulate and propagate metabolic distress. Although ATP-linked respiration in ME/CFS remains elevated, it may reflect an unproductive “futile cycling” response to oxidative or proteostatic damage, where ATP is expended to mitigate stress but fails to support contractile machinery.

In contrast, LC-19-treated samples demonstrated a more consistent pattern with attempted muscle remodeling and adaptation. The upregulation of pathways involved in hypertrophy suggests that the muscle may initially engage in compensatory regeneration or hypertrophy following serum exposure. Simultaneously, the increase in macromolecule biosynthesis, amide/peptide metabolic processes, and nitrogen compound biosynthesis reflects a high metabolic demand in these patients. It suggests that cells in LC-19 individuals may be caught in a chronic state of repair or inflammation, leading to sustained anabolic processes. However, the absence of a meaningful improvement in contractile force implies that these structural changes are either insufficient or maladaptive, potentially involving

aberrant myofibrillogenesis, fibrosis, or energetically unsustainable growth. Given that protein synthesis is one of the most ATP-intensive cellular processes, the combination of enhanced translational activity and mitochondrial inefficiency likely creates an energy sink, further intensifying the functional deficit.

Metabolic reprogramming was also a prominent feature observed for both diseased conditions. Upregulation of *ENO3* and *AGL* indicate a shift towards glycolysis possibly due to mitochondrial dysfunction. Enhanced enolase activity compensates for compromised OXPHOS but promotes lactic acidosis, exacerbating IL-6 release. Increased *AGL* expression suggests rapid glycogen breakdown to sustain ATP production under stress conditions. However, this reliance on glycolysis is energetically inefficient and unsustainable over time.

To this end, a particularly interesting finding is the early elevation of IL-6 specifically in tissues exposed to patient sera, with no comparable increase in untreated controls. This indicates that the sera of ME/CFS and LC-19 patients contain soluble factors that actively trigger inflammatory signaling within muscle tissue. IL-6 is a pleiotropic cytokine with known effects on muscle metabolism and homeostasis (285–288). Its rapid upregulation in 48 hours coincides with the peak of OCR and onset of mitochondrial fragmentation, supporting a model where IL-6 acts as both a metabolic and catabolic stressor (285,289). Mechanistically, IL-6 has been shown to impair OXPHOS within 24 hours in human myotubes, induce ROS, and activate STAT3-mediated transcription of E3 ubiquitin ligases such as MuRF1 and Atrogin-1, which drive proteolysis (290,291). These actions likely converge to initiate the observed myotube thinning and loss of function over time.

The time-dependent profile of degeneration reveals distinct pathological phases. During the acute phase (0–48 hours), mitochondrial hyperfusion and elevated OCR dominate, likely reflecting an adaptive bioenergetic response. However, by 48 hours, IL-6 levels surge, triggering mitochondrial dysfunction, and diversion of translational resources away from contractile proteins. The subacute phase (>48 hours) is characterized by visible reducing in myotube diameter, and

progressive mitochondrial fragmentation, possibly driven by ROS accumulation and disrupted calcium handling, both known downstream consequences of IL-6 signaling.

The absence of force impairment or myotube atrophy in tissues treated with purified patient IgGs underscores that antibodies, alone, while capable of inducing mild mitochondrial fragmentation, are insufficient to recapitulate the full phenotype in this model. This implies that non-IgG serum constituents such as cytokines, immune complexes, metabolic byproducts, or extracellular vesicles could also be the co-mediators of early dysfunction. Furthermore, recent work has implicated circulating mitochondrial DNA, lactate, and acylcarnitines in the sera of ME/CFS patients as possible inducers of mitochondrial stress and systemic fatigue, highlighting future avenues for exploration.

Collectively, the data suggests that skeletal muscle exposed to ME/CFS and LC-19 sera undergoes a staged transition from early compensatory adaptation to inflammatory and metabolic collapse. This trajectory is driven by an intersection of mitochondrial dysfunction, unbalanced protein turnover, and IL-6-mediated catabolism. Importantly, the force reduction observed by 48 hours arises not solely from structural atrophy but from an *acute triad of energetic crisis, contractile machinery degradation, and mitochondrial destabilization*, initiated before overt morphological decline (Figure 38).

These findings have several translational implications. Targeting IL-6 may represent a therapeutic opportunity to blunt the early inflammatory-catabolic cascade. Enhancing mitochondrial quality control, through agents that promote mitophagy or stabilize mitochondrial networks, could mitigate the progression of bioenergetic failure. From a diagnostic standpoint, serum IL-6 levels or mitochondrial biomarkers could serve as early indicators of disease severity or treatment response, offering patient stratification tools in both ME/CFS and post-viral fatigue syndromes such as LC-19.

From Raman spectroscopy analyses using sera from a different cohort of patients and tissues derived from primary myoblasts, sera from ME/CFS patients consistently led to reduced signals of key mitochondrial components, including cytochromes, amino acids, and unsaturated fatty acids which is consistent with prior reports of mitochondrial impairment in ME/CFS. Notably, sera from cryotherapy-treated patients (CFS_Cryo) partially restored these spectral deficits, with increased signals for lactate, glucose, and cytochromes, indicating enhanced mitochondrial function, an effect corroborated by improved oxygen consumption metrics.

Real-time metabolic analysis using MitoXpress Intra nanoprobe demonstrated reduced basal oxygen consumption in muscle tissues exposed to ME/CFS sera relative to controls, mirroring clinical observations of diminished aerobic capacity, but contrary to the original results based on Mito Stress test. This difference could be because of a different cohort of patients or the use of primary myoblasts instead of immortalized muscle progenitor cells used earlier. Cryotherapy was found to partially rescue this phenotype, aligning with reports of improved cardiovascular and autonomic function following treatment. These findings are consistent with broader biomarker studies using Raman spectroscopy, which differentiate ME/CFS from healthy and disease controls with up to 91% accuracy (292). Interestingly, conventional mitochondrial respiration assays in isolated cells failed to detect such differences, emphasizing the importance of using complex, tissue-relevant models.

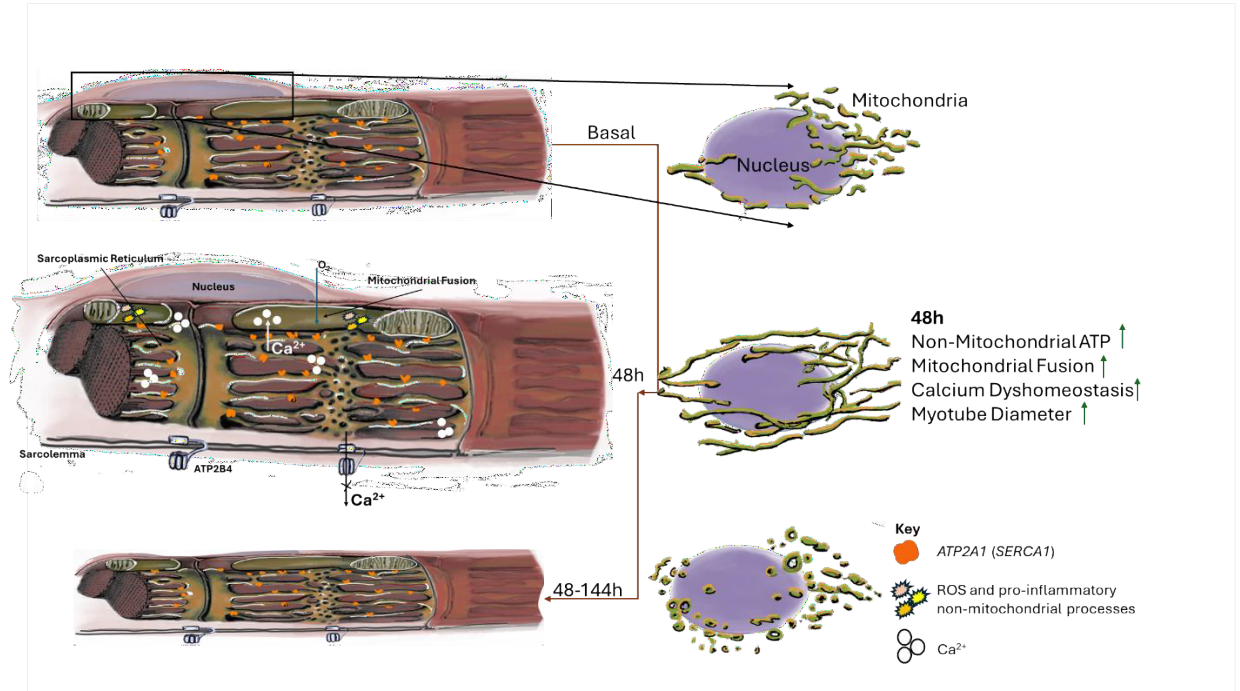
Collectively, these results suggest that cryotherapy may attenuate ME/CFS-related metabolic dysfunction by modulating circulating factors, potentially through reduced oxidative stress or improved redox homeostasis. However, the transient nature of cryotherapy's benefits, as observed in previous clinical trials, highlights the need for cautious interpretation and further longitudinal investigation (293). This study bridges cellular bioenergetics with systemic pathophysiology.

ME/CFS and Long COVID-19 are complicated multi-symptomatic conditions which have severe physical manifestations with a dysregulated energy balance at

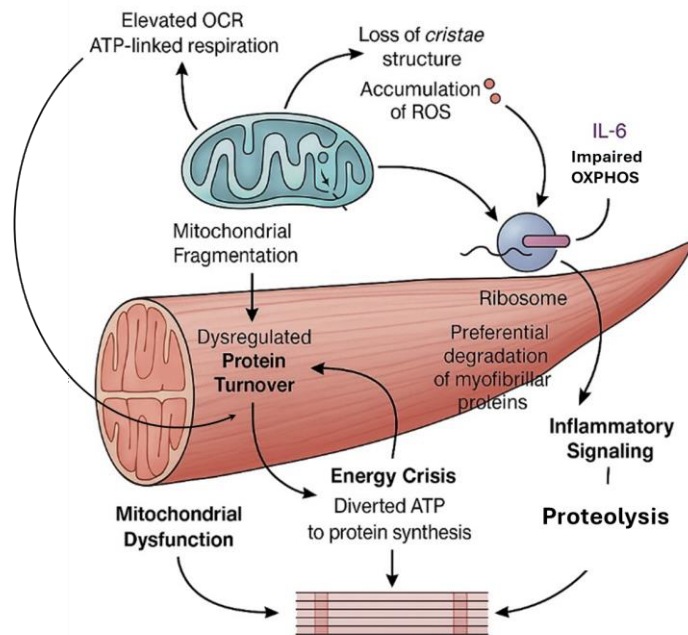
their core. Within a single cell or tissue type various metabolic and homeostatic processes appear to be altered (hyperregulated during short exposures). It is hypothesized that at short exposures, there comes a point in which the tissue attempts to resist against stress but when the stress is prolonged, the resistance gives in, allowing rapid deterioration. This research presents the first 3D *in vitro* model to study performance profiles of ME/CFS and LC-19 patients without distressing them directly. These investigative insights provide pivotal findings regarding disease progression but are limited by small sample size. This work not only presents a new avenue for ME/CFS and LC-19 research but also posits an important question regarding the nature of systemic factors that are responsible for inducing this stress response.

Limitations of this work include the inherent constraints of *in vitro* modeling, which lacks neuromuscular junction input, vascular factors, and systemic hormonal regulation. However, the controlled environment allows for precise dissection of cell-intrinsic responses to defined serum components. Future studies incorporating longer-term exposure, single-cell resolution, and functional imaging of calcium and ROS dynamics could provide deeper mechanistic clarity. Altogether, these data provide a mechanistic foundation for understanding muscle weakness in ME/CFS and LC-19 and offer multiple points of intervention for future therapeutic development.

Section A: ME/CFS and Long COVID-19: Discussion and Conclusion



A



B

Figure 38: Graphical representation of progressive pathological changes A) Temporal changes in 3D in Vitro skeletal muscles due to sera exposure B) The acute triad of energetic crisis, contractile machinery degradation, and mitochondrial destabilization

4A.2. Conclusion

Key inferences drawn from this section are:

1. **ME/CFS and LC-19 patient sera induce contractile dysfunction in Skeletal Muscle tissues:** Significant contractile impairment was observed in 3D *in vitro* skeletal muscles tissues upon exposure to patient sera for both long and short exposures, indicating early mechanisms of muscle dysfunction. Both conditions, however, presented distinct metabolic signatures.
2. **Differential Maximum Contractile Performance:** LC-19 tissues reached peak contractile performance at lower stimulation frequencies (25 Hz) compared to controls and ME/CFS, indicating muscle fatigue, while ME/CFS showed a more severe general impairment in contractile strength across the range of frequencies tested.
3. **Transcriptional Landscape:** ME/CFS tissues show an upregulation of genes related to protein translation without enrichment of muscle contractile or mitochondrial genes, suggesting misallocation of translational resources. LC-19 tissues show enriched muscle remodeling pathways, mitochondrial structural and functional genes, indicating different repair capacities and adaptation strategies.
4. **Transposon-Assisted Adaptation again stress:** Transposon expression appeared to be assisting in upregulating stress responses and muscle structural adaptation.

5. **Mitochondrial Dysfunction:** Both conditions initially display mitochondrial hyperfusion but progress to fragmentation, indicating impaired mitochondrial quality control. ME/CFS shows elevated oxygen consumption rate (OCR) and ATP-linked respiration, suggesting mitochondrial overactivation or "burnout". Persisting muscle weakness indicates that the energy is being re-routed to maladaptive hyper protein synthesis.
6. **Metabolic Reprogramming:** Both ME/CFS and LC-19 sera induce a shift towards glycolysis, evidenced by upregulation of *ENO3* and *AGL*, indicating a compensatory mechanism for mitochondrial dysfunction.
7. **IL-6 Involvement:** Early elevation of IL-6 in tissues exposed to patient sera suggests that soluble factors in ME/CFS and LC-19 trigger inflammatory signaling in muscle tissue.
8. **IgG Antibodies Insufficiency:** Purified patient IgGs alone are insufficient to fully replicate the phenotype in the model, suggesting other serum constituents co-contribute to early dysfunction.
9. **Multi-Phasic Degeneration:** Skeletal muscle undergoes a staged transition from compensatory adaptation to inflammatory and metabolic collapse, driven by mitochondrial dysfunction, unbalanced protein turnover, and possibly IL-6-mediated catabolism.
10. **Cryotherapy Benefits (Raman Spectroscopy):** Sera from cryotherapy-treated ME/CFS patients partially restored mitochondrial components, suggesting enhanced mitochondrial function, which correlated with improved oxygen consumption metrics.
11. **Real-Time Metabolic Analysis:** Using MitoXpress nanoprobe, the study showed reduced basal oxygen consumption in muscle tissues exposed to ME/CFS sera, which cryotherapy partially rescued, aligning with reports of improved cardiovascular and autonomic function following treatment.

12. Potential Therapeutic Targets: Targeting IL-6, enhancing mitochondrial quality control, and managing metabolic imbalances could represent therapeutic opportunities. Serum IL-6 levels or mitochondrial biomarkers could serve as indicators of disease severity

Section B: Steroid Myopathy

This work has been published in Disease Models and Mechanisms as:

Mughal S, Sabater-Arcis M, Artero R, Ramón-Azcón J, Fernández-Costa JM. *Taurine activates the AKT-mTOR axis to restore muscle mass and contractile strength in human 3D in vitro models of steroid myopathy.* **Dis Model Mech.** 2024 Apr 1;17(4):dmm050540. doi: 10.1242/dmm.050540. Epub 2024 Apr 24. PMID: 38655653; PMCID: PMC11073513.

Chapter 1B: Introduction and Overview

Skeletal muscles are the largest and most adaptable protein reservoirs with a capacity to regulate their mass, protein turnover and fiber cross-sectional area in response to a variety of external and internal stimuli (294). A well-regulated balance between proteolytic and proteogenic pathways is essential to maintain a healthy muscular function (295). During muscular atrophy, proteolytic pathways are hyperactivated which result in a low protein turnover, a steep decline in muscle mass and loss of contractile function (296).

Excessive loss of muscle mass is also associated with poor disease prognosis (296). This holds particularly true for anti-inflammatory treatment protocols requiring extended use of oral or intravenous corticosteroids. Corticosteroid-induced muscular atrophy, also called glucocorticoid or steroid myopathy, is a toxic, iatrogenic, noninflammatory condition arising due to prolonged glucocorticoid use (297). It is known to manifest through either catabolic, anti-anabolic or both routes. Contrary to other muscular myopathies, in patients with steroid myopathy standard diagnostic biomarkers such as creatine kinase, aspartate aminotransferase, lactate dehydrogenase and aldolase levels are often normal in the early disease stages. These markers may only elevate in critically ill patients making it difficult to get a timely diagnosis (298).

Initially discovered in patients being treated for Cushing's disease with corticosteroids, steroid myopathy proceeds through hyperactivation of the ubiquitin-proteasome pathway (297,299). Muscle Atrophy F-box (MAFbx) or Atrogin-1 and Muscle Specific Ring Finger Protein 1 or MURF1 are two E3 ubiquitin ligases upregulated in the atrophying muscle. Therefore, these proteins are important markers in studying muscle mass regulation due to corticosteroid or glucocorticoid exposure (300).

The glucocorticoid receptor (GCR) gene (NR3C1 NM_000176.3) is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily (301). It is expressed in all major cell types to enable the pleiotropic effects of glucocorticoids. In the absence of a ligand, the receptor is transcriptionally inactive

and remains restricted in the cytoplasm as a multi-protein complex (301). This complex consists of chaperone proteins and immunophilins that regulate conformational change to enable high-affinity ligand binding and subsequent nuclear translocation (302). Prior to nuclear translocation, chaperone proteins dissociate exposing two nuclear localization signals (NL1 and 2). Once inside the nucleus, GCR's central-DNA Binding Domain (DBD) binds to Glucocorticoid Responsive Elements (GRE). These elements are imperfect palindromic sequences that allow GCR dimerization and subsequent transcription of multiple genes by recruiting RNA Polymerase II (303).

The GCR can initiate catabolic effects by inducing the expression of Forkhead Transcription Factors O1 (FOXO1) which upregulates Atrophy F-box (MAFbx) or Atrogin-1 and Muscle Specific Ring Finger Protein 1 or MURF1, thereby upregulating protein catabolism in the muscle (296,304). GCR's anti-anabolic effects suppress the stimulatory effects of insulin, IGF-I (insulin-like growth factor-I), and amino acids on two key cellular components: eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) (305). These components directly regulate protein synthesis through the mTOR (mammalian target of rapamycin) pathway (299). This exclusive and highly coordinated interaction between the catabolic and anabolic mechanisms is essential to understand how distortion of balance may impact the muscle's structural and functional capacity.

Amino acid supplementation is known to enhance isometric muscle strength and improve ambulatory dysfunction (306). The semi-essential micronutrient, Taurine (TAU), is known for improving physical endurance by modulating excitation-contraction coupling in skeletal muscles and forms a crucial part of the cytoprotective mechanisms in the cell (307–309). According to Uozumi et al., 2006 skeletal muscle stores 70% of total Taurine reserves in the body and expression of Taurine Transporter (TauT) regulates positively under the myogenic program. In the same study, a higher muscular regeneration activity was also directly associated with higher Taurine levels (310). The exact mechanism of Taurine's amelioration capacity for steroid myopathic skeletal muscle tissues, however, remains unexplored.

While extensive research has been conducted to identify the mechanistic pathophysiology involved in muscular myopathies, contemporary research utilizes samples obtained from 2-D cell cultures or rodents. Not only do these procedures require exhaustive resources, provide no information on the *in situ* functional implications but also quite often have limited reproducibility in humans (311). Moreover, the percentage of FDA approved drugs to the monetary investment spent annually on drug development and validation studies continues to decrease monotonically. 3-D *in vitro* tissues and organoids bioengineered from human derived cells have garnered immense interest in mechanistic and pharmacological studies due to their better reproducibility and similarity to the *in vivo* microenvironmental conditions (312,313).

In this work we developed 3-D bioengineered skeletal muscle tissues to quantitatively map steroid myopathy. We used Dexamethasone (DEX) to induce steroid myopathy and then studied the isolated and combined effect of Taurine's amelioration capacity. While regulation of metabolism-volume coupling through glucocorticoids has been studied in mouse models and 2-D cell cultures (300). There is at present no 3-D model that relates the underlying molecular pathology to the functional capacity of a human muscle. Our 3-D bioengineered skeletal muscle model for the first time replicated human steroid myopathy *in vitro*, particularly GCR translocation, and employed it as a drug testing platform to study mechanistic action of Taurine.

Overall, our findings confirm that high systemic levels of dexamethasone hyperactivates the ubiquitin-proteasomal degradation of the skeletal muscles. This causes a steep decline in maximum contractile force and an increase in fatigue. Moreover, upregulation of the p-AKT/p-mTOR axis by Taurine in the diseased tissues not only promotes protein synthesis and restoration of functional and structural phenotypes but does so possibly by inhibiting GCR recruitment onto the GREs through mTOR

Chapter 2B: Materials and Methods

2B.1. Mold design and fabrication

Master molds for the 3D hydrogel casting platforms were designed using Fusion 360 by Autodesk (312). In this protocol, the final polydimethylsiloxane (PDMS) (10:1) molds were made using PDMS (5:1) intermediary negative molds (Fig. S1A).

2B.2. 3D printing by Digital Light Projection

SolusProto (Reify3D, CA, USA), an opaque high-temperature-resistant resin, was used to print master molds with a Direct Light Projection 3D printer (Solus DLP 3D Printer, Reify3D) at low resolution (80×45 mm with a pixel distance of 45 μ m). The design was converted to a Standard Triangle Language (STL) format. Initial layer thickness was maintained at 30 μ m and the light exposure time was set at 2 s per layer at an intensity of 7. A buffer time of 2 s allowed the platform to settle against turbulence before it continued moving downwards. After printing, the molds were thoroughly sonicated and washed with RESINAWAY (Monocure 3D, CA, USA) and MilliQ water and dried with nitrogen. The molds were then cured in ultraviolet light for 45 min. To remove any uncured resin from the casting cavity, the molds were placed for 1 h at 120°C on the hot plate (on an overturned glass Petri plate covered with an overturned big beaker to avoid cracking). Prior to silanization, the thermocured master molds were activated with ozone plasma for 30 s and silanized by chemical vapor deposition in a vacuum desiccator with a few drops of trichloro (1H,1H,2H,2H-perfluorooctyl)-silane (PFOTS, Sigma-Aldrich) (placed separately in an open Petri dish) for 1 h.

2B.3. Fabrication of negative molds

The PDMS-negative mold was made by mixing the elastomer and curing agent (two parts polymer: base and curing agent, Sylgard 184, Dow Corning, MI, USA) (5:1). Prior to pouring on the master molds, the mixture was degassed in a vacuum desiccator to remove bubbles. After pouring, the molds were again degassed for 10 min in the desiccator to remove any entrapped bubbles and they were cured overnight at 75°C. Bubbles entrapped in the pillars were removed using

a 0.41 mm dispensing tip syringe when needed. The negative mold was peeled and washed thoroughly with ethanol and MilliQ water, dried with nitrogen and silanized with a few drops of PFOTS (placed separately in an open Petri dish) for 1 h.

2B.4. Fabrication of the PDMS platforms

The final PDMS platforms were made by mixing the elastomer and curing agent in a ratio of 10:1. The mixture was thoroughly mixed and degassed for 10-15 min at 0.7 MPa vacuum pressure. The uncured PDMS mixture was poured on the silanized negative molds and placed in a plastic Petri dish. The molds were again degassed to remove any trapped bubbles. For curing, the molds were left at 75°C overnight covered with a glass slide to achieve a smooth surface. After demolding, the PDMS platforms were obtained using an 8 mm biopsy punch. All the PDMS platforms were thoroughly washed with sonication in MilliQ water, detergent and isopropanol. Prior to encapsulation, the PDMS platforms were treated with filtered 5% Pluronic F-127 (Sigma-Aldrich) in PBS for 2 h at 4°C to facilitate hydrogel detachment. The pluronic acid was removed using a filter paper without touching the interior of the platform. The treated PDMS platforms were sterilized in ultraviolet light for 15 min.

2B.5. Cell culture

Human immortalized muscle precursor cells were procured from the Institut NeuroMyoGene, Lyon, France (314). Specifically, line` control 2-E4 was used with a population-doubling time of 3.55 days and 99.40% of CD56+ cells. The cells were cultured in skeletal muscle basal growth medium (SMC-GM, C23060, PromoCell), consisting of supplemental mix (C23060, PromoCell), 1% penicillin-streptomycin (10,000 U ml⁻¹ , 15140-122, Thermo Fisher Scientific) and 10% fetal bovine serum (10270-106, Thermo Fisher Scientific). For differentiations, the cells were cultured in Dulbecco's modified Eagle medium (DMEM), high glucose, GlutaMAX (Gibco, Thermo Fisher Scientific), 1% v/v penicillin-streptomycin-glutamine (P/S-G, 100×, Gibco, Thermo Fisher Scientific) and 1% v/v insulin-transferrin-selenium-ethanolamine supplement (ITS-X, 100×, Gibco, Thermo Fisher Scientific). Tissue

fabrication A previously designed protocol was adopted to fabricate functional skeletal muscle tissues (315). Briefly, for encapsulation, the human muscle precursor cells were trypsinized and resuspended in skeletal muscle growth medium. The cells were encapsulated at a density of 2.5×10^7 cells ml⁻¹ in 30% v/v Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning), 2 U ml⁻¹ thrombin from human plasma (Sigma-Aldrich) and 4 mg ml⁻¹ fibrinogen from human plasma (Sigma-Aldrich). During hydrogel casting, care was taken to avoid bubbles and cold plasticware was used to prevent polymerization of Matrigel. The mixture was spread as homogenously as possible between the pillars without grazing the surface. After hydrogel introduction, all PDMS platforms were incubated at 37°C for 30 min before adding skeletal muscle growth medium supplemented with 1 mg ml⁻¹ of 6-amino-caproic acid (ACA, SigmaAldrich). After 48 h, the growth medium was replaced with differentiation medium (DM) supplemented with 1 mg ml⁻¹ ACA (DM/ACA). Subsequently, half of the DM/ACA was replenished every 2 days.

2B.6. Drug treatments

Steroid myopathy induction using DEX To develop the atrophy model, after 6 days of differentiation, the tissues were treated with 100 μ M of DEX (D4902-100MG, Merck Life Science, Germany) in DM/ACA for 24 h as previously reported for C2C12 cells by Shimizu et al., 2017. A stock solution of 10 mM DEX in absolute ethanol was prepared and diluted with DM/ACA to make a final concentration of 100 μ M. The same volume of absolute ethanol was used to analyze the effect, if any, of the dissolvent.

2B.7. Evaluation of independent taurine effect

For taurine treatment, 100 mM stock solution of taurine (T0625-25G, Merck Life Science) was prepared in MilliQ water and diluted to 1 mM in DM/ACA. The same volume of MilliQ was used as control. Taurine-supplemented DM/ACA was added on the sixth day of differentiation for 24 h to observe whether the drug had any effect on healthy tissues.

2B.8. Rescue treatment with Taurine

To investigate the atrophy amelioration capacity of taurine, two rescue regimes were adopted: (1) entirely replacing the DEX-containing medium with DM/ACA containing 1 mM taurine for another 24 h, or (2) continuing DEX treatment for 48 h and adding 1 mM taurine after 24 h. The final concentrations of DEX and taurine were 50% of their initial values. MilliQ water was used as a control in both cases. Contractile force analysis and immunofluorescence studies were performed on day 8 of differentiation, post treatment.

2B.9. Electrical pulse stimulation

Post treatment, the bioengineered skeletal muscle tissues were placed in a 12-well plate with custom-built graphite electrodes connected to a pulse generator (Multifunction Generator WF1974, NF Corporation). An XL S1 cell incubator maintained at 37°C and 5% CO₂ was used to acquire brightfield images of the plate on top of a Zeiss Axio Observer Z1/7 microscope. The tissues were electrically stimulated to induce both twitch and tetanic contractions. Square pulses of 1 to 50 Hz in an electrical field strength of 1 V mm⁻¹ were applied for 10 s with a 10% duty cycle. All experiments were performed with fresh DM/ACA.

2B.10. Video analyses and force measurement

Based on the theory of linear bending, the Euler–Bernoulli's beam-bending equation was used to calculate the force exerted by each tissue. Readings were recorded from both pillars and the average force of contraction was calculated using Fiji/ImageJ.

$$F = \frac{6\pi ED^4}{64a^2(3L - a)} \cdot d$$

$$F = k \cdot d$$

where: ***F*** is force exerted by the tissue; ***E*** is the Young's modulus of the PDMS (calculated to be 1.6 ± 0.1 MPa.); ***D*** is diameter of the post; ***a*** is location of the tissue on the pillar, ***L*** is the height of the post; and ***d*** is displacement of the pillar. Spring constant ***k*** was calculated using the above parameters to be 3.54 N m^{-1} (312).

Post stimulation, the tissues were either fixed in 10% Formalin solution for 30 minutes at room temperature to be cryosectioned for Immunofluorescence staining or frozen in liquid nitrogen for RNA extraction and Western Blot.

2B.11. Cryosectioning and Immunofluorescence

Post fixation in 10% Formalin, the tissues were embedded in OCT (Optimal Cutting Temperature) compound in plastic Cryomolds® using chilled Isopentane. Transverse sections ($15 \mu\text{m}$) were obtained by sectioning with a Leica CM1900 cryostat. The sections were transferred to SuperFrost Plus™ Adhesion slides (Fisher Scientific) and stored at -20°C .

Prior to staining, the tissue sections were encircled with a PAP pen. PBS was used to hydrate the sections before a 15-minute permeabilization step with PBS-T (0.1% Triton-X in PBS). Tissue sections were then blocked for 30-40 minutes at room temperature in blocking buffer (0.3% Triton-X and 3% Donkey Serum in PBS) followed by incubation with primary antibodies in blocking buffer overnight at 4°C . After this incubation, the sections were washed three times with PBS-T (5 minutes per wash) and incubated with Fluorophore tagged secondary antibodies and phalloidin in blocking buffer for 40-60 minutes at room temperature. Sections were then washed three times with PBS-T (5 minutes per wash) and mounted using VECTASHIELD Plus Mounting Medium with DAPI (Palex). After placing coverslips, corners of the slides were sealed with a transparent enamel sealant.

2B.12. Imaging and Quantitative Morphological Analyses

Sections were analyzed using Brightfield and Confocal Imaging with ZEISS Axio Observer Z1/7 and ZEISS LSM800 respectively. 20X and higher magnifications

were used to procure images from the confocal microscope. After acquisition, images were processed and analyzed using Fiji, ImageJ.

To calculate myotube diameter, SAA or Phalloidin and DAPI channels were used. Images were binarized and free hand tool was used to mark the boundary for obtaining Ferret's diameter ($n \geq 50$ per image, at least 3 images were procured per tissue).

Localization of total GCR and FOXO1 content was checked relative to DAPI, Phalloidin and SAA signals. Separate channels shown in Figure S4. To evaluate colocalization of the Glucocorticoid receptor JACoP Plugin on Fiji, ImageJ was used (316,317). Mander's M1 Colocalization coefficients were obtained and compared for different treatment conditions (318).

2B.13. RNA Extraction and qRT-PCR

Sterilized pestles were used to homogenize the tissues in Qiazol reagent, and the total RNA was extracted using miRNeasy Micro Kit (QIAGEN) and manufacturer's instructions. One microgram of RNA was digested with DNaseI (Invitrogen) and retrotranscribed with SuperScriptII (Invitrogen), using random hexanucleotides. For each biological replicate, qRT-PCR reactions from 10 ng of cDNA were carried out per triplicate using TaqMan probes (Applied Biosystems; Table S3). GAPDH was used as the endogenous control after comparing its stable expression across different treatment regimens (Figure S6). Thermal cycling was performed in StepOne Plus RT-PCR System (Applied Biosystems). Relative expression to the endogenous gene and the control group was obtained by the $2^{-\Delta\Delta Ct}$ method. Pairs of samples were compared using a two-tailed t-test ($\alpha = 0.05$), applying Welch's correction when necessary.

2B.14. Western Blot

For total protein extraction, samples were homogenized in RIPA buffer (Fisher Scientific, Waltham, Massachusetts, USA, 10230544) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science, Indianapolis, IN, USA,

4906837001). Total protein was quantified using a BCA assay kit (Thermo Scientific Pierce, Grand Island, NY, USA, 10741395) following the manufacturer's instructions. For specific protein detections, 25 µg of total protein were denatured for 5 min at 100 °C, electrophoresed on 12-15% SDS-PAGE gels, and transferred onto 0.45 µm nitrocellulose membranes (Cytiva, Little Chalfont, UK, GE10600002). Membranes were blocked for 1 h at RT with blocking solution containing 5% non-fat dried milk in PBS-T (8 mM Na₂HPO₄, 150 mM NaCl, 2 mM KH₂PO₄, 3 mM KCl, 0.05% Tween 20, pH 7.4) for non-phosphorylated proteins and for phosphorylated proteins we used as blocking solution 5% BSA in PBS-T. Membranes were incubated in agitation overnight with blocking solution at 4 °C with the corresponding primary antibody described in Supplementary Table 3. Primary antibodies were detected using goat HRP-conjugated anti-rabbit-IgG (1 h, 1:3500, Sigma-Aldrich, San Luis, Missouri, USA, A0545) or goat HRP-conjugated anti-mouse-IgG (1:3500, Sigma-Aldrich, San Luis, Missouri, USA, B7264) incubated for 1 h at RT. Immunoreactive bands were detected using enhanced chemiluminescence (SuperSignal™ West Pico PLUS, 34579) western blotting substrate. Membranes were stripped to remove both the primary and secondary antibodies. For that, membranes were wash with water for 5 min, then incubated with a stripping solution (8 g/L NaOH and 10 g/L SDS in MiliQ Water) during 10 min and finally with water for 5 min. Then the membranes were blocked again with blocking solution containing 5% non-fat dried milk in PBS-T for 1h at RT. Finally, anti-GAPDH-binding protein-horseradish peroxidase (HRP) antibody was used as a loading control incubating the membranes 1h at RT. Images were acquired with an Amersham ImageQuant 800 and were quantified using ImageJ Fijii software (NIH) (Sabater-Arcis et al., 2021) The corresponding primary antibodies used are described in Supplementary Table 3. Uncropped bands are shown in Figure S7-10.

2B.15. Statistical Analysis

Statistical analyses were performed using Prism 9.5 software (GraphPad). All data was tested for normality by using the Shapiro-Wilk test ($p \geq 0.05$). The comparisons between more than two groups were performed by One-Way ANOVA.

For two groups unpaired, parametric Student t-tests with Welch's correction was applied while for non-normal data sets, non-parametric Student T-tests with Mann Whitney U test was performed. In case of ANOVA, if a significant trend was observed, post-hoc tests with 95% confidence interval were applied ($p < 0.05$).

3B. Results

3B.1. 3D Bioengineered Tissues Atrophy following Dexamethasone Treatment

A basic hallmark of steroid myopathy is muscle weakness due to rapid proteolysis (298). 3-D *in vitro* skeletal muscle models provide an opportunity to study the functional and mechanistic implications of disease progression. To fabricate skeletal muscle 3D tissues, we encapsulate human myogenic precursor cells in a Matrigel-Fibrin matrix. Before initiating differentiation, the cells were allowed to aggregate together to form essential cell-cell contacts. A 3-D *in vitro* skeletal muscle tissue has longitudinally aligned myofibers and mature muscle marker sarcomeric actinin (SAA) (Figure S1B, D), formed due to the pillars' passive tension extending through the compacting matrix. All tissues derived from human myogenic precursor cells showed efficient matrix compaction post encapsulation and longitudinally aligned, well-differentiated myofibers were observed stretching between the two pillars after 6 days (Figure 1A). The hence formed tissues were then treated with Dexamethasone (DEX) 100 μ M and EtOH for 24 hours after 6 days of differentiation (Figure 1A). We used EtOH as a control as it was the Dexamethasone diluent to rule out any changes due to the vehicle effect. Prolonging the exposure of tissues to Dexamethasone for 48 hours caused most of the tissues to break, therefore, making it impossible to record their contractile profiles. All control tissues (with $\leq 1\%$ of EtOH), and DEX treated tissues were well-compacted but some broken myotubes were observed at the margins for DEX-treated tissues. This should be because myotubes at the peripheral margins are more exposed to external stimuli than those deeply embedded in the center (Figure 1B). DEX (100 μ M) treated tissues exhibited a lower maximum force of contraction compared to untreated and EtOH controls. The same trend was observed for both twitch and tetanic stimulations (Figure 1C-F). Twitch or isolated contractions are induced by exposure to low-frequency (1-2 Hz) pulses. On the contrary, tetanic contractions are sustained contractions at higher frequencies (25-50 Hz), that help indicate the muscle's maximum contractile force. An electric pulse stimulation scheme incorporating both types of frequencies allowed us to study the differential contraction dynamics of the tissues in response to different drugs (Figure S1D). The difference in maximum contractile force between EtOH

controls and Untreated tissues was not statistically significant. Nevertheless, the drop in strength observed for EtOH samples indicates that although minimal, the toxic effect exists.

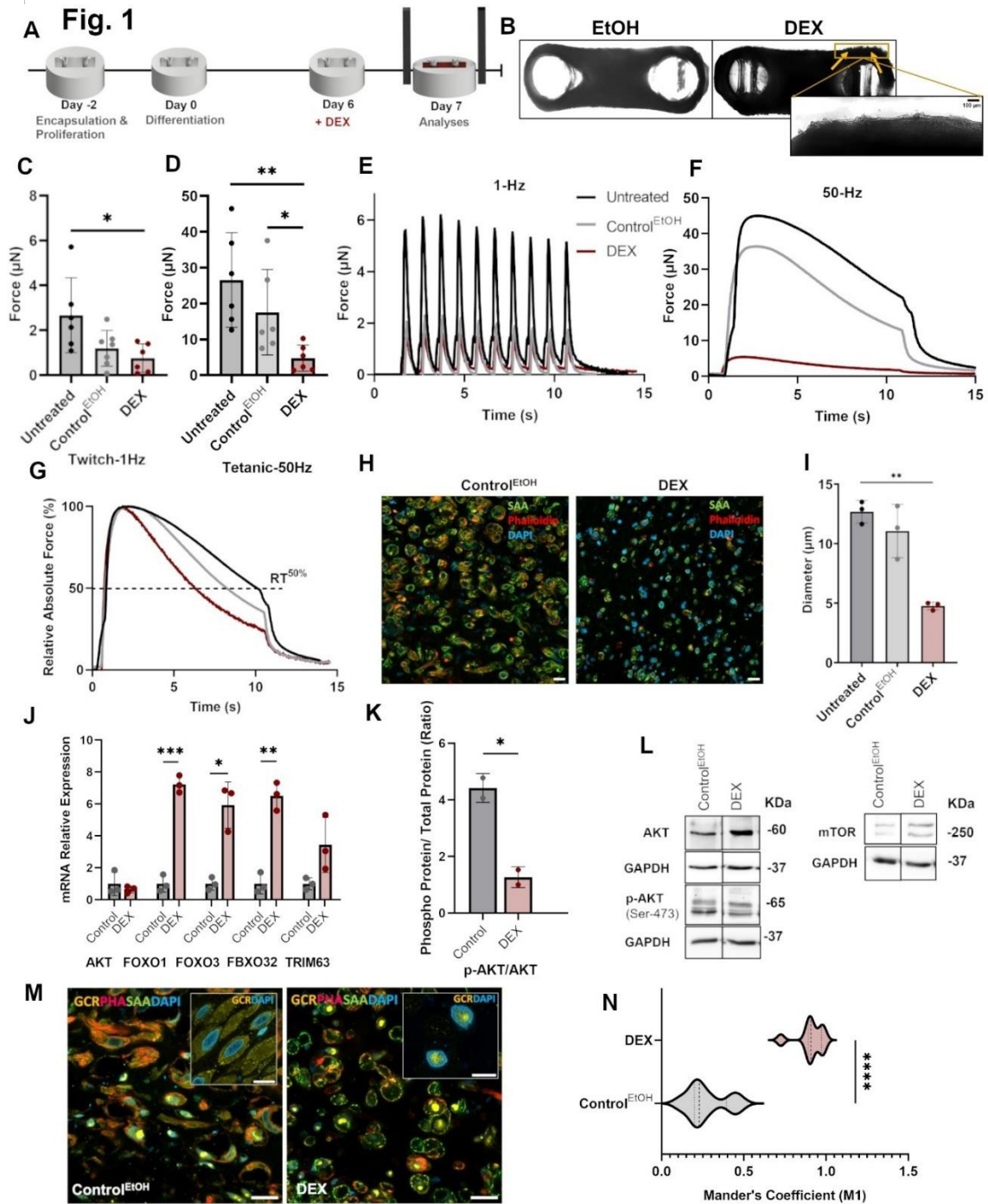


Figure 1: 3-D in vitro Bioengineered Human Skeletal Muscle Tissue Model of Steroid Myopathy (A) Scheme of tissue fabrication and induction of Steroid Myopathy (scale bar 100 μ m). (B) Top-view Brightfield Images

representative for each treatment condition. Arrows point to broken myotubes (C) Maximum Twitch and (D) Tetanic forces $n = 6$ tissues per condition (One-Way ANOVA; $p \leq 0.05$ $** \leq 0.01$). (E) Comparative Twitch Spectrum at 1 Hz (F) Comparative Tetanic Spectrum at 50 Hz. (G) Relative Absolute Force at 50-Hz. (H) Representative Confocal Images of transverse cross sections stained with α -sarcomeric actinin (SAA), phalloidin (f-actin) and DAPI (nuclei) (Scale bar= 20 μm). (I) Myotube Diameters $n = 3$ tissues. (J) qRT-PCR based Relative expression of atrogene mRNAs. (K) Quantification of Western Blot for p-AKT/AKT and p-mTOR/mTOR (L) Representative Western Blots. (M) Representative Confocal Images of transverse cross sections stained for α -sarcomere actinin (SAA), phalloidin (PHA), Glucocorticoid Receptor (GCR) and DAPI (Scale bar= 20 μm and 10 μm). Unmerged corresponding images shown in Fig S4-A. (N) Mander's Coefficient (M1) (Scale bar) $n = 6$. (Statistical Analysis: Unpaired Student t-test with Welch's correction $p \leq 0.05$ $** \leq 0.01$ $*** \leq 0.001$ $**** \leq 0.0001$). All experiments were repeated atleast three times to confirm reproducibility.

A drop in strength or force of contraction in skeletal muscles is a direct indicator of dysregulated volume-mass ratio which compromises fiber contractile properties (319). The data obtained from Electric Pulse Stimulation (EPS) indicated fatigue in the Dex-treated tissues which is another characteristic of a weak contractile apparatus (Figure 1G). The significant difference in RT50 between the myopathic and control tissues is further quantified in Figure S3A. A decrease in myotube diameter for myopathic tissues, quantified through immunofluorescence, further corroborated this trend (Figure 1H-I). Subsequent analysis of GCR translocation in the nucleus showed a higher internalization of the receptor in DEX tissues compared to the controls (Figure 1M, S4A) as indicated by Mander's M1 coefficient which measured the total components of GCR-channel that overlapped with the components of DAPI.

To further analyse these signs of atrophy due to DEX, we quantified the mRNA levels of atrogenes. qRT-PCR data of atrogenes implicated in the IGFR-1/AKT/FOXO pathway indicated elevated levels of FOXO1 and FOXO3 RNAs in DEX treated tissues compared to controls. Both FOXO1 and FOXO3 have GRE elements, enhancing gene transcription significantly. Protein Kinase B (AKT) transcript levels, however, remained the same in all treatment conditions confirming that glucocorticoids do not affect genetic transcription of AKT. TRIM63 (MURF1) and not FBXO32 (Atrogin-1) contain GRE.

To investigate the AKT pathway, we performed western blot analyses to quantify alterations at the protein level. The results indicated that p-AKT(Ser473)/AKT (total) decreased in DEX treated tissues which explains the observed increase in FOXO-dependent FBXO32 and TRIM63 transcription.

Moreover, the levels of p-mTOR (Ser2448)/mTOR(total) also decreased significantly in all DEX-treated human skeletal muscle tissues (Figure 1K-L). Uncropped western bands shown in Figures S7-10. mTOR is a nutritional sensor in skeletal muscles known to regulate hypertrophy and muscle mass. Interestingly, these findings indicate both catabolic and anti-anabolic routes of atrophy induction. While DEX upregulates both E3-ubiquitin ligases; FBXO32 by increasing the levels of FOXO transcription factors only, and TRIM63 through both FOXO-responsive elements and GREs, it simultaneously downregulates the p-AKT and p-mTOR expression, by upregulating REDD1 and KLF15 as demonstrated previously (Shimizu et al., 2011). The protocol, therefore, successfully induced steroid myopathy in 3-D *in vitro* human skeletal muscle tissues.

3B.2. Dexamethasone Replacement with Taurine Partially Restores Functional Capacity

To check Taurine's restoration capacity for tissues with steroid myopathy, we completely removed glucocorticoid-supplemented media from the tissues after 24 hours and replaced it with fresh media containing either MiliQ water (control) or Taurine (1 mM) (Figure 2A). The tissues were maintained in the new media for 24 hours before analyses. Changing the media did not affect tissue compaction as clearly observed in Figure 2B. Removal of DEX in both cases improved force and myotube diameter overall (Figure 2C-F and 2H-I). Taurine's restoration capacity was, however, significantly higher compared to the control (Figure 2C-F and 2H-I). Moreover, the control tissues indicated fatigue, calculated through time taken to achieve 50% relaxation or RT50% (Figures 2G and S3B). Comparison of nuclear translocation of GCR indicated a significantly higher GCR localization in the nucleus of control tissues compared to Taurine-treated tissues (Figure 2M-N, S4B).

To check if Taurine induced any effects at the transcription level, qRT-PCR was performed to check for any changes in the ubiquitin-proteasome pathway. qRT-PCR of mRNA showed similar levels of AKT in TAU treated and control tissues. A partial decrease in FOXO1 and significant decrease in FOXO3 levels was observed in favor of TAU supplementation. The levels of TRIM63 and FBXO32 decreased

overall but remained partially different between the two groups (Figure 2J). The data suggests that as long as glucocorticoids remain in the system, FOXO1 and 3 transcriptions would be promoted and consequently FBXO32 mRNA levels will increase. Removal of glucocorticoids is not sufficient to restore, even partially, the contractile strength and protein turnover in the myopathic tissues. Supplementation with Taurine helped to significantly reverse the damage inflicted by 24-hour glucocorticoid treatment compared to control tissues by significantly reducing the levels of FOXO3 which partially reduced FBXO32 transcription.

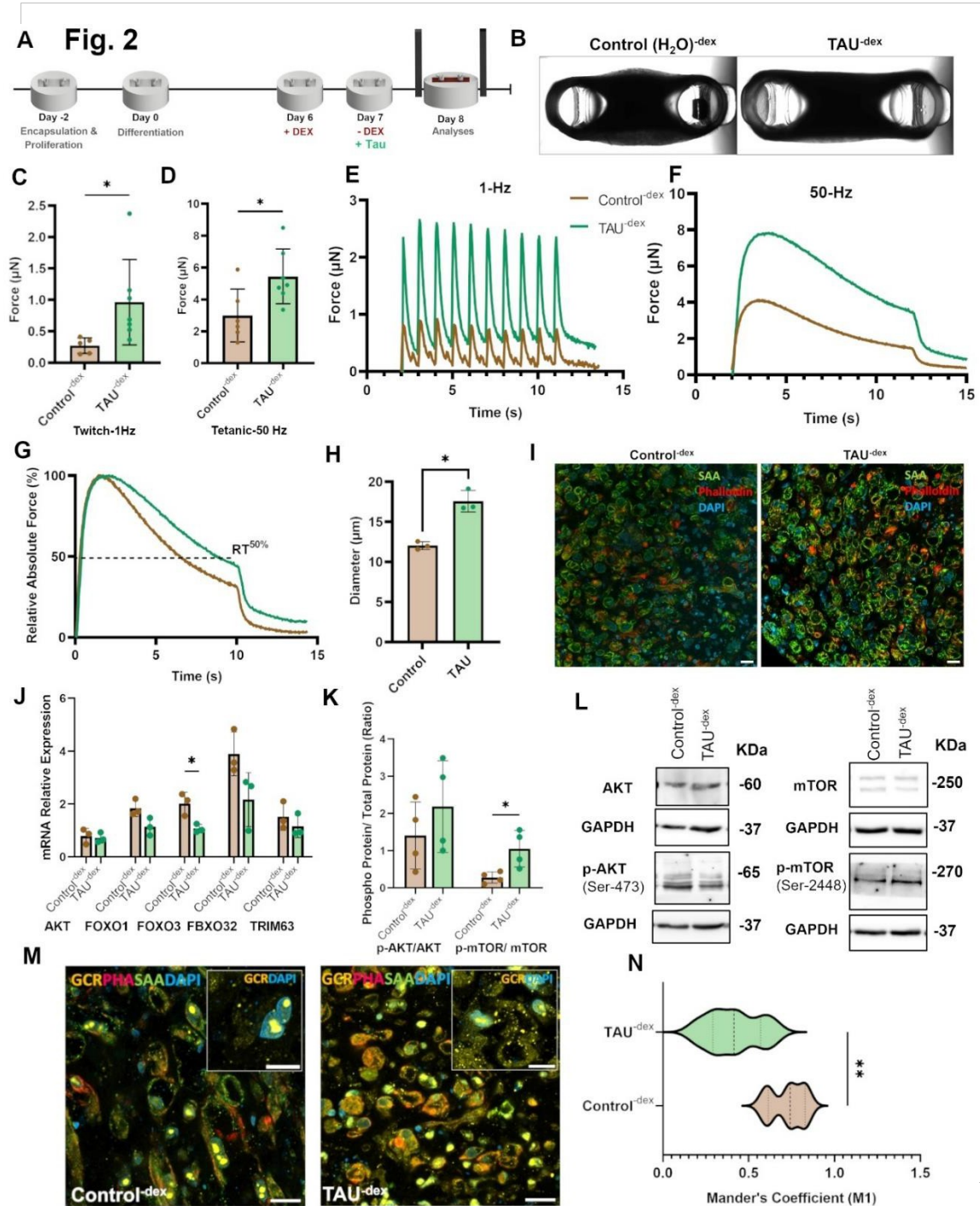


Figure 2: Effect of Taurine on Dexamethasone treated tissues (**A**) Scheme of Myopathy induction and Taurine treatment (**B**) Top-view Brightfield Images representative for each treatment condition (**C**) Maximum Tetanic and (**D**) Twitch forces $n = 6$ tissues per condition (**E**) Comparative Tetanic Spectrum at 50 Hz (**F**) Comparative Twitch Spectrum at 1 Hz (**G**) Time to reach half relaxation (RT_{50%}) (**H**) Representative Confocal Images of transverse cross sections stained with α -sarcomeric actinin (SAA), phalloidin (f-actin) and DAPI (nuclei) (Scale bar = 20 μ m) (**I**) Myotube Diameters $n = 3$ tissues (**J**) qRT-PCR based Relative expression of atrogene mRNAs (**K**) Quantification of Western Blot for p-AKT/AKT and p-mTOR/mTOR in Arbitrary Units (**L**) Representative Western Blot (**M**) Representative Confocal Images of transverse cross sections stained with α -sarcomeric

*actinin (SAA), phalloidin (PHA), Glucocorticoid Receptor (GCR) antibodies and DAPI (Scale bar= 20 μ m and 10 μ m). $p \leq 0.05$. Unmerged corresponding images shown in Fig S4-B. (N) Mander's Coefficient (M1), $n= 6$. (Statistical Analysis: Unpaired Student t-test with Welch's correction $p \leq 0.05$ $**\leq 0.01$). All experiments were repeated at least three times to confirm reproducibility.*

At the protein level, p-AKT(Ser473)/AKT (total) protein expression remained the same in both groups while the p-mTOR levels were significantly improved in TAU supplemented group (Figure 2K-L). Uncropped western bands shown in Figures S7-10. This indicates that Taurine upregulates p-AKT and p-mTOR, promotes protein synthesis while downregulating transcription of atrogenes in the steroid myopathic human skeletal muscle tissues. These findings also substantiate that only replenishing fresh media, and removing glucocorticoids from the system is insufficient to restore protein synthesis in myopathic tissues. While removal of glucocorticoids may help in reducing transcription of atrogenes, it is not enough for the restoration of protein synthesis cascades through the AKT pathway.

3B.3. Taurine's Restoration Capacity is more pronounced with Dexamethasone in the medium

We checked Taurine's ability to competitively regulate protein turnover and contractile properties with glucocorticoids in the system (Figure 3A). Treating myopathic tissues with TAU in the presence of DEX (1:1), does not have an effect on the shape of the tissues (Figure 3B) but the tissues presented a significant restoration of contractile force for both tetanic and twitch contractions compared to control tissues with DEX and MiliQ water (1:1) (Figure 3C-F). The myotube diameter was also found to increase along with an improvement in muscle fatigue (Figures 3H-I, 3G and S3C). As demonstrated by the Mander's Coefficient (M1), Taurine supplementation was not capable to expel GC-GCR from the nucleus (Figure 3M-N, S4C), confirming that Taurine does not affect nuclear translocation of the receptor and that this translocation is predominantly driven by glucocorticoids in the system. AKT mRNA levels remained the same as did the levels of FOXO1 and FOXO3 which further confirmed that Taurine does not directly affect the transcriptional regulation of these factors. Their transcription is solely driven by GREs indicating that the presence of glucocorticoids in the system will keep the transcription of these factors ongoing. TRIM63 and FBXO32 were, however, significantly reduced with TAU in the

medium while p-AKT(Ser473)/AKT (total) protein levels increased substantially as did the levels of p-mTOR (Ser2448)/mTOR(total) (Figure 3K-L). Uncropped western bands shown in Figures S7-10. These findings suggest that an upregulation of phosphorylated Protein Kinase B (AKT) and mTOR prevented GRE-mediated transcription of the two ubiquitin ligases. It appeared that TAU activated AKT and mTOR with glucocorticoids still in the system, more so compared to treating myopathic tissues with no DEX in the system.

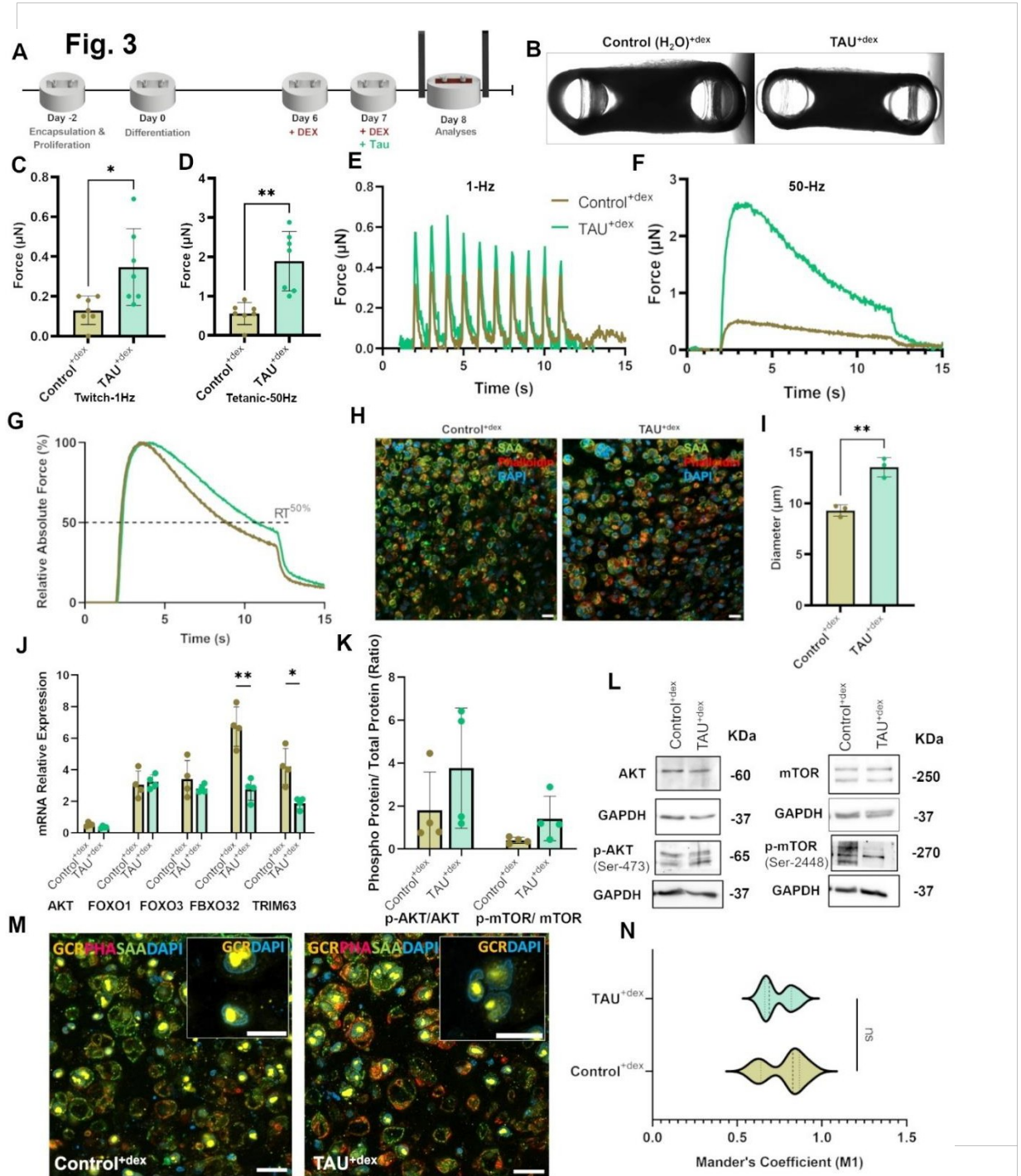


Figure 3: Taurine Treatment concurrently with Dexamethasone (A) Scheme of Myopathy induction and Taurine treatment (B) Top-view Brightfield Images representative for each treatment condition (C) Maximum Tetanic and (D) Twitch forces n= 6 tissues per condition (E) Comparative Tetanic Spectrum at 50 Hz (F) Comparative Twitch Spectrum at 1 Hz (G) Time to reach half relaxation (RT-50%) (H) Representative Confocal Images of transverse cross sections stained with α-sarcomeric actinin (SAA), phalloidin (f-actin) and DAPI (nuclei) (Scale bar= 20 µm) (I) Myotube Diameters n= 3 tissues (J) qRT-PCR based Relative expression of atrogene mRNAs (K) Comparative concentration of p-AKT/AKT and p-mTOR/mTOR in Arbitrary Units (L) Bands from Western Blot (M) Representative Confocal Images of transverse cross sections stained with α-

sarcomeric actinin (SAA), phalloidin (PHA), Glucocorticoid Receptor (GCR) antibodies and DAPI (Scale bar= 20 μm and 10 μm) $p \leq 0.05$. Unmerged corresponding images shown in Fig S4-C. (**N**) Mander's Coefficient (M1) $n=6$. (Unpaired Student t-test with Welch's correction $p^* \leq 0.05$ $^{**} \leq 0.01$). All experiments were repeated at least three times to confirm reproducibility.

3B.4. Taurine raises p-AKT levels in Healthy Skeletal Muscle Tissues

To validate our findings, we checked the effect of TAU on healthy tissues. After allowing the tissues to proliferate in growth media for 2 days, the growth media was changed to differentiation media. On day 6 of differentiation, the tissues were treated with Taurine (1mM) (Figure 4A). All treated tissues formed well-compacted and differentiated tissues capable of contraction on electric stimulation as observed under brightfield microscopy (Figure 4B). Interestingly, neither did Taurine increase the force nor induce hypertrophy in healthy tissues (Figure 4C-H). No differences were observed between the controls and Taurine treated healthy tissues. Moreover, the RNA levels of atrogenes also remained at baseline as did the protein expression levels of p-mTOR (Figure 4I-J). There was, however, a significant increase in the levels of p-AKT, marking it as a direct target of the amino acid (Figure 4J). For all the above treatments (including the myopathic tissues) total FOXO1 protein was found localized within the cytoplasm (Figure S2) perhaps due to the electric pulse stimulation performed on all tissues prior to their fixation (Soto et al., 2023). These findings highlight that Taurine effectively regulates the AKT/mTOR axis through p-AKT in our *in vitro* bioengineered 3-D skeletal muscle tissues to counter damage due to downregulation of the phosphorylation cascade and subsequent activation of the ubiquitin-proteasome system.

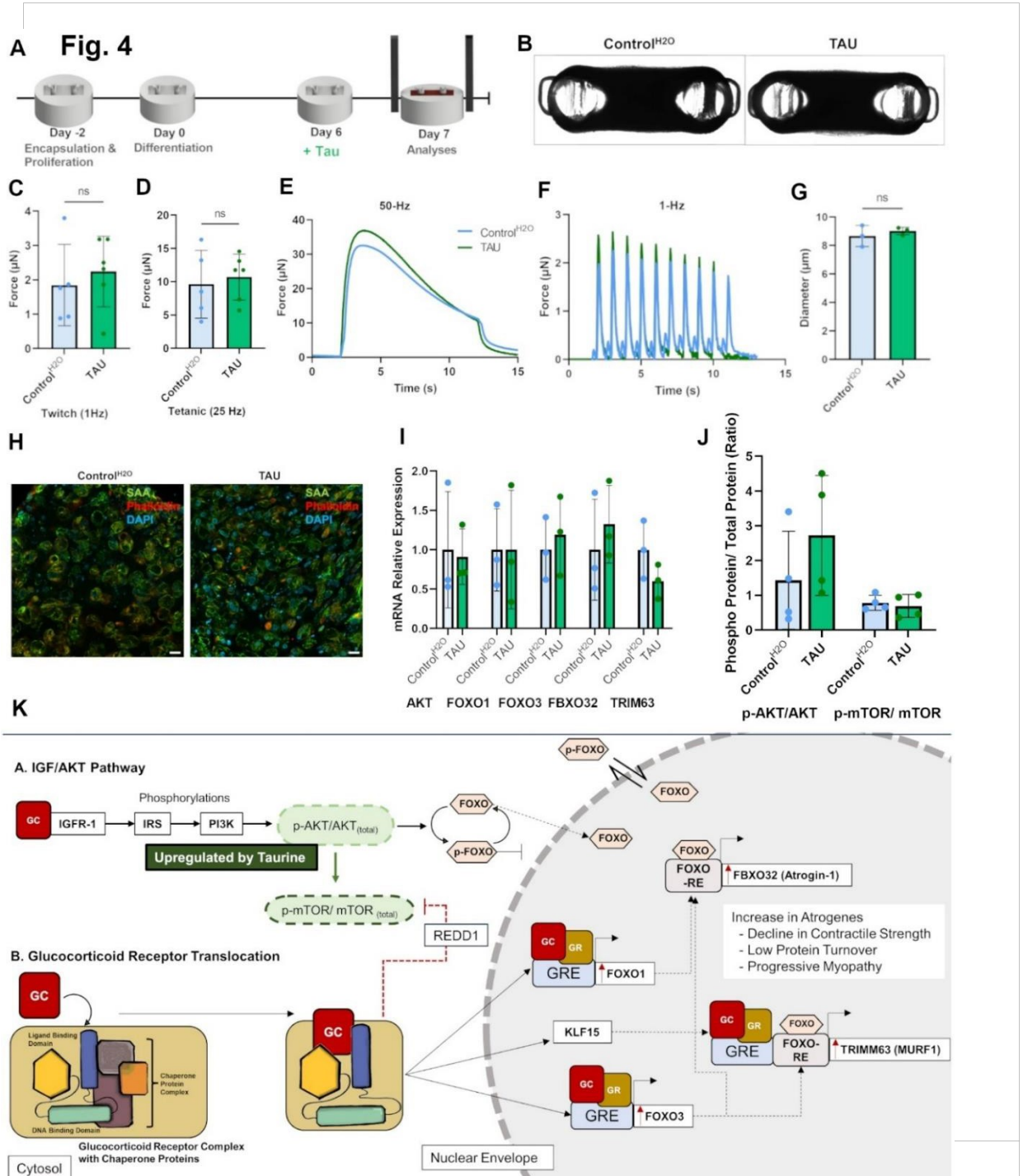


Figure 4: Overall regulation of the IGF/AKT pathway by taurine. (A) Scheme of Taurine treatment on healthy tissues (B) Top-view Brightfield Images representative for each treatment condition (C) Maximum Tetanic and (D) Twitch forces $n = 6$ tissues per condition (E) Comparative Tetanic Spectrum at 50 Hz (F) Comparative Twitch Spectrum at 1 Hz (G) Myotube Diameters $n = 3$ tissues (H) Representative Confocal Images of transverse cross sections stained with α -sarcomeric actinin (SAA), phalloidin (PHA) and DAPI at 20X (Scale bar= 20 μ m) (I) qRT-PCR based Relative expression of atrogene mRNAs (J) Comparative concentration of p-AKT/AKT and p-mTOR/mTOR proteins in Arbitrary Units (K) Pathways implicated by Glucocorticoid exposure and Taurine's point of action. (Statistical Analysis: Unpaired Student t-test with Welch's correction $p \leq 0.05$). All experiments were repeated atleast three times to confirm reproducibility.

4B. Discussion

An accurate identification of steroid myopathy relies on a strong clinical suspicion for patients with moderate to chronic exposure of corticosteroids. Elevated systemic corticosteroid levels might stem from excessive endogenous corticosteroid production caused by adrenal tumors, or due to an excess of exogenous steroid administration aimed at managing various health conditions like asthma, Chronic Obstructive Pulmonary Disease (COPD), and rheumatoid arthritis (298,299). Steroid myopathy causes muscle weakness and wasting but without inducing any pain. There are no signs of inflammation and normal levels of creatine kinase, aldolase, aspartate aminotransferase and lactate dehydrogenase are found in the patients (298). Abrupt corticosteroid withdrawal is the immediate response to stop disease progression and morbidity, but this approach does not help in restoring muscle strength.

Corticosteroids hinder the transport of amino acids into cells, suppress the production of muscle IGF-I, and decrease the differentiation of precursor cells into muscle fibers (297). As a result, this inhibition leads to reduced protein synthesis and impaired myogenesis. Contemporary literature points to the functional cooperativity between GCRs, GREs and FOXOs in regulating the expressions of atrogenes which eventually control a muscle's protein turnover (297–299,319). While FOXOs have been singled out as major catabolic regulators, the direct and indirect influence of GCRs on transcriptional regulation of atrogenes and mTOR driven hypertrophic cascades cannot be ignored. Amino acid supplementation has been largely considered to control osmoregulation and protein stability within a muscle (308). However, their contribution to managing and countering signaling cascades warrants further investigation. Contextual to their contractile performance, it is essential to understand how the synchronization of atrophy pathways is orchestrated in skeletal muscle tissue and subsequently the restoration of balance between protein synthesis and degradation. Through the application of a functional 3-D muscle model, we reproduced the pathophysiology of steroid myopathy in human skeletal muscle tissue and subsequently investigated the mechanistic regulation of the nutritional sensor by Taurine supplementation.

Tissues exposed to Dexamethasone underwent ubiquitin-proteasomal degradation as indicated by elevated levels of TRIM63 and FBXO32 through GCR-GRE and FOXO-mediated transcription. As a result, there was a significant drop in both tetanic and twitch contractile forces after 24 hours of exposure. Prolonging the exposure to 48 hours caused most of the tissues to break, therefore, making it impossible to record their contractile profiles. In an atrophying skeletal muscle, there is a downregulation of the signaling cascade downstream of Protein Kinase B (AKT). This permits de-phosphorylation of FOXOs (1 and 3) allowing their translocation to the nucleus, subsequent attachment to FOXO-responsive elements and transcription of E3 Ubiquitin ligases TRIM63 and FBXO32 (Figure 4K). A second pathway, driven by primarily the glucocorticoid receptor also operates simultaneously alongside AKT downregulation (Figure 4K).

Once a glucocorticoid receptor bound with the ligand internalizes in the nucleus, it initiates transcription by binding to GREs (301). While both TRIM63 and FBXO32 have FOXO-responsive elements, only the former has a GRE sequence. A significant increase in RNA levels was observed for FBXO32 after 24-hour treatment with DEX but not in the levels of TRIM63, which increased after an additional 24-hour exposure with DEX (Figure 1J).

In addition to these atrogenes, GCR also induces transcription of KLF15 and REDD1 which downregulate the signaling cascade of kinases controlled by mTOR as apparent by the protein expression levels of mTOR in atrophying tissues (Figure 1J) (319). Besides controlling hypertrophy and protein synthesis, mTOR also suppresses GR-mediated transcription by inhibiting recruitment onto GREs (320). Downregulation of both AKT and mTOR in conjunction with upregulation of FOXO (1 and 3) transcription disrupted protein turnover, hampered amino acid import, and induced proteolytic degradation in the myopathic myotubes resulting in weaker and thinner tubes.

While the induction of steroid myopathy via Dexamethasone has effectively produced myopathic tissues; however, it is imperative to acknowledge the potential

impact of ethanol (EtOH) as a vehicle in this experimental design. As clearly observed (Figure 1), there is a significant difference in all parameters between the control and dexamethasone. Moreover, the dose-response curve of EtOH in muscle progenitor cells also indicated 100% viability at 1% or lower concentrations while the toxic response increased beyond 5% (v/v) of EtOH (Figure S5). Furthermore, evidence from literature also suggests that lower concentrations of EtOH do not adversely impact muscle structure and performance (Bláha and Rusnak, 2004; MERCK, 2023; MSD, 2023). Chronic use at higher concentrations, on the other hand, may have deleterious effects. *In vivo* studies undertaken in mice suggest atrophy at chronic dosages with at least 33% (v/v) consumption (Ni et al., 2013). Therefore, at concentrations of 1% (v/v) or lower no deleterious vehicle effects can be attributed to our myopathic model.

Steroid myopathy was first discovered in a 24-year-old patient undergoing intravenous hydrocortisone treatment for asthma exacerbation. While the treatment cured her airway obstruction; her muscles were unable to support her weight. After the treatment was suspended, she was able to walk unassisted after 3-4 weeks but the weakness in her distal limb muscles persisted (321). This proves that immediate withdrawal of corticosteroids is an effective emergency intervention but not enough to sufficiently improve muscle function and structure long-term. The same was also observed in our model when we evaluated the response of our myopathic tissues to glucocorticoid-free media. Although the contractile profile of these tissues improved, the effect was not significant. Moreover, tissues were fatigued, and lost strength as evidenced by their reduced time to reach half relaxation. This quicker relaxation is symptomatic of a flaccid, weak tissue which was further confirmed by thinner myotubes and elevated levels of FBXO32 (Atrogin-1). Levels of TRIM63, however, remained the same as controls after media replenishment. On the contrary, when myopathic tissues were treated with Taurine-supplemented, glucocorticoid-free media, we observed a significant improvement in contractile strength and myotube diameter compared to controls without Taurine (Figure 2). Upon further investigation, Taurine was able to significantly lower the levels of FOXO3 while there was a partial reduction in the transcription of other atrogenes. Phosphorylated protein expressions

of both AKT and mTOR increased but the former was not significant compared to myopathic tissues in glucocorticoid-free media. Complete cessation of glucocorticoid use with Taurine supplementation can sufficiently improve muscle strength and protein turnover.

Complete removal of systemic corticosteroids is often not a practically viable solution (307). In these cases, it is pivotal to not only provide symptomatic treatment to patients but also to check myopathic progression. To this end, after induction of myopathy, we kept the tissues in media containing Dexamethasone during the treatments (Figure 3A). While glucocorticoids remained in the medium, Taurine significantly rescued not only the functional and physiological parameters but also significantly lowered the transcription of FBXO32 and TRIM63. This was inferred to be a direct consequence of an increase in p-mTOR and p-AKT levels. p-AKT phosphorylated FOXO proteins and inhibited their translocation to the nucleus while p-mTOR upregulated protein synthesis, myotube hypertrophy and restricted GCR attachment to the GREs resulting in a two-fold drop in the transcription levels of FBXO32 and TRIM63 (320).

It is pivotal to highlight that a corticosteroid treatment is often given to induce immunosuppression in patients with inflammatory conditions or those receiving an organ transplant, in such cases it is undesirable to induce inflammation by inhibiting GCR recruitment to GREs. Taurine's anti-inflammatory potential has been demonstrated before (322–325). As demonstrated in *mdx* mice models, not only is Taurine non-inflammatory but also protects against neutrophil invasion in myotubes by scavenging HOCl produced by these immune cells to Taurine chloramines (TauCl) which are less reactive than the former and prevent against the further production of pro-inflammatory cytokines (325). Increase in the muscle levels of HOCl causes oxidative damage, compromising the muscle's strength and stability (Schaffer and Kim, 2018). The myeloperoxidase-catalyzed reaction contributes to the anti-inflammatory effects of taurine. TauCl, formed during this reaction, also mitigates an increase in nitric oxide and prostaglandin E2, reduces the activity of matrix metalloproteinases, and triggers leukocyte apoptosis, thereby effectively

countering acute inflammation (322). It is, therefore, beneficial in the context of immunosuppression to have high Taurine content not only in the myotubes but also in the neutrophils. Due to its established anti-inflammatory capacity, Taurine is commonly used in patients with inflammatory conditions such as arthritis (324). In line with this contextual evidence, our model also indicates that while there is a marginal tendency due to the effect of m-TOR upregulation, there is no significant reduction in the translocation of the GCR-GRE complex to the nucleus (Figure 3M-N, S4C). Instead, Taurine, largely exerts its protective effects against steroid myopathy by regulating the AKT/mTOR axis through p-AKT upregulation (Figure 4-J).

Therefore, combinatorial therapy of glucocorticoids with Taurine supplementation offers a more feasible solution to initiate protein synthesis in muscle tissues with steroid myopathy without having to clear the system of glucocorticoids. These results also indicate that a combinatorial therapy of glucocorticoids with Taurine for patients undergoing treatment with steroids for a different health condition such as COPD, or only Taurine in the case of adrenal tumors, can help protect patients against progressive myopathy. Furthermore, conclusions from the two rescue regimens, also warrant in favor of Taurine's restoration capacity instead of an effect due to lowering of steroid dosage. The first rescue experiment proves that when the media containing dexamethasone to induce damage was entirely replaced and Taurine added, there was a significant restoration in force. The second treatment protocol, on the other hand included Dexamethasone and Taurine at (1:1), again establishing the superior restoration capacity of Taurine compared to the vehicle. This suggests, that while minor improvement is observed upon lowering or even entirely removing dexamethasone from the system, it is not as significant as when Taurine is added.

To validate Taurine's mechanistic regulation, we checked if isolated Taurine treatment to healthy tissues brought any changes (Figure 4). Interestingly, treating healthy skeletal muscle tissues with Taurine did not increase contractile strength,

average myotube diameter or bring any change to the levels of atrogenes. It did, however, significantly increase the protein expression of p-AKT.

Existing models of steroid myopathy use 2-D cell cultures of C2C12 cells or study contractile profiles of muscles excised from mice (296,304,311,326). While these models have immensely added to the existing knowledgebase, they cannot be completely relied upon to study disease progression and develop therapeutic interventions for humans due to their limited ability to reproduce biological complexity. For instance, data from 2-D rodent cell culture demonstrates an increased expression of TRIM63 levels but not of FBOX032 following Dexamethasone treatment tissues (319). Yet this trend is reversed in our model of human precursor cells derived 3-D.

3-D *in vitro* skeletal muscle engineering has been employed to fabricate models for a variety of complex diseases and has been proven to be an efficient platform for drug testing and identification of therapeutic targets (312,327–329). Our model not only identified mTOR and AKT as the key metabolic targets of muscle mass regulation by Taurine but also related it directly to the detailed regulation of contractile profiles for tissues with steroid myopathy. To the best of our knowledge, this is the first 3D skeletal muscle model derived from human muscle precursor cells which replicates steroid myopathy at functional and molecular levels. It also explores the protective mechanism of Taurine supplementation against steroid myopathy. Therefore, opening the door to exploring Taurine's therapeutic potential for patients facing a diagnosis of progressive steroid myopathy.

Supplementary Information

Taurine activates the AKT-mTOR axis to restore muscle mass and contractile strength in human 3-D *in vitro* bioengineered models of Steroid Myopathy.

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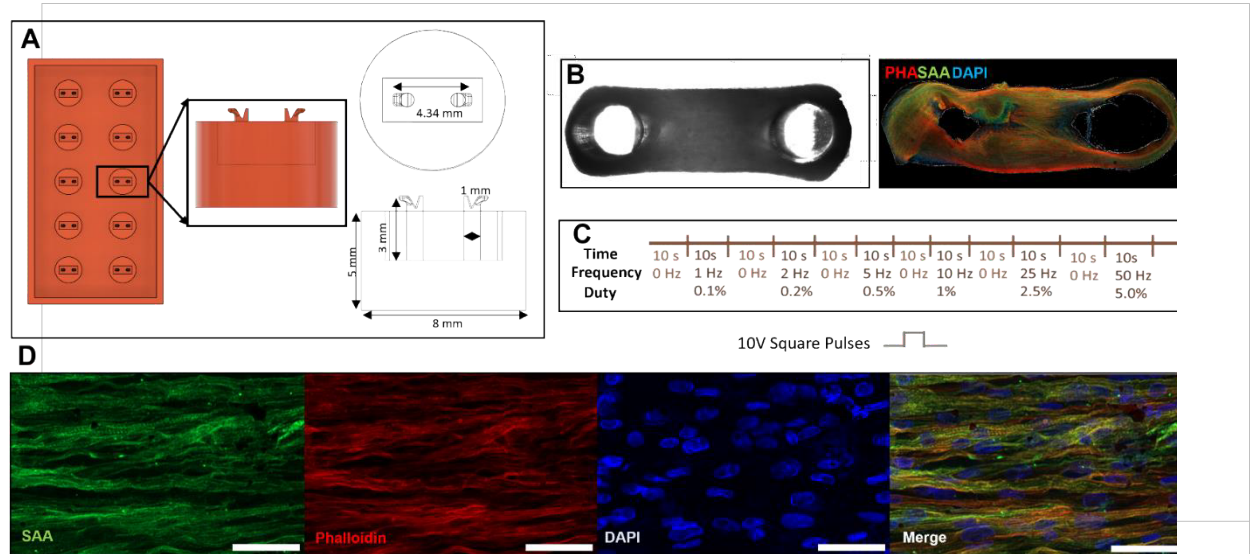


Figure S1: Fabrication of 3D *in vitro* Skeletal Muscle Tissues **(A)** 3-D mold design and Soft-lithographic process to fabricate PDMS Platforms **(B)** Representative Brightfield and Confocal images (stained for α -sarcomeric actinin (SAA), phalloidin (f-actin) and DAPI (nuclei)) of a 3-D Skeletal Muscle Tissue **(C)** Scheme of Electric Pulse Stimulation including frequencies for Twitch and Tetanic Contractions. **(D)** Representative Longitudinal Confocal Image of a 3-D Skeletal Muscle Tissue stained for α -sarcomeric actinin (SAA), phalloidin (f-actin) and DAPI (nuclei) (Scale bar= 50 μ m).

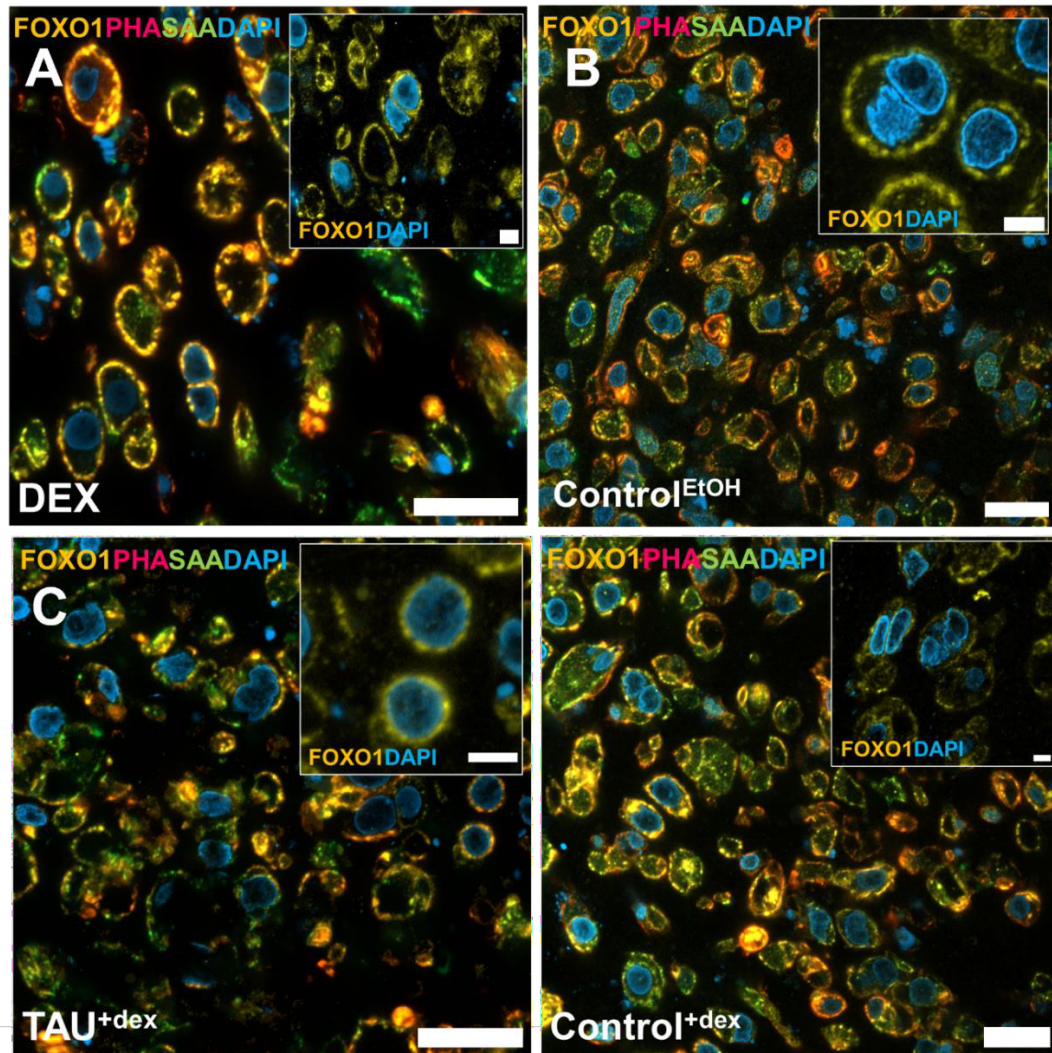


Figure S2: Immunostaining for FOXO1 in transverse tissue sections stained for FOXO1 (yellow), Phalloidin for f-actin (red), α-sarcomeric actinin (Green), and DAPI (blue) **(A)** Tissues treated with 100uM Dexamethasone for 24 hours **(B)** Control Tissues for A **(C)** Tissues concurrently treated with Dex and Tau (1:1) **(D)** Control tissues for combinatorial therapy of Dexamethasone with Taurine (Scale bar = 20 μm and 5 μm).

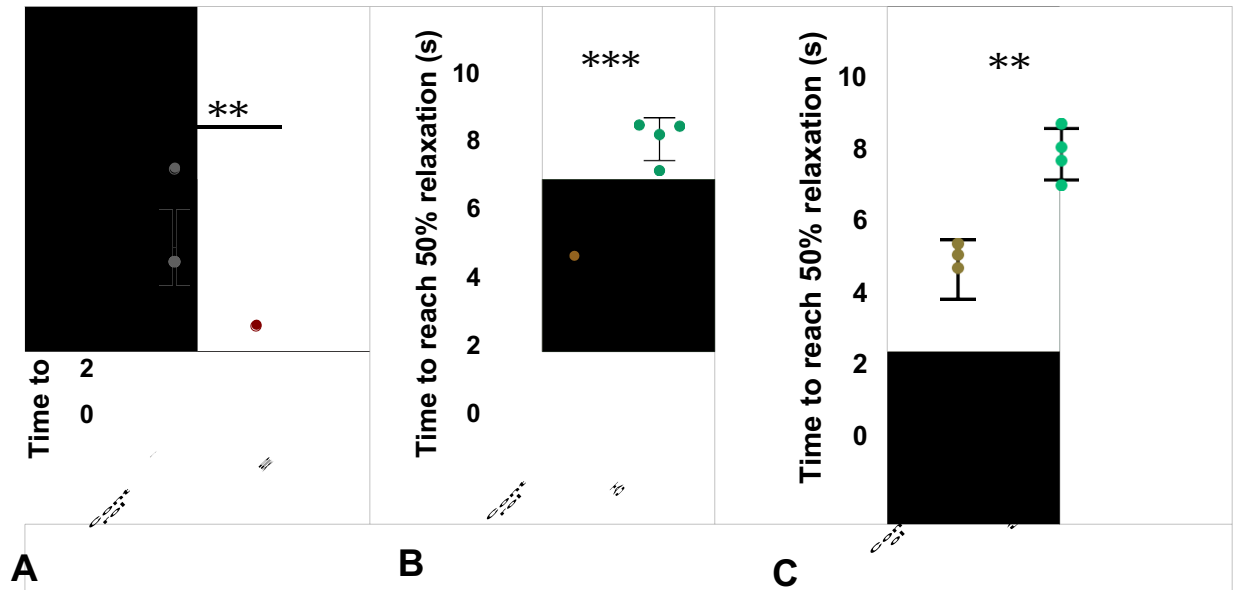


Figure S3: Quantification of Time to reach half Relaxation for **A)** Myopathic Tissues and Vehicle Control **B)** Taurine treated and Control Myopathic tissues without Dexamethasone **C)** Tissues treated concurrently with Taurine and Dexamethasone (1:1) and Control. *Statistical Analyses:* A) unpaired, non-parametric student T-test was performed with Mann Whitney U test B and C) Unpaired, parametric Student's T-test with Welch's correction ($p^{**} \leq 0.01$ $^{***} \leq 0.001$).

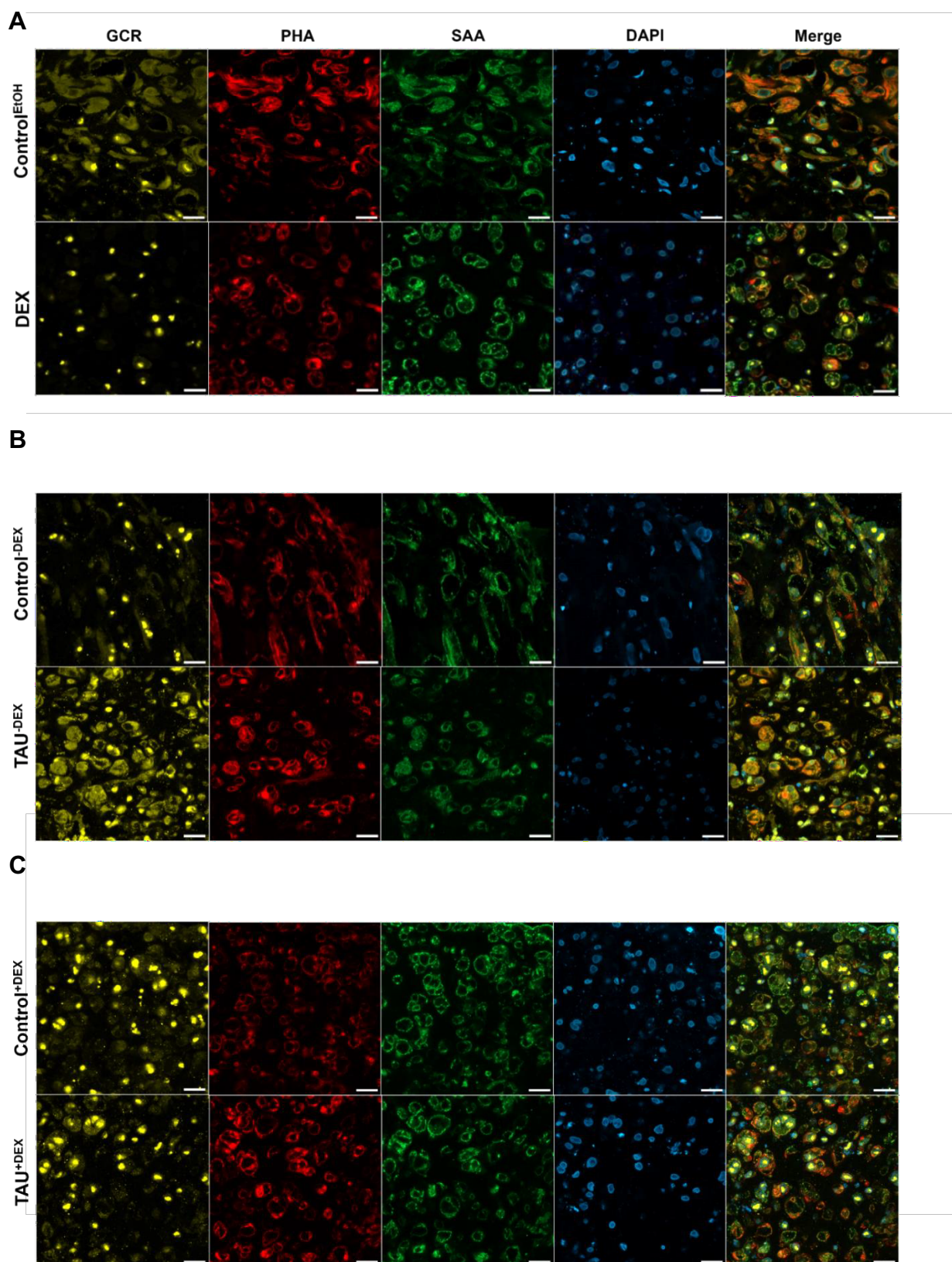


Figure S4: Unmerged Immunostaining images for GCR in transverse tissue sections stained for GCR (yellow), Phalloidin for f-actin (red), α -sarcomeric actinin (Green), and DAPI (blue) **(A)** Tissues treated with Vehicle (EtOH) and 100uM Dexamethasone for 24 hours **(B)** Control and Taurine treated Myopathic Tissues with Dexamethasone in the system **(C)** Tissues concurrently treated with Dex and Tau (1:1) (Scale bar = 20 μ m). The panels correspond to figures 1M, 2M and 3M respectively.

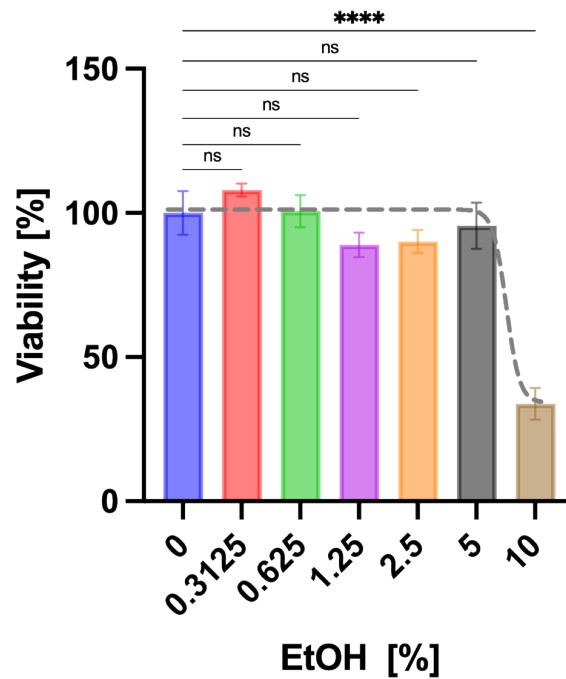


Figure S5: Dose-response curve of EtOH expressed as percentage viability at percentage (v/v) of EtOH in Differentiation Medium. (Statistical Analysis: One-Way ANOVA with Tukey's post-hoc test at 95% confidence interval).

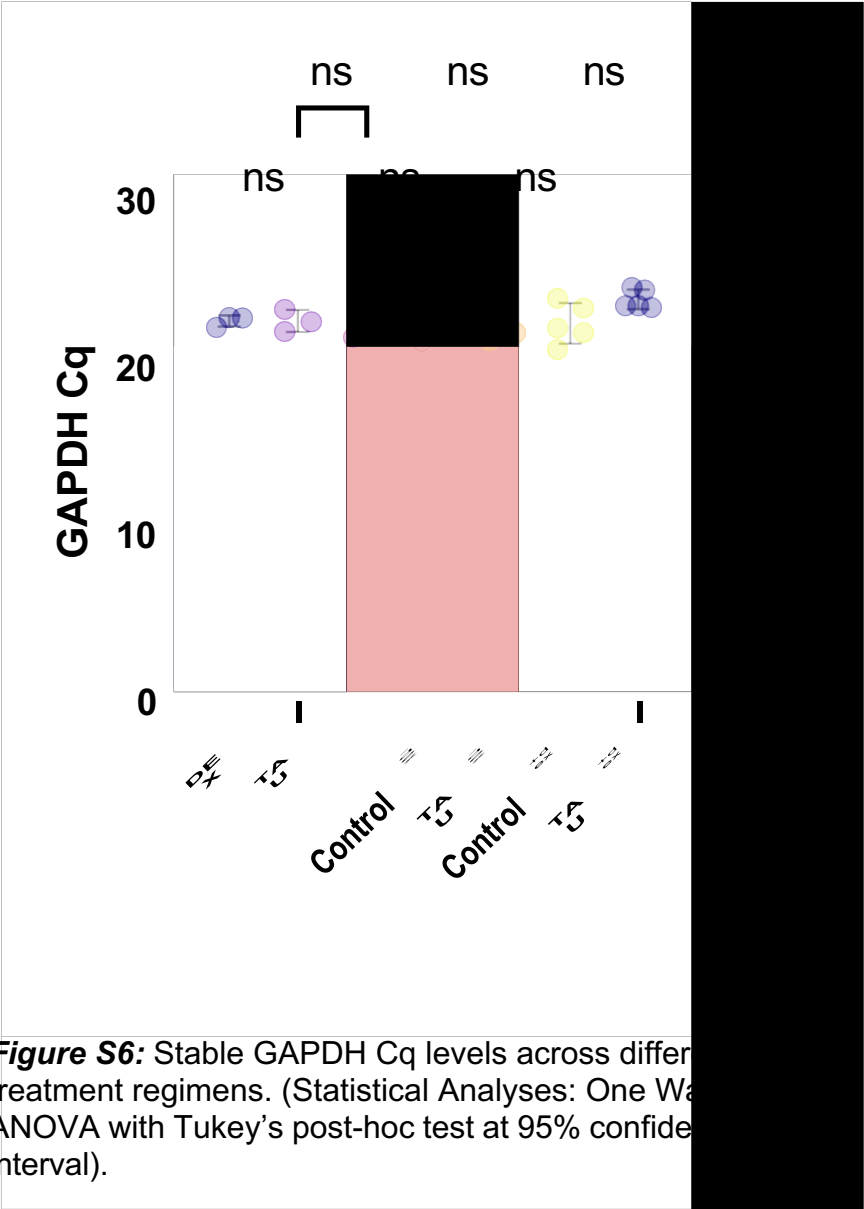


Figure S6: Stable GAPDH Cq levels across different treatment regimens. (Statistical Analyses: One Way ANOVA with Tukey's post-hoc test at 95% confidence interval).

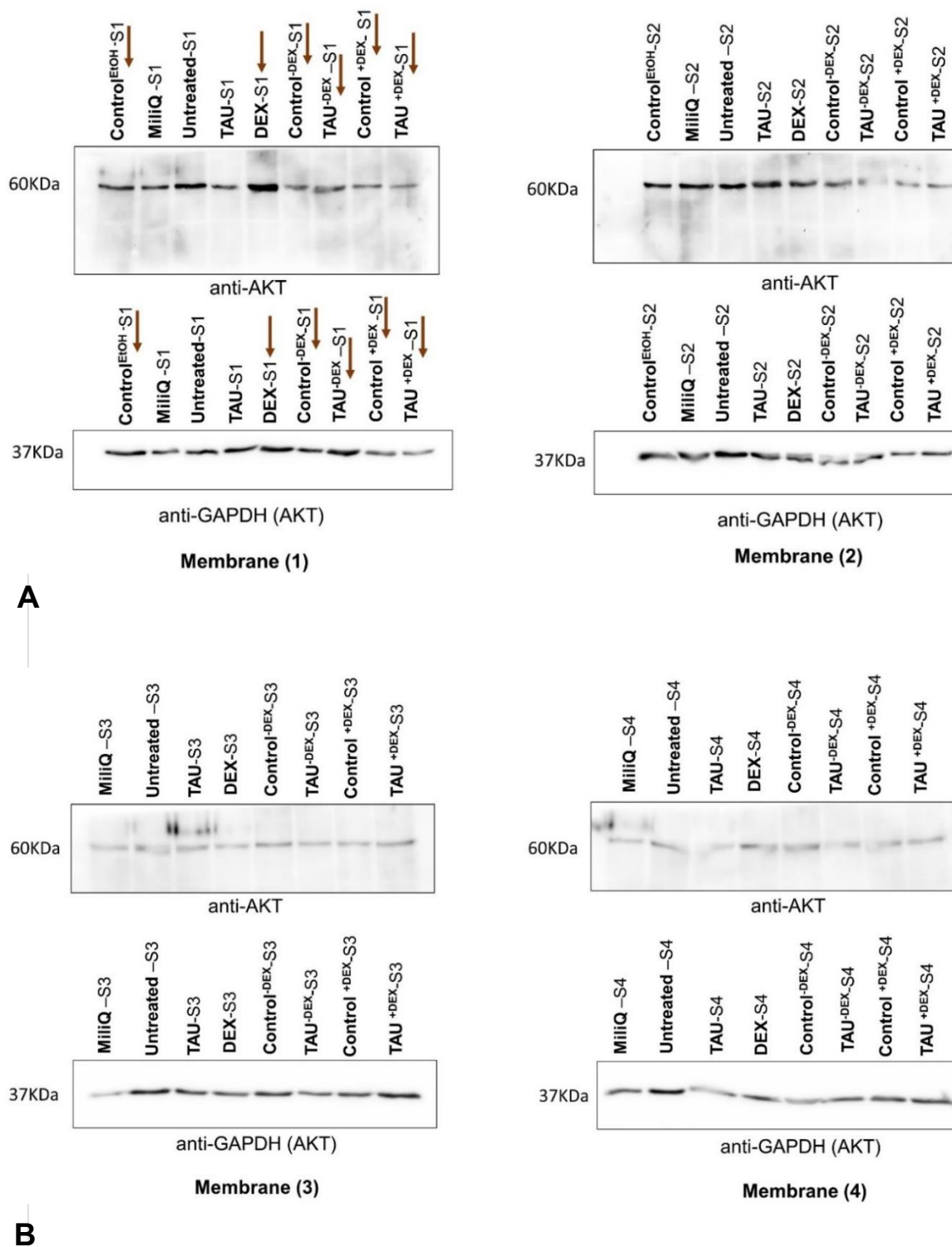


Figure S7: Uncropped western blots for anti-AKT **A)** anti AKT with corresponding GAPDH bands membranes 1-2. **B)** anti AKT with corresponding GAPDH bands membranes 3-4, corresponding to Figures 1,2,3, and 4. Arrows indicate representative bands shown in the manuscript (Figures 1L, 2L and 3L).

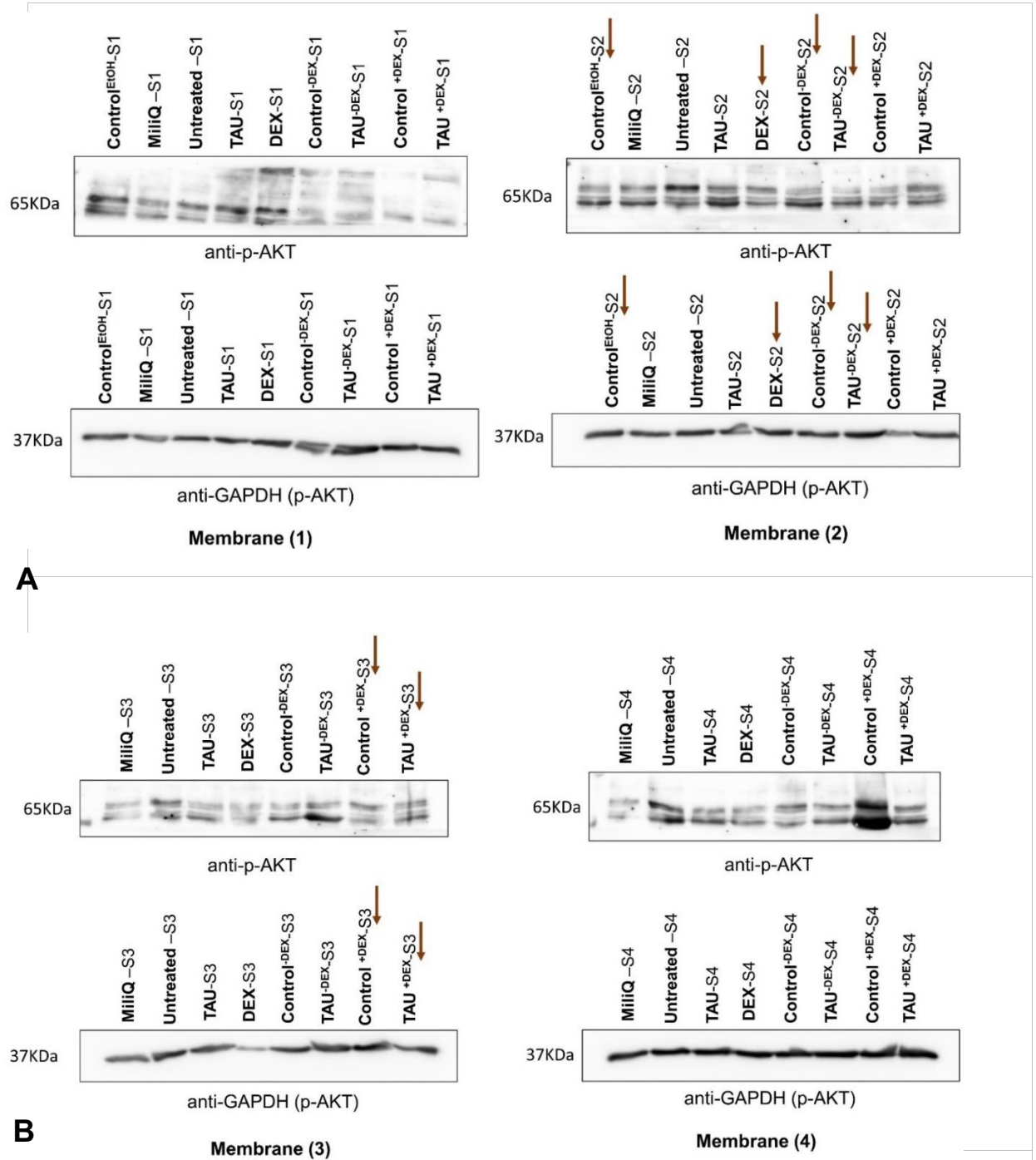


Figure S8: Uncropped western blot for anti p-AKT **A)** anti p-AKT with corresponding GAPDH bands membranes 1-2. **B)** anti p-AKT with corresponding GAPDH bands membranes 3-4 corresponding to Figures 1,2,3, and 4. Arrows indicate representative bands shown in the manuscript (Figures 1L, 2L and 3L).

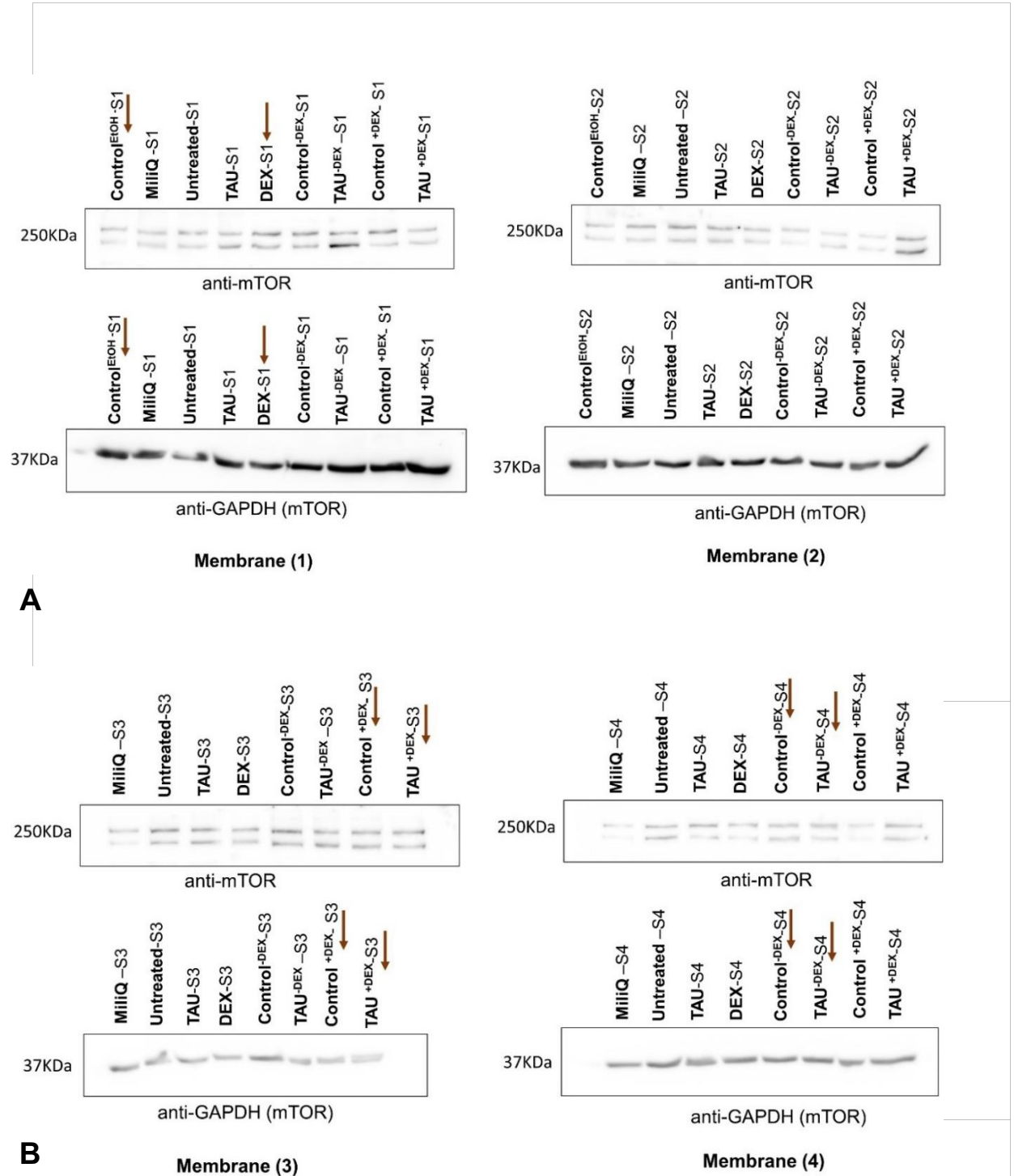
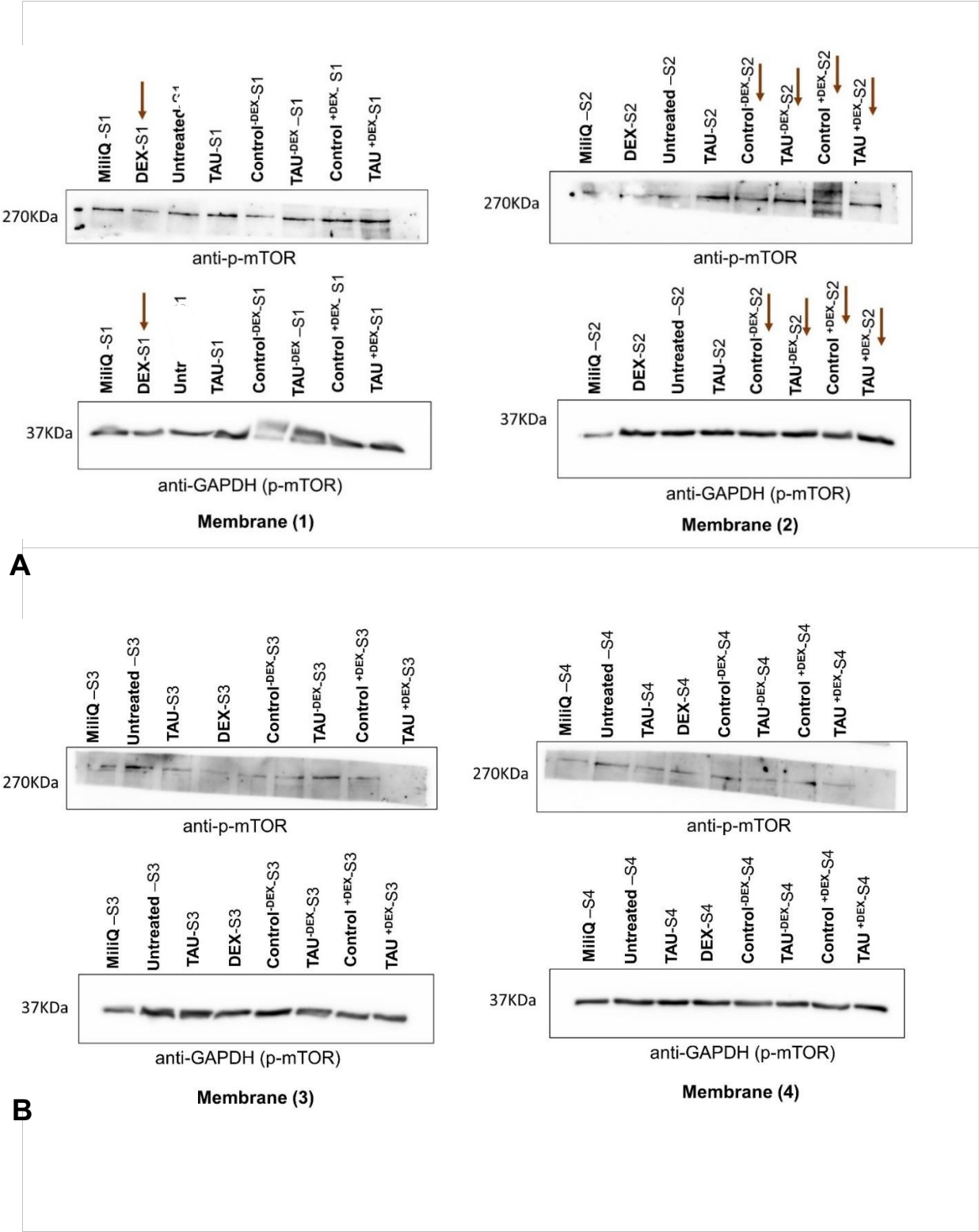
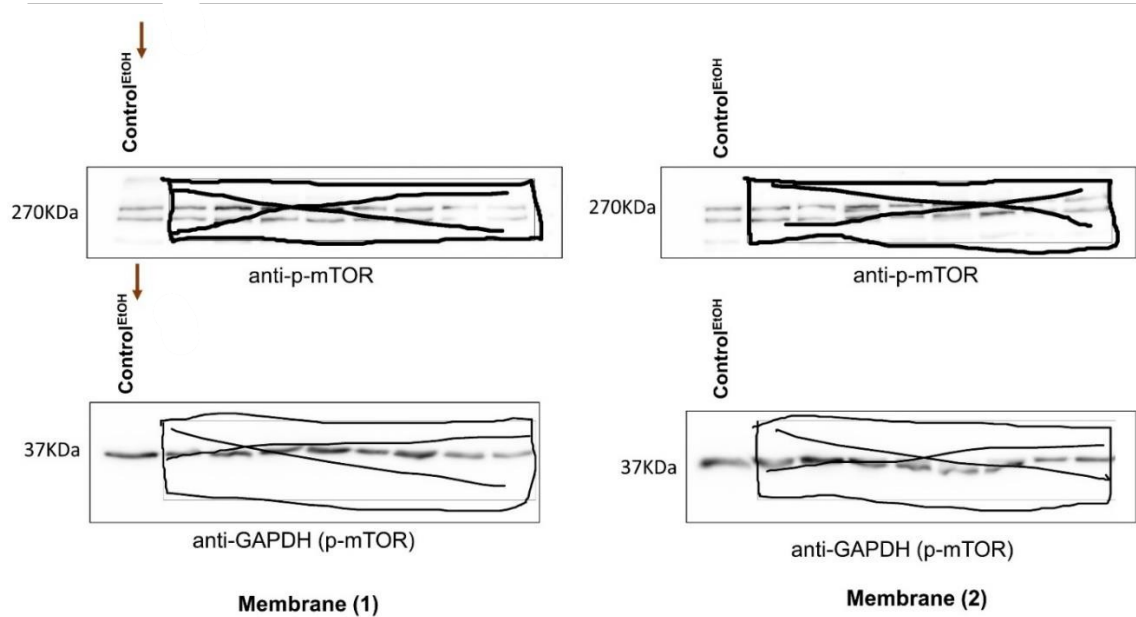


Figure S9: Uncropped western blot for anti m-TOR **A)** anti m-TOR with corresponding GAPDH bands membranes 1-2. **B)** anti m-TOR with corresponding GAPDH bands membranes 3-4 corresponding to Figures 1,2,3, and 4. Upper bands were used for m-TOR quantification. Arrows indicate representative bands shown in the manuscript (Figures 1L, 2L and 3L).





C

Figure S10: Uncropped western blot for anti p-mTOR **A)** Anti p-mTOR with corresponding GAPDH bands membranes 1-2 **B)** Anti p-mTOR with corresponding GAPDH bands membranes 3-4 corresponding to Figures 1,2,3, and 4. **C)** Anti p-mTOR with corresponding GAPDH bands membranes 1-2 3for Control^{EtOH}. The GAPDH in these membranes is the same as for AKT. After transfer, the membranes were cut, blocked, and incubated with the corresponding primary antibodies, anti-AKT or anti-p-mTOR, given their markedly different sizes. Arrows indicate representative bands shown in the manuscript (Figures 1L, 2I and 3L). p-mTOR blots for Dex and Control^{EtOH} and their quantification was not shown in the manuscript (Fig 1K-L) because the blots were on different membranes. Regardless, the quantification indicated significant difference as well.

Supplementary Table 1: Antibodies and probes

Name	Target	Working Dilution	Company
Monoclonal mouse Anti- α -Actinin (Sarcomeric)	Sarcomeric α -Actinin	1:200	Merck Life Science #A7811
Alexa Fluor™ 594 Phalloidin	F-actin	1:400	Invitrogen #A12381
Polyclonal Rabbit Glucocorticoid Receptor Primary Antibody	Glucocorticoid receptor	1:100	Invitrogen #PA1-512
Polyclonal donkey anti-mouse IgG, Alexa Fluor™ 488- conjugated secondary antibody.	Mouse IgG	1:200	Invitrogen #A21202
Polyclonal donkey anti-rabbit IgG, Alexa Fluor™ 647- conjugated secondary antibody.	Rabbit IgG	1:200	Invitrogen #A32795

Supplementary Table 2: Antibodies for Western Blots

Protein name	Host species	Antibody dilution	Size	Reference ID	Company
Phospho-mTOR (Ser2448)	Mouse	1:3500	250-279 kDa	67778-1-IG	Proteintech
mTOR	Mouse	1:2000	250 kDa	66888-1-IG	Proteintech
Phospho-Akt (Ser473)	Rabbit	1:1000	60-65 kDa	#9271	Cell Signaling Technology (Danvers, MA, USA)
AKT	Rabbit	1:1000	60 kDa	#9272	Cell Signaling Technology (Danvers, MA, USA)
GAPDH-HRP	Mouse	1:3500	37kDa	Sc-365062	Santa Cruz, Dallas (Texas, USA)

Supplementary Table 3: Primer Sequences for Gene Expression Analysis

Gene Name	TaqMan™ Gene Expression Assay (FAM) ID	Company
FOXO1	Hs00231106_m1	Thermofisher Scientific
FOXO3, FOXO3B	Hs00921424_m1	Thermofisher Scientific
GAPDH	Hs99999905_m1	Thermofisher Scientific
AKT1	Hs00178289_m1	Thermofisher Scientific
TRIM63	Hs00822397_m1	Thermofisher Scientific
FBXO32	Hs01041408_m1	Thermofisher Scientific

Chapter 3: General Discussion

Cell culture techniques have been in constant evolution from 1885 when Wilhelm Roux extracted cells from chicken embryo and successfully maintained them in a saline solution for 13 days (330). The second significant milestone in cell culture was by Harrison's efforts to grow tissue samples in test tubes while studying the growth of nerve fibers in frogs. The media composition he used included blood clots, and agar in saline solution. Harrison also introduced the hanging drop technique which involved culturing cells in plasma drops on glass slides which allowed the cells to gather in a nutrient rich environment (331). In 1916, groundwork for long-term culture was laid by Carrel and Burrows who evidenced that enriching culture media with embryo extracts helped in achieving reproducibility and longevity in cells. The ability to propagate cells indefinitely was introduced to science in 1951 by George Gey who established the HeLa cell line. In parallel, attempts to improve media composition continued (332). In the 1970s and 1980s media types, like defined and serum-free, were introduced. Gradually, researchers began to explore extracellular matrix components like laminin, collagen and Matrigel to support cell anchorage, differentiation and morphogenesis (333). Human science began to heavily rely on these monolayer cell cultures, as they were easier to maintain and gave quick results. Despite their importance, however, these two-dimensional cell culture techniques have significant limitations such as the space constraints forcing cells to grow and establish cell-cell contacts in a single dimension, forced polarity and activation of stress responses, lack of nutrients, pH, waste and gaseous gradients and absence of biomechanical cues to enable efficient differentiation (119). These concerns catalyzed efforts towards the development of 3D cell culture techniques that resembled more closely to the *in vivo* environment by allowing cell growth in three dimensions and enabling cell-ECM interactions (334). Furthermore, the use of static or dynamic perfusion systems helped in further strengthening this physiological relevance. With the advancement of technology different fabrication techniques were developed such as liquid overlay, hydrogel encapsulation, bioreactors, scaffold-mediated growth and 3-D bioprinting. Depending on the cell types, different fabrication techniques and materials are used to form functional 3D

dimensional *in vitro* tissues or organoids with a single or multiple cell types (335,336).

For studying and modelling skeletal muscle pathophysiology in different diseases, 3D tissues hold primary importance as they allow growing diverse types of cells, the scaffolds are biomimetic and can be tuned to achieve the required stiffness (337). Furthermore, the ability to introduce dynamic tension to simulate physiological stretching helps in achieving myotube formation, differentiation and contractility. These systems also allow monitoring of sarcomere organization, stimulated and spontaneous contraction as well as analyses of transcriptomic, proteomic and metabolic profiles. They also allow the ability to incorporate biocompatible sensors within the tissues or on microfluidic platforms to monitor tissue secretions in response to an external challenge real time (338). Considering the extensive benefits of 3D tissue models, it is possible to not only study diseases with unknown etiologies but also to establish disease models of already characterized diseases to find and test new therapeutic interventions. Both types of studies can be undertaken using either primary cells, immortalized progenitors or iPSCs.

Clearly, 3D *in vitro* tissues and organoids are rapidly transforming disease modelling, particularly idiopathic metabolic muscle diseases (339). These conditions have unknown etiologies and unexplained abnormalities in muscle metabolism which ultimately impact muscle structure and function. They frequently manifest as muscle weakness, fatigue, exercise intolerance, and rhabdomyolysis (340). Their unknown etiologies and pathophysiology present a significant challenge to develop therapeutic interventions and diagnostic techniques. Skeletal muscles are metabolically active, energy intensive organs capable of undergoing metabolic and structural adaptations in the presence of stress to sustain essential cellular functions. Given the central role of skeletal muscle in systemic glucose and lipid metabolism, as well as mitochondrial respiration, understanding the root of these dysfunctions requires a model that accurately reproduces structural, functional, and metabolic complexity of native muscle tissue. In idiopathic metabolic myopathies, these

metabolic pathways appear to be dysregulated without a clear cause, leading to significant functional impairments (341). Two-dimensional cultures of myotubes do not sufficiently replicate sarcomere alignment, mitochondrial organization, or the mechanical cues necessary for proper metabolic signaling. In contrast, 3D muscle-constructs formed by embedding human primary or induced pluripotent stem cell (iPSC) within extracellular matrix scaffolds such as fibrin or collagen, develop organized, contractile myofibers with robust mitochondrial networks and more physiologically accurate responses to metabolic stimuli (121). These engineered tissues support real-time assessment of mitochondrial function through oxygen consumption and glycolytic flux assays, structural analyses and quantification of real time force of contraction. One particularly well-characterized idiopathic metabolic condition is statin-induced myopathy, which affects a subset of individuals treated with cholesterol-lowering drugs. These patients often report muscle pain, weakness, and fatigue, and in rare cases, develop rhabdomyolysis. Traditional models fail to explain why only some individuals are susceptible (342). However, 3D muscle constructs exposed to statins exhibit myofiber atrophy, mitochondrial dysfunction, and increased reactive oxygen species, closely resembling patient biopsies. Co-treatment with mitochondrial-targeted antioxidants or Rho kinase inhibitors has shown potential in reversing these effects *in vitro*, offering mechanistic insights into potential therapeutic strategies (343).

This thesis investigated two distinct yet similar axes of muscle pathophysiological conditions. The first section conducted a thorough investigation of two idiopathic diseases (ME/CFS and LC-19) and reproduced extensive time-dependent profiles of both, in 3D *in vitro* skeletal muscle tissues. This is the first account of these disease models in human derived 3D *in vitro* tissues that provide key insights otherwise difficult to achieve using conventional 2D systems and even through patients' biopsies. As extensively discussed throughout the length of this work, ME/CFS and LC-19 are metabolically complicated multi-symptomatic diseases that are usually triggered by previous viral infections (169). The results showed that at 48 hours, patient tissues are suffering from energy deficit (particularly prominent in ME/CFS samples). They are actively compensating against stress by metabolic

adaptations distinct from those in LC-19 samples, but these attempts are insufficient to support contractile performance. The data from this study revealed interesting early and evolving mechanisms of skeletal muscle dysfunction induced by ME/CFS and LC-19 patient sera. The marked reduction in contractile force observed after 48 hours of exposure, especially in ME/CFS-treated tissues, appears to be the result of converging disruptions in mitochondrial bioenergetics, protein homeostasis, and inflammatory signaling. These molecular disturbances appeared to occur in a time-dependent manner, where early compensatory adaptations give way to maladaptive remodeling and catabolic stress. Both ME/CFS and LC-19 samples demonstrated upregulation of genes associated with protein translation and synthesis, yet the functional outcomes diverged. In ME/CFS, this increased translational activity was not supported by enrichment of muscle contractile or mitochondrial genes. Instead, there was a depletion of transcripts linked to sarcomeric structure and mitochondrial machinery. This suggests a misallocation of translational resources, where protein synthesis could be redirected toward stress-responsive proteins such as, chaperones or acute-phase proteins, at the expense of functional myofibrillar components. Contrary to ME/CFS samples, LC-19 tissues had sufficient enrichment of not only protein synthesis but also mitochondrial components, ETC and striated muscle adaptations. The Mito Stress test revealed a paradoxical elevation of oxygen consumption rate (OCR) and ATP-linked respiration in ME/CFS-treated tissues, which would typically imply heightened mitochondrial activity. However, rather than reflecting efficient bioenergetic output, this profile likely represents a state of mitochondrial overactivation or stress-induced hypermetabolism. An increase in protein-leak and upregulation of protein translation pathways imply that in addition to being mis-directed to protein synthesis, the ETC is producing ROS by excessive proton leak and dysregulation of $\text{Na}^+/\text{Ca}^{2+}$ homeostasis.

The early elevation of IL-6 in patient samples indicates that there are some factors in the patient sera that trigger the release of the cytokine. Its rapid upregulation in 48 hours coincides with the peak of OCR and onset of mitochondrial fragmentation, supporting a model where IL-6 acts as both a metabolic and catabolic stressor (285,289). Mechanistically, IL-6 has been shown to impair OXPHOS within

24 hours in human myotubes, induce ROS, and activate STAT3-mediated transcription of E3 ubiquitin ligases such as MuRF1 and Atrogin-1, which drive proteolysis (290,291). These actions likely converge to initiate the observed myotube thinning and loss of function over time. Over longer exposure duration, the myotubes underwent progressive myotube atrophy and mitochondrial fragmentation which was reflected by progressive decline in muscle force of contraction and reduction in RT50 values. Furthermore, gene expression analysis indicated that both diseased tissues underwent metabolic reprogramming with an increased inclination towards glycolysis.

The disease phenotypes observed with serum exposure were not reproduced by exposure to purified patient IgGs, which indicates that IgGs alone are not sufficient to induce muscle damage.

Furthermore, Raman spectroscopy analyses showed that sera from ME/CFS patients consistently led to reduced signals of key mitochondrial components, including cytochromes, amino acids, and unsaturated fatty acids which are consistent with prior reports of mitochondrial impairment in ME/CFS. Notably, sera from cryotherapy-treated patients partially restored these spectral deficits, with increased signals for lactate, glucose, and cytochromes, indicating enhanced mitochondrial function. However, the transient nature of cryotherapy's benefits, as observed in previous clinical trials, highlights the need for cautious interpretation and further longitudinal investigation (293).

The second section develops the first 3D skeletal muscle model of steroid myopathy using human derived immortalized muscle precursor cells. Corticosteroid-induced muscle atrophy is a common side effect of exogenous corticosteroids taken for the treatment of Asthma, COPD, and allergic rhinitis, skin conditions, rheumatoid arthritis, lupus, Crohn's disease, modulation of immune systems, hormonal replacement and inflammatory Bowel disease (344). It also develops because of elevated levels of endogenous corticosteroids due to underlying adrenal tumors (299).

Dexamethasone is a commonly prescribed glucocorticoid for immunosuppression, hormonal replacement and as an anti-inflammatory agent (345). It works by binding to the glucocorticoid receptor and modulating gene expression. The GCR can initiate catabolic effects by inducing the expression of Forkhead Transcription Factors O1 (FOXO1) which upregulates Atrophy F-box (MAFbx) or Atrogin-1 and Muscle Specific Ring Finger Protein 1 or MURF1, thereby upregulating protein catabolism in the muscle (296,304). GCR's anti-anabolic effects suppress the stimulatory effects of insulin, IGF-I (insulin-like growth factor-I), and amino acids on two key cellular components: eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) (305). These components directly regulate protein synthesis through the mTOR (mammalian target of rapamycin) pathway (299). This exclusive and highly coordinated interaction between catabolic and anabolic mechanisms is essential to understand how distortion of balance may impact the muscle's structural and functional capacity.

Amino acid supplementation is known to play an important role in enhancing skeletal muscle recovery, growth and functional capacity (346). Furthermore, amino acid assimilation and uptake is faster and more efficient compared to whole proteins. The semi-essential micronutrient, Taurine (TAU), is known for improving physical endurance by modulating excitation-contraction coupling in skeletal muscles and forms a crucial part of the cytoprotective mechanisms in the cell (307–309).

In this section steroid myopathic 3D invitro skeletal muscle tissues were developed by exposing healthy tissues to dexamethasone and then evaluating their contractile and structural properties to confirm muscle atrophy. The activation of atrophic pathways was reflected by a high pAKT/AKT ratio and an upregulation of gene expression for *FOXO1*, *FOXO3*, *FBXO32* and *TRIM63*. The model also showed an increased internalization of the Glucocorticoid-receptor complex in the nuclei. After validating the steroid myopathy model, two different strategies were employed to check taurine's capacity to restore muscle function, 1) By replacing dexamethasone completely from the media and adding taurine, 2) Adding taurine in the presence of dexamethasone. Taurine administration concurrently with

dexamethasone upregulated phosphorylated AKT (p-AKT) and mTOR, restoring protein synthesis and suppressing proteolytic pathways. This dual action preserved contractile strength and muscle mass despite ongoing steroid exposure. Notably, discontinuing corticosteroids alone failed to fully reverse muscle damage, whereas taurine supplementation enabled functional recovery even without dose reduction.

This doctoral thesis presents two types of disease models – idiopathic and pathognomonic (well-known diseases with symptoms that definitively diagnose them). The complex adaptation mechanisms underlying structural and functional changes in the myotubes when exposed to ME/CFS and LC-19 sera were thoroughly investigated in 3D skeletal muscle tissues in a patient specific manner. Key findings included distinct adaptation strategies in ME/CFS and LC-19 treated tissue samples. Both conditions, however, revealed a significant upregulation in protein translation. Protein synthesis is an energy-intensive process, the combination of enhanced translational activity and mitochondrial inefficiency likely creates an energy sink, further intensifying the functional deficit (347). Furthermore, the levels of IL-6 presented an interesting axis to further downstream analyze the disease progression. Steroid myopathy was successfully induced in 3D invitro skeletal muscle tissues and Taurine's restorative capacity was evaluated after identifying the mechanistic pathway of action.

While the findings of this thesis present a convincing case to bring 3D *in vitro* tissues from bench to bedside, there are limitations which present opportunities for future work. The tissue model can be made more physiologically relevant to study ME/CFS and LC-19 by introducing a co-culture with motor neurons to study the effect on neuromuscular junctions. Furthermore, immune cells can be introduced to study the impact on inflammation. It is also important to identify the nature of systemic factors which induce stress and impair function of the skeletal muscles. In subsequent studies, sample size should be increased to enable patient stratification on the basis of transcriptomic, functional, structural and metabolic phenotypes. The model can also be employed to develop efficient therapeutic interventions and diagnostic techniques.

Chapter 4: General Conclusions

Key conclusions from this thesis are presented below:

1. **Three-dimensional (3D) skeletal muscle tissue models offer superior physiological relevance for studying idiopathic metabolic muscle diseases compared to traditional two-dimensional (2D) cultures.** Unlike 2D models, 3D constructs replicate native muscle architecture, support sarcomere alignment, and enable real-time assessment of contraction and metabolic function. These models successfully demonstrated early and progressive impairments in muscle tissue exposed to sera from patients with ME/CFS and LC-19, revealing distinct adaptive responses and metabolic dysregulation not captured in simpler systems.
2. **Tissue samples treated with ME/CFS and LC-19 sera showed no significant differences in key phenotypic and transcriptomic parameters.** While the trend was statistically insignificant, the force of contraction for ME/CFS tissues compared to LC-19 were lower.
3. **Idiopathic metabolic diseases such as ME/CFS and LC-19 trigger complex, time-dependent disruptions in muscle energy homeostasis, mitochondrial integrity, and protein synthesis.** The data showed that ME/CFS samples, in particular, suffered from early energetic deficits and mitochondrial stress responses, such as hyperactivation of oxygen consumption, protein leak, and ROS production, without appropriate transcriptional support for muscle contractility. In contrast, LC-19 tissues showed a more integrated adaptive response. These findings underscore the multifactorial nature of idiopathic myopathies, where metabolic, structural, and inflammatory signals converge to cause functional decline.
4. **Steroid-induced myopathy can be effectively modeled in 3D skeletal muscle constructs, enabling the investigation of molecular mechanisms.** Dexamethasone treatment led to activation of catabolic pathways through FOXO transcription factors and downregulation of AKT levels.

5. **Taurine's potential to ameliorate steroid myopathy through AKT/mTOR pathway was modelled and evidenced in 3D skeletal muscle tissues using two supplementation strategies.** Taurine supplementation showed significant restorative effects by reactivating protein synthesis and improving muscle contractile performance, even in the continued presence of corticosteroids, providing a potential strategy to preserve muscle function during glucocorticoid therapy.
6. **This research highlights the potential of 3D skeletal muscle models as a platform for personalized disease modeling, therapeutic screening, and mechanistic studies in idiopathic and pathognomonic muscle diseases.** These models enabled the detection of subtle, patient-specific responses to patient sera or controlled inducing of disease condition. The profiling included transcriptomic shifts, cytokine signaling (e.g., IL-6 elevation), mitochondrial dysfunction, structural analyses, and mapping receptor translocation to the nucleus. The work paves the way for future improvements, including co-culture with motor neurons and immune cells, stratification of patient subgroups, and development of precision interventions.

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Appendices

Appendix 1: Coding Scripts

1. Force Analyses

Using ImageJ/Fiji to extract stack profile data from videos

#Caution to be taken while drawing the line or rectangular selection on the pillar's edge

```
macro "Stack profile Data" {
    if      (!(selectionType()==0      ||      selectionType==5      ||
selectionType==6))

        exit("Line or Rectangle Selection Required");

    setBatchMode(true);

    run("Plot Profile");
    Plot.getValues(x, y);
    run("Clear Results");
    for (i=0; i<x.length; i++)
        setResult("x", i, x[i]);
    close();

    n = nSlices;
    for (slice=1; slice<=n; slice++) {
        showProgress(slice, n);
        setSlice(slice);
        profile = getProfile();
        sliceLabel = toString(slice);
        sliceData = split(getMetadata("Label"), "\n");
        if (sliceData.length>0) {
```

```

        line0 = sliceData[0];
        if (lengthOf(sliceLabel) > 0)
            sliceLabel = sliceLabel+ " (" + line0 + ")";
    }
    for (i=0; i<profile.length; i++)
        setResult(sliceLabel, i, profile[i]);
}

setBatchMode(false);

updateResults;
}

```

Using Python x Jupyter Notebook in Visual Studio to quantify and extract Contractile force

Step1: Generating Results File

Importing libraries

```

import matplotlib
import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
import seaborn as sns
import scipy
import tkinter as tk
from tkinter.filedialog import askdirectory
import os
from scipy.ndimage import uniform_filter1d
from matplotlib.pyplot import figure
import math

```


#Generation of Results. CSV files for each data file. It is important to check that the Frames per second in the script are the same as in the original video recording. It is also important to check that the pixels to μm scale correspond to the magnification of the videos.

```
path = askdirectory(title='Select Folder') # shows dialog box
and return the path

files = next(os.walk(path))[2]

for file in files:

    directory = path + "/" + file

    print(directory)

    df = pd.read_csv(directory) #read file

    values = []

    time = []

    for (columnName, columnData) in df.items(): #find position
at each frame

        max_value = df[columnName].max()

        values.append((df.loc[df[columnName] == max_value
] ['x'].mean()))

    df1= pd.DataFrame()

    df1['pixels'] = values[2:]

    df1['frames']= range(1, len(df.columns)-1)

    df1['time (sec)'] = df1['frames']/82.28 #check fps

    df1['distance (pixels)'] = abs(df1["pixels"] -
df1["pixels"][0:400].mean()) #subtract mean initial values

    df1['distance (uM)'] = df1['distance (pixels)'] / 0.4626
#pixels to um (scale in imagej from .czi videos
1pixel=0.4626um)

    df1['distance (uM) smooth'] =
uniform_filter1d(df1['distance (uM)'],size=15)
```

```
df1.to_csv(path+"/"+ "result"+file) #save files at the same
folder but with a "result" at the beginning (change this as
you wish)
```

Step 2: Graphical Representations

Plotting each result file as generated in the previous script

```
path = askdirectory(title='Select Folder') # shows dialog box
and return the path
```

```
files = next(os.walk(path))[2]
```

```
x= 0
```

```
figure(figsize=(100, 10), dpi=80)
```

```
for file in files:
```

```
    directory = path + "/" + file
```

```
    data = pd.read_csv(directory) #read file
```

```
    sns.set_theme(style="ticks", font_scale=1.7)
```

```
    g = sns.lineplot(x="time (sec)",y="distance (uM) smooth",
data=data, label=file)
```

```
    plt.xlabel("Time (seconds)")
```

```
    plt.ylabel("Displacement (\u03bcm)")
```

```
    # g.set(ylim=(0, 0.000005))
```

```
    # g.set(xlim=(46, 65))
```

```
g.figure.set_size_inches(8, 4)
```

```
sns.despine()
```

```
x=x+1
```

```
if x == 1:
```

```
plt.legend(bbox_to_anchor=(2, 1.05))
```

```
plt.show()
```

```
figure(figsize=(100, 10), dpi=80)
```

```
x = 0
```

Step 3: Normalizing tissue area (Tissue position on the pillar, Hydrogel cross sectional area and effective cross-sectional area calculated using Cellpose previously)

```
# Assuming coord is the existing DataFrame
```

```
# coord = ...
```

```
# Here, the cross-sectional area is approximated based on the
z-positions of the .txt file. The position of the tissue was
approximated by manually changing the z-axis to identify the
top, middle and bottom of the tissue. Also, the height of the
tissue on the pillar, is estimated (av_dist_basetissue)
```

```
# Check if the required columns exist in the coord DataFrame
```

```
required_columns = ['sample', 'replicate', 'name',
                    'top_tissue', 'bottom_tissue', 'middle_tissue', 'top_pillar']
```

```
missing_columns = [col for col in required_columns if col not
                    in coord.columns]
```

```
if missing_columns:
```

```
    print(f"Missing columns in coord DataFrame:
{missing_columns}")
```

```
else:
```

```
    df = pd.DataFrame()
```

```
    df['Condition'] = coord['sample']
```

```
    df['Replicate'] = coord['replicate']
```

```
    df['name'] = coord['name']
```

```

                                df['cross-section1']      =
list(math.pi*((abs(coord['top_tissue']
                                coord['bottom_tissue']))/2)**2)
                                -

                                df['cross-section2']      =
list(math.pi*(abs(coord['top_tissue']
                                coord['middle_tissue'])**2))
                                -

                                df['cross-section3']      =
list(math.pi*(abs(coord['bottom_tissue']
                                coord['middle_tissue'])**2))
                                -

                                df['cross_section']      = list((df['cross-section1'] +
df['cross-section2'] + df['cross-section3'])/3)

                                df['dist_basetissue1'] = list(3250 - ((coord['top_pillar']
- ((coord['top_tissue'] + coord['bottom_tissue'])/2))))

                                df['dist_basetissue2'] = list(3250 - ((coord['top_pillar']
- coord['middle_tissue'])))

                                df["av_dist_basetissue"] = list((df["dist_basetissue1"] +
df["dist_basetissue2"])/2)

#Saving the data in a .csv file. Can also be done interactively
using the tkinter function (import tkinter as tk)

Example:      df.to_csv(r'\\10.5.119.12\home\Projects\Chronic
Fatigue Syndrome and Long COVID-19\BP-IgG Exps\E1 and
E2\Displacement\E1 and E2_eCSA.csv')

#Loading the data

all_coord= pd.read_csv(r'\\10.5.119.12\home\Projects\Chronic
Fatigue Syndrome and Long COVID-19\BP-IgG Exps\E1 and
E2\Displacement\E1 and E2_eCSA\EPS_1_2_eCSA.csv')

#Checking data frame

all_coord

Step 4: Calculating Force, Specific Force and Displacement for
each data file.

# Saves in the same result files

#Here, the CSA can be changed use the estimated cross-sectional
area () "cross_section" column in the all_coord file), whole

```

CSA or eCSA (eCSA data has to be manually added in the all_coord file from the Cellpose generated data)

#The force measurements take into account the "a" in the displacement to force formula, and the dimensions of the standard casting molds

```
path = askdirectory(title='Select Folder') # shows dialog box
and return the path
```

```
files = next(os.walk(path))[2]
```

```
E = 1.6 #young's modulus (MPa)
```

```
for file in files:
```

```
    for x in all_coord["name"]:
```

```
        if file[6:-4] == x:
```

```
            print(file)
```

```
            directory = path + "/" + file
```

```
            df = pd.read_csv(directory)
```

```
                a = float(all_coord[all_coord.name ==
x]["av_dist_basetissue"])
```

```
                csa = float(all_coord[all_coord.name == x]["CSA"])
```

```
                    df['force (uN)'] =
(6*math.pi*1.6*pow(800,4))/(64*pow(a,2)*(3*3250-
a))*df['distance (uM) smooth']
```

```
                    df["specific force (uN/mm2)"] = (df['force
(uN)']/csa)/0.000001
```

```
                    df.to_csv(directory)
```

Step 5: Calculating Maximum Values

```
path = askdirectory(title='Select Folder') # select folder with
"result..." files
```

```
files = next(os.walk(path))[2]
```

```
forces_all = pd.DataFrame()
```

```

fps = 82.28

time = [0,20,40,60,80,100] #change if the timings are different

freq = ["1Hz","2Hz","5Hz","10Hz","25Hz","50Hz"]

all = dict(zip(time, freq))

for file in files:

    forces = pd.DataFrame()

    directory = path + "/" + file

    data = pd.read_csv(directory) #read file

# Can be modified based on the file name and the information
needed

    forces['Condition'] = [file[6:-7]]

    forces['Replicate'] = [file[-6:-4]]


    for t,f in all.items(): #t = time f =freq

        df = pd.DataFrame()

                                largest =
data.iloc[int(t*fps):int((t+20)*fps)][ 'distance (uM)
smooth'].nlargest(20) #find higher values in each frequency

        for item,n in largest.items():

                                df =
pd.concat([df,data.iloc[item].to_frame().transpose()],
ignore_index=True)

                                force = df['distance (uM) smooth'].max()-
data.iloc[int((t+3)*fps):int((t+9)*fps)][ 'distance (uM)
smooth'].mean() #subtract the baseline value

        forces[f] = [force]

        forces['tetanic_index'] = ((forces['50Hz']+
forces['25Hz'])/2)/ ((forces['1Hz']+ forces['2Hz'])/2)

    forces_all= pd.concat([forces_all,forces])

```

```
forces_all.to_csv((path+"/"+ "max_displacement.csv"))
print(forces_all)
```

Step 6: Graphing Maximum Values

All new files generated for maximum values (maximum_specific_force.csv) have to be moved to a new folder and then navigated to plot each of the file.

```
data = pd.read_csv(r'\\10.5.119.12\home\Projects\Chronic
Fatigue Syndrome and Long COVID-19\BP-IgG Exps\E1 and
E2\Displacement\E1 and E2_eCSA\Data\Results\Max
values\max_displacement.csv')
```

```
freqs = ["1Hz", "2Hz", "5Hz", "10Hz", "25Hz", "50Hz", "tetanic_index"]
```

```
color = ["darkkhaki", "goldenrod", "darkolivegreen",
"lightblue", "steelblue", "lightslategray"]
```

```
for i in freqs:
```

```
# Draw the bar chart
```

```
    ax = sns.barplot(
        data=data,
        x="Condition", y=i, palette=color,
        alpha=.7,
        errorbar="sd", capsize = 0.03, errwidth = 0.8,
    )
```

```
# Get the legend from just the bar chart
```

```
handles, labels = ax.get_legend_handles_labels()
```

```
# Draw the stripplot
```

```
sns.stripplot(
    data=data,
    x="Condition", y=i,
```

```

        dodge=True,                edgecolor="black", palette=color,
        linewidth=.75,
        ax=ax,
    )

    # Adjust the bar chart legend back

    # ax.set_ylim([0, 3])

    plt.ylabel(" max displacement at "+i , fontdict={'family':
'arial'},fontweight='bold')

    plt.xlabel("",fontdict={'family': 'arial'})

    # plt.ticklabel_format(axis="y", style="sci",
scilimits=(0,0))

    sns.despine()

    plt.show()

```

Step 7: Data Grouping (of technical replicates) for maximum values and plotting per biological replicate (serum)

```

import pandas as pd

import os

import matplotlib.pyplot as plt

import seaborn as sns

from tkinter.filedialog import askopenfilename

# Step 1: Allow opening a .csv file interactively

file_path = askopenfilename(title="Select a CSV File",
filetypes=[("CSV Files", "*.csv")])

if not file_path:

    raise FileNotFoundError("No file selected!")

```



```

# Load the data
data = pd.read_csv(file_path)

# Step 2-12: Map conditions to their respective groups
group_mapping = {
    "A": "HC_01", "B": "HC_02", "C": "HC_03", "D": "HC_04",
    "E": "HC_05", "F": "Untreated", "G": "ME_01", "H": "ME_02",
    "I": "ME_03", "J": "ME_04", "K": "ME_05"
}

data["Group"] = data["Condition"].map(group_mapping)

# Step 3: Ensure required columns exist
required_columns = ["Condition", "1Hz", "2Hz", "5Hz", "10Hz",
                    "25Hz", "50Hz"]

if not all(col in data.columns for col in required_columns):
    raise ValueError(f"The input CSV must contain the following
columns: {'', '.join(required_columns)}")

# Step 4: Compute averages for each group and frequency
(without SDs)

grouped = data.groupby("Group").agg(
    {"1Hz": "mean", "25Hz": "mean", "50Hz": "mean"}
).reset_index()

# Flatten the column names
grouped.columns = ["Group", "1Hz Mean", "25Hz Mean", "50Hz
Mean"]

```

```

# Step 5: Save the grouped data to a new Excel file in the same
directory as the original file

output_file      =      os.path.join(os.path.dirname(file_path),
"Grouper_Data.xlsx")

grouped.to_excel(output_file, index=False)


print(f"Grouper data saved as {output_file}.")


# Step 6: Plot bar charts for each frequency (1Hz, 25Hz, 50Hz)
without SDs

def plot_bar_chart(data, frequency, output_file):
    # Create the bar chart for the given frequency

    plt.figure(figsize=(10, 6))

    sns.barplot(
        x="Group",
        y=f"{frequency} Mean",
        data=data,
        palette="viridis"
    )

    plt.title(f"{frequency} Mean Values", fontsize=16)
    plt.xlabel("Group", fontsize=12)
    plt.ylabel(f"{frequency} Mean", fontsize=12)
    plt.xticks(rotation=45)
    plt.tight_layout()

    # Save the plot

```

```

plt.savefig(output_file)

plt.close()

# Plot bar charts for each frequency (1Hz, 25Hz, 50Hz) without
SDs

plot_bar_chart(grouped, "1Hz",
os.path.join(os.path.dirname(file_path),
"1Hz_Bar_Chart.png"))

plot_bar_chart(grouped, "25Hz",
os.path.join(os.path.dirname(file_path),
"25Hz_Bar_Chart.png"))

plot_bar_chart(grouped, "50Hz",
os.path.join(os.path.dirname(file_path),
"50Hz_Bar_Chart.png"))

print("Bar charts saved as 1Hz_Bar_Chart.png,
25Hz_Bar_Chart.png, and 50Hz_Bar_Chart.png.")

```

Step 8: Grouping on the basis of Conditions/ Treatment Groups

```

import pandas as pd

import os

import matplotlib.pyplot as plt

import seaborn as sns

from tkinter.filedialog import askopenfilename

# Allow opening a .csv file interactively

file_path = askopenfilename(title="Select a CSV File",
filetypes=[("CSV Files", "*.csv")])

if not file_path:

    raise FileNotFoundError("No file selected!")

```

```

# Load the data

data = pd.read_csv(file_path)


# Map conditions to their respective groups

group_mapping = {

    "A": "Healthy Control IgGs", "B": "Healthy Control IgGs",
    "C": "Healthy Control IgGs", "D": "Healthy Control IgGs",

    "E": "Healthy Control IgGs", "F": "Untreated", "G": "ME
IgGs", "H": "ME IgGs",

    "I": "ME IgGs", "J": "ME IgGs", "K": "ME IgGs"

}

data["Group"] = data["Condition"].map(group_mapping)


# Ensure required columns exist

required_columns = ["Condition", "1Hz", "2Hz", "5Hz", "10Hz",
"25Hz", "50Hz"]

if not all(col in data.columns for col in required_columns):

    raise ValueError(f"The input CSV must contain the following
columns: {'', '.join(required_columns)}")


# Compute averages for each group and frequency (without SDs)

grouped = data.groupby("Group").agg(

    {"1Hz": "mean", "25Hz": "mean", "50Hz": "mean"}

).reset_index()


# Flatten the column names

```

```

grouped.columns = ["Group", "1Hz Mean", "25Hz Mean", "50Hz
Mean"]

# Save the grouped data to a new Excel file in the same
directory as the original file

output_file      =      os.path.join(os.path.dirname(file_path),
"Groupped_Data.xlsx")

grouped.to_excel(output_file, index=False)

print(f"Groupped data saved as {output_file}.")

# Plot bar charts for each frequency (1Hz, 25Hz, 50Hz) without
SDs

def plot_bar_chart(data, frequency, output_file):
    # Create the bar chart for the given frequency

    plt.figure(figsize=(10, 6))

    sns.barplot(
        x="Group",
        y=f"{frequency} Mean",
        data=data,
        palette="viridis"
    )

    plt.title(f"{frequency} Mean Values", fontsize=16)
    plt.xlabel("Group", fontsize=12)
    plt.ylabel(f"{frequency} Mean", fontsize=12)
    plt.xticks(rotation=45)
    plt.tight_layout()

```

```

# Save the plot

plt.savefig(output_file)

plt.close()

# Plot bar charts for each frequency (1Hz, 25Hz, 50Hz) without
SDs

plot_bar_chart(grouped, "1Hz",
os.path.join(os.path.dirname(file_path),
"1Hz_Bar_Chart.png"))

plot_bar_chart(grouped, "25Hz",
os.path.join(os.path.dirname(file_path),
"25Hz_Bar_Chart.png"))

plot_bar_chart(grouped, "50Hz",
os.path.join(os.path.dirname(file_path),
"50Hz_Bar_Chart.png"))

print("Bar charts saved as 1Hz_Bar_Chart.png,
25Hz_Bar_Chart.png, and 50Hz_Bar_Chart.png.")

```

2. Calculating myotube Diameter and Cross-sectional Area using Cellpose

Export single Channel .TIFF files for each channel to be used (SAA, F-actin). Can be done using batch mode in ZEN software.

Step 1: Rename all generated overlays to add _label.png at the end using the following python code:

```

import os

import tkinter as tk

from tkinter import filedialog

def remove_suffix_from_files(folder_path):
    suffix = "_label_cp_masks.png"
    for filename in os.listdir(folder_path):
        if filename.endswith(suffix):

```

```

        # New filename after removing the suffix
        new_filename = filename[:-len(suffix)] +
"_label.png"    # Removing "_label_cp_masks.png" and keeping
".png"

        # Full paths
        old_file = os.path.join(folder_path, filename)
        new_file = os.path.join(folder_path, new_filename)

        # Rename the file
        os.rename(old_file, new_file)

        print(f"Renamed: {filename} -> {new_filename}")

def open_folder_and_rename_files():
    # Open a dialog to choose a folder
    folder_selected = filedialog.askdirectory()

    if folder_selected:
        remove_suffix_from_files(folder_selected)
    else:
        print("No folder selected")

# GUI for folder selection
root = tk.Tk()

root.withdraw()    # Hide the main Tkinter window

# Call the function to open the folder dialog and rename files
open_folder_and_rename_files()

```

Use Image J/FIJI LabelstoROIs Generator

Finally, the generated PNG masks were saved in the same directory as the corresponding TIFF files, and the *LabelstoROIs* plugin was launched in FIJI. By loading the folder, multiple images were selected and

processed in batch mode. For each TIFF file, the analysis produced a CSV file containing the selected measurements for each detected particle, a JPEG image displaying the segmented myotube outlines, and a comprehensive results table based on the specified pixel erosion setting (set to 0 px for this work). Output will be the .CSV and .JPEG files.

ImageJ/ FIJI macro to calculate effective cross-sectional Area (eCSA). It can be done using .TIFF files from either SAA, F-actin or any other border protein.

```
// Select the directory containing the images

dir = getDirectory("Choose a Directory");

// Debug output: check the constructed directory

print("Selected Directory: " + dir);

setBatchMode(true); // Improve performance by running in batch
mode

// Get all image files in the directory

list = getFileList(dir);

// Loop through all files in the directory
for (i = 0; i < list.length; i++) {
    // Only process .tif files
    if (endsWith(list[i], ".tif")) {
        open(dir + list[i]); // Open the image file

        // Process the image
```



```

run("Median...", "radius=1");

run("Auto Threshold", "method=Default white");

run("Fill Holes");

run("Close-");

setOption("BlackBackground", true);

run("Convert to Mask");

run("Set Measurements...", "area standard feret's
display add redirect=None decimal=3");

// Analyze particles

run("Analyze Particles...", "size=3-Infinity display
include summarize overlay");

// Save overlay image as JPEG

overlayImageName = dir + "overlay_" +
list[i].replace(".tif", ".jpeg");

saveAs("jpeg", overlayImageName);

// Extract measurements from the results table

area = getResult("Area", 0); // Get the area of the
first particle

feretsDiameter = getResult("Feret's", 0); // Get
Feret's diameter of the first particle

// Check if measurements are valid

if (isNaN(area)) {

    area = 0; // Use 0 for no measurement

}

```

```

        if (isNaN(feretsDiameter)) {
            feretsDiameter = 0; // Use 0 for no measurement
        }

        // Debug output: print the results for verification
        print("Processed: " + list[i] + ", Area: " + area + ",
Feret's Diameter: " + feretsDiameter);

        // Close the image
        close();
    }
}

```

```

// Clean up
setBatchMode(false);

```

3. Mitochondrial Network Analyses

Export PNG files

Step 1: Use the MitoAnalyser macro to pre-process images and analyze skeleton

```

// Set the path to your images
inputFolder = getDirectory("Choose a Directory");

// List all PNG files in the directory
list = getFileList(inputFolder);
setBatchMode(true);

```

```

// Loop through all files
for (i = 0; i < list.length; i++) {
    fileName = list[i];

    // Skip non-PNG files
    if (endsWith(fileName, ".png")) {
        open(inputFolder + fileName);

        // Apply median filter
        run("Median...", "radius=1");

        // Thresholding (Example: Otsu method for binary
thresholding)
        setAutoThreshold("Otsu dark");
        run("Convert to Mask");

        // Run Analyze Skeleton
        run("Analyze Skeleton (2D/3D)", "flood=0");

        // Save the results
        saveAs("Results", inputFolder + "Results_" + fileName
+ ".csv");

        // Close the current image
        close();
    }
}

```

```

}

// Return to normal mode
setBatchMode(false);

Save all the generated files in a separate folder

Averaging and plotting
import pandas as pd
import tkinter as tk
from tkinter import filedialog

# Create a Tkinter root window (it won't be shown)
root = tk.Tk()
root.withdraw() # Hide the root window

# Open a file dialog to select the CSV file
file_path = filedialog.askopenfilename(
    title="Select the CSV File",
    filetypes=[("CSV files", "*.csv")]
)

# Check if a file was selected
if not file_path:
    print("No file selected. Exiting.")
    exit()

```

```

# Load the selected CSV file

df = pd.read_csv(file_path)


# Define groups and labels

groups = {

    'HC_IgG_01': [

        'Results_A_03_01.csv',          'Results_A_03_02.csv',
        'Results_A_03_03.csv',          'Results_A_03_04.csv',
        'Results_A_03_05.csv',          'Results_A_03_06.csv',
        'Results_A_04_01.csv',          'Results_A_04_02.csv',
        'Results_A_04_03.csv'

    ],

    'HC_IgG_02': [

        'Results_B_01_01.csv',          'Results_B_01_02.csv',
        'Results_B_01_03.csv',          'Results_B_01_04.csv',
        'Results_B_01_05.csv',          'Results_B_03_01.csv',
        'Results_B_03_02.csv',          'Results_B_03_03.csv',
        'Results_B_03_04.csv',          'Results_B_03_05.csv',
        'Results_B_03_06.csv'

    ],

    'HC_IgG_03': [

        'Results_C_02_01.csv',          'Results_C_02_02.csv',
        'Results_C_02_03.csv',
        'Results_C_02_04.csv'

    ],

    'HC_IgG_04': [

```

```

        'Results_D_02_01.csv',          'Results_D_02_02.csv',
'Results_D_02_03.csv'

    ],

    'HC_IgG_05': [

        'Results_E_02_01.csv',          'Results_E_02_02.csv',
'Results_E_02_03.csv',

        'Results_E_02_04.csv',          'Results_E_02_05.csv',
'Results_E_02_06.csv',

        'Results_E_02_07.csv',          'Results_E_02_08.csv',
'Results_E_02_09.csv'

    ],

    'Untreated': [

        'Results_F_01_01.csv',          'Results_F_01_02.csv',
'Results_F_02_01.csv',

        'Results_F_03_01.csv',          'Results_F_03_02.csv',
'Results_F_03_03.csv',

        'Results_F_03_04.csv', 'Results_F_03_05.csv'

    ],

    'ME_IgG_01': [

        'Results_G_01_01.csv',          'Results_G_01_02.csv',
'Results_G_01_03.csv',

        'Results_G_01_04.csv',          'Results_G_01_05.csv',
'Results_G_01_06.csv',

        'Results_G_01_07.csv', 'Results_G_01_08.csv'

    ],

    'ME_IgG_02': [

        'Results_H_01_01.csv',          'Results_H_01_02.csv',
'Results_H_02_01.csv',

        'Results_H_02_02.csv',          'Results_H_02_03.csv',
'Results_H_02_04.csv',

```

```

        'Results_H_02_05.csv',
'Results_H_02_07.csv',

        'Results_H_04_01.csv',
'Results_H_04_03.csv',

        'Results_H_04_04.csv'

    ],

    'ME_IgG_03': [

        'Results_I_01_01.csv',
'Results_I_01_03.csv',

        'Results_I_01_04.csv'

    ],

    'ME_IgG_04': [

        'Results_J_01_01.csv',
'Results_J_02_02.csv',

        'Results_J_02_03.csv',
'Results_J_02_05.csv',

        'Results_J_02_06.csv',
'Results_J_02_08.csv',

        'Results_J_03_01.csv'

    ],

    'ME_IgG_05': [

        'Results_K_03_01.csv',
'Results_K_04_02.csv',

        'Results_K_04_03.csv',
'Results_K_04_05.csv',

        'Results_K_04_06.csv',
'Results_K_04_08.csv',

        'Results_K_04_09.csv'

    ]

}

```

```

results = []

# Process each group
for label, files in groups.items():

    # Filter the DataFrame for the current group
    group_df = df[df['File Name'].isin(files)]

    # Calculate average and standard deviation
    avg_branch_length = group_df['File Average Branch
Length'].mean()

    sd_branch_length = group_df['File Average Branch
Length'].std()

    # Store the results
    results.append({
        'Group Label': label,
        'Average Branch Length': avg_branch_length,
        'Standard Deviation': sd_branch_length
    })

# Convert results to a DataFrame
results_df = pd.DataFrame(results)

# Save the results to a CSV file
output_file = 'MitoIgGSummary.csv'

```



```
results_df.to_csv(output_file, index=False)
```

```
print(f"Results have been saved to {output_file}")
```

4. Transcriptomics

1. Gene Ontology using gProfiler

Navigate to .CSV file of Differentially expressed gene set without the version numbers (ENSEMBL IDs)

#Libraries

```
!pip install gprofiler-official
```

```
import pandas as pd
```

```
import numpy as np
```

```
import tkinter as tk
```

```
from tkinter import filedialog
```

```
import matplotlib.pyplot as plt
```

```
import seaborn as sns
```

```
from gprofiler import GProfiler
```

```
# Initialize Tkinter for file selection
```

```
root = tk.Tk()
```

```
root.withdraw()
```

```
# Open file dialog to select CSV file
```

```
file_path = filedialog.askopenfilename(title="Select Gene List  
CSV", filetypes=[("CSV Files", "*.csv")])
```

```
if not file_path:
```

```
    print("No file selected. Exiting.")
```

```

exit()

# Load the gene list (assuming genes are in the first column)
df = pd.read_csv(file_path)
genes = df.iloc[:, 0].dropna().tolist() # Remove NaN values

# Perform GO enrichment analysis
gp = GProfiler(return_dataframe=True)
go_results = gp.profile(organism="hsapiens", query=genes)

# Save results
output_file = "GO_enrichment_results.csv"
go_results.to_csv(output_file, index=False)

print(f"GO enrichment analysis completed. Results saved to
{output_file}.")

# Filter results for BP, CC, and MF
go_bp = go_results[go_results["source"] == "GO:BP"].sort_values("p_value").head(35)

go_cc = go_results[go_results["source"] == "GO:CC"].sort_values("p_value").head(35)

go_mf = go_results[go_results["source"] == "GO:MF"].sort_values("p_value").head(35) #35 to display top
35 GO terms, can be modified as needed

# Function to create a horizontal bubble plot
def plot_go_enrichment(go_data, title, filename):
    plt.figure(figsize=(9, 8))

```

```

# Sorting so lowest p-value is at the top
go_data = go_data.sort_values("p_value")

# Generate y-labels with GO terms and codes
y_labels = go_data["name"] + " (" + go_data["native"] +
")"

# Bubble size corresponds to number of genes in the pathway
bubble_size = go_data["intersection_size"] * 20 # Scale
factor for visibility

# Scatter plot (bubble plot)
scatter = plt.scatter(
    -np.log10(go_data["p_value"]), # X-axis: -log10(p-
value)
    y_labels, # Y-axis: Pathway names
    s=bubble_size, # Bubble size corresponds to number of
genes in pathway
    c=-np.log10(go_data["p_value"]), # Color based on -
log10(p-value)
    cmap="twilight", # Color map
    alpha=0.6, # Transparency
)

# Titles and labels
plt.xlabel("-log10(p-value)")
plt.ylabel("GO Terms (with GO ID)")

```

```

plt.title(title)

# Add color bar
cbar = plt.colorbar(scatter)
cbar.set_label("-log10(p-value)")

# Save plot
plt.tight_layout()
plt.savefig(filename, dpi=300, bbox_inches="tight")
plt.show()

# Generate and save the three plots
plot_go_enrichment(go_bp, "Top 35 GO:BP Enrichment Terms",
"GO_BP_bubble_plot.png")
plot_go_enrichment(go_cc, "Top 35 GO:CC Enrichment Terms",
"GO_CC_bubble_plot.png")
plot_go_enrichment(go_mf, "Top 35 GO:MF Enrichment Terms",
"GO_MF_bubble_plot.png")

```

2. Volcano plots for Differential Gene expression

```

import pandas as pd
import numpy as np
import matplotlib.pyplot as plt
import seaborn as sns
from tkinter import Tk, filedialog

# Allow interactive file selection

```

```

Tk().withdraw() # Hide main Tkinter window

file_path = filedialog.askopenfilename(title="Select the
Differential Expression CSV File",

                                         filetypes=[("CSV files",
".*.csv"), ("Text files", "*.txt")])

# Check if a file was selected
if not file_path:
    print("No file selected. Exiting.")
    exit()

# Load the dataset
df = pd.read_csv(file_path)

# Ask the user for column names
log2fc_col = input("Enter the column name for Log2 Fold Change:
")

pval_col = input("Enter the column name for p-value (or
adjusted p-value): ")

gene_col = input("Enter the column name for Gene Names: ")

# Thresholds
log2fc_threshold = 1 # Change if needed
pval_threshold = 0.05 # Change if needed

# Add a column for significance categorization
df['Significance'] = 'Not Significant'

```

```

df.loc[(df[log2fc_col] > log2fc_threshold) & (df[pval_col] <
pval_threshold), 'Significance'] = 'Upregulated'

df.loc[(df[log2fc_col] < -log2fc_threshold) & (df[pval_col] <
pval_threshold), 'Significance'] = 'Downregulated'

# Select top 3 upregulated and top 3 downregulated genes

top_up = df[df['Significance'] == 'Upregulated'].nlargest(3,
log2fc_col)

top_down = df[df['Significance'] ==
'Downregulated'].nsmallest(3, log2fc_col)

# Plotting the volcano plot

plt.figure(figsize=(10, 6))

# Scatter plot for regular points

sns.scatterplot(data=df, x=log2fc_col, y=-
np.log10(df[pval_col]), hue='Significance',

palette= { 'Upregulated': 'Crimson',
'Downregulated': 'Teal', 'Not Significant': 'grey'},
alpha=0.6, s=30)

# Scatter plot for top upregulated and downregulated points
(larger with black borders)

sns.scatterplot(data=top_up, x=log2fc_col, y=-
np.log10(top_up[pval_col]), color='#D73027',
edgecolor='black', s=100, linewidth=1.5, label="Top
Upregulated")

sns.scatterplot(data=top_down, x=log2fc_col, y=-
np.log10(top_down[pval_col]), color='#1A9850',
edgecolor='black', s=100, linewidth=1.5, label="Top
Downregulated")

```

```

# Label the top genes

for _, row in top_up.iterrows():

    plt.text(row[log2fc_col], -np.log10(row[pval_col]),
row[gene_col], fontsize=9, ha='right', color='black')

for _, row in top_down.iterrows():

    plt.text(row[log2fc_col], -np.log10(row[pval_col]),
row[gene_col], fontsize=9, ha='left', color='black')

# Draw threshold lines

plt.axhline(-np.log10(pval_threshold), linestyle='--',
color='black', alpha=0.7, label=f"p = {pval_threshold}")

plt.axvline(log2fc_threshold, linestyle='--', color='black',
alpha=0.7, label=f"log2FC = ±{log2fc_threshold}")

plt.axvline(-log2fc_threshold, linestyle='--', color='black',
alpha=0.7)

# Labels and title

plt.xlabel("Log2 Fold Change", fontsize=12)

plt.ylabel("-Log10 p-value", fontsize=12)

plt.title("Volcano Plot of Differential TE Expression",
fontsize=14)

plt.legend(title="TE Regulation", loc='upper right')

# Show the plot

plt.show()

```

3.3. Transposable Elements overlap and Annotation Scripts

Having the right directory/folder open in the environment is important.

```
cd "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs CNT/CFS vs CNT
DETs DOWN"
```

Using BedTools

Overlap between genes and DEts (WSL Conda Powershell in Administrative mode)

```
bedtools intersect -a "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs
CNT/CFS vs CNT DEts DOWN/genes.bed" \
```

```
-b "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs CNT/CFS vs CNT
DEts DOWN/CFS vs CNT DEts DOWN locations.bed" \
```

```
> "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs CNT/CFS vs CNT
DEts DOWN/DOWNoverlap_output.bed"
```

(Sometimes the .BED conversion might give problems, it will seem okay but perhaps because of some spacing between columns.

Optional: In case it happens, use the following troubleshooting script:

Objective:

1. Ensure the file is **tab-delimited**.
2. Ensure the chromosome coordinates (columns 2 and 3) are **integer values** (no non-numeric values).
3. Verify the file is correctly formatted for bedtools.

Find intersection by Running bedtools intersect

```
bash
```

```
bedtools intersect -a "/mnt/c/Users/smughal/Downloads/DETs and
DEGs/CFS vs LC-19/genes.bed" \
```

```
-b "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs LC-
19/CFS_vs_LC_UP_TEs_cleaned.bed" \
```

```
> "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs LC-
19/UPoverlap_output.bed"
```

Gene Annotation

Annotating regions that directly overlap genes:

```
bedtools intersect -a "/mnt/c/Users/smughal/Downloads/DETs and
DEGs/CFS vs CNT/CFS vs CNT DETs DOWN/DOWNoverlap_output.bed" \

-b  "/mnt/c/Users/smughal/Downloads/DETs    and    DEGs/CFS    vs
CNT/CFS vs CNT DETs DOWN/genes.bed" \

-wa -wb > "/mnt/c/Users/smughal/Downloads/DETs and DEGs/CFS vs
CNT/CFS                vs                CNT                DETs
DOWN/DOWNdirectoverlapannotated_overlap.bed"  #Naming can be
changed as needed
```

(it is best to process these files locally as sometimes mounting an external directory or server can give problems)

Extract Gene IDs after annotation and use python script to clean the list (remove version numbers) before entering it in g:profiler

3. Distribution of Different classes of Transposable Elements in a dataset

```
import pandas as pd

import matplotlib.pyplot as plt

import tkinter as tk

from tkinter import filedialog

def extract_te_type(te_name):

    """Extract the transposon type from the last colon-
    separated field."""

    return te_name.split(":")[-1]

# Open file dialog to select the Excel file

root = tk.Tk()

root.withdraw()
```

```

file_path = filedialog.askopenfilename(title="Select an Excel
file", filetypes=[("Excel files", "*.xlsx;*.xls")])

if file_path:

    # Read the Excel file

    df = pd.read_excel(file_path, usecols=[0], header=None,
names=["TE"])

    # Extract TE types

    df["Type"] = df["TE"].apply(extract_te_type)

    # Count occurrences of each TE type

    type_counts = df["Type"].value_counts()

    # Save results as CSV

    output_file = file_path.replace(".xlsx",
"_TE_counts.csv").replace(".xls", "_TE_counts.csv")

    type_counts.to_csv(output_file, header=["Count"])

    print(f"Saved TE counts to {output_file}")

    # Plot bar chart

    plt.figure(figsize=(8, 5))

    type_counts.plot(kind='bar', color='skyblue',
edgecolor='black')

    plt.xlabel("Transposon Type")

    plt.ylabel("Count")

    plt.title("Distribution of Transposon Types")

```

```
plt.xticks(rotation=45)
plt.tight_layout()
plt.show()
else:
    print("No file selected.")
```


Appendix 2: Other works published

Integrated Bioluminescent Immunoassays for High-Throughput Sampling and Continuous Monitoring of Cytokines

Eva A. van Aalen, Bas J. H. M. Rosier, Tom Jansen, Simone F. A. Wouters, Robin T. Vermathen, Harmen J. van der Veer, José Yeste Lozano, Sheeza Mughal, Juan M. Fernández-Costa, Javier Ramón-Azcón, Jaap M. J. den Toonder, and Maarten Merckx*

Cite This: *Anal. Chem.* 2023, 95, 8922–8931

Read Online

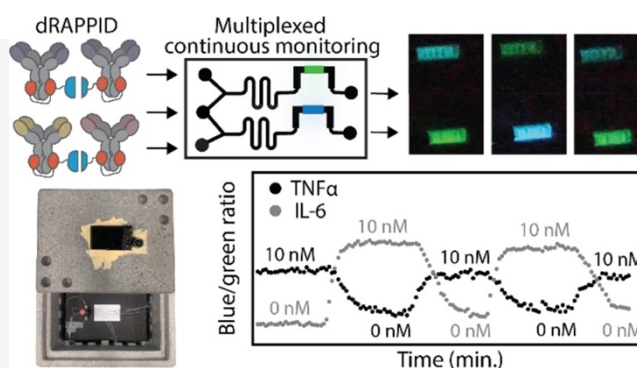
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ABSTRACT: Immunoassays show great potential for the detection of low levels of cytokines, due to their high sensitivity and excellent specificity. There is a particular demand for biosensors that enable both high-throughput screening and continuous monitoring of clinically relevant cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α). To this end, we here introduce a novel bioluminescent immunoassay based on the ratiometric plug-and-play immunodiagnostics (RAPPID) platform, with an improved intrinsic signal-to-background and an >80-fold increase in the luminescent signal. The new dRAPPID assay, comprising a dimeric protein G adapter connected via a semiflexible linker, was applied to detect the secretion of IL-6 by breast carcinoma cells upon TNF α stimulation and the production of low concentrations of IL-6 (~18 pM) in an endotoxin-stimulated human 3D muscle tissue model. Moreover, we integrated the dRAPPID assay in a newly developed microfluidic device for the simultaneous and continuous monitoring of changes in IL-6 and TNF α in the low-nanomolar range. The luminescence-based read-out and the homogeneous nature of the dRAPPID platform allowed for detection with a simple measurement setup, consisting of a digital camera and a light-sealed box. This permits the usage of the continuous dRAPPID monitoring chip at the point of need, without the requirement for complex or expensive detection techniques.



INTRODUCTION

Cytokines are small proteins that are secreted by cells to regulate complex signaling events. Proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α), play an important role in inflammation¹ and are involved in inflammation-related diseases, such as in Crohn's disease,² atherosclerosis,³ and several types of cancer.^{4–8} Elevated cytokine levels in patients with cancer are often related to tumor expansion and differentiation and engender poor prognosis.^{9–12} Furthermore, cytokine concentrations can change over time due to disease progression or treatment, for example, in acute inflammation or sepsis.^{13,14} The levels of cytokines such as TNF α , IL-6, and IL-1 β are elevated in sepsis, and their concentration profile is correlated with disease severity.^{15–17} Continuous detection of cytokine concentrations could aid clinicians in health monitoring and treatment selection and adjustment (i.e., personalized medicine).^{13,17,18} Besides applications in diagnostics, continuous monitoring of cytokines could improve our understanding of in vitro models such as organ-on-chip models, by providing temporally resolved information about the presence and concentration of relevant cytokines.^{19–21} In recent years, organs-on-chips

have shown to be effective model systems for fundamental research in the mechanism of organ physiology as well as in determining therapeutic effectiveness, drug screening, and studying disease progression.^{22–24}

Monitoring cytokine secretion is challenging due to the relatively small size of cytokines and the low physiological concentrations (picomolar range).^{25–27} Currently, ELISA and related (sandwich) immunoassays that rely on highly specific antibody-based target binding are widely used for the detection of cytokines.^{26,28} Although these heterogeneous methods allow for sensitive detection of analytes, they require a time-consuming workflow with several washing and incubation steps and expert operators.^{29,30} Therefore, ELISA is less suitable for tracking the dynamics of cytokine release nor adapted for high-throughput screening of cytokine levels

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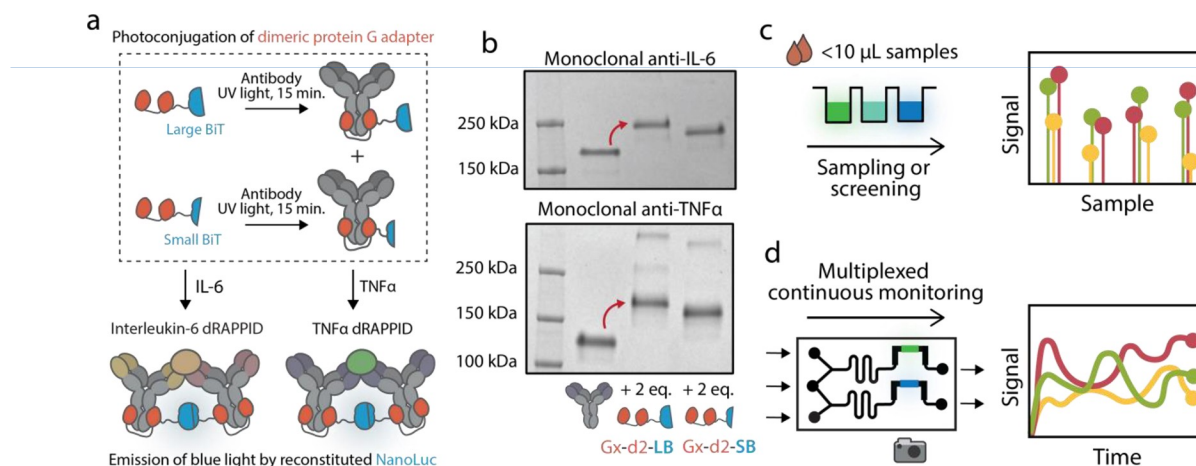


Figure 1. Development and implementation of the dimeric protein G RAPPID. (a) Schematic overview of the photoconjugation of the dimeric protein G adapters to anti-TNF α and anti-IL-6 antibodies. Reactions were performed in PBS (pH 7.4), with a 1:2 molar ratio of antibody to adapter, and were exposed to ultraviolet (UV) light ($\lambda = 365$ nm) for 15 min. (b) Nonreducing SDS-PAGE analysis of the photoconjugation described in (a), illustrating the efficient photoconjugation. The dimeric protein G RAPPID (dRAPPID) was applied to (c) detect the concentration of IL-6 in the culture medium from two different cell models and (d) continuously monitor the simultaneous presence of IL-6 and TNF α by using a herein-developed microfluidic chip.

Alternative technologies that allow multiplex detection of cytokines in solution such as Luminex still require expensive and sophisticated detection technology.^{31,32} So far, few immunoassays have been developed that allow continuous monitoring of biomarkers. Soh and co-workers recently reported a fluorescence-based sandwich immunoassay for continuous monitoring of glucose and insulin by using a microfluidic device and bead-based immunoassays.³³ Alternatively, the Prins lab applied the principles of antibody-based antigen detection based on the detection of single-particle mobility (biosensing by particle mobility, BPM).³⁴

Bioluminescence-based detection shows great promise for sensitive continuous monitoring in point-of-need settings. Unlike fluorescence, which requires external excitation and suffers from autofluorescence and scattering, bioluminescence can be performed directly in complex samples and does not require dedicated detection equipment. The recently established bioluminescent ratiometric plug-and-play immunodiagnosics (RAPPID) platform relies on analyte-induced complementation of split NanoLuc (NLuc) luciferase fragments, which are covalently coupled to analyte-specific antibodies through photoconjugation with a protein G adapter.^{35–38} The simple mix-and-measure workflow of RAPPID does not require sample preparation or washing steps, thereby making the sandwich immunoassay easy to handle and enabling in-solution measurements. Furthermore, the assay can be performed with low sample volumes and allows for fast analyte detection, due to the fast binding kinetics of antibodies.

Here, we adopted the RAPPID platform for highly sensitive detection of the cytokines TNF α and IL-6. To this end, we introduced a new dimeric protein G adapter that binds to both Fc domains on a single antibody, which increases photoconjugation efficiency and yields more homogeneous antibody-sensor conjugates (Figure 1a,b). Importantly, we show that the dimeric adapters reduce background luminescence which results in an increase in sensitivity. This dimeric RAPPID format (dRAPPID) was subsequently used to measure subnanomolar IL-6 levels both in traditional cell cultures of breast cell lines and in tissue-engineered 3D muscle models (Figure 1c). Furthermore, we designed and developed a

dedicated microfluidic chip that allows continuous monitoring of analyte concentrations by enabling continuous mixing of sensor components of the dRAPPID assay with samples. We applied this continuous monitoring chip for the multiplexed continuous monitoring of TNF α and IL-6 (Figure 1d).

EXPERIMENTAL SECTION

Cloning. pET28 plasmids encoding Gx-d2-LB and Gx-d2-SB were obtained by performing overhang PCR and subsequent restriction using XhoI, KpnI, and SacI (Figures S2 and S3). Successful cloning was confirmed via Sanger Sequencing (BaseClear).

Protein Expression. dRAPPID sensors were expressed as described in ref 35. Briefly, *Escherichia coli* BL21 (DE3) cells were transformed with pET28 plasmids encoding Gx-d2-LB or Gx-d2-SB together with a pEVOL vector (a kind gift from Peter Schultz, Addgene plasmid # 31190) encoding a tRNA/tRNA synthetase pair to incorporate the unnatural amino acid para-benzoyl-phenylalanine (pBpA, Bachem, 104504-45-2) at the amber stop codons.³⁹ The cells were cultured at 20 °C overnight, subsequently harvested by centrifugation, and lysed using Bugbuster and benzonase (Novagen). Purification of the expressed Gx-d2-LB and Gx-d2-SB was done using Ni²⁺ affinity chromatography followed by Strep-Tactin purification. The purity of the two proteins was determined using SDS-PAGE analysis (Figure S4). The correct incorporation of two unnatural amino acids was confirmed by Q-ToF LC-MS (Waters MassLynx v4.1), using MagTran v1.03 for MS deconvolution. The observed mass for the Gx-d2-LB and Gx-d2-SB was 45862.81 Da (expected: 45863.04) and 29593.36 Da (expected: 29593.52), respectively.

Photoconjugation. Gx-d2-SB and Gx-d2-LB were photoconjugated to infliximab (obtained via the Maxima Medisch Centrum pharmacy in Veldhoven, The Netherlands) and anti-IL-6 (10395-mhK23 and 10395-R508, ordered from SinoBiological). Photoconjugation was performed with 1 μ M antibody and 2 μ M Gx-d2-SB or Gx-d2-LB in PBS (pH 7.4) for 15 min, by using a Thorlabs M365LP1 ($\lambda = 365$ nm) UV-lamp coupled with a Thorlabs LEDD1B T-Cube LED Driver (continuous wave, current limit 1.2A, 80% intensity), as shown in Figure S5.

After conjugation, the crosslinked sensor components were not further purified and stored at 4 °C until use.

dRAPPID Assays. The dRAPPID assays were done in buffer (PBS (pH 7.4), 0.1% (w/v) BSA) or in Dulbecco's modified Eagle's medium (DMEM, from Gibco) with phenol red, supplemented with 4.5 g/L D-glucose, 0.58 g/L L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Life Technologies). An assay mixture of 1 nM antibody-LB and 10 nM antibody-SB was incubated with a target analyte for 60 min prior to the addition of 1000-fold diluted NLuc substrate (Promega, N1110). For ratiometric detection, 50 or 17 pM of calibrator luciferase was added to the sensor mixture for IL-6 or TNF α detection, respectively. The assays were executed in nontreated white Thermo Scientific 384-well plates (Cat. no 262360) in a total volume of 20 µL. The luminescent signals were recorded between 398 and 653 nm on a Tecan Spark 10 M plate reader, with a 25 nm bandwidth, at room temperature. The blue-to-green ratios were calculated by dividing the blue light emission at 458 by the green light emission at 518 nm. Detection with a digital camera (Sony DSC-Rx100 III) was done in a gray Styrofoam box. The LOD was calculated using eq 1, in which SD is the standard error of the y -intercept, by linear regression of the luminescent signal related to a selection of low cytokine concentrations.

$$\text{LOD} = 3.3 \times \frac{\text{SD}}{\text{slope}} \quad (1)$$

Off-Line dRAPPID Assay of Secreted IL-6. MDA MB 231 and MCF-7 cells were cultured in DMEM (with phenol red, supplemented with 4.5 g/L D-glucose, 0.58 g/L L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin) in Falcon corning T25 culture flasks (Nunc EasYFlask Cell Culture Flasks) at 37 °C with 5% CO₂ until a confluency of 80% was reached. Subsequently, 200,000 cells were seeded in a CELLSTAR 12-well cell culture plate (Greiner, cat.-no. 665180) and grown overnight. The next day, the cell culture medium was removed and fresh DMEM, with 0.5 nM TNF α or without TNF α , was added to the cells. After 0, 6, 12 and 24 h, 10 µL culture medium samples were removed from the cells and IL-6 dRAPPID (1 nM antibody-LB and 10 nM antibody-SB) was added to these culture medium samples. After an incubation step of 60 min, the substrate of NLuc was added (1000-fold diluted) and the luminescent signal was measured on a Tecan Spark 10 M plate reader. Culturing of the 3D muscle tissues was performed according to ref 49. The muscle tissues were stimulated with 10 µg/mL lipopolysaccharide (LPS) or left untreated. Culture medium samples (10 µL) were removed from the muscle tissues after 2, 4, and 24 h of LPS stimulation and mixed with 0.1 nM anti-IL-6-LB and 1 nM anti-IL-6-SB. After 1 h of incubation, 1000-fold diluted NLuc substrate was added, and the luminescent signal was measured with a plate reader (Infinite 200Pro). To test for significant differences in IL-6 secretion between TNF α stimulated and nonstimulated cells at the same timepoint, and IL-6 secretion between different timepoints in LPS-stimulated and nonstimulated tissues, a paired t-test was used. Statistical analysis was performed in MATLAB (Mathworks, USA).

Development of Microfluidic Devices. The photolithography mask was designed in AutoCAD software (Figure S6) and then printed via CAD/Art Services, Inc. The SU-8 master wafer was fabricated by patterning ~100 µm-thick SU-

82075 photoresist (MicroChem) on a silicon wafer. UV exposure was done with an exposure energy of 230 mJ/cm² and an irradiation of 20 mW/cm². The development of the wafer was performed for 30 min in Mr. Developer 600 (Microresist, Germany). The final polydimethylsiloxane (PDMS) chip was fabricated by soft lithography. Accordingly, SYLGARD 184 base and curing agent (Dow, USA) were mixed in a 10:1 ratio (w/w) and placed in a vacuum desiccator to remove air bubbles. The mixed and degassed mixture was poured over the SU-8 master wafer to form a ~5 mm-thick PDMS layer. The PDMS was subsequently cured at 65 °C for 6 h. After this curing step, the PDMS chip was removed from the SU-8 wafer and the inlet and outlet holes were punched with a 1.2 mm puncher (Uni-Core). To bond the PDMS to a glass slide (24 × 50 mm), plasma treatment (in a Quorum K1050X asher) was used. Plasma treatment of the glass slide and PDMS chip was done with oxygen plasma for 30 s with an RF power of 20 W. Immediately after treatment, the glass and PDMS were brought into contact and placed at 65 °C for 1 h. Before performing the continuous monitoring experiments, the detection chambers, 6 mm of polyethylene tubing (from scientific Commodities Cat. Nr. BB31695-PE/5, with an inner diameter of 0.034 inch) and two metal adapters, were added into the punched holes.

Continuous Measurements in Microfluidic Chips.

Food coloring experiments were performed in buffer (PBS (pH 7.4), 0.1% (w/v) BSA) with red food coloring (TRS Foods, UK, 25 mg/mL). PHD ULTRA CP Syringe Pumps (Harvard Apparatus) and 1 mL syringes (Henke-Ject) were used to inject buffer with food coloring (analyte inlet) and buffer without food coloring (sensor inlets and NLuc substrate inlet) into the microfluidic chip, with flowrates of 1, 2, and 4 µL/min for the sensor inlet, analyte sample inlet, and NLuc substrate inlet, respectively. The microfluidic chip was placed in a light-proof black Styrofoam box and polyethylene tubing (from Scientific Commodities) was used to connect the chip to the syringes in the pumps. The sensor, analyte, and substrate were injected for 30 min at these flow rates before measurements started, to ensure steady flow in the chip. Pictures of detection chambers were taken with a Sony DSC-Rx100 III digital camera, with an integration time of 30 s and an ISO value of 6400. Continuous monitoring experiments with dRAPPID were executed as described above but with 1 nM of antibody-LB, 10 nM of antibody-SB, 250-fold diluted NLuc substrate, and 60 or 27 pM of calibrator luciferase for IL-6 dRAPPID or TNF α RAPPID, respectively. Switching between 0 nM analyte and 10 nM analyte (both in PBS (pH 7.4), 0.1% (w/v) BSA) was done using a IDEX V-101D manual flow switching valve.

RESULTS AND DISCUSSION

Development of Dimeric RAPPID for IL-6 and TNF α Detection. The classic RAPPID platform comprises one photo-cross-linkable protein G adapter domain (Gx) connected via a semiflexible linker to either large BiT (LB) or small BiT (SB), the two fragments of split NLuc.³⁶ Subsequent crosslinking to antibodies typically generates a mixture of non-, once-, and twice-conjugated antibodies, resulting in background luminescence by undesired NLuc complementation due to binding of a free Gx-LB or Gx-SB to an unoccupied heavy chain (Figure S7). To further reduce the background signal and enable the detection of low concentrations of cytokines, we here established a new class of dimeric RAPPID

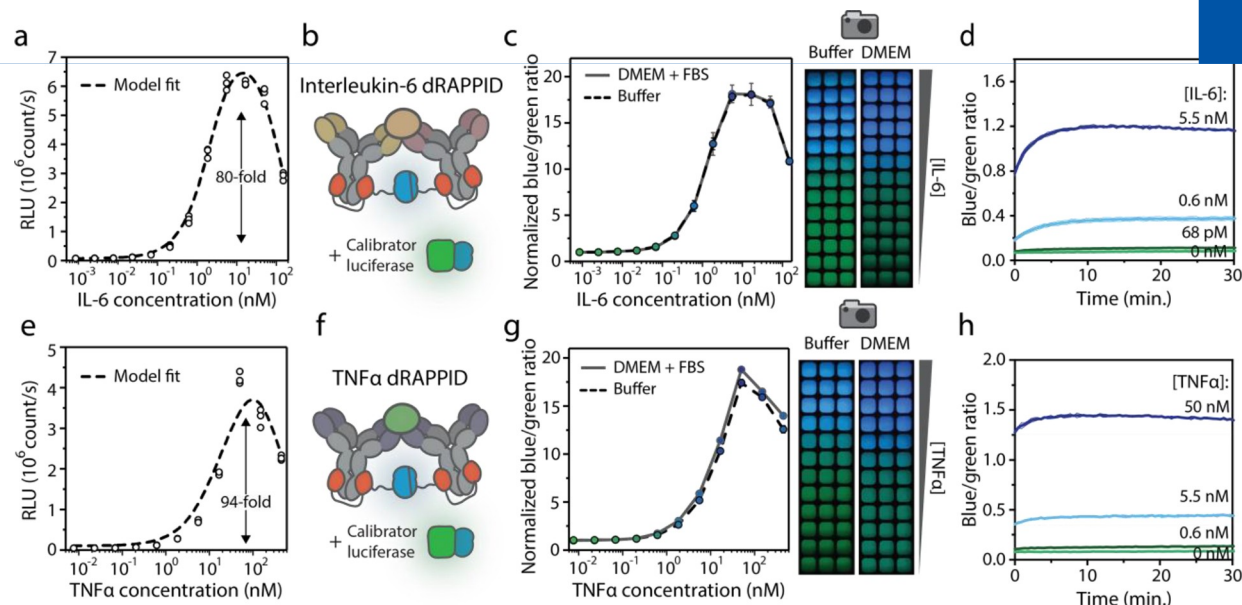


Figure 2. Performance of the protein G-dimer RAPPID assays. (a) Intensimetric detection of IL-6 in buffer (PBS (pH 7.4), 0.1% (w/v) BSA). RLU, relative luminescence units. (b) Schematic overview of the ratiometric IL-6 dRAPPID assay. (c) Ratiometric detection of IL-6 in buffer (PBS (pH 7.4), 0.1% (w/v) BSA) or in the cell culture medium (DMEM with 10% FBS) with 50 pM concentration of the calibrator luciferase. The same samples were used for detection with the plate reader (left) and with a digital camera (right). (d) Ratiometric sensor output over time at various concentrations of IL-6. All components (sensors, IL-6, substrate, and calibrator luciferase) were added at $t = 0$. (e) Intensimetric TNF α dRAPPID in buffer (PBS (pH 7.4), 0.1% (w/v) BSA). RLU, relative luminescence units. (f) Schematic overview of the ratiometric TNF α dRAPPID assay. (g) Ratiometric detection of TNF α in buffer and DMEM (+10% FBS) with 17 pM calibrator luciferase. The measurements in the plate reader (left) and the pictures with the digital camera (right) were done with the same samples. (h) Ratiometric response over time after one-step incubation of all TNF α dRAPPID components. The reaction mixtures in (a), (c), (e), and (g) consisted of 1 nM Ab-LB and 10 nM Ab-SB and were incubated for 60 min at room temperature prior to the addition of 1000 $\times$ diluted NLuc substrate. Data points in (a) and (e) represent technical replicates, with $n = 3$ independent preparations of the target analyte. Experimental data were fitted to a thermodynamic model (dotted lines, Supporting Information). Values in (c) and (g) depict mean $\pm$ s.d. of technical replicates, with $n = 3$ independent preparations of the target, and the lines represent the mean. Lines in (d) and (h) represent mean $\pm$ s.d. of technical replicates, with $n = 3$ independent preparations of the analyte.

(dRAPPID) sensors. The dRAPPID sensor proteins consist of two photo-cross-linkable protein G adapters, connected via a semiflexible linker that was designed to optimally span the distance between the two heavy chains of one antibody, fused to an LB or an SB ($K_D = 2.5 \mu\text{M}$) domain.³⁶ The Gx-d2-SB and Gx-d2-LB proteins were expressed in *Escherichia coli* and purified using Ni^{2+} affinity chromatography followed by Strep-Tactin purification (Figure S4). The sensor components and the monoclonal anti-IL-6 or anti-TNF α antibodies were mixed in a 1:2 molar ratio, ensuring the binding of the two protein G adapters of one sensor component to the Fc-domain of an antibody in a 1:1 stoichiometry. Subsequent short UV light irradiation (15 min, $\lambda = 365 \text{ nm}$) of the mixture results in the covalent crosslinking of antibody to the sensor (Figures 1a and S5). SDS-PAGE analysis showed an improved photoconjugation efficiency and a more homogeneous reaction mixture compared to classic RAPPID sensors with only one protein G adapter (Figures 1b and S7a).^{33,35} The two minor bands corresponding to higher molecular weight species in Figure 1b can be attributed to two sensor components conjugated to one antibody and one sensor protein photo-crosslinked to two antibodies. Furthermore, in this new dRAPPID platform, both heavy chains of the IgG antibody are occupied by a protein G adapter, preventing the binding of a protein G-dimer with the complementary fragment of split NLuc and thus resulting in less background luminescent activity (compare Figure S7b with Figure S7c). This low background signal is particularly important for the detection of analytes such as cytokines, which are generally present in low concentrations.

After confirming the efficient photoconjugation of the sensor components to the IL-6 antibodies (using a commercially available ELISA-pair binding distinct epitopes), we tested the performance of the new IL-6 dRAPPID sensor in PBS buffer. Increasing concentrations of IL-6 were added to 1 nM of anti-IL-6 Gx-d2-LB (Ab-LB) and 10 nM of anti-IL-6 Gx-d2-SB (Ab-SB) and incubated for 60 min at room temperature. Subsequently, the furimazine substrate of NLuc was added and the luminescent signal was measured with a plate reader. Binding of the dRAPPID sensor components to the IL-6 target resulted in NLuc complementation and an IL-6-dependent increase in blue light emission. Figure 2a shows that the IL-6 dRAPPID exhibited an 80-fold increase in the luminescent signal and the detectable IL-6 concentration regime spanned three orders of magnitude. At IL-6 concentrations exceeding $\sim 10 \text{ nM}$, the luminescent signal decreased again due to the hook effect.³⁵ Fitting a thermodynamic model of the system generated a bell-shaped curve that closely resembled the data in Figure 2a and yielded antibody affinities of ~ 2.3 and $\sim 120 \text{ nM}$ for 10395-mhK23(-SB) and 10395-R508(-LB) (Figure S1a).⁴⁰ Next, we translated this intensimetric IL-6 dRAPPID assay into a ratiometric version by adding the green light-emitting calibrator luciferase, a genetic fusion of NLuc to mNeonGreen (Figure 2b).^{35,41} The ratio of blue light, emitted by the complemented NLuc of the sensor components, and green light of the calibrator luciferase gives a stable ratiometric signal that is insensitive to changes in environmental factors such as pH and temperature and does not suffer from the signal decrease over time as a result of substrate depletion.

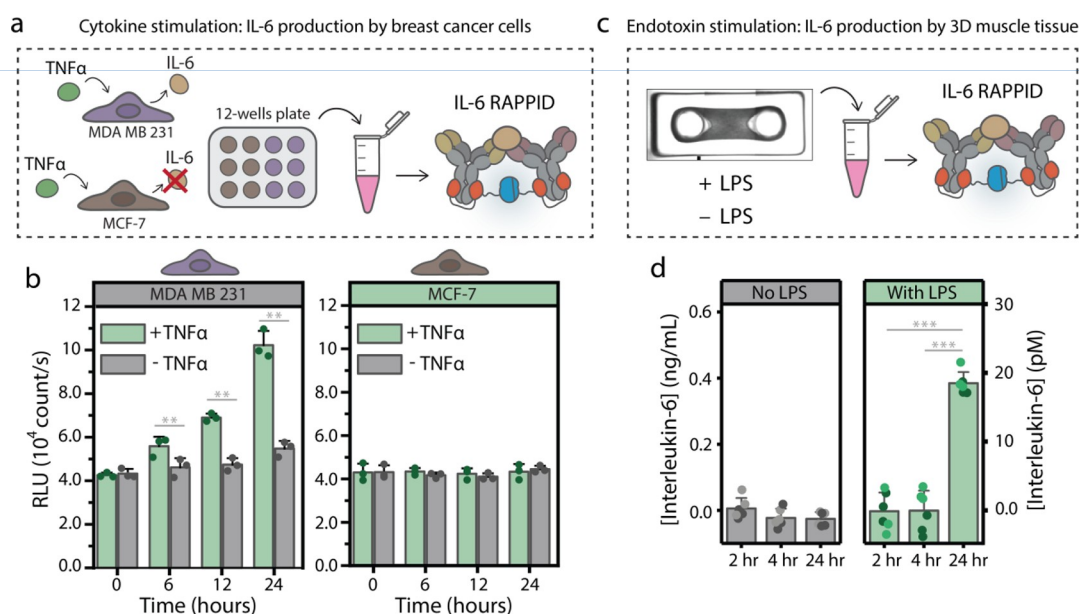


Figure 3. Off-line measurements of IL-6 in the cell culture medium. (a) Schematic representation of IL-6 detection in the cell culture medium of cytokine-stimulated breast cancer cells. MDA MB 231 and MCF-7 cells were cultured in a 12-well plate and were either stimulated with 0.5 nM TNF α or left untreated. Culture medium samples were taken after 0, 6, 12, and 24 h of TNF α stimulation and measured with IL-6 dRAPPID (1 nM Ab-LB and 10 nM Ab-SB) in a plate reader after 60 min of incubation. (b) IL-6 detection in the cell culture medium of MDA MB 231 and MCF-7 cells. Individual data points ($n = 3$) are shown, and bars represent mean values with s.d. RLU, relative luminescence units. (c) Schematic representation of IL-6 detection in the culture medium of human 3D muscle tissue. Muscle cells were cultured in a hydrogel in a PDMS mold, and cells were stimulated with 10 μ g/mL lipopolysaccharide (LPS) or left untreated. IL-6 dRAPPID (0.1 nM Ab-LB and 1 nM Ab-SB) was added to the cell culture medium, and after 60 min of incubation the luminescent signal was measured with a plate reader. (d) IL-6 detection with dRAPPID of the 3D human muscle tissue. $n = 2$ biological replicates, $n = 3$ technical replicates and bars represent mean $\pm$ s.d. Asterisks represent a significant difference (** = $p \leq 0.01$, *** = $p \leq 0.001$).

Figure 2c displays the performance of the ratiometric IL-6 dRAPPID under standard buffer conditions (PBS (pH 7.4), 0.1% (w/v) BSA) and in the cell culture medium (DMEM with 10% FBS). dRAPPID performed in buffer and DMEM yielded similar bell-shaped dose-response curves and the IL-6-dependent blue-to-green ratios were detectable both with a plate reader and with a simple digital camera. The kinetics of the complex formation between dRAPPID components and IL-6 was fast, reaching a stable blue-to-green ratio after ~ 10 min (Figure 2d).

Next, we analyzed the performance of the new TNF α dRAPPID assay, consisting of the same anti-TNF α antibody (infliximab) conjugated to either Gx-d2-LB or Gx-d2-SB. The sensor components were incubated for 60 min (1 nM Ab-LB and 10 nM Ab-SB), to ensure complex formation, with increasing concentrations of TNF α . Figure 2e shows that this intensimetric TNF α dRAPPID assay exhibited a dose-response curve with a maximal 94-fold increase in luminescent signal. Fitting of a thermodynamic model to the intensimetric titration data yielded an antibody affinity of ~ 155 nM for TNF α (Figure S1b). The ratiometric TNF α assay displayed a ~ 19 -fold increase in the blue-to-green ratio, both in buffer and culture medium, upon increasing concentrations of TNF α (Figure 2f,g). The ratiometric signal reached a stable signal within ~ 5 min, demonstrating fast complex formation between TNF α and the sensor components (Figure 2h). Furthermore, by decreasing the concentration of the dRAPPID sensor components to 0.1 nM of Ab-LB and 1 nM of Ab-SB, the limit-of-detection could be lowered to 4.5 and 18 pM for IL-6 and TNF α , respectively (Figure S6). The low background signal of the

platform allows for fast and sensitive detection of cytokines, due to low background luminescence, and performs excellent both in buffer and culture media.

Sampling of Cell-Secreted Interleukin-6. The low background signal of the new class of RAPPID variants, with the ensuing low limit-of-detection, makes dRAPPID well suited to measure the low concentrations of cytokines secreted by cells. Furthermore, the excellent performance in the culture medium, the minimal sample handling, and the mix-and-measure workflow makes the dRAPPID assay a promising tool for high-throughput screening. To demonstrate this, we measured IL-6 secretion levels after stimulation with a cytokine or an endotoxin, for two different cell models. First, we used dRAPPID to detect IL-6 secretion of breast carcinoma cells after cytokine stimulation. MDA MB 231 cells are highly invasive cancer cells and are known to have an upregulated and cytokine-responsive IL-6 expression compared to other breast cancer cell lines such as MCF-7 that do not respond to this type of stimulation.^{42–44} MDA MB 231 and MCF-7 cells were cultured in a 12-well plate, stimulated with TNF α or left untreated, and samples of the cell culture medium were taken at different time points ($t = 0, 6, 12$, and 24 h). The levels of secreted IL-6 in these samples were subsequently determined by adding IL-6 dRAPPID and measuring the corresponding luminescent signal (Figure 3a). Figure 3b shows that stimulation with TNF α resulted in the accumulation of IL-6 in the cell culture medium of the MDA MB 231 cells, demonstrated by the increase in luminescent signal over time. Untreated MDA MB 231 cells did not display this increase in luminescent signal, suggesting that stimulation is needed for elevated IL-6 secretion of these cancer cells. As expected, the

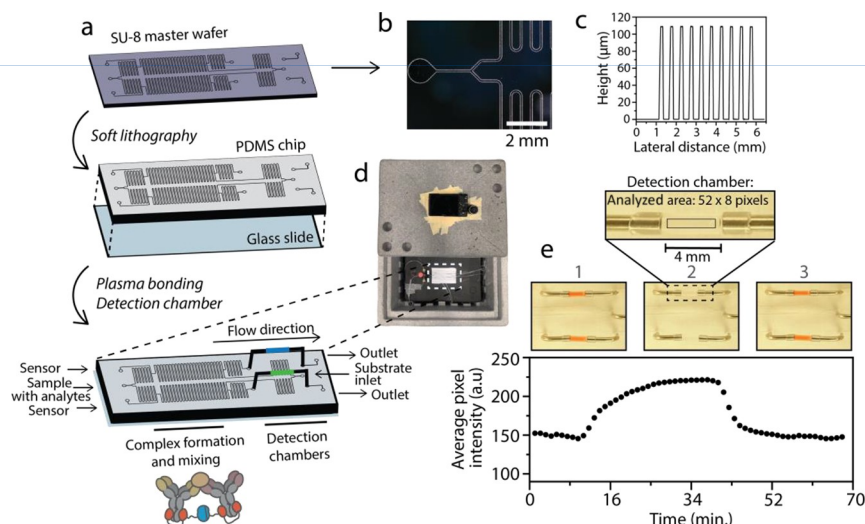


Figure 4. Fabrication and testing of the continuous monitoring microfluidic chip. (a) Fabrication of the microfluidic chip using photolithography and soft lithography. The microfluidic chip has two sensor inlets, one sample inlet, one NLuc substrate inlet, and two outlets. Complex formation and mixing of the analytes and the sensors take place in the long serpentine channel, and detection of the luminescent signal occurs in the detection chambers with a digital camera. (b) Digital microscope image of the SU-8 master wafer. (c) Contact profilometer analysis of the microchannels of the SU-8 master wafer. (d) Measurement setup, consisting of a light-tight box in which the chip is positioned and that has a hole for placing a conventional digital camera (Sony DSC-RX100 III). (e) Performance of the continuous monitoring chip. Switching between different analyte inputs, buffer (PBS (pH 7.4), 0.1% (w/v) BSA) without and buffer with red food coloring (25 mg/mL), is done with a V-101D flow switching valve. Switching occurred at $t = 1$ min and $t = 25$ min. Flow rates of 1, 2, and 4 $\mu\text{L}/\text{min}$ for sensor inlets, analyte sample inlets, and NLuc substrate inlets, respectively, were used. Data represent the average pixel intensity measured in the analyzed area (52×8 pixels, shown in the inset) of the detection chamber.

MCF-7 cells did not secrete IL-6 upon TNF α stimulation, as the luminescent signal stayed constant and did not increase over time (Figure 3b).

A 3D tissue construct more accurately represents the cells in an in vivo environment and is hence a better model to study topics like disease physiology and drug discovery than a more traditional 2D cell culture.^{45,46} To investigate the compatibility of dRAPPID with advanced cell culturing systems, we stimulated a human 3D muscle tissue model with lipopolysaccharide (LPS) and measured the corresponding IL-6 secretion of the muscle cells with dRAPPID. LPS is an endotoxin that stimulates the expression of proinflammatory cytokines in many different types of tissues and cells, including skeletal muscle cells.^{47,48} We fabricated the 3D muscle models as described previously and subsequently stimulated them with LPS.⁴⁹ Culture medium samples (10 μL) were removed after 2, 4, and 24 h of LPS stimulation (Figure 3c). After analyzing these samples with IL-6 dRAPPID, we found that after 24 h of LPS stimulation, the muscle tissue secreted ~ 18 pM (corresponds to ~ 0.4 ng/mL) of IL-6 (Figure 3d). Muscle tissue without LPS stimulation did not show an increase in secreted IL-6 levels over time. The low-picomolar sensitivity of the dRAPPID platform would also allow the detection of IL-6 levels secreted by other in vitro cell models such as monocytes, astrocytes, adipose tissue, and muscle tissues, where accumulated concentrations can reach low-picomolar to mid-picomolar levels.^{21,50–52} Furthermore, the dRAPPID assay allows the quantification of analytes in volumes as low as 10 μL , making high-throughput screening possible in situations where high temporal resolution is necessary or when screening of many conditions in parallel is desired.

Development of a Microfluidic Chip for Continuous Monitoring. The continuous monitoring of cytokines shows potential in both healthcare and research applications. Hence,

we developed a microfluidic device for continuous sensing and integrated dRAPPID for IL-6 and TNF α detection. Accordingly, we used a standard microfluidic device fabrication protocol with an SU-8 master wafer, soft lithography, and sealing with a glass slide to develop the polydimethylsiloxane (PDMS) microfluidic chip shown in Figure 4a. Microscope inspection of the SU-8 master showed the correct fabrication of the microchannels (Figure 4b) and measurements with a contact profilometer confirmed that the features had the desired height of ~ 100 μm (Figure 4c). Because the signal strength of the bioluminescent output is directly related to the z-height of the detection chamber, we decided to implement external detection chambers consisting of polyethylene tubing and two metal adapters to obtain the fully assembled microfluidic chip (Figure S9). The PDMS chip contained four different inlets, two intended for dRAPPID sensors, enabling multiplexed detection of two different biomolecules, one inlet for the sample containing the analytes of interest and one for the substrate of NLuc (Figure 4a). After the sample has entered the microfluidic chip, the fluid splits and mixes with the different dRAPPID sensors in the long serpentine channels, resulting in the formation of a complex between the target and sensor. Before the sensor-analyte mixture enters the detection chamber, it is mixed with the substrate of NLuc and the luminescent signal of the reconstituted NLuc, indicating the presence of the target analyte, can subsequently be observed in the detection chambers. The detection of this luminescent signal is done by using a simple light-tight box with a hole for a digital camera, enabling continuous monitoring of biomolecules without expensive and sophisticated equipment (Figure 4d). New dRAPPID sensors and samples are continuously injected into the chip, refreshing the detection chamber with newly formed complexes and thereby enabling the continuous detection of changes in analyte concentration.

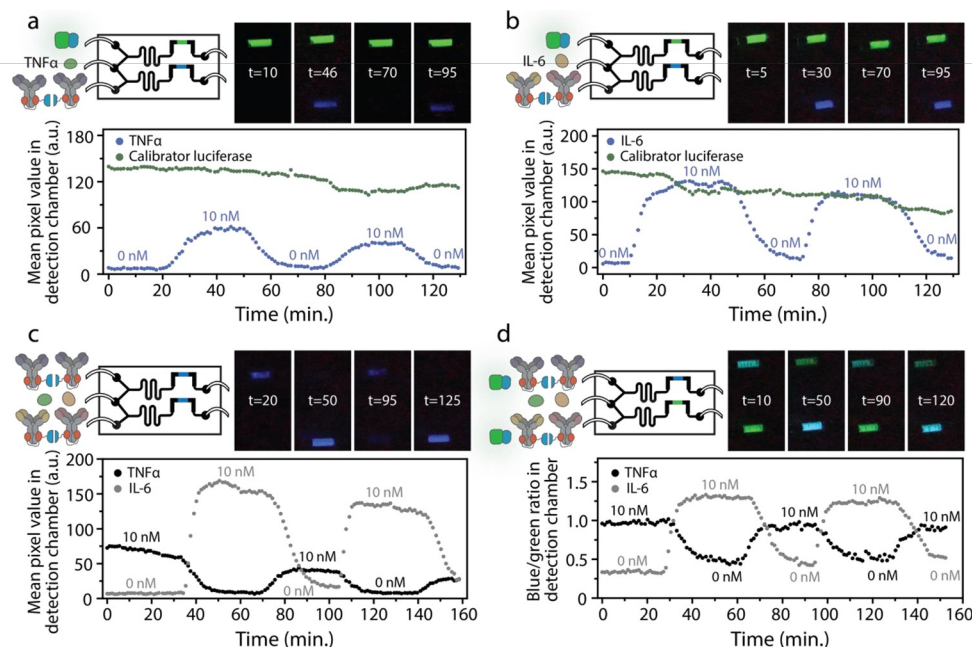


Figure 5. Multiplexed detection of interleukin-6 and TNF α in the dRAPPID continuous monitoring microfluidic chip. (a) Intensiometric continuous detection of TNF α . The calibrator luciferase (60 pM) was added to the upper sensor inlet, TNF α dRAPPID was injected in the lower sensor inlet, and the TNF α concentration, in the analyte inlet, was switched between 0 and 10 nM. (b) Intensiometric continuous detection of IL-6. The green calibrator luciferase (75 pM) was injected in the upper sensor inlet and the IL-6 dRAPPID in the lower inlet. 10 or 0 nM of IL-6 was injected into the analyte inlet, and the corresponding blue light response in the lower detection chamber was measured with a digital camera. (c) Multiplexed intensiometric detection of IL-6 and TNF α . TNF α dRAPPID was added to the upper sensor inlet, and IL-6 dRAPPID was injected into the lower sensor inlet. Both IL-6 and TNF α were added in the sensor inlet, either at 0 or 10 nM, and the responsive change in blue light emission was detected in both detection chambers. (d) Multiplexed ratiometric detection of IL-6 and TNF α . TNF α dRAPPID with 27 pM calibrator and IL-6 dRAPPID with 60 pM calibrator were injected in the upper and lower sensor inlet, respectively. All dRAPPID assays were performed with 1 nM Ab-LB, 10 nM Ab-SB, and 250 $\times$ diluted substrate in buffer (PBS (pH 7.4), 0.1% (w/v) BSA).

Using optimized flowrates (Figure S10), which enabled ~ 3 min of complex formation between sensor and analyte in the serpentine channels, we next determined the performance and the temporal resolution of the microfluidic chip by injecting buffer samples with or without red food coloring (Figure 4e). We started by injecting buffer with red food coloring in the analyte inlet (1 in Figure 4e), switched to buffer without food coloring, resulting in the disappearance of the red color (2 in Figure 4e), and finally switched back to buffer with red food coloring (3 in Figure 4e). The average pixel intensity in the analyzed area of the detection chamber changed accordingly and complete replenishment of the detection chamber took ~ 16 min (Figure 4e). The gradual change of color in the detection chamber, instead of a sharp transition, is due to Taylor dispersion.^{53,54}

Multiplexed Continuous Monitoring of Interleukin-6 and TNF α . To demonstrate that the continuous monitoring chip, in combination with dRAPPID, can be used to detect changes in concentrations of relevant biomolecules, we next applied the chip to monitor IL-6 and TNF α levels. Using the same flow rates and setup as with the food coloring experiments (Figure 4e), we first injected TNF α as an analyte in the sample inlet and TNF α dRAPPID in the lower sensor inlet of the microfluidic chip. The calibrator luciferase, unresponsive to the analyte concentration, was added to the upper sensor inlet of the chip to determine the effect of the long continuous measurement on the luminescent signal in the detection chambers. We started by adding buffer without TNF α into the chip (0 nM TNF α), resulting in the expected absence of a blue luminescent signal in the lower detection

chamber in Figure 5a. Switching to 10 nM TNF α caused an increase in blue signal, due to complex formation between TNF α and the dRAPPID sensor and the consequential complementation of the split NLuc. Switching back to 0 nM TNF α and subsequently increasing the concentration to 10 nM resulted in the disappearance and appearance of the blue signal in the lower detection chamber, respectively, demonstrating the continuous detection of changes in TNF α concentrations. As expected, the green luminescent signal of the calibrator luciferase in the upper detection chamber did not respond to the changes in injected TNF α concentration. A $\sim 20\%$ decrease in the signal of both the calibrator luciferase and the dRAPPID sensor was observed over time, however, which is likely due to slow auto-oxidation of the NLuc substrate over the course of the experiment. The blue and green signals decrease with a similar slope, enabling the normalization of the blue responsive signal by the green constant signal and thereby eliminating the effect of NLuc substrate degradation (Figure S11a). Similarly, we injected IL-6 and IL-6 dRAPPID in the microfluidic chip and observed the expected change in blue light in response to different IL-6 concentrations (Figure 5b). Normalization of this responsive blue light by the constant green luminescent signal in the upper detection chamber resulted in a more stable signal over time (Figure S11b).

Next, to demonstrate the simultaneous detection of two relevant biomolecules, we added TNF α dRAPPID in the upper sensor inlet and IL-6 dRAPPID in the lower sensor inlet (Figure 5c). The injected sample contained either 0 nM IL-6 and 10 nM TNF α , resulting in a blue signal in the upper

detection chamber, or 10 nM IL-6 and 0 nM TNF α , giving a blue signal in the lower detection chamber. Switching between these samples resulted in the expected alternating blue light responses in the detection chambers. Finally, to create a more stable luminescent signal over time and eliminate the effects of NLuc substrate oxidation, we added the calibrator luciferase to the two dRAPPID sensors, enabling multiplexed ratiometric detection of IL-6 and TNF α (Figure 5d). The captured light in the detection chambers changed from blue to green, depending on the concentrations of the target analytes. The plotted blue-to-green ratios were constant over time, as switching back to the same analyte concentration yielded similar blue-to-green ratios. These results demonstrate that the dRAPPID continuous monitoring chip can be used to detect two relevant biomolecules simultaneously and that ratiometric detection yields stable signals over time, enabling sensitive and accurate real-time measurements.

CONCLUSIONS

Herein, we developed a new class of RAPPID sensors, with a low intrinsic background luminescent signal, to detect picomolar concentrations of clinically relevant cytokines. The individual sensor components of the dRAPPID system contain two photo-cross-linkable protein G domains to ensure conjugation to both heavy chains of the target-specific IgG. This format enables more efficient and essentially complete formation of antibody-conjugates at 1:1 stoichiometry and precludes the binding of free protein G luciferase fragments to remaining Fc binding sites, ensuring that the observed luciferase activity only results from analyte-induced NLuc complementation. Subsequently, we applied this dimeric RAPPID to detect cytokines in two different contexts: (1) sampling of concentrations of IL-6 secreted by cells and (2) the multiplexed continuous detection of IL-6 and TNF α concentrations in a microfluidic chip.

Continuous monitoring with the dRAPPID microfluidic chip relies on the continuous inflow of new dRAPPID sensors. Therefore, fast, reversible binding between antibody and target may not be required. This broadens the scope of antibodies that can be used, as high affinity and slow dissociation kinetics do not impede their usage. In the current setup, it takes ~15 min before the complete detection chamber is refreshed by a new sensor-analyte complex, which might hamper the detection of fluctuations on a very short time scale of minutes. However, a time resolution of 15 min would most likely be sufficient to detect pathophysiological changes in cytokine concentrations. The 15 min delay could be decreased by further reducing the volume of the detection chamber, increasing the flowrates or by introducing active mixing. The continuous sensing platform shows potential to detect biomarkers in in vitro cell models, demonstrated by the minimal difference in ratiometric signal between measurements in buffer and culture medium and the low picomolar sensitivity of the dRAPPID sensors. For measurements in more complex media, such as blood plasma, we expect that samples need to be diluted prior to mixing with the sensor, to decrease possible viscosity-related problems.

The herein-developed continuous sensing platform demonstrates the feasibility of integrating the dRAPPID sensors for point-of-need monitoring. The simple measurement setup, with a light-tight box and a digital camera, and the large variety of available RAPPID sensors allow the detection of a wide panel of biomarkers outside of traditional laboratories. The few

continuous monitoring immunoassay-platforms that have been developed so far do not reach picomolar sensitivity or require relatively expensive and complex detection techniques.^{33,34,55,56} In the future, the dRAPPID microfluidic chip can be applied to detect biomarkers in in vitro models, increasing our knowledge about disease progression, therapeutic effectiveness, and organ or cell physiology. Furthermore, the continuous sensing platform can be used in healthcare monitoring, for example, for continuously measuring inflammation markers in the intensive care unit. The detection of sepsis-related biomarkers such as IL-6, procalcitonin, and C-reactive protein, with serum concentrations that can exceed ~70, ~600, and ~400 pM, respectively, could facilitate early diagnosis of sepsis and predict disease severity.^{13,57–59}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.3c00745>.

Amino acid and DNA sequences of the pG-d2-LB and SB, SDS-PAGE gels of the expression of pG-d2-LB and pG-d2-SB, fitting of intensimetric data to a thermodynamic model, the photoconjugation set-up, microfluidic mask design, background signal of the different sensor proteins, limit-of-detection measurements, flow rate determination and normalized signals of the continuous monitoring chip (PDF)

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Author Contributions

E.V.A. and B.R. designed the study, performed experiments, analyzed the data and wrote the manuscript. T.J. contributed to the continuous monitoring experiments and S.W. designed the Gx-dimer proteins and did initial characterization. R.V. cloned the dRAPPID sensor components and developed the photoconjugation setup. H.V.D.V. compared the intensimetric dRAPPID data with a thermodynamic model. S.M. and J.F. designed and contributed to the 3D muscle tissue work. J.Y.L. designed the microfluidic chip and J.R. supervised the microfluidic chip development work. J.D.T. conceived the cancer cell work and analyzed the data. M.M. conceived, designed, and supervised the study and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Notes

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Organ-on-Chips: trends and challenges in advanced systems integration

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Abstract

Organ-on-chip platforms combined with high throughput sensing technology allow bridging gaps in information presented by 2-D cultures modelled on static microphysiological systems. While these platforms do not aim to replicate whole organ systems with all physiological nuances, they try to mimic relevant structural, physiological, and functional features of organoids and tissues to best model disease and/or healthy states. The advent of this platform has not only challenged animal testing but has also presented the opportunity to acquire real-time, high-throughput data about the pathophysiology of disease progression by employing biosensors. Biosensors allow monitoring the release of relevant biomarkers and metabolites as a result of physicochemical stress. It, therefore, helps conduct quick lead validation to achieve personalized medicine objectives. The organ-on-chip industry is currently embarking on an exponential growth trajectory. Multiple pharmaceutical and biotechnology companies are adopting this technology to enable quick patient-specific data acquisition at substantially low costs.

Keywords: organ-on-a-chip, biosensors, disease models, microphysiological systems, microfabrication

Introduction

The organs-on-chips (OOC) market is expected to reach \$350.8 million by 2030, with a Compound Annual Growth Rate of nearly 60% since 2015^[1]. This constant market growth has been driven but not limited to the global demand to ban cosmetic animal testing. As evidenced by the EU directive 76/768/EEC^[2], Mexican general law of health directive 465 Bis^[3], the US Food and Drug Administration Modernization Act of 2011, and the Humane Research and Testing Act (HR 1744)^[4]. Recent advances in microfluidics, additive manufacturing, and 3-D cell culture have significantly affected the OOC technological development and expansion. The growing number of partnerships and collaborations between pharmaceutical companies and OOC manufacturers to develop high-throughput and scalable drug testing platforms to minimize financial losses due to late-stage drug failure has accelerated efforts to design and develop OOC platforms^[1]. The Pharmaceutical industry is afflicted with the limitation of high throughput screening and clinical scalability of contemporary drug discovery procedures. Therefore, it is pertinent to try to reproduce patient-specific tissues and organ systems in vitro and integrate them with high throughput sensing platforms.

This perspective highlight the most relevant advances in the last few years on biosensor integration in OOC platforms to achieve sensitive and real-time monitoring of metabolites and relevant biomarkers. The perspective also discusses potential challenges and opportunities to achieve clinically scalable, high-throughput multiorgan-on-a-chip systems with real-time monitoring using novel integrated imaging techniques and novel optical biosensors.

1. OOC general overview and state of the art

Each year the pharmaceutical industry spends billions of dollars on a single drug candidate to get it from the bench to the market. The conventional route of drug discovery includes testing for safety and efficacy in animal models, which offers negligible to minimal reproducibility in humans^[5]. About 60% of animal-tested drugs are rendered inefficacious in clinical trials. Typical examples include vaccines for tuberculosis, Hepatitis C, and Hu5c8 monoclonal antibodies, which passed the animal testing phase but failed to reproduce similar efficacy in humans or elicited a toxic response ^[6,7]. Equally disturbing is that multiple drugs

that could have been efficacious in humans never made it to clinical trials. Due to this weak predictive power of animal testing and the high costs incurred, the need for alternatives became a severe concern for the pharmaceutical and biotechnology industry. Attempts were then made to establish mammalian cell cultures with nutrient provisions. These static 2-D cell cultures were, after that, extensively used to study underlying pathophysiological cues which determine drug responses but eventually were also proven incapable of accurately replicating the *in vivo* biochemical and physiological environment *in vitro*^[7]. OOC technology rapidly developed and occupied an important niche in personalized medicine and drug development (see Figure 1). It uses different types of cells withdrawn from human donors to encapsulate them in a suitable biomaterial, thereby allowing them to proliferate and differentiate in a suitable microphysiological system^[8]. Therefore, the technology presents different techniques for co-culturing cells to form organoids or placing tissues together with suitable barriers to study crosstalk between tissue or organ systems.

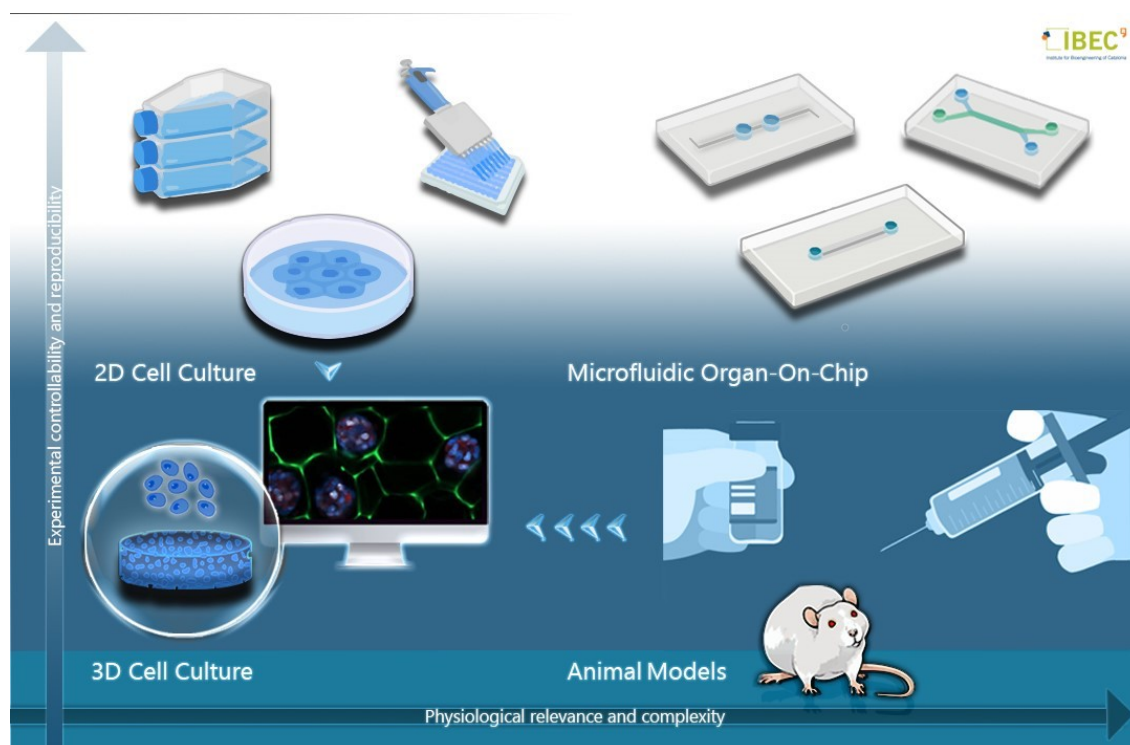


Figure 1. Relevance of OOC devices as platforms to replicate human biology. Preclinical studies employ in-vitro cell cultures and in-vivo animal models for drug testing. 2D models represent fast and high-throughput tools for drug testing. However, 3D models try to mimic advanced physiological tissue environments. Therefore, it is expected to be more accurate on drug responses. Animal models represent the gold standard in drug testing, but differences in physiological mechanisms between animals and humans promote a lack of accuracy and reproducibility of results. Microfluidic OOC devices allow controlling cell culture parameters to mimic organ microenvironments. As a

consequence, provide a more physiologically relevant environment to interrogate human biology. Adapted icons BioRender.com

The choice of a suitable biomaterial considers different physical-chemical characteristics: diffusion to and from the environment, biocompatibility, topography, and micromechanical properties such as porosity, swelling, topography, and stiffness^[9]. Once mature and functional, these organoids can be assembled on microfluidic sensing platforms to allow for *in situ* biosensing applications. Microfluidic platforms allow maintaining the organoid in environments similar to their natural cellular microenvironment^[10]. Initially, the efforts focused on designing and developing the right microfluidic system ensuring appropriate perfusion and tissue functionality. However the objective has since evolved to bring the models as close as possible to replacing animal models^[7,9]. Whether the aim is to reproduce the respiratory crackle sounds or establish an oxygen gradient across tissue interfaces, single and multiorgan on-chip platforms have currently occupied a top niche in research on interdisciplinary science.

2. 2D Vs. 3D In Organs-on- Chip, State of the Art

2.1. 2-D Culture

Beginning from Wilhelm Roux's "*Entwicklungsmechanik*" in 1885, developmental biology has undergone multiple transformational phases to be what it is at the moment^[11]. The term "*Entwicklung*" stood for "*development by natural causation*", which favors the functional attributes of biological development over time in the presence of natural biological cues. The last set forth the precedence of 2-D culture to study a plethora of biochemical and functional changes accompanying growth in a specific cell type. In Roux's words, "*We now wish to learn what this extensive play of changing shapes is good for*"^[11].

A 2-D culture involves adhering to and maintaining a proliferative mono-layer cell culture^[12]. It is sometimes also pivotal to keep cells at a certain specific maturation stage to ensure proliferative growth. However, stimulation of proliferative growth to keep the proliferative factors is also associated with a

decline in tissue-specific functions, leading to questionable data. For a wide variety of cells sourced from solid tissues, adherence to the substrate is a common and critical step before initiating proliferation^[12]. For anchorage, cells release matrix proteins that attach to the conditioned plastic substrate, followed by receptor-mediated cell adherence^[13]. It is important to mention that different cell types produce several matrix elements that contribute to the extracellular matrix (ECM). For example, epithelial cells release laminin while fibrocytes release collagen type I and fibronectin. This ECM is subsequently responsible for regulating the phenotypic expression of cells^[13]. The complex and dynamic composition of the ECM is, therefore, an important variable to consider while controlling a specific cellular phenotype. The lack of a suitable perfusion system results in nutrient depletion and accumulated toxic waste over time which must be frequently replenished and removed^[9,10].

2.2. Microphysiological Systems and 3-D Organoids

The need to better sustain functionality over extended periods and achieve biomimicry, gave birth to micro-physiological systems that allowed the development of 3-dimensional encapsulation of tissues in hydrogels or materials mimicking the ECM^[14–16]. These scaffolds provide essential support to the cells and help restore and repair damaged tissues^[17]. The employed biomaterials used for making these scaffolds are porous and permeable materials to confer flexibility and easy diffusion. Due to this porosity, they help release biological cues and bioactive molecules such as cytokines, antibiotics, inhibitors, stimulators, and other externally added drugs^[15]. The scaffold's charge also plays an essential role in improving the proliferation capacity. For example, increasing the positive charge helps the cells spread and proliferate due to their negatively charged membranes. The elastic modulus of the scaffold is another important property that determines the ease of adhesion, differentiation, and overall morphology of the tissue construct. These scaffolds can be made artificially or sourced from natural sources such as the IKVAV, YIGSR laminin-derived sequences, and self-assembling peptides^[18,19]. The ability of hydrogels to mimic viscoelastic and topographical cues makes them the material of choice for developing scaffolds.

3-D tissue constructs grown in a static environment can mirror histological and functional attributes, particularly for drug metabolism studies. In order to develop

organ systems or organoids *in vitro*, multiple types of tissues or cells must be co-cultured. The first-ever organ on chip recapitulated the lung alveolus using soft lithography inspired by the microchip industry. This chip's concept comprises two-channelled PDMS structure separated by a porous membrane^[20]. This porous partition was coated with the ECM to allow the growth of human alveolar epithelial cells on one side and vascular endothelial cells on the other. It helped to mimic not only vascular perfusion but also the liquid-air interface present in the lungs. From there on, multiple organ chips were developed using different microengineering approaches. For instance, the most convenient format to develop tissue-tissue interfaces are trans-wells with a porous partition serving as a barrier between two cell types. The well with co-culture is usually accompanied by a reservoir and a flow channel beneath the chambers to help with nutrient perfusion^[21]. A more extensive fluidic coupling can also be installed for multi-fluid transfer and collection^[21,22]. To facilitate high-resolution imaging and visualization, the material of these chips is preferred to be transparent.

Since the first use of human lung alveolar epithelial cells to develop the lung alveolus chip, the model has been used to study bacterial infections, toxic exposure to nanoparticles, perfusion of chemotherapeutic drugs such as IL-2, and pulmonary edema toxicity due to it^[23]. Observations from these experiments shed light on the importance of mechanical stimulation and thereby the inference that dynamic conditions best mimic pulmonary toxicity disease conditions compared to static^[20]. Therefore, work on multiple axes has aroused growing interest in organ-on-chip devices, such as developing models for the brain and blood-brain barrier in conjunction with gut and microbiota to study the dynamic relationship between gastrointestinal microbiota and the gut-brain axis. The overall system has three types of cells to mimic brain function: neurons, glial cells, and astrocytes. Caco-2 cells performed the gut function, and for the immune system, they used macrophages and lymphocytes. Meanwhile, endothelial cells recapitulate the blood-brain barrier^[24].

2.2.1 Single Organ-on-chip Models

2.2.1.1 Tumor-on-Chip

OOC platforms have been popular for studying cancer's underlying mechanisms and multifaceted disease pathology. In particular, these models help to mimic the tumor microenvironment in solid and liquid forms. The tumor microenvironment includes chemokines, stromal cells, and immune suppressor cells^[25]. For example, a single organ-on-chip was designed to mimic pancreatic cancer to investigate the interaction between the ductal adenocarcinoma, vascular system, and activin signaling. This model helped explain hypovascularity, which leads to low drug delivery and poor chemotherapeutic outcomes for an aggressive type of cancer. To develop tumor biomimetic models several different factors have to be considered such as the oxygen gradients responsible for inducing *in vivo* intravasation and optimum perfusion. Sung's group developed 3-D tumor models based on spheroids to model breast cancer invasion by employing the technique of surface tension pumping^[26]. This allowed them to bring about sequential loading of cells at different time points. Thereby providing an important model to study the spatial and temporal cues that determine metastasis, tumour invasion and cell migration. More recent attempts have developed a 3-D model with controlled perfusion systems to model the microvascular system of the tumour microenvironment. The Lung Tumor chips developed by Hassell et al., using the human NSCLC cells helped to explain cancer dormancy and resistance to tyrosine kinase-based inhibitors^[27]. Brain tumor chips developed using U87 glioblastoma cells allowed screening drugs such as pitavastatin and irinotecan. These and multiple other tumor models exemplify that the OOC technology can be used to study signaling mechanisms, the mechanobiology of disease progression, and immune suppression leading to poor therapeutic outcomes. In parallel, the pharmacokinetic (PK) and pharmacodynamic (PD) studies of different drug candidates on the disease models can help understand their dose-response relationship and their administration dosage. PK and PD studies using multiorgan systems have been carried out for nicotine, fluorouracil, cisplatin, amiodarone, and several other drugs ^[7,28].

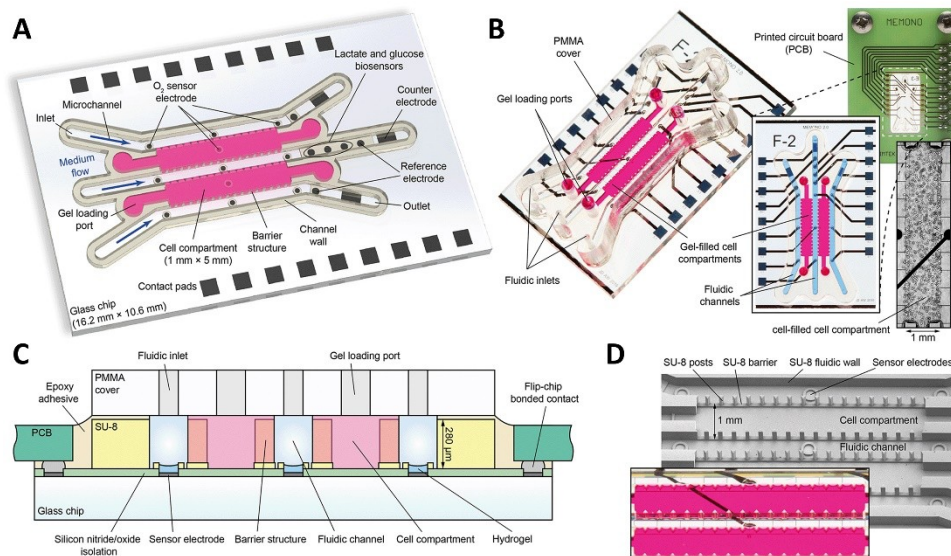


Figure 2: Overall design of the system, arrangement and fabrication A) Top-view of the glass cell culture system for the production of spheroids with microchannels for nutrient supply and fluidic control, in conjunction with electrochemical metabolite sensors for oxygen, glucose and lactate to enable *in situ* monitoring B) An assembled device C) Cross-sectional schematic D) SEM micrograph of patterned SU-8 structures^[29].

2.2.1.2 Hepatic Models

Ex-vivo human hepatic models allow for studying drug metabolism and pathology of drug-induced damage by mimicking the spatiotemporal characteristics of the organ^[30]. In addition to the conventional 2-D micropatterned systems using a combination of hepatocytes and 3T3-J2 fibroblasts^[31], 3-D designs have been developed that employ static hepatic spheroids in conjunction with perfusion systems to introduce nutrients, withdraw metabolites in real-time, establish oxygen gradients, and shear stress^[32]. One important biomarker to study the proper functioning of ex-vivo hepatic models is the cytochrome P450^[30]. This enzyme helps synthesize cholesterol, steroids, and prostacyclins, and the source of hepatocyte retrieval has influenced its activity. For example, primary human hepatocytes and cryopreserved human hepatocytes had higher CYP450 activity than animal and iPSC-derived hepatocytes^[33]. Some non-perfusion hepatic systems include HepatocPac, PDMS Stencil, Microarray chip, micropatterned fibrous mat, RegeneTox, and GravityTRAP^[34]. On the other hand, perfusion-based systems include DILI Train, HUREL Tox, Flux, and Viral chips. The HUREL chips have eight microfluidic channels arranged in parallel while two are connected in series. The material of the biochip is polystyrene which allows cell seeding to develop mono and co-cultures. The flow rate within the chip was

optimized to 4.5uL/min/chip using peristaltic pumps^[35]. Under flow conditions, the hepatocytes exhibited higher metabolic activity compared to static cultures. The platform can also reduce unwanted adsorption of hydrophobic drugs, often a common problem in PDMS platforms^[34,35]. While these advancements contribute to technology, there can still be architectural improvements in the microstructure of hepatic models and the incorporation of fluorescent biomarkers within cells to provide an automated quantification of requisite outputs *in situ*.

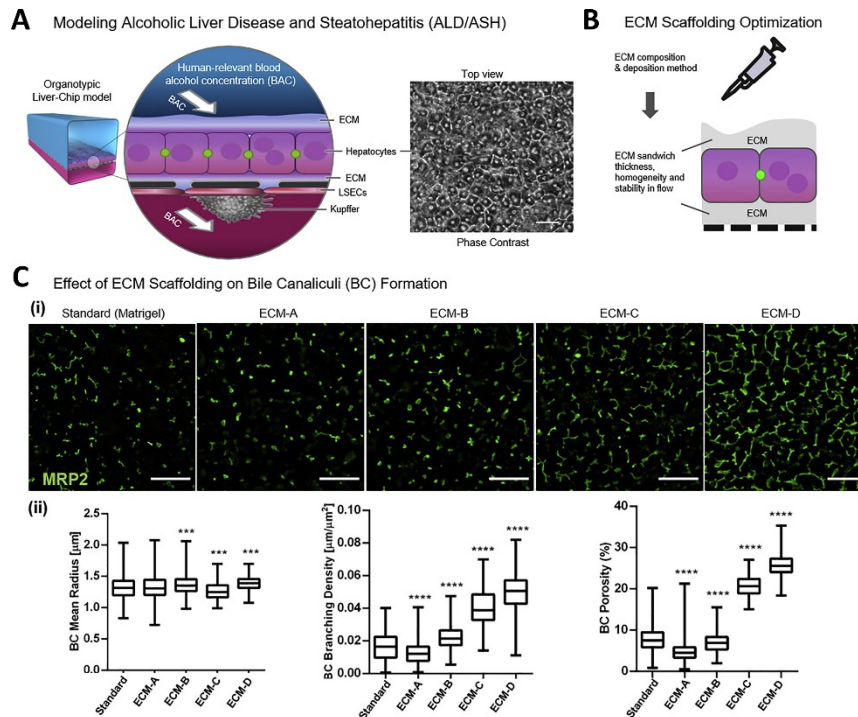


Figure 3: Alcohol associated Liver Disease Liver-Chip^[36]
[37];

[7]. 2.2.1.3 Skeletal Muscle Models

Contemporary gold standard techniques for *in vitro* characterization of muscles are based on studying the expression of myogenic markers and fusion indices. While this information is indispensable for observing and studying differentiation in culture, it does not help in understanding the physiological processes such as the force of contraction, opening, and closing of the Ca^{2+} -gated channels. Progressively degenerative diseases such as muscular dystrophies, amyotrophic lateral sclerosis, atherosclerosis, and other inflammatory illnesses affecting muscles have been studied using muscle-on-chip platforms with the requisite perfusion strategy. Platform design, choice of biomaterials, and integration with

the proper testing platforms can help to model and study the pathology of multiple diseases such as diabetes mellitus, implicating skeletal muscles. The most widely used approaches for generating *in vitro* skeletal muscles include micropatterning and microcontact printing, bioprinting, and electrospinning. Other techniques include E-field assisted printing, Microfluidic extrusion, droplet-emulsion assisted patterning, and dielectropatterning. Geometric cues such as anisotropy of scaffold fibers help form better-aligned sarcomeres and enhance cell maturation. Electrical stimulation with varying frequencies can also be used to micropatterned substrates to enhance myogenic differentiation further. Biomaterials such as PEG-DA, GelMA^[38], CMCA, PEDOT^[39], and Matrigel-Fibrinogen have been used to micropattern and cast hydro or cryogels for cell encapsulation to eventually form biochemically and physiologically viable *in vitro* muscle organoids^[40].

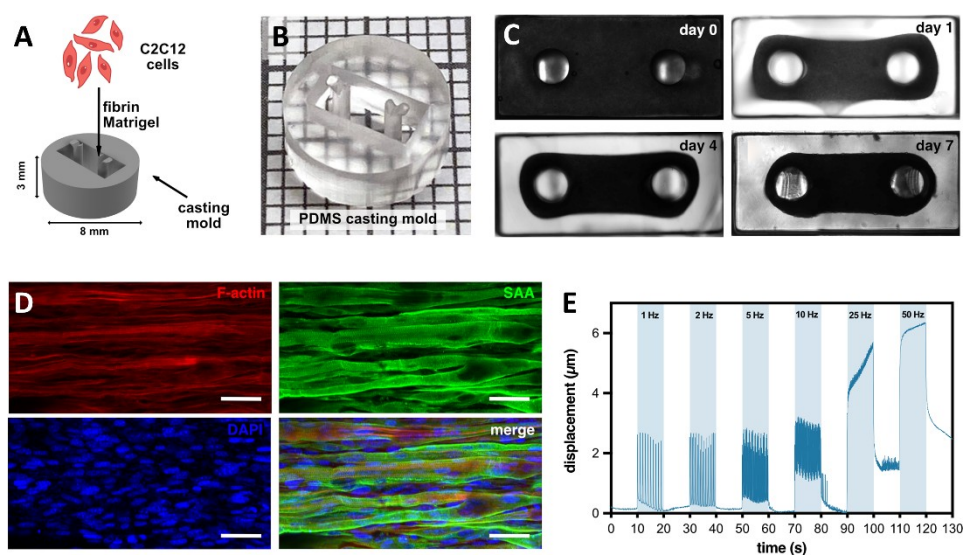


Figure 4: 3D bioengineered skeletal muscles^[40]

2.2.1.4 Other Models

Induced pluripotent stem cell-derived cardiomyocytes cultured in ECM were grown within a single-channel microfluidic system on micro-array electrodes to record the electrophysiological responses *in situ*^[33]. This platform helped to evaluate the cardio-toxic response to drugs such as terfenadine and doxorubicin. Other models utilized micropatterning through soft lithography, microcontact printing, electrospinning and 3D bioprinting to patterns HUVECs, rat-derived cardiomyocytes and fibroblasts. The materials employed by these models include PAAM, Collagen, GELMA and MeTro. All of these models helps to understand

the effect of material properties such as stiffness on cellular attachment, proliferation, alignment and communication, An important component of the heart-on-chip models is the microactuator which allows introduction of external stimuli to enable cell maturation. These actuators can be electrical or mechanical. In the case of electrical actuators multiple types of electrodes can be used, such as indium tin oxide, gold, graphite, titanium and platinum. There are two types of mechanical microactuators; pneumatic and electromagnetic. Mihic et al., discovered that cyclic stretching helped drive the maturation of stem cell-derived cardiomyocytes and the formation of 3-D tissue^[41]. To induce stretching they introduced a non-contact electromagnetic force by fixing custom-built stainless-steel clamps at both ends of the tissue construct, it was possible to control the frequency, pattern and time duration of the stretching cycles.

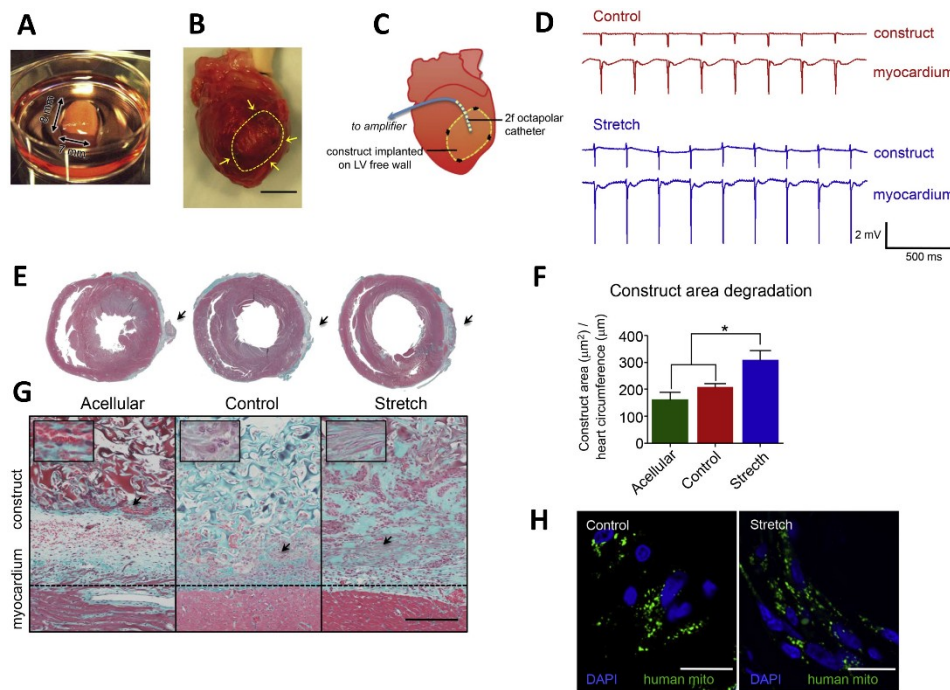


Figure 5: Implantation and characterization of hESC-CM constructs on Rat Epicardium^[41].

Optimizing microfluidic parameters to promote villi formation for developing intestinal models also warrants significant mention. Efforts have been made to create intestinal models with or without endothelium. Compared with static flow, dynamic flow has been found to assist in the development of villi and in increasing the number of goblet cells and secretion of the mucous membrane. Increasing or

decreasing receptors such as ACE-2, mucous production, and associated morphological and inflammatory changes in response to external stimuli or treatment can help understand disease progression^[7].

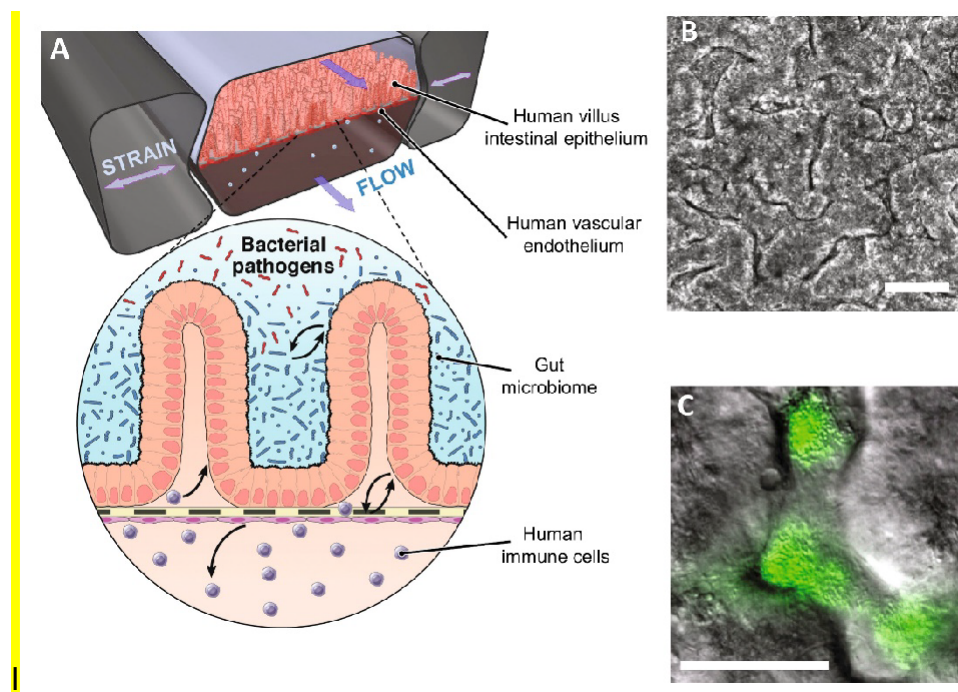


Figure 6: Intestine-on-a-chip^[42]

2.2.2 Multiorgan-on-chip models

While single organoids models can provide important insights into disease progression as evidenced in the previous section, to accurately model and study multi-factorial or multi-organ-based disease pathophysiology, it is essential to allow physiological communication between two or more different types of organs. Efforts have been made to establish co-cultures, vasculature, and multiorgan on-chip (MOC) platforms with the recirculating vascular flow. Every organ in the body functions within its physiological boundaries. Therefore, while designing multiorgan platforms, it is essential to maintain and preserve the independent physiological space of each organ^[43]. Therefore, establishing endothelial barriers and promoting organ-organ crosstalk through the vascular fluid is critical to achieving functional integration. When designing endothelial barriers, shear stress is an important hemodynamic parameter. Adequate shear stress is essential to maintain tight barrier function and to form gap junctions. The

barrier thus formed allows tissues to communicate through the release of cytokines and exosomes through the vascular fluid. Ronaldson-Bouchard et al. interlinked hepatic, cardiac, bone, and skin tissues on a multiorgan platform and studied their crosstalk via recirculating vascular fluid across endothelial barriers^[43]. This approach allows the development of personalized disease models for each patient to identify early disease biomarkers and establish patient-specific toxicity profiles. Mechanistic models to study pharmacokinetics are limited by flow or permeability rates. To simulate mechanistic PK models for multi-compartment/ organ systems, it is, therefore, essential to model vascular flow as a closed loop, the endothelial barrier as a porous membrane, and the topography as close as possible to real-time organ morphology. Assuming a closed-loop vascular system, it is also important to consider the conservation of mass and volume in mechanistic studies. Multiple studies are continuing to explore possibilities to improve technical and physiological nuances in disease models and eventually move towards personalized treatment approaches.

Even physically distant, the interaction between organs is essential for the function of the human body. Chemical factors are released from one cell to another to maintain the body's homeostasis. This continuous feedback loop system is tightly regulated at local (i.e., paracrine signals) and systemic levels (i.e., hormones). With the advance in technology applied to human health, it was possible to identify players that affect other organs using blood and lymphatic flow, such as miRNA, extracellular vesicles, cytokines, peptides, etc. When this communication is not correct, many diseases can result. A dysfunctional organ typically occurs in the early stage of the disease, but when it reaches a more advanced stage, the involvement of multiple organs occurs ^[44]. The understanding of cross-organs communication must be pursued to decode the mechanisms underlying the disease to find potential biomarkers and targetable players for diagnostic and therapeutic purposes. A tremendous leap forward has been accomplished in replicating *in vitro* many pathological processes in OOC devices on such a smaller scale by using the organ-to-organ communication information gathered. These systems can be employed to demonstrate the causality of an organ on a different organ during disease development ^[45]. Initially, these systems were used for toxicity studies, but over the years, they became an

essential tool for personalized medicine ^[46]. The complexity of these systems is determined by organ-to-organ biology that must be replicated. MOC can include cell lines ^[45], Induced pluripotent stem cells (IPS) ^[47], and primary cells ^[48] representing different organs. The next step of complexity is reached when cellular integration is considered. The cells can be either cultured in 2D ^[49], in circulation ^[47], encapsulated in specialized scaffolds ^[45], separated by a barrier ^[50], or including an air-liquid interface condition ^[51]. Since there are no standard rules, principles, or a clear outcome, all these MOCs present various limitations that must be solved before this technology can be integrated for diagnostic or treatment reasons.

Primarily, the MOCs have a wide range of measurable outcomes such as can be mechanostructural (i.e., force ^[52] and architecture ^[53]), physicochemical (i.e., oxygen ^[54] and charge ^[55]), biochemical (i.e., hormones ^[56] and cytokines ^[57]), and particles production (i.e., extracellular vesicles ^[58]). However, the results are difficult to reproduce since the MOC only works using the setup and sophisticated instrumentations present in the laboratory where it was developed. Second, most publications do not show the variability in the fabrication process and its impact on the microfluidic of MOC, which impacts the experimental success ratio and, more importantly, the cell phenotype. Third, MOCs are built using no Good-Manufacturing Protocol (GMP) processes, which prevents them from being applied to patients. Obtaining GMP certification is a long and expensive process that few laboratories can afford. Fourth, the cells, tissues, organoids, and 3D printed tissues employed to build a MOC have their own set of supplements in the medium (i.e., glucose content, amino acids pool, insulin, transferrin etc.) designed for the specific organ. However, when multiple organs are co-cultured, such organ-specific supplements can affect the metabolism of the downstream organ. Fifth, intensive collaboration is essential between engineers, biologists, and computational scientists to analyze and interpret the results. Overall, the MOCs have a disruptive potential to give us more information about drugs and compounds, reduce animal experimentation, speed up the diagnostic and drug discovery pipeline, and unveil important cues about human biology and disease progression. With the reverse-engineering approach, MOCs allow us to progressively exclude both players to reproduce in vitro a specific pathologic

phenotype and biases introduced by operator sampling, genetic components, and physical variables.

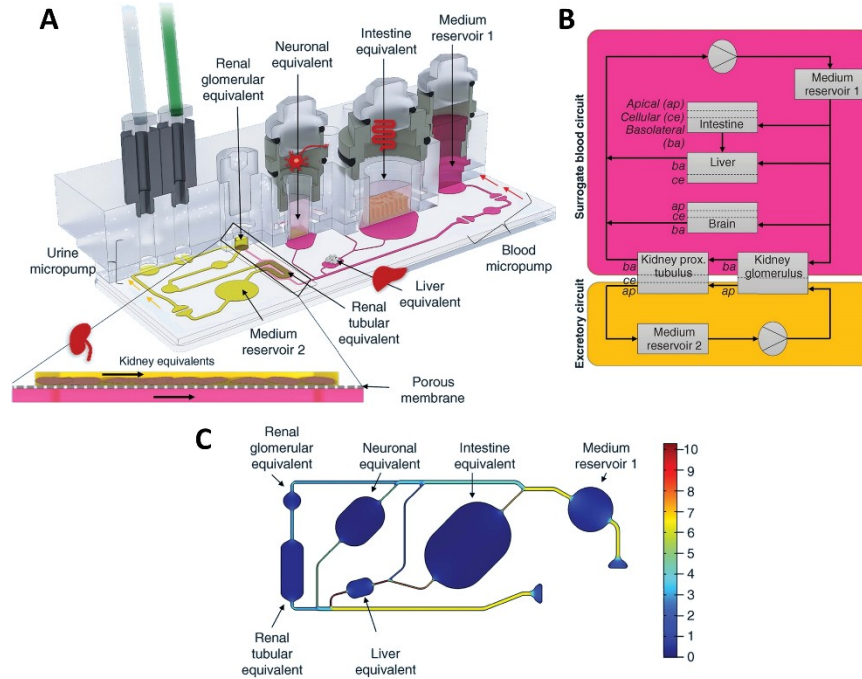


Figure 7: The Four-organ-on-chip Microfluidic Model schematic A) Physiologically relevant model of the four-organ-on-chip B) Yellow: Excretory system and Pink: blood circuit C) Distribution of the wall shear stress^[59]

3. Biosensing integration in OOC

In 2009, the first microfluidic perfusion system was designed to conduct functional analyses and quantify insulin production from pancreatic beta cells following a glucose challenge through ELISA^[60]. Within an organ system, at a specific time, multiple factors are at play, such as the pH, oxygen gradient, temperature, chemicals such as hormones, cytokines, and metabolites such as glucose, lactate, and calcium^[61]. These culture parameters offer the diagnostic potential to monitor cell behavior changes using various techniques. Biosensors can be briefly defined as integrated analytical tools that allow the quantitative analysis of biochemical interactions with high accuracy in a few minutes with minimal sample volume and pretreatment. Biosensors have high miniaturization and integration potential for developing Lab-on-a-chip devices^[62]. Consequently, they are highly suitable to be integrated for real-time monitoring in 2D cell

culture^[63] and OOC devices^[64]. A biosensor usually uses antibodies, aptamers, or enzymes as bioreceptors/biorecognition elements. Among the different transduction methods for biosensing in OOC devices, mainly electrochemical and optical transducers have been reported due to their well-known potential for direct and label-free sensitive biodetection.

Over the last few years, the number of biosensors to detect relevant analytes and biomarkers secreted by OOC devices has steadily increased. However, there have been limited advances in integrating biosensors with OOC devices to achieve fully integrated and autonomous platforms (see Figure 2). Although the integration in a whole single chip of biosensors and 3D organoids would be desirable and would represent an evolution in the technology, there are still different challenges to surpass. We have observed over the last years systems that include biosensors integrated into the OOC device or in a modular way interconnected in line with the organ on chip. The selected integration is highly related to the transduction method, the type of analyte/biomarker to detect, and the physicochemical stability of the biorecognition elements. Among the challenges to surpass, microfluidics is an indispensable module for performing simultaneous analysis with low sample consumption. Consequently, an appropriate design of microfluidics pathways, chambers, etc., can influence the speed of the reactions, sensitivity, and mass transfer^[65]. On the other hand, surface functionalization that allows the target's sensitive and selective bio/chemo-recognition while decreasing non-specific binding from different molecular elements present in cell-culture media is highly desirable^[66]. Also, it is crucial to consider that a biosensor with potential recycling/regeneration^[67] or a wide working range for multiple biodetection cycles would be ideal.

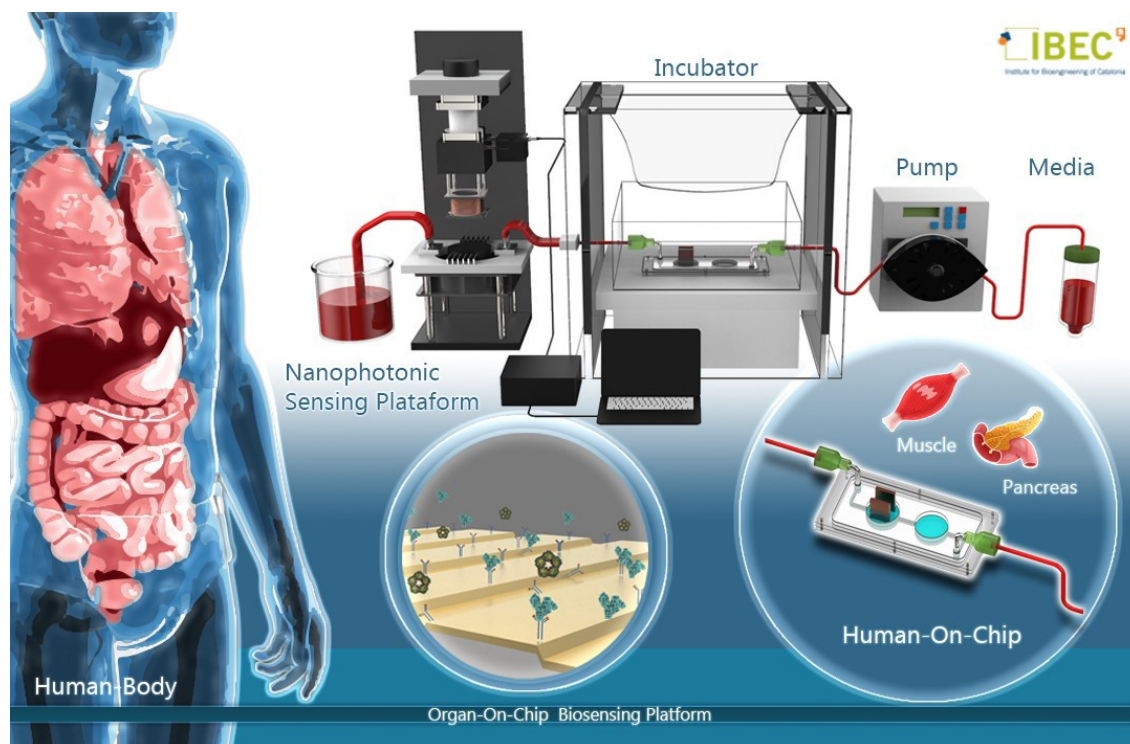


Figure 7. Schematic illustration integrating a multi-OOC device with nanocrystal-based plasmonic biosensors for direct, label-free, and real-time biodetection of relevant biomarkers. Integrated platforms must mimic relevant environmental conditions while providing opportunities to couple novel sensing technologies. Microfluidic devices with the potential for multiorgan coupling for crosstalk are desirable.

3.1 Breakthroughs in Biosensing integration in OOC

As mentioned before, there have been limited advances in integrated biosensing platforms for *in situ* biodetection of segregated biomarkers or metabolites from OOC devices; these are described mainly in a modular integration in line with the organoids rather than full integration in a chip. Due to the wide availability and variety of commercial electrodes, most technological advances have been achieved using electrochemical biosensing. Recently, the Ramon-Azcon group has presented a multiplexed modular in-line platform based on gold electrochemical biosensors for the biodetection of IL-6 and TNF- α secreted by electrical and chemical stimulation on a muscle OOC platform using monoclonal antibodies as biorecognition elements^[68]. Although the electrochemical biosensors achieved sensitivity in the order of ng/mL, the biodetection was based on a "sandwich" assay using a secondary recognition antibody, increasing the complexity and not being a direct assay. The Khademhosseini research group has developed other examples of modular in-

line electrochemical biosensors in OOC platforms. One first approach allows the multiplexed monitoring of Creatine Kinase, Albumin, and GST- α on a heart-liver OOC model using monoclonal antibodies as biorecognition elements with a sensitivity in the order of ng/mL^[69]. A subsequent approach allowed the monitoring of Creatine Kinase in a heart OOC model using aptamers as biorecognition elements. The electrochemical biosensors based on aptamers achieved a superior shelf life and biosensing performance (in the order of pg/mL) compared to those based on monoclonal antibodies^[70]. In the case of metabolites, Weltin et al. presented a brain cancer OOC device with integrated on chip electrochemical biosensors for the multiplexed biodetection of glucose and lactate using glucose and lactate oxidases immobilized in a membrane and detecting the formation of hydrogen peroxide^[71]. Both biosensors achieved sensitivity in the μ M range and allowed them to obtain a measurement in less than a minute with high stability, selectivity, and reversibility. More recently, Bavli et al. integrated on chip enzyme-based commercial electrodes to integrate them and monitor glucose and lactate on a liver OOC model with sensitivity near μ M^[72]. The biosensing system was automated to perform the perfusion of the biosensors with the potential to achieve multiplexed measurement every 200 seconds.

Finally, considering the potential benefits of optical biosensors in terms of direct, label-free, and real-time multiplexed biosensing Ramon-Azcon group has presented different optical biosensing platforms based on plasmonic metamaterials for monitoring multiple biomarkers secreted from OOC. In a first approach, rod-like gold nanoantennas obtained by electron-beam lithography were used to monitor insulin secretion from a pancreas OOC model under low/high glucose concentrations^[64]. The proposed optical biosensor allowed direct, label-free, and multiplexed in-line monitoring of insulin secretion from OOC. However, the achieved sensitivity was in the order of ng- μ g/mL, unsuitable for detecting other biomarkers, usually in the order of pg-ng/mL. In a recent approach, nanograting-like plasmonic metamaterials based on commercial Blu-Ray optical discs^[73] were used to detect in-line the IL-6 and Insulin secretion in a pancreas-muscle OOC device simultaneously. The proposed biosensor platform not only achieved sensitivity in the pM range but also provided direct, label-free, and multiplexed biosensing with a high-throughput, lithography-free

fabrication method^[64]. **Table 1** summarizes the main relevant integrated biosensors applied to OOC platforms, including information like detection method, detected biomarker, sensing performance, integration level, cell culture type (2D-3D), and the features of the proposed biosensors.

Table 1. Research papers overview using integrated biosensors in OOC platforms.

OOC platform Biosensor type	Bioanalytes	Integration Level and Features
Brain Cancer Electrochemical ^[74]	Glucose and Lactate	Integrated on Chip-(2D cell culture) Engineered enzymatic electrodes with LOD in μM range
Liver Electrochemical ^[75]	Glucose and Lactate	Modular interconnected-(3D cell culture) Commercial enzymatic electrodes with LOD in μM range and fully automatized
Muscle Electrochemical ^[76]	IL-6 and TNF- α	Modular interconnected-(3D cell culture) ommercially available electrodes with LOD in nM range and multiplexed detection with indirect assay (sandwich)
Liver-Heart Electrochemical ^[77]	Creatine Kinase, Albumin, and GST- α	Modular interconnected-(3D cell culture) Engineered electrodes with monoclonal antibodies biorecognition elements in nM range and fully automatized
Heart Electrochemical ^[78]	Creatine Kinase	Modular interconnected-(3D cell culture) Improved shelf life and biosensing performance using aptamers with LOD in pM range
Pancreas Optical ^[79]	Insulin	Modular interconnected-(3D cell culture) Direct, label-free, and multiplexed optical biosensing with high-resolution lithographic fabrication and LOD in μM order
Muscle-Pancreas Optical ^[80]	IL-6 and Insulin	Modular interconnected-(3D cell culture) Direct, label-free, and multiplexed optical biosensing with lithography-free fabrication and LOD in pM range
Breast Cancer Electrochemical^[29] 1	*Oxygen, Glucose and Lactate	Integrated on Chip-(3D cell culture) Engineered enzymatic electrodes with LOD in μM range, system combine bio and chemosensors

Abbreviations: Limit of detection (LOD)*Oxygen detection based on chemosensing

As previously described, in most examples of biosensors integration in OOC devices, biosensors are modular in-line interconnected to the OOC device. The selection of modular platforms can be highly correlated in some cases with the complexity of integration of the transduction method and the physicochemical stability of the biorecognition interfaces. On the other hand, different sensors and chemosensors integrated into chips have been described considering that external stimuli or internal abnormalities can manifest in several ways: cell confluency or adhesion, temperature changes, pH, oxygen and carbon dioxide levels, among others.

Integrated electrical sensors based on the detection of impedance are mainly reported. The electrical impedance measurement in trans-epithelial electrical resistance (TEER) is widely used to evaluate the integrity and differentiation of 2D in-vitro cell cultures; the electrical impedance across the epithelium is correlated to the junction forces between neighboring cells in a cell monolayer. Odijk et al.^[81] and Henry et al.^[82] have reported the development of integrated electrodes for impedance measurement over several days and weeks in 2D-based intestine and lung-on-chip, respectively. Collagen was selected in both models to promote cell adhesion to electrodes. On the other hand, oxygen and nitric oxide sensing integrated on-chip has been reported based on electrochemical sensing and optical chemosensing. Li et al.^[83] used core-shell titanium carbide/carbon nanowire arrays for highly specific nitric oxide electrochemical monitoring in a 3D blood vessel on-chip model. The sensor achieved a LOD in the nM level and a response time of milliseconds. In oxygen sensing, Grant et al.^[84] integrated optical chemosensors based on commercial nanoparticles engineered to measure oxygen gradients on an intestine on-chip model. The oxygen monitoring was based on a commercial fiber coupled detection system and allowed continuous detection over the 72 hrs duration of the experiment. Finally, Dornhof et al.^[29] integrated bio and chemosensing on a single chip using electrochemical sensing in a 3D breast cancer model. In one part, the enzyme detection of glucose and lactase; in the other part, the amperometric oxygen sensor proposed was based on the electrochemical reduction of oxygen species in a platinum electrode. All the sensors achieved a LOD in the μM range. Considering the vast number of materials for sensing and chemosensing purposes, we can expect, as in the last example, a hybrid combination of bio/chemosensors in organ on chip platform for a multiparametric monitoring.

3.2 Perspective in biosensing and imaging for OOC integrated platforms

As mentioned, real-time monitoring of biomarkers, metabolites, and morphological changes is desirable to study the influence of physical and chemical stimuli in 2D and 3D cultures and crosstalk between the different cell types (see Figure 2). Thoroughly integrated platforms combining real-time biosensing and imaging under relevant cell-culture conditions are required for this aim. Although, as mentioned previously, all integrated on-a-chip systems would be desirable and a technological breakthrough, the complexity, associated fabrication costs, and fixed design would be relevant limitations. However,

developing modular/brick systems (LEGO-like) would be a high-throughput, versatile, cost-effective solution.

As demonstrated in the last years, evanescent-wave optical biosensors (especially those based on plasmonic metamaterials) offer indisputable advantages in terms of sensitivity (up to aM-fM level), miniaturization, and multiplexing capabilities (multi-spot array capabilities) over other technologies ^{[[85]} in developing fully integrated OOC real-time biosensing platforms ^{[[64]}. The latest advances in nanolithography have exploded the development of meta-plasmonic biosensors with many potential materials and designs. However, in most nano lithographic technologies, there are still challenges and questions about their potential to achieve large-scale and high-throughput novel devices outside the laboratory. As we recently proposed, lithography-free methods like glancing angle deposition and thermal dewetting can help surpass these challenges and, even more, enhance the capabilities of conventional lithographic techniques ^{[[86]}.

In the case of the biorecognition layer, new biofunctionalization strategies are required, considering that a biosensor's final performance implies a direct correlation between the transducer and the biorecognition elements. Traditional biofunctionalization methods are truly time-consuming and require multiple steps. Engineered biorecognition elements with high specificity, long-term stability, the potential for direct attachment, and several detection cycles like aptamers would be ideal. These specific biorecognition elements must be accompanied by antifouling features that minimize non-specific absorptions from the cell culture media components. It looks like engineered polymer brushes and other blocking elements like serum from different host species can significantly decrease non-specific absorptions and cross-reactivity issues^{[[87]}.

Additionally, to biosensing platforms, with the latest advances in high-performance CMOS image sensors with affordable magnification elements (between 500-1000x) in handheld digital microscopes, it could be feasible to achieve integrated OOC platforms that combine real-time microscopy and biosensing information. Under relevant culture conditions, it is possible to study the morphological changes and biomarkers released from cells to drugs and physicochemical stimuli. As a first step, conventional broadband bright/dark-field can provide relevant information from cell morphological changes. However, new

techniques like multispectral imaging^[88], where certain compounds in cells have a high absorption from light, can bring to light information complex to detect by the human eye, improving the contrast of details and the real-time tracking of physiological events^[89]. Finally, the massive amount of information generated in real-time monitoring allows machine learning to be a handy tool in collecting and analyzing data. While also, with feedback from sensors, intelligent control of culture or experimental conditions can be possible.

[43][43][44][45][46][45][47][48][49][47][45][50][51][52][53][54][55][56][57][58][85][64][86][87][88][89]4. High throughput, clinical applicability scalability

Despite their numerous benefits, organ-on-a-chip platforms are complex systems with significant biological and technical complexities, which have constantly increased over the past two decades^[90]. To reproduce biological models that resemble native conditions, OOCs require the coordination of individual multidisciplinary elements. For instance, the material choice, sterilization technique, surface modification, and peripheral equipment (pumps, incubators, sensors, among others) are tied to one another^[91]. Similarly, the phenotypes observed during an experiment run inside an OOC are the outcome of the interaction between single elements, each adding variability and propagating the experimental error^[90]. Due to this modular nature, OOC devices deal with numerous sources of variability that influence reproducibility. OOC devices, therefore, show low reproducibility, like many other disciplines^[92]. A significant challenge lies in reducing background variability from multiple elements to detect and measure the systems' response to the treatment of the study.

The manufacturing process can be optimized at different stages, and there is still massive room for improvement if OOCs to reach commercial availability^[93]. Tools like checklists that help verify the dimensions and features of the device have been suggested to improve reproducibility in OOC fabrication. This could help researchers to assess weak spots in their fabrication processes. Additionally, commonly used prototyping materials like PDMS have poor scalability, and it is necessary to transfer initial designs to automated fabrication.

Large-scale manufacturing employs techniques like injection molding, and alternative materials compatible with these technologies are often expensive.

Recently has been substantial efforts to standardize the experimental work in OOC devices. Technology-related, production-related, and market-enabling aspects of OOC can be used to determine the main tasks towards standardization in fields like metrology, performance characterization, and quality ^[94]. Therefore, scientists must collate the primary sources of variability and prioritize standardization tasks according to importance and feasibility. Researchers should, for instance, aim to standardize fluid, gas, and temperature control, which is a highly feasible and essential task. The recommendation of optical readouts and the definition of quality controls for ECM fabrication (concerning mechanical properties, composition, and batch-to-batch variability) are further examples of highly feasible and vital tasks in OOC technologies^[94].

One of OOC technologies' most interesting potential applications is the drug discovery screening process. However, drug screening needs high-throughput (HT) platforms to assay a significant number of compounds simultaneously, which has not been already achieved for the OOC. HT assays require pharmacological relevance, reproducibility, and low-cost, high-quality outputs. For example, standard HT drug screenings using cell cultures imply the use of up to 1536 well plates that allow to assay 10000-100000 compounds in 24 hours. The small number of HT-OOC platforms available to date reflects that several challenges are still present in OOC technology and must be addressed to establish real HTS platforms^[95–97]. These challenges include the limitation in the fabrication scale-up process due to the chip materials, the automatization of the injection of media and drugs, and the integration of robust analytical devices as real-time biosensing platforms. The fabrication processes must be standardized to achieve reproducible protocols using materials that permit multiplexing.

Moreover, media and compounds must be automatically and precisely introduced and extracted in the HT-OOC to allow the parallelization of the assays while reducing variability. To achieve this, complex microfluidics designs must be implemented in HT-OOC devices. Finally, the outputs of the drug screening must be robust by integrating the analytical devices that allow multiplexing readouts, including optical, electrochemical sensors, fluorescence markers, and

biochemical readouts. HT-OOC devices integrating these biosensing platforms would enable the assay of compounds in biomimetic systems, which would be a step forward in the field of drug discovery.

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A.



Sensors and Biosensors in Organs-on-a-Chip Platforms

3

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Abstract

Biosensors represent a powerful analytical tool for analyzing biomolecular interactions with the potential to achieve real-time quantitative analysis with high accuracy using low sample volumes, minimum sample pretreatment with high potential for the development of in situ and highly integrated monitoring platforms. Considering these advantages, their use in cell-culture systems has increased over the last few years. Between the different technologies for cell culture, organs-on-a-chip (OOCs) represent a novel technology that tries to mimic an organ's functionality by combining tissue engineering/organoid with microfluidics. Although there are still challenges to achieving OOC models with high organ mimicking relevance, these devices can offer effective models for drug treatment development by identifying drug targets, screening toxicity, and determining the potential effects of drugs in living beings. Consequently, in the future, we might replace animal studies by offering more ethical test models. Considering the relevance that different physiological and biochemical parameters have in the correct functionality of

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cells, sensing and biosensing platforms can offer an effective way for the real-time monitoring of physiological parameters and, in our opinion, more relevant, the secretion of biomarkers such as cytokines, growth factors, and others related with the influence of drugs or other types of stimulus in cell metabolism. Keeping this concept in mind, in this chapter, we focus on describing the potential use of sensors and biosensors in OOC devices to achieve fully integrated platforms that monitor physiological parameters and cell metabolism.

Keywords

Sensors · Biosensors · Organ-on-a-chip · Screening · Pre-clinical platforms

3.1 Biosensors

According to market projections, the biosensors market is valued at USD 25.5 billion in 2021 and can reach USD 36.7 billion by 2026 [1]. Between the different applications, point-of-care (i.e., insulin monitoring, pregnancy test) represents the main segment of the market (about 57%); however, there is a remarkable increase in biosensors for nonmedical applications, for example, in the development of cell-culture systems. Among the different biosensors suitable for cell-culture systems, electrochemical biosensors are still the most relevant due to their high-throughput quantification and analysis of biochemical interactions. However, optical biosensors have emerged with the fastest growth over recent years. This rise in optical biosensing technology is related to a wide analytical coverage [1].

3.1.1 Definition of a Biosensor and Classification

How can a biosensor be described? The International Union of Pure and Applied Chemists (IUPAC) describes a biosensor as “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals” [2]. From this description, we can define two main features: a biosensor device requires a biological recognition element in direct contact with a transducer and transforms a biorecognition or biophysical event mainly into a signal that later can be measured. Although there are many aspects involved in the design of a biosensor, we can describe three main parts: the first part is the biorecognition material (i.e., antibody, aptamer, or enzyme) that gives the potential to detect the analyte with high specificity and selectivity; the second part is the transducer (i.e., optical, electrochemical, mechanical) that transforms the biorecognition event in a physical quantity, which later can be measured and analyzed by an electronic device that monitors the results for the final user (see Fig. 3.1) [3].

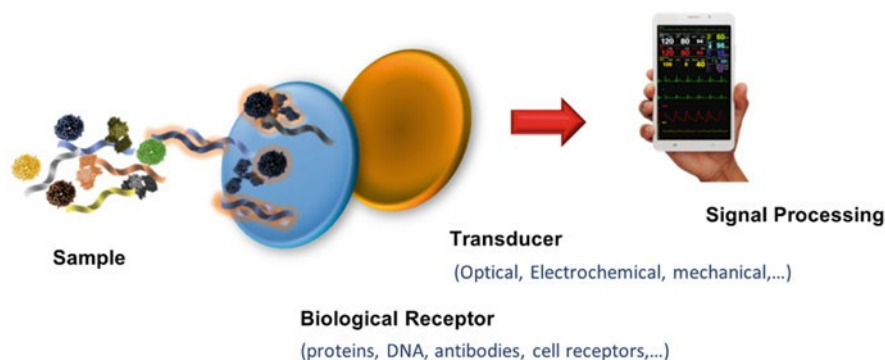


Fig. 3.1 Representation of the main parts of a biosensor device. The analyte, the biorecognition material, the transducer, and the signal processing system

On the other hand, there are expected attributes from a biosensor. These attributes range from the potential to detect and quantify biomolecular interactions in minutes, using minimum sample pretreatment and reagents, to the potential to be portable and used by non-trained personnel. Biosensors are considered as analytical tools with a high potential to achieve miniaturized and integrated Lab-on-a-Chip (LOC) platforms and end-user devices [4]. As a consequence of these potential attributes, the biosensors field has been extensively investigated and developed over the recent few years and, year-to-year emerge novel point-of-care and end-user devices.

There are different transduction methods for biosensors; however, only three technologies have relevance in the market [5]: electrochemical, mechanical, and optical biosensors.

Electrochemical biosensors have been the gold standard over the years in biosensing. The working principle of these biosensors is to detect electrochemical changes with biorecognition events. When the biorecognition layer covers the working electrode and an electric potential is applied, electroactive species conversion into electrical changes can be quantified (see Fig. 3.2a). Electrochemical biosensors allow simple and high-throughput fabrication with compact instrumentation [8].

The principal example of this technology in our lives is the glucose biosensor. This biosensor emerged in 1962 and is based on the amperometric detection of an enzymatic reaction related to hydrogen peroxide generation [8]. Over the last years, there have been significant improvements in electrochemical biosensors. However, there are still many changes to overcome, most of them related to novel materials that improve the limited sensitivity of the biosensors and their stability [9, 10].

Mechanical biosensors also have been widely reported and employed. The working principle of mechanical biosensors is to detect the biorecognition events as superficial mass changes. Between the different mechanical biosensors, the quartz crystal microbalance (QCM) biosensor is the most representative. QCM has a native resonant frequency. This frequency changes with mass changes in their surface, in this case, when the analyte binds to the biorecognition molecule (see Fig. 3.2b). This change in resonant frequency is proportional to the amount of analyte bound [11]. Among their main advantages is the possibility to achieve label-free and

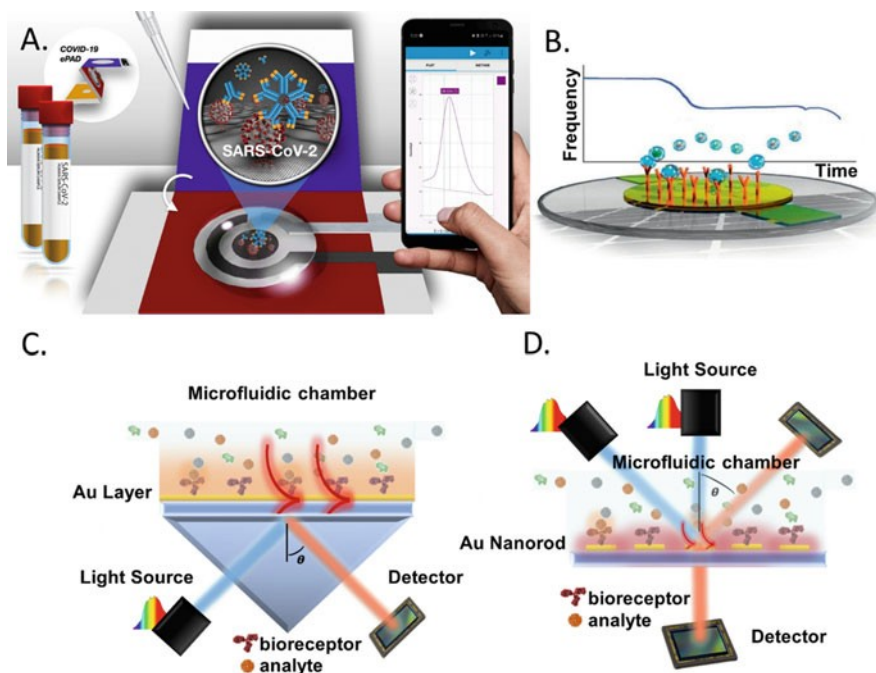


Fig. 3.2 Schematic illustration of the working principles of the three leading biosensing technologies in the market: (a) electrochemical (Adapted and reprinted with permission from [6]. # Copyright 2021, Elsevier Inc.), (b) mechanical quartz crystal microbalance (Adapted and reprinted with permission from [7]. # Copyright 2015, Elsevier Inc.), and optical (c) prism coupled SPR and (d) transmission/reflection LSPR

multiplexed biodetection. However, the mechanical nature of their working principle decreases their sensitivity and potential uses; between them in the biodetection of bacteria and cells [12]. Also, liquid environments without thermal control lead to erroneous readings [13, 14].

Although optical biosensors have been widely studied over the last decades, the last advances in nanomaterials boosted their growth in recent years. These biosensors detect variations in intensity, wavelength, and refractive index between others of the propagated light with biointeractions [15]. Between the different detection mechanisms, most optical biosensors measure refractive index changes [16]. Refractometric biosensing uses the evanescent field, which acts as a probe to detect refractive index changes near the surface [17]. The amount of analyte in the biorecognition event is proportional to the optical changes [18]. As mentioned before, these biosensors only detect superficial changes and, as a consequence, there is a limited interference of other compounds of the media [19]. Unlike electrochemical and mechanical biosensors, optical biosensors have a high sensitivity and do not suffer from electronic or mechanical interferences. Although there are different optical biosensors, we can summarize two principal technologies: those

based on dielectric waveguides like interferometers [20] and resonators [19], and those based on plasmonic modes. Plasmonic biosensors are the most widely used optical sensors to study multiple biorecognition events in the last decades [21]. Unlike semiconductor materials, noble metals (mainly gold) have a well-established surface biofunctionalization based on thiol-based surface chemistry with high chemical stability [15].

Contemporary biosensing techniques have sought through years to achieve favorable figures of merit such as high sensitivity, limits of detection, signal-to-noise ratio, specificity, and binding capacity by employing different techniques to design integrated biosensors. Attempts to design an all-encompassing biosensor have been futile and instead took recourse to focus on achieving at least one of these facets of device performance. Revolution in immunochemistry and biomarker detection began in the 1970s when Faulk and Taylor conjugated an antibody to gold nanoparticles to visualize salmonella antigens in direct electron microscopy. After Seeman presented DNA as a structural molecule in 1980, Mirkin and Alivisatos explored the possibility of using DNA to produce aggregated gold nanoclusters. Taking inspiration, Boal explored the utility of 2 nm gold nanoparticles protected by self-assembled monolayers to serve as building blocks for micro and eventually macro-scale constructs. Multiple studies, henceforth, used controlled and self-assembly of gold nanostructures to develop different types of biosensors, including the electrochemical and optical types. These devices work by amplifying the optical or electrical signal generated of the interaction within gold and the biorecognition elements. Therefore, two properties of gold nanoparticles have mainly been interesting; surface plasmon resonance and electrical conductivity. For optical biosensors, multiple factors allowed for accounting and tuning the sensor's performance. These included the particles' size and shape, interparticle distance, and the refractive index of the surrounding media/environment. In 1983, Lieberg, for the first time, used surface plasmon resonance (SPR) for label-free detection of biomolecular interactions and gas sensing. The technique was presented as a hassle-free, cheap way to detect analytes without any expensive machinery. Biacore™'s optical sensors deserve a special mention in the SPR optical sensor field [22]. They have various options that allow measuring the specificity, affinity, thermodynamic parameters, biologically active concentration of the analyte, and the association and dissociation constants. Their technology couples a microfluidic system with an exchangeable sensor chip. Biacore™'s optical SPR sensors have been in use since the 1990s to answer questions about bio-specific interactions [22].

The working principle of plasmonic biosensors, also called SPR biosensors, can be summarized. SPR is an electromagnetic (EM) wave that occurs when incident light hits a noble metal surface. At specific conditions in momentum or wavelength between others, a portion of the light energy couples with the surface electrons of the metallic layer, which move due to excitation [23]. The electrons' oscillation generates an exponentially decaying evanescent field which is highly sensitive to refractive index changes, especially near the surface. Consequently, the biorecognition events close to the surface generate variations of the different optical properties of the propagated light, such as intensity, wavelength, or phase, which can

be monitored and quantified. Plasmonic phenomena can be generated through grating couplers [24] or by waveguides [25], although the most common way is using a coupling prism, usually known as Kretschmann configuration (see Fig. 3.2c). However, this configuration considerably reduces the potential for multiplexing and miniaturization of plasmonic sensors [15].

The need for “bulky” coupling elements for SPR generation can be overcome using metallic nanostructures in the sub-wavelength size range instead of thin metallic films (see Fig. 3.2d), generating the so-called localized surface plasmon resonance (LSPR) [15]. Meanwhile, plasmonic waves propagate through the surface of a continuous metallic film; localized surface plasmons are confined excitations of the conduction electrons of metallic nanostructures. These modes arise naturally from the light scattering of sub-wavelength conductive nanostructures in an oscillating electromagnetic field. Another consequence of the nanostructured surfaces is that plasmon resonances can be excited by direct light illumination than conventional SPR [26]. The presence of edges in nanostructures strongly scatters light at a specific wavelength range. Analogous to conventional SPR, LSPR can be exploited for biosensing applications, as the wavelength depends on the surrounding media’s refractive index. The binding on the surface of the nanostructures results in a refractive index change, causing a shift in the extinction peak wavelength, which can be maximized by optimizing the nanostructure characteristics (i.e., metal type and geometry).

Table 3.1 summarizes relevant emerging examples of different biosensors based on the leading transduction technologies (optical, mechanical, and electrochemical).

Table 3.1 Examples of emerging biosensing technologies applied to relevant clinical diagnosis				
Type of Biosensor	Material	Biomarker	Limit of detection	Application
Electrochemical [27]	Au nanoparticles /graphene modified carbon electrodes	microRNA (21,155, and 210)	Up to 0.04 fM in serum	Breast cancer
Electrochemical [28]	ZnO/Au electrodes	Cytokines (IL-6, IL-8, IL-10, TRAIL, and IP-10)	Up to 1 pg/mL in plasma	Sepsis
Optical [29]	Au nanopillars	SARS-CoV-2 virus particles	370 virus/mL in PBS	SARS-CoV-2
Optical [30]	Au nanoprisms	microRNA (10b,64,145,143 and 490-5p)	100 aM in plasma	Bladder cancer
Mechanical [31]	Au electrodes	MDA MB 231 human breast cancer cells	12 cells/mL	Breast cancer
Mechanical [32]	Graphene oxide/ Au nanoparticles coated Au electrodes	Carcinoembryonic antigen	Up to 0.06 ng/mL in serum	Colorectal, ovarian, and breast cancer

Most of the presented examples directly detect human fluids and present relevant clinical diagnosis applications, including cancer diagnosis.

3.1.2 Biosensors and Cancer Research

Multiple biomarkers have been considered for cancer detection, such as circulating tumor cells (CTCs), circulating DNAs and micro RNAs, proteins, and exosomes. Of these, the CTCs and protein biomarkers have been utilized in the design of multiple biosensor devices. Current techniques popularly used for detecting and quantifying these markers include ELISA and sequencing kits commercially available, such as SafeSeqS, PCR, and TamSeq (Tagged Amplicon Sequencing) [33]. While these techniques are reliable, they have considerable limitations such as a lengthy multi-step protocol, impossibility to achieve device miniaturization, integration, and multiplex detection. Other techniques such as FACS, Dynamic Light Scattering (DLS), and NTA require complex machinery and are, therefore, restricted to the laboratory. Nanoplasmonic sensing techniques such as SPR and LSPR, therefore, present viable solutions to these issues. These optical sensing techniques depend on the optical shift observed as a change in local refractive index within a comparatively small sample size. They are label-free and can provide measurements free from the interference of the surrounding medium, particularly LSPR. SPR presents the capability to measure surface depths between 100–200 nm suitable for detecting circulating tumors cells, proteins, and exosomes but not nucleic acids, while the LSPR technique has a sensing depth of 5–20 nm, thereby providing the opportunity to detect nucleic acids directly. Both SPR and LSPR are capable of detecting low concentrations of the sample analyte. One of their key differences is that while the interaction of monochromatic light source on planar conductive thin film creates an evanescent wave as observed in SPR [34], the interaction of the electromagnetic light source with conductive metallic nanoparticles with sizes smaller than the wavelength of incident light causes localized surface plasmon resonance, which is a collective, non-propagative oscillation of the conduction band free electrons [33]. This enhances the local electromagnetic field in the vicinity of the particle surface. In other words, any change in the local environment of the dielectric will directly affect the nanoparticle's polarizability and the optical extinction spectrum [35]. Such changes will alter the electron wave function delocalization of the nanoparticles, resulting in proportional changes to the LSPR pattern, allowing the opportunity to quantify biorecognition events [36].

The thermal convection method was used to deposit gold nanoislands from a colloidal gold solution on a glass slide [37]. Their design allowed the detection of exosomes via vn96 polypeptide through LSPR in an MCF7 conditioned cell-culture media. Nevertheless, the binding capacity was only theoretically, and not practically, proved.

A parallel gold nanorod LSPR, microfluidic-based, portable lab-on-a-chip sensor was developed with eight channels and 32 sensing sites for real-time detection of four cancer biomarkers, including alpha-fetoprotein (AFP) and prostate-specific antigen [38]. The device can simultaneously measure analyte concentrations as low as 500 pg/mL in a solution with 50% human serum in all eight channels. This design bridges substantial gaps in sensors miniaturization, reliability, sensitivity, integration, and compactness. The researchers claimed the device to be ready for the transition from lab to market. There are, however, noticeable differences between LOD values for different biomarkers attributed to the uneven and different performance of antibody pairs.

Like proteins and exosomes, miRNAs continue to hold great promise for the non-invasive detection of cancer. An ssDNA functionalized gold nano prism-based LSPR sensor capable of achieving sensitivity as low as 140 zp/M was also developed [36]. Their design could detect a single base-pair mismatch in the ssDNA and miRNA duplex by sensing a significant change in the LSPR properties. The researchers assayed four types of miRNAs obtained from 50 uL of bladder cancer patients' plasma, namely microRNA-10b, -182, -143, and -145. This work substantiated the utility of noble metallic nanoparticles to achieve device miniaturization, particularly for cancer detection [36].

3.2 Sensors and Biosensors in Organs-on-a-Chip Platforms

3.2.1 Organs-on-a-Chip Definition

Studies on two-dimensional *in vivo* cell cultures and animal models have contributed to significant cellular and molecular biology accomplishments, but the progress has been slow. The ratio of FDA-approved drugs to the billions of dollars spent on research development programs is unprofitably low and decreases monotonically. To curtail this drop, contemporary drug development approaches require substantial advancements in tools and techniques to achieve efficiency with cost-effectiveness. The role played by animal models in drug development to unveil essential aspects of physiology and biochemistry has been pivotal to evaluate drug response. Nevertheless, the anatomic and physiologic difference between humans and animal models is hard to ignore and often becomes a bone of contention to predict a patient's response. This is evidenced by the fact that from the drugs that fail in the clinical trials, 60% are inefficacious and 40% are due to toxicity. It is therefore impractical and illogical to use animal models for developing a patient-specific personalized treatment. Currently, efforts are in place to get the organs-on-a-chip (OOCs) (see Fig. 3.3) approved by the National Institute of Health Sciences for clinical research and translation [40].

An OOC device has cells harvested from the patient or a healthy donor in a 3-dimensional microfluidic cell-culture platform to the input culture medium, drug of choice, and harvest the products of cellular metabolism. The output can then be tested either with sensors integrated into the chip or by off-chip analyses. These

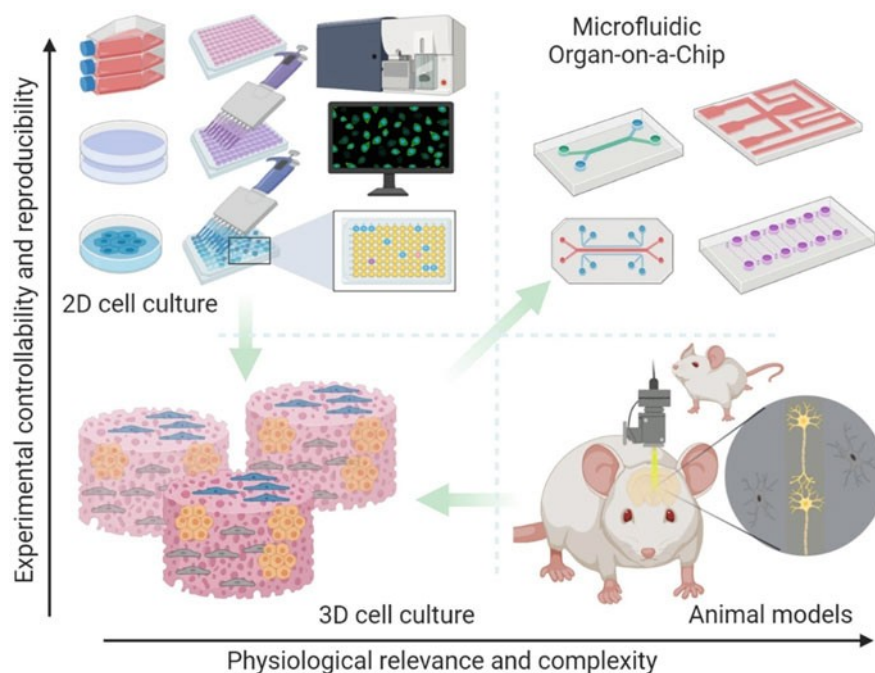


Fig. 3.3 Relevance of OOC devices as platforms to controllably and systematically interrogate human biology. Adapted and reprinted with permission from [39]. # Copyright 2021, Elsevier Inc.

microfluidic chips allow growing the organs in an environment mimicking the microenvironment within the human body. The organs or organoids are allowed to culture in a hydrogel, exchanging nutrients and metabolites, thereby monitoring drug response in situ. Therefore, choosing the biomaterial to encapsulate the organs/organoids of choice is essential to design a platform similar to the patient's or model's microenvironment.

3.2.2 Clinical Applications

After developing lung-on-a-chip as the first OOC model, many OOC platforms were developed for disease modeling, toxicology studies, pathophysiological evaluation, drug response testing under varying external and internal conditions (see Fig. 3.4).

3.2.2.1 Pancreas-on-a-Chip

Type 2 Diabetes caused 1.5 million deaths in 2019 alone. While the cause of type 1 diabetes is a genetic predisposition to the disease, the leading cause of type 2 diabetes is excess body weight, lack of exercise, and unchecked dietary intake. Islets of Langerhans or beta cells play a pivotal role in regulating glucose metabolism. However, static culture techniques do not bode well with the Islet functionality

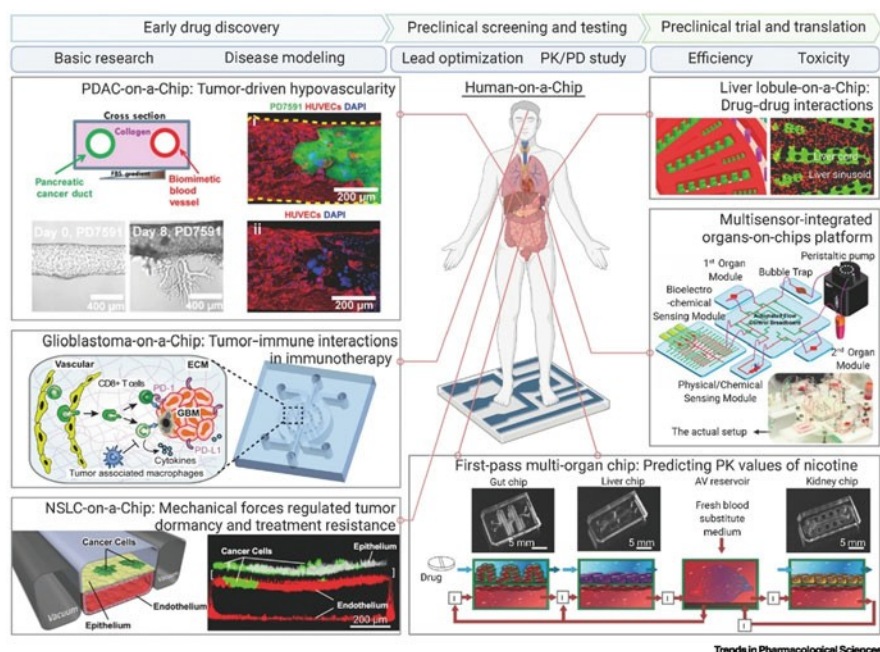


Fig. 3.4 The prospective multi-functional utility of OOC platforms disease modeling in drug development and as an alternative of research with animal models. Adapted and reprinted with permission from [39]. # Copyright 2021, Elsevier Inc.

because they cannot mimic the *in vivo* physiology and the continuous perfusion offered by microfluidic platforms to the 3-dimensional organs or organoids. Therefore, the Islets of Langerhans mounted on microfluidic platforms are the culture of choice for studying disease pathology and drug response. The cells can be harvested from diseases and healthy donors. At present, glucose-stimulated insulin secretion (GSIS) assays which are primarily enzymatic in nature, are conducted to evaluate islet functionality. However, the OOC technology offers the opportunity to integrate detection of *in situ* response with different types of sensors. Numerous research teams have come up with varied designs to address complexities about organ growth and detection mechanisms. In 2009, Mohammed et al. came up with the first microfluidic perfusion design to achieve multimodal characterization, which included functionality analysis of mitochondrial potential and simultaneous quantification of insulin production through ELISA under a glucose challenge [41]. Their chosen combination of analyses provided a more reliable course for assessing islet quality before transplantation than the standard assays in practice at the time. Over time the integration and real-time evaluation of islet cell integrity and functionality have continued to improve. Perrier used integrated electrochemical biosensors based on a microarray of platinum black electrodes to study ionic fluxes generated with variable glucose concentrations [42]. This approach allowed to detect changes in

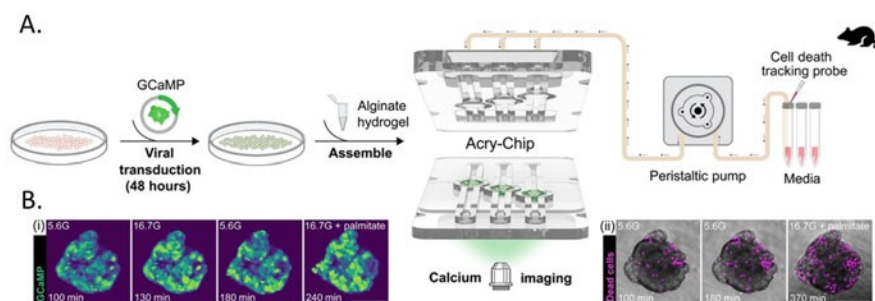


Fig. 3.5 Scheme of the monitoring of glucolipotoxicity stimuli on in situ Ca^{2+} signaling and viability of hydrogel-embedded pancreatic islets. (a) Schematic representation of the experimental workflow. (b) Time-course snapshots of a representative (i) GCaMP islet and (ii) accumulated dead. Adapted and reprinted with permission from [44]. # Copyright 2021, American Association for the Advancement of Science

islet function proportional to glucose within only 40 μs . The integrated islet-on-a-chip with an LSPR sensor device developed in 2021 allows studying in situ response of the microtissues or islets-on-a-chip to external stimuli [43]. Moreover, the GSIS assays are now acknowledged to have low potency to predict islet cell potency in insulin production. In another example, a microfluidic device has been used to test the glucolipotoxicity stimuli on in situ Ca^{2+} signaling and the viability of the cells embedded in a hydrogel has been tested (see Fig. 3.5) [44].

Another critical concern while designing the islet or pancreas on a chip is to control differentiation, optimize culture and flow conditions to minimize shear stress, and improve the exchange of nutrients. Multiple researchers have attempted to address these complications by approaches such as dynamic culturing to achieve matrix reconstitution [45], employing a multilayer perfusion system to optimize differentiation of pluripotent stem cells (iPSCs) [46], developing a system with self-guided trapping sites to optimize both real-time monitoring and functionality analyses [47] and using scaffolds based on the right biomaterial such as cellulose to enhance the differentiation, formation, and functionality of pancreatic pseudo-islets [48].

Researchers from the Edmonton group [49] demonstrated that islet transplantation in 7 patients allowed them to achieve significant insulin independence coupled with steroid-free immunosuppression. After that, islet cell transplantation has been considered to be a promising approach, particularly for type 1 diabetes. However, the transplantation process requires manual handling and is often coupled with pro-inflammatory signals and ischemic damage. Pancreas-on-a-chip allows not only to precisely control the flow conditions but also to check the vitality of islets post-isolation. They also provide an opportunity to assess the growth and function of stem cell-derived beta cell function. Other checks against size heterogeneity prevalent between different islets, physical protection, and control of nutrient flow can also be controlled within the platform to achieve standardization (Table 3.2).

Table 3.2 Pancreas-on-a-chip models with their fabrication technology

Objective	Technique
Multimodal characterization	Functionality analysis of mitochondrial potential and a simultaneous quantification of insulin production through ELISA under glucose changes [41].
To study islet ionic fluxes in response to nutrient stimulation	Array of platinum black electrodes to detect changes in islet function proportional to glucose within only 40 μ s [42].
To optimize matrix reconstitution	Dynamic culturing [45].
To optimize differentiation of iPSCs to organoids	A multilayer perfusion system [46].
To achieve real-time monitoring and functionality evaluation	Development of self-guided trapping sites [47].
To optimize the formation and function of pseudo-islets	Using scaffolds based on cellulose [48].
To study in situ response of islet cells to external stimuli	Integration of an LSPR sensor [43].

3.2.2.2 Muscle-on-a-Chip (Muscular Dystrophy)

Muscle-on-a-chip models have been developed to study the underlying pathologies of progressively degenerative diseases such as Duchenne Muscular Dystrophy, amyotrophic lateral sclerosis (ALS), diabetes mellitus, endothelial inflammation, and atherosclerosis. These models provide an opportunity to develop personalized treatment options alongside studying the underlying biochemical pathologies by detecting cytokines, transcription factors, and other relevant biomarkers such as dystrophin. While immunostaining allows for studying immunohistochemistry, electrical stimulation allows evaluating contractile muscle response under multiple external stimuli, ranging from chemical to electrical impulses.

To achieve these objectives, contemporary research on muscle-on-a-chip has aimed to find the right biomaterial, perfusion strategies to enhance organoid formation and function, and integration with sensors to achieve real-time monitoring. The cells used can be both myoblasts and satellite cells harvested from both human donors and mice. Human amniotic mesenchymal stem cells (hAMCs) have also recently shown potential for myogenic differentiation [50]. There are, however, specific differences in the culture or encapsulation protocols and components of the growth/differentiation medium to be used for each of these cell types. There are also innate differences in the functionality of these cells. For example, skeletal muscle tissue organoids made from mice C2C12 cells show spontaneous contractions instead of those harvested from human cells, which show stimulated or induced contractions.

The applicability of PEG-DA and GelMA was proved as promising biomaterials for dielectropatterning of cells [52] (see Fig. 3.6), after which a nano-biosensor was developed to evaluate glucose consumption by GelMA hydrogel encapsulated skeletal muscle models under electrical stimulation at different time durations [53]. They found that skeletal muscle organoids exhibited maximum glucose

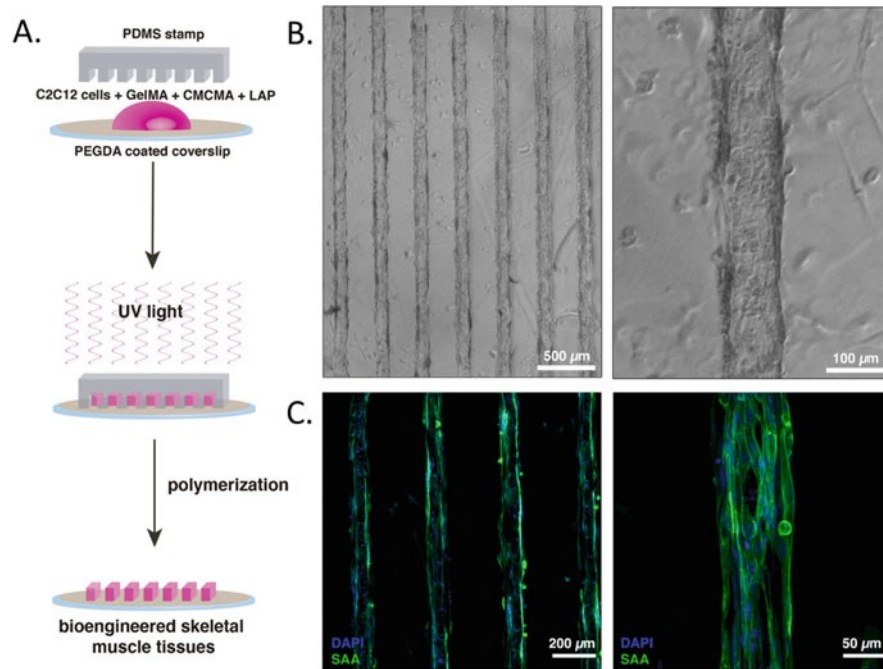


Fig. 3.6 Bioengineered 3D skeletal muscles in GelMA hydrogels. (a) Fabrication protocol to obtain 3D skeletal muscle tissues hydrogels. (b) Microscope images of cell-laden micropatterned hydrogels. (c) Representative confocal microscopy images of 3D skeletal muscle microtissues stained for the muscle maturation marker sarcomeric α -actinin (SAA, green) and nuclei (DAPI, blue). Adapted and reprinted with permission from [51]. # Copyright 2021, Walter de Gruyter GmbH

consumption in the initial 3 h, and the chosen biomaterial offered low viscosity and helped maintain cell viability [53]. Geometric orientation and alignment have been found to be extremely important for the proper development of myotubes. Towards this end, extracellular matrix conditions, type of scaffold, and patterning techniques play an essential role. Components in the chosen biomaterial act as geometric cues to achieve proper alignment, as observed in Matrigel, which has Collagen I, II, and these components create a tensile stretch necessary to achieve cell alignment into tubules and elasticity [54]. The fabrication approaches to achieve this alignment are also multiple such as 3D printing (bioprinting) [55], microfluidic extrusion/electrospinning [55, 56], bio-casting, 3-D and 2-D bioprinting [57], thermal gelation, micro-molding [58], photo mold patterning [59], micropatterning (3D and 2D), dielectropatterning [52], droplet-emulsion assisted patterning, e-field assisted printing [55], cryogelation and hydrogelation. All these techniques facilitate alignment and cell orientation and help identify biochemical, micromechanical, and geometric cues to enhance myotubes' differentiation, maturation, growth, and regeneration (Table 3.3).

Table 3.3 Skeletal muscle-on-a-chip models with their biofabrication technology

Objective	Technique
To identify the biochemical and microphysical cues involved in myoblast alignment	Electrospinning and 3-D printing to co-culture HUVECs and myoblasts [55].
To induce morphological retention and stimulate cellular alignment in myoblasts	Thermogel-assisted bioprinting [57].
To test the applicability of PEG-DA and GelMA for cell-patterning	Dielectropatterning [52].
To evaluate glucose consumption, GelMA encapsulated myoblasts under electrical stimulation	Nano-biosensor integrated with GelMA hydrogel encapsulated myoblasts coupled with electrical stimulation [53].
To bioengineer an all-encompassing 3-D skeletal muscle model of myotonic dystrophy type 1 and subsequent drug testing	Photomold patterning of GelMA and CMCMA hydrogel laden with cells [59].
To develop a new approach for vascularization of in vitro synthetic tissues	Micromilling-molding [58].
To enhance skeletal muscle development and evaluate the effect of agrin treatment on growth dynamics	Microfluidic extrusion and photo-crosslinking [56].
To design a new process to achieve better alignment and differentiation	E-field assisted printing [60].

3.2.2.3 Liver-on-a-Chip

The liver is not only the largest but also the most important internal organ of the human body. Its primary functions include detoxification, metabolism of macronutrients, drugs, and hormones, glycogenesis, bile production, and inflammatory response control. This organ is known for its regenerative capacity but is sufficiently vulnerable to drug toxicity. Liver-on-a-chip platforms allow mimicking the spatiotemporal characteristics and subsequently evaluating drug response outside the actual human body. At present, there are three primary research paradigms: disease modeling, drug toxicology, and organ physiology and regenerative capacity.

Current approaches involve multiple strategies to fabricate liver-on-a-chip devices. Among these, a few studies have even attempted to synergistically associate artificial intelligence with OOC to study disease pathology, such as that for NAFLD [62] (see Fig. 3.7). A few of these have been summarized in Table 3.4.

Similarly, OOCs for other organs such as the lung [65, 66], heart [67], gut [68, 69], kidney [70], and brain [71] also exist, as do the co-culture and multiple organ systems. There is, at present, exhaustive literature evidencing research undertaken using different approaches to elucidate drug responses, toxicology, physiology, biochemistry, regenerative capacity, and inter-organ communication.

3.2.2.4 Tumor-on-a-Chip

Billions of dollars are spent every year in research and development for unmasking underlying disease pathology in cancer and the development of viable drug targets. Conventional techniques, such as 2-D and 3-D cell cultures and animal models, are unsuitable as they cannot precisely replicate the tumor microenvironment, notably

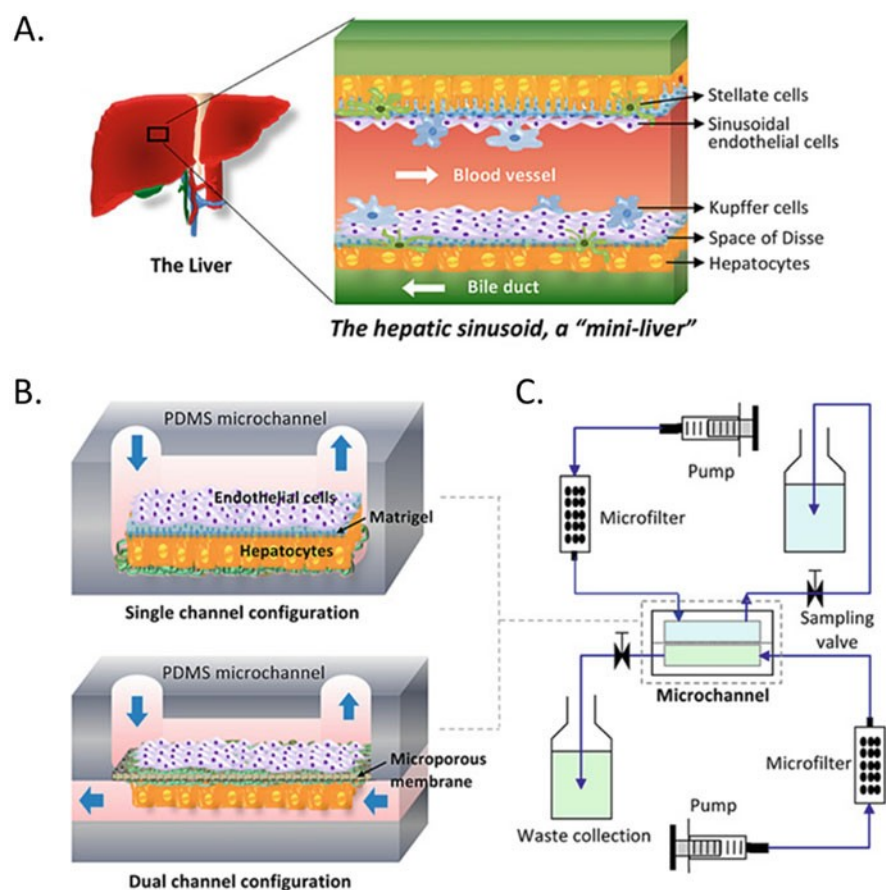


Fig. 3.7 Design of a microfluidic platform for the liver sinusoid model. (a) The liver sinusoid functional unit, (b) two proposed microfluidic configurations; and (c) bioreactor circuit for continuous perfusion of media and waste collection. Adapted and reprinted with permission from [61]. # Copyright 2015, John Wiley & Sons

Table 3.4 Liver-on-a-chip models with their biofabrication technology

Objective	Technique
To fabricate in vitro integrated models for evaluation of hepatic safety and metabolism of drugs	3-D liver OOC and spheroid culture for hepatotoxic screening and metabolic profiling using PDMS based microchannels [63].
To model the first-pass metabolism of a flavonoid using a co-culture model	Soft-lithography to model to develop a live co-culture first-pass metabolism [64].
To model liver sinusoid	Soft-lithography (single and dual-channel designs) to support long-term primary liver cultures [61].

Table 3.5 Tumor-on-a-chip models with their biofabrication technology

Objective	Technique
To evaluate the possibility of 3D multicellular structure bioprinting for investigating the response of paclitaxel on breast cancer spheroids	A dual nozzle bio-deposition system to bioprint cells and spheroids using different bioinks based on matrigel, gelatin, alginate, and collagen [73].
To address obstacles to high-throughput screening assays	Production of tumor spheroids using ultra low-attachment microplates and microwell arrays of hydrogels [74].
To precisely model complexity of in vivo pathophysiology of colorectal cancer and efficacy of drug loaded nanoparticles in a dose-dependent regime	A PDMS microfluidic platform fabricated by photolithography. The endothelial invasion of the core was quantified using bright-field microscopy followed by gene expression studies [75].

the extracellular matrix. The tumor microenvironment plays an essential role in cancer progression. This is where tumor-on-chip devices provide the opportunity and flexibility to replicate tumor microenvironment as close as possible to the in vivo environment, such as creating biomimetic microfluidic channels to mimic the oxygen gradients with spatial and temporal resolution. This oxygen gradient is known to be pivotal for metastasis. Therefore, the tumor-on-chip models can be used to mimic angiogenesis, metastasis, and transition from early to advanced forms by epithelial to mesenchymal transition [72]. A few models of tumor-on-chip are tabulated below (Table 3.5).

3.2.3 Drugs/Dosage Tested on Organs-on-a-Chip Technology

As previously stated, one of the purposes of developing OOCs is to model disease phenotype for drug development. Table 3.6 shows a few examples of drugs that have been tested for their response in different organ systems. A database by The North Columbian 3R's Collaborative (<https://www.na3rsc.org/mps-tech-hub/>) provides easy access to commercially available OOC platforms along with exhaustive literature and previous studies.

3.2.4 Integration of Biosensors in OOC Platforms

Although the number of examples applying different biosensors to detect relevant biomarkers in OOC devices is steadily increasing, there are limited advances in the achievement of fully integrated and self-operative devices, with complete integration in compact autonomous platforms and their validation in real environments.

Integration and high-throughput analysis are required to succeed in the development of relevant biosensor devices for OOC platforms. Microfluidics is, in this sense, an indispensable module to provide simultaneous analysis and assure low

Table 3.6 Commercially available OOC systems used in drug assays

Drug	Model	Objective
Aurora B kinase AZD2811 inhibitor	Bone marrow-on-chip	To elucidate the clinical toxicity profile of the drug in question [40].
Cisplatin and cyclosporine	Kidney-on-chip	Drug-induced nephrotoxicity and linkage with glucose accumulation [76].
AntagomiR-23b	DM1 skeletal-muscle-on-chip	Protects and helps in restoring both molecular and structural characteristics of DM1 myotubes [59].
Inhibitor empagliflozin	Kidney-on-chip	Significantly reduced cisplatin and cyclosporine-mediated kidney damage due to glucose accumulation [40].
Nimesulide and troglitazone	Liver-on-chip	To evaluate toxicity dose-dependent profiles of nimesulide and troglitazone (direct hepatotoxic agents) [77].
Trovaflaxacin	Liver-on-chip	To evaluate indirect immune-mediated hepatotoxicity [78].
Acetaminophen, amiodarone, troglitazone, and rotenone	Liver-on-chip	To evaluate drug-induced hepatotoxicity by disruption of mitochondria [79].
Fialuridine and acetaminophen	Liver-on-chip and 3D hepatic spheroids	To analyze and compare model sensitivities of two drugs by measuring cytotoxic profiles [63].
Apigenin	Gut-on-chip	To verify the success of first-pass metabolism by analyzing the metabolites [64].

sample and reagent consumption. Appropriate dimensions and geometries in microfluidic pathways can enhance the diffusive mixing and, consequently, the speed and accuracy of reactions. Performance improvements like reduced measurement times, improved sensitivity, higher selectivity, and parallelism can be obtained by integrating an appropriate microfluidic system. On the other hand, the appropriate surface biofunctionalization that allows sensitive and selective biorecognition while minimizing non-specific adsorptions from cell-culture media is highly desirable. Also, it is necessary to remark that most of the proposed surface biofunctionalization methods are based on single-use/detection, limiting the platforms' capability. New surface functionalization methods that allow biosensor regeneration for several biodetection cycles would be ideal.

The impact of external stimuli or internal abnormalities manifests in multiple ways: changes in temperature, the optical density of the extracellular media, pH, levels of oxygen and carbon dioxide, metabolites such as glucose and lactate, and chemicals such as hormones and cytokines, and changes in the cell behavior. Table 3.7 shows only a few different techniques adopted to monitor these parameters for multiple objectives.

Over the last years, there have been limited examples of integrated biosensing systems for in situ biodetection of segregated biomarkers from OOC devices. Considering the high availability of commercial electrodes for the development of

Table 3.7 Integrated sensors for respective culture parameters

Parameter	Technique
Temperature	To monitor any physiologic changes in iPSC culture medium using an integrated CMOS smart sensor [80].
pH	To measure temperature, oxygen, and pH simultaneously using a combination of silica beads and fluorescent dyes for florescent based sensing [81].
pH	To measure carbon dioxide and oxygen using a pH sensitive fluorescent indicator within ethyl-cellulose [82].
pH	To dynamically measure cancer metabolism based on potentiostatic analysis [83].
O ₂	To study the respiratory kinetics in adherent (HeLa) cells using a Clark-type oxygen chip [84].
O ₂	To develop sensitive miniaturized amperometric oxygen sensors [85].
O ₂	To study hepatocyte polarization and metabolic function based on the involvement of non-parenchymal cells (NPCs) using luminescence-based sensor spots (building on the ability of oxygen to quench luminescence) [86].
O ₂	To study oxygen gradients and oxygen consumption in 2-D and 3-D hydrogel cultures using amine functionalized polystyrene bead-based oxygen sensor. Monitoring was performed using FireStingO2 optical sensor at 1 Hz [87].
CO ₂	To measure temperature, oxygen, and pH simultaneously using a combination of silica beads and fluorescent dyes for florescent based sensing [81].
CO ₂	To measure carbon dioxide and oxygen using pH sensitive fluorescent indicator within ethyl-cellulose [82].
Metabolites:	
Glucose and lactate	To dynamically measure cancer metabolism using an enzyme immobilized hydrogel for amperometric measurements [83].
Glucose and lactate	To measure mitochondrial function in liver-on-a-chip model using imbedded amperometric sensors for glucose and lactate [79].
Glucose and lactate	To selectively detect ascorbate, glucose, and lactate using dehydrogenase based sensing mechanism adsorbed onto single-walled carbon nanotubes [88].
Calcium	To study activity dynamics and electrical activity in neurons using a microfluidic/microelectrode array [89].

electrochemical biosensors, most of the developed biosensing systems are based on this technology. Recently, Ramon-Azcon research group presented a multiplexed integrated platform based on electrochemical biosensors for high sensitivity biodetection of IL-6 and TNF- α in a muscle-on-chip model platform with electrical stimulation [90]. Although they reach a sensitivity in the order of ng/mL, it is based on an amplified biodetection (sandwich assay) using a secondary recognition antibody, increasing the complexity of the bioassay. Other examples of electrochemical-based biosensing devices have been developed by Khademhosseini research group (see Fig. 3.8). The first device was based on the multiplexed monitoring of creatine kinase, albumin, and GST- α in a Heart-Liver-on-Chip model using monoclonal antibodies [92]. The system was able to detect changes of the biomarkers with a sensitivity of ng/mL produced by the presence of acetaminophen as a model for liver toxicity. The second device was based on the single monitoring of creatine kinase in a heart-on-chip model using biorecognition based on aptamers [93]. The system

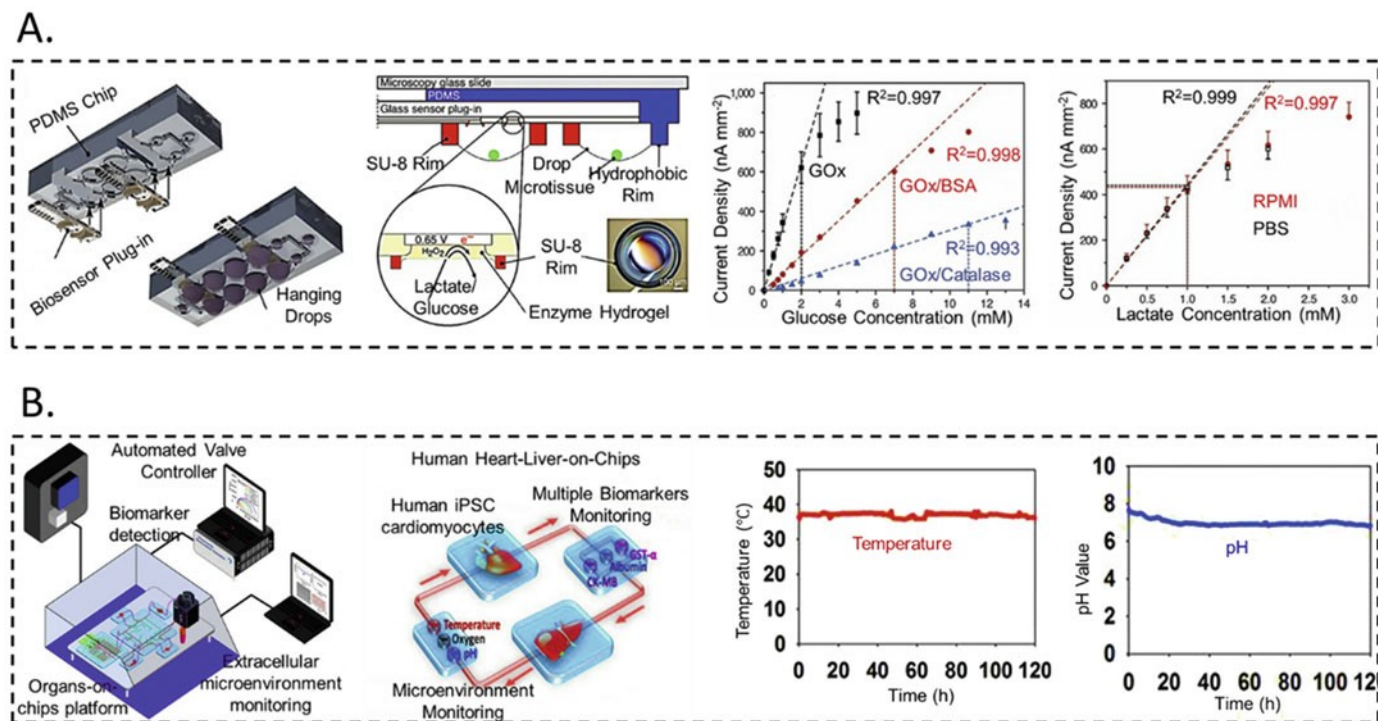


Fig. 3.8 Examples of different types of integrated sensors and chemosensors in OOC platforms. (a) Glucose oxidase (GOx) or lactate oxidase (LOx) transforms glucose or lactate to produce hydrogen peroxide, which is electrochemically detected on the electrode surface. Right: Glucose/lactate calibration curves showing the sensitivity and linearity. (b) Multiplexed biophysical sensing platform in the monitoring of temperature and pH for OOC applications. Right: Continuous measurements of temperature and pH within the integrated heart-liver-on-chips. Adapted and reprinted with permission from [91]. # Copyright 2021, Elsevier

Table 3.8 Examples of emerging biosensing systems in the monitoring of relevant biomarkers in OOC devices

Type of biosensor	OOC	Biomarker	Limit of detection
Electrochemical [90]	Muscle	IL-6 and TNF- α	ng/mL
Electrochemical [92]	Liver/heart	Creatine kinase, albumin, and GST- α	ng/mL
Electrochemical [93]	Heart	Creatine kinase	pg/mL
Optical [43]	Pancreas	Insulin	μ g/mL

based on aptamers shows a high shelf life for up to 7 days while antibodies biosensing performance decreases over time. Even more, the aptamer biosensor showed a superior biosensing performance with a sensitivity in the order of pg/mL in cell-culture media. The heart model was based on the secretion of creatine kinase under doxorubicin, an anticancer drug used as a model to induce cardiac injury.

Finally, a novel optical biosensing platform has recently been presented by Ramon-Azcón group based on nanoplasmonic biosensors for monitoring insulin secretion from pancreatic islets spheroids under the presence of different concentrations of glucose in a Pancreas-on-Chip model [43]. The nanoplasmonic biosensor based on gold rod-shaped nanoantennas allows multiplexed monitoring of insulin secretion with a sensitivity of 0.85 μ g/mL. Although the sensitivity is worst compared to other biosensors, the system allows label-free real-time monitoring without sample pretreatment and amplification. The optical readout is based on simple transmission configuration, increasing the potential for highly integrated monitor systems. Table 3.8 summarizes the most relevant emerging examples of different biosensing platforms applied to OOC devices. On the other hand, in Fig. 3.9, we present the schemes of two examples of all integrated OOC/biosensing platforms.

3.3 Conclusion and Perspectives

Biosensors are a rising technology in their integration with OOC devices to achieve fully operative and autonomous platforms that can effectively monitor physiological parameters and the secretion of biomarkers by the cell to a physical and chemical stimulus. Although there are still challenges to surpass, mainly related to the integration and multiplexing biosensing, the new advances in surface biofunctionalization can minimize cross-reactivity, non-specific adsorption and allow biosensors regeneration for real-time multiplexed detection. On the other hand, effective microfluidic designs with the correct flow components can create compact and modular systems. Due to its high versatility and straightforward integration in OOC platforms, biosensors technology is an exciting candidate for developing sensing platforms for real-time cell monitoring in OOC devices.

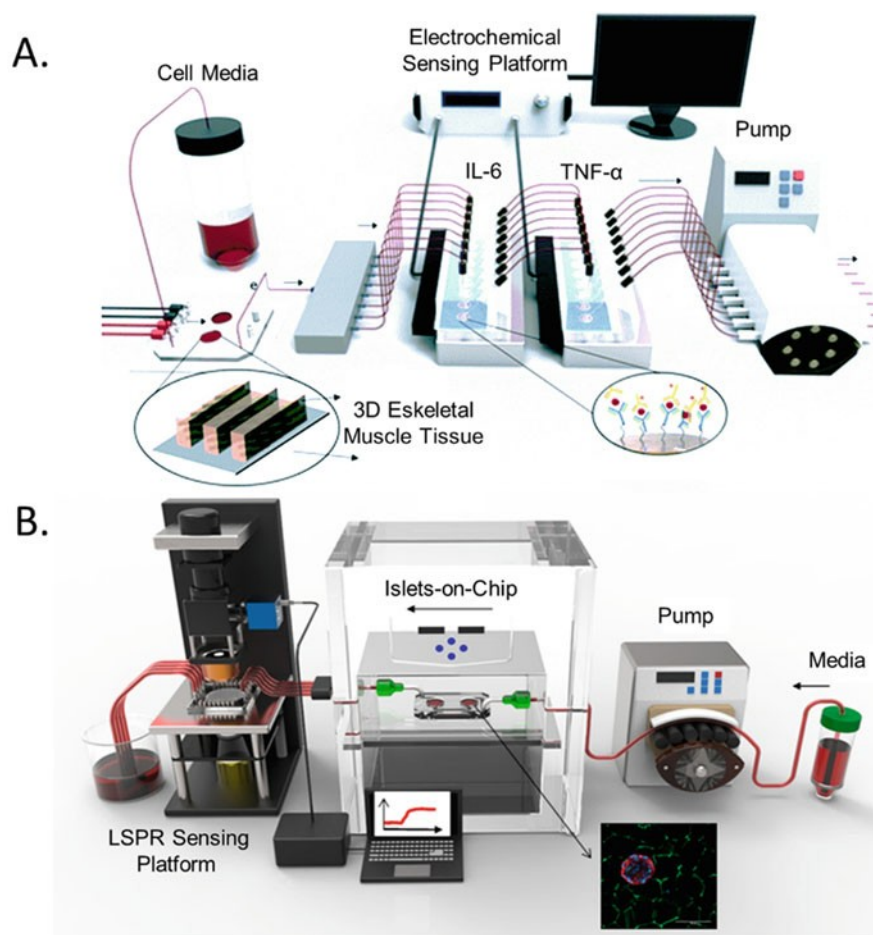


Fig. 3.9 Schematic illustration of OOC systems with integrated biosensing platforms based on (a) electrochemical (Adapted and reprinted with permission from [90]. # Copyright 2019, Royal Society of Chemistry) and (b) optical (Adapted and reprinted with permission from [43]. # Copyright 2021, MDPI) detection

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