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Study of the components and adherence to the Mediterranean diet on functional and cognitive ageing: from dietary assessment to a metabolomic approach

Alba Tor Roca

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Facultat de Farmàcia i Ciències de l'Alimentació

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Alba Tor Roca

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Universitat de Barcelona

Facultat de Farmàcia i Ciències de l'Alimentació

Programa de Doctorat en Alimentació i Nutrició

Study of the components and adherence to the Mediterranean diet on functional and cognitive ageing: from dietary assessment instruments to a metabolomic approach

Memòria presentada per Alba Tor Roca per optar al títol de Doctor per la
Universitat de Barcelona

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2023

*«i et tinc a vora meu com la poma escollida
que es torna groga i vella i encara fa perfum.»*

Josep Carner, Els fruits saborosos

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Abstract

Global population ageing represents many opportunities but also a great challenge for science and health policy, as this increase in longevity is intended not to lead to a worse health status or a lower quality of life. For this reason, there is a need to study factors that can influence health status throughout life and in old age, especially in the prevention and management of chronic diseases and disability. Healthy dietary patterns, such as the Mediterranean diet, have generally been shown to reduce the risk of chronic diseases, as well as the risk of mortality. The effects of healthy dietary patterns can be explained by the synergy of factors that composes it, such as phytochemicals and unsaturated fatty acids. However, evidence provided by both clinical and epidemiological studies regarding physical and cognitive functions is still limited and mixed. This is partly due to the methods traditionally used to collect dietary data, which are susceptible to measurement errors. For this reason, the use of dietary biomarkers represents an opportunity not only to obtain more objective measures but also to take into account other relevant aspects such as the bioavailability of food compounds and the metabolic intervariability of individuals.

Thus, the hypothesis stemming from this doctoral thesis is that high adherence to a dietary pattern rich in phytochemicals and unsaturated fatty acids, such as the Mediterranean diet, may contribute to determining the physical and cognitive health status of older adults. This work aims to study the effect of the Mediterranean diet and its components, evaluated through dietary assessment instruments and/or metabolomic tools, on physical and cognitive function in adults aged 65 years or over. This doctoral thesis has been reflected and documented in five scientific publications.

In the framework of the FoodBall Project, the phytochemical profile of legumes was investigated, and their composition was analyzed in a second stage. Metabolomics data analysis tools were used to visualize qualitative differences of phytochemicals between pulses, showing beans as those with the greatest number of described phytochemicals. Beans had the most discriminant profile, while lentils, chickpeas, and peas revealed a high number of common compounds. The intervention study with pulses, which aimed to characterize the composition of bioactive compounds of legumes and to identify biomarkers of their consumption, revealed a broad list of candidate metabolites. Some metabolites and their families were found to be specific for certain pulses, while others appeared to be more generic indicators of legume consumption.

Pulses are one of the main protein sources of the Mediterranean diet, which was evaluated through the MEDAS questionnaire to investigate the relationship between adherence to this pattern and response to an exercise and health education program to prevent hospital associated disability in older patients within the AGE-CAR-PLUS clinical trial. The work showed that while intervention proved effective in preventing hospital associated disability, patients with low adherence to the Mediterranean diet would probably benefit more from it than patients with higher adherence. In the study, the concentration of urinary total polyphenols was also used as a biomarker of polyphenol intake and as a proxy for the Mediterranean diet, and similar results were obtained. In the fourth study, within the InCHIANTI prospective cohort, it was observed that fish consumption was inversely associated with the risk of cognitive decline, one of the main causes of disability and dependency in the elderly. In this same vein, a greater adherence to the Mediterranean diet, assessed through an *a priori* Mediterranean diet metabolomic score, was retrospectively associated with lower odds of cognitive decline in older adults from the Three-City Study.

In conclusion, the results obtained in this doctoral thesis provide evidence that adherence to a healthy diet pattern rich in phytochemicals and unsaturated fatty acids, such as the Mediterranean diet, may contribute to the prevention of cognitive and functional decline in older people. In addition, they reinforce the need to refine nutritional metabolomic techniques for their full application in future research, in the study of both the diet-health relationship in aging as well as the value of bioactive compounds from a food group of high nutritional and health relevance such as legumes and fish.

Resum

L'envelliment poblacional a nivell global suposa moltes oportunitats però també un gran repte per a la ciència i les polítiques de salut, ja que es pretén que aquest augment de la longevitat no impliqui un pitjor estat de salut ni una menor qualitat de vida. Per aquest motiu, sorgeix la necessitat d'estudiar els factors que poden influenciar en l'estat de salut al llarg de la vida i durant la vellesa, especialment en la prevenció i maneig de les malalties cròniques i la discapacitat. En aquest sentit, els patrons dietètics saludables, com la dieta mediterrània, han demostrat reduir generalment el risc de patir malalties cròniques, així com el risc de mortalitat. Els efectes dels patrons dietètics saludables es poden explicar per la sinergia dels factors que els componen, tals com els fitoquímics i els àcids grassos insaturats. No obstant, l'evidència aportada tant per estudis clínics com per estudis epidemiològics en relació a la funció física i cognitiva és encara limitada i ambivalent. Aquest fet és degut, en part, als mètodes usats tradicionalment per recollir les dades dietètiques, els quals són susceptibles a errors de mesura. Per aquest motiu, l'ús de biomarcadors dietètics suposa una oportunitat per no només obtenir mesures més objectives, sinó també per tenir en compte altres aspectes rellevants com la biodisponibilitat dels compostos alimentaris i la intervariabilitat metabòlica dels individus.

Així doncs, la hipòtesis que se'n deriva en aquesta tesis doctoral és que una alta adherència a un patró dietètic ric en fitoquímics i àcids grassos insaturats, tal com la dieta mediterrània, pot contribuir en determinar l'estat de salut funcional i cognitiu dels adults majors. El present treball té com a objectiu l'estudi de la dieta mediterrània i dels seus components, estimats a través d'eines d'avaluació dietètica i/o metabolòmiques, en la funció física i cognitiva d'adults de 65 anys o majors. Aquesta tesis doctoral s'ha reflectit i queda constància en cinc publicacions científiques.

En el marc del Projecte FoodBall, es va procedir a investigar el perfil fitoquímic dels llegums i a analitzar la composició d'aquests en una segona fase. Es van usar eines d'anàlisi de dades metabolòmiques per visualitzar diferències qualitatives de fitoquímics entre llegums, mostrant les mongetes com els llegums amb major nombre de compostos descrits. Les mongetes tenien el perfil fitoquímic més discriminant, mentre que les llenties, els cigrons i els pèsols van revelar un gran nombre de compostos comuns. L'estudi d'intervenció amb llegums, que tenia per objectiu caracteritzar la composició de compostos bioactius dels llegums i identificar biomarcadors del seu consum, va revelar una àmplia llista de metabòlits candidats. Alguns

metabòlits i les seves famílies es van trobar específicament en certs tipus de llegums, mentre que altres van semblar ser indicadors més genèrics del consum de llegums.

Els llegums són una de les principals fonts proteiques de la dieta mediterrània, la qual es va avaluar a través del qüestionari MEDAS per investigar la relació entre l'adherència a aquest patró i la resposta a un programa d'exercici i educació sanitària per prevenir la discapacitat associada a l'hospitalització en pacients grans dins de l'estudi clínic AGE CAR-PLUS. El treball va demostrar que, si bé la intervenció va resultar eficaç per prevenir la discapacitat associada a l'hospitalització, els pacients amb baixa adherència a la dieta mediterrània probablement es beneficiarien més d'aquesta que els pacients amb major adherència. En l'estudi també es va utilitzar la concentració de polifenols totals en orina com a biomarcador del consum de polifenols i *proxy* de la dieta mediterrània, i es van obtenir resultats similars. En el quart estudi, emmarcat en la cohort prospectiva d'InCHIANTI, es va observar que el consum de peix estava inversament associat al risc de declivi cognitiu, una de les principals causes de discapacitat i dependència en la gent gran. En aquesta mateixa línia, una major adherència a la dieta mediterrània, avaluada a través d'una puntuació metabolòmica *a priori* de dieta mediterrània, es va associar retrospectivament amb una menor probabilitat de declivi cognitiu en persones adultes de l'Estudi de les Tres Ciutats.

En conclusió, els resultats derivats d'aquesta tesis doctoral aporten evidència de que l'adherència a un patró dietètic ric en fitoquímics i àcids grassos insaturats, com la dieta mediterrània, pot contribuir a la prevenció del declivi cognitiu i funcional de la gent gran. A més, reforcen la necessitat de refinar les tècniques nutricionals metabolòmiques per la seva completa aplicació en la recerca futura, tant en l'estudi de la relació dieta-salut en l'envelliment com del valor en compostos bioactius de grups d'aliments de gran rellevància nutricional i sobre la salut com són els llegums i el peix.

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Glossary

24HR	24-hour dietary recall	FFQ	food frequency questionnaire
3C	Three-City		
ADLs	activities of daily living	FoodBAI	Food Biomarker Alliance
AGECAR-PLUS	Activity in Geriatric acute CARE Plus	F-C	Folin-Ciocalteu
		GAEs	gallic acid equivalents
ANCOVA	analysis of covariance	GC	gas chromatography
BFI	biomarker of food intake	HA	hippuric acid
BMI	body mass index	HAD	hospitalization-associated disability
CD	cognitive decline		
CVD	cardiovascular disease	HCA	hierarchical cluster analysis
D-CogPlast	Dietary modulators of Cognitive ageing and brain Plasticity	HR	high-resolution
		InCHIANTI	Invecchiare in Chianti
		IQR	interquartile range
DASH	Dietary Approaches to Stop Hypertension	IS	internal standard
		LC	liquid chromatography
DHA	docosahexaenoic acid	LCFA	long-chain fatty acids
EL	enterolactone		
ES	external standard	MDS	Mediterranean diet score
EPA	eicosapentaenoic acid	MDMS	Mediterranean diet metabolomic score
EPIC	European Prospective Investigation into Cancer and Nutrition	MEDAS	Mediterranean Diet Adherence Screener
		MedDiet	Mediterranean diet
FDR	false discovery rate		

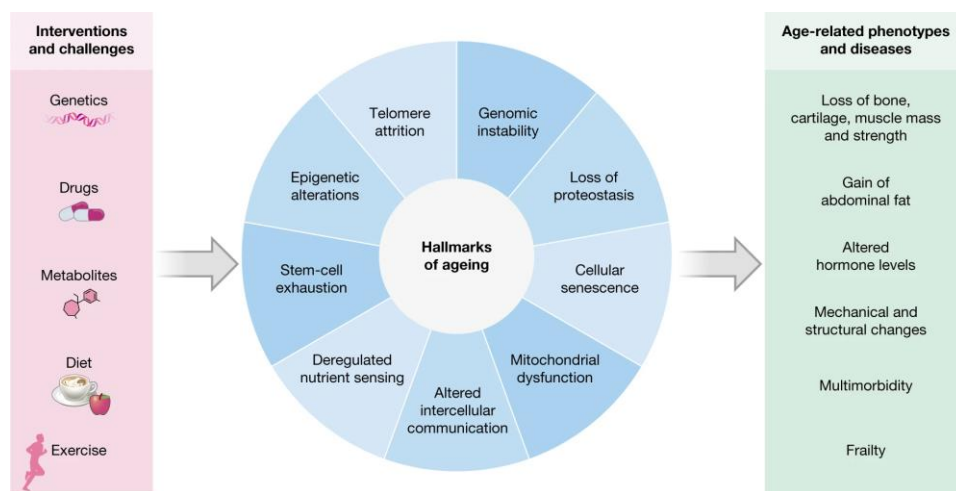
MIND	Mediterranean-DASH Intervention for Neurodegenerative Delay	SD	standard deviation
MMSE	Mini-Mental State Examination	SFA	saturated fatty acid
MS	mass spectrometry	SPE	solid phase extraction
MUFA	monounsaturated fatty acid	TMT	Trail Making Test
QC	quality control	UroA-G	3,8-dihydroxy-urolithin glucuronide
PCA	principal component analysis	UTP	urinary total polyphenols
PREDIMED	Prevención con Dieta Mediterránea	3,4-DHBA	3,4-dihydroxybenzoic acid
PUFA	polyunsaturated fatty acid	3,4-DHPAA-S	3',4'-dihydroxyphenylacetic acid sulfate
		3,4-DHPV-S	5-(3',4'-dihydroxyphenyl)- γ - valerolactone sulfate

1. Background

1.1. Ageing

At the biological level, the ageing process has been considered as the convoluted natural set of structural and physiological changes (also known as the hallmarks of ageing) which arise from the impact of the gradual accumulation of molecular and cellular damage over time. These changes include shifts in body composition, hormone level alterations, changes in blood pressure and lipids, and reduced responsiveness to hormones (Figure 1). This leads to a progressive decline in both physical and cognitive abilities, an increased susceptibility to diseases, and eventual mortality (Partridge et al., 2018). However, these changes are neither homogenous, consistent, nor linear, but may reflect a mix of genetics and influencing external factors such as the physical and social environments and the individual's behaviors and lifelong choices (World Health Organization, 2015).

Figure 1. The hallmarks of ageing and their contribution to the etiology of specific age-related phenotypes and diseases.



Source: Partridge et al., 2018.

1.1.1. Epidemiology of ageing

In 2019, the global population aged 65 years and over was 703 million people, representing 9% of the world's population. This number is projected to reach nearly 1.5 billion by 2050. Moreover, the number of persons aged 80 years or older nearly tripled between 1990 and 2019, and it is projected to triple again between 2019 and 2050 worldwide (United Nations - Department of Economic and Social Affairs, 2019).

Population ageing is driven by the reductions in fertility rates and the increase in life expectancy associated with the historically unprecedented socioeconomic development and with advances in nutrition, medicine, and public health. This progress has led to shifts in the age structure of populations as successive cohorts live longer and have fewer children (United Nations - Department of Economic and Social Affairs, 2019).

The increase in life expectancy is not only due to improved survival at younger ages but also at older ages, particularly in high-income settings. For instance, the life expectancy at birth in Europe in 2000 was 72.5 years, while the life expectancy at 60 was 19.6 years. In 2019, the life expectancy at birth and at 60 was 78.2 years and 22.5 years, respectively (World Health Organization, 2020).

1.1.2. Ageing and health

These changes imply a variation in population cause of death, as the leading cause in older age are non-communicable diseases, especially in high-income settings. Moreover, a greater lifespan does not necessary imply experiencing better health. In fact, recent epidemiological evidence reveals that changing age-related demographics have dramatically augmented the health burden of chronic non-communicable diseases and attributable disability (Engchuan et al., 2019). This trend might be attributed to the fact that ageing is still generally associated with a greater prevalence of impaired physical performance and cognitive function, which leads to associated morbidity and ultimately lowers quality of life and increases dependency (Chang et al., 2019; Partridge et al., 2018).

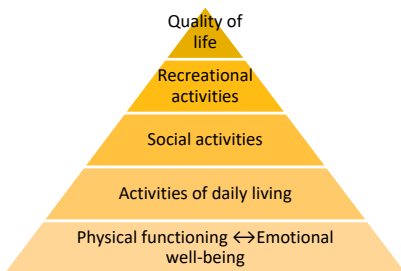
1.1.2.1. Physical function

Physical functioning is of critical importance in quality of life in old age. Adequate levels of physical function are essential for independently performing both activities of daily living (ADLs), such as self-care tasks and transferring, and instrumental ADLs (IADLs), such as housework and managing money. Physical functioning is not only important for participation in recreational and social activities but also for emotional wellbeing. Moreover, limitations in physical functioning are highly predictive of important clinical outcomes, such as falls, morbidity, institutionalization, and mortality in older persons (Painter et al., 1999).

The ability to perform daily living tasks involves the complex integration of multiple physiological systems such as the neuromotor, musculoskeletal, and cardiorespiratory systems. At the same time, there are many factors that affect the ability to perform basic movements (e.g., walking,

sitting, and standing) and more complex activities. Such factors include physical fitness (i.e., cardiorespiratory fitness, muscle function, and flexibility), clinical conditions (e.g., weakness, pain, depression, cognitive function, orthopedic problems), sensory (e.g., vision and hearing), environmental (e.g., social support and education level), and behavioral factors (e.g., physical activity, nutrition, smoking, self-confidence) (Painter et al., 1999).

Figure 2. *The quality-of-life pyramid.*



Source: Adapted from Painter et al., 1999.

In order to evaluate the functional independence in older population, several tools have been proposed over time. The Barthel Index (BI) is one of the instruments widely used to this end, as it measures the ability of a person to independently perform ten basic ADLs: feeding, transferring from bed to chair and back, walking on level surfaces, stair climbing, bowel and bladder control, using the toilet, bathing, grooming (e.g., tooth brushing, shaving), and dressing. The BI essentially scores on a 3-point scale, and the sum score ranges from 0 (total dependence) to 100 (complete independence) points (Mahoney & Barthel, 1965). Although studies evaluating responsiveness and sensitivity of the BI have produced mixed results, it is considered a quick and reliable scale with tested validity and predictive validity (Cohen & Marino, 2000; Rix et al., 2022).

1.1.2.1.1. Hospital-associated disability

Physical function might be a critical aspect during hospitalization in older adults, since acute medical illness that necessitates hospitalization may often deteriorate functional capacity even when such illness is successfully treated. This leads to the subsequent inability to live independently and complete ADLs (Covinsky et al., 2011). Hospitalization-associated disability (HAD; i.e., loss of the ability to perform one or more ADLs independently at discharge) occurs in approximately one-third of patients older than 70 within the first 24-48 hours after admission, especially the most fragile (Cunha et al., 2019; Fimognari et al., 2017; Loyd et al., 2020).

Moreover, HAD is associated with an increased length of stay, higher use of resources, institutionalization, hospital readmissions, long-term disability, and mortality (Dharmarajan et al., 2020; Resnick & Boltz, 2019). Hospitalization periods are also associated with an increased risk of cognitive decline and dementia (Mathews et al., 2014).

HAD arises from the mobility barriers during hospitalization and numerous risk factors have been identified for its development (Dijkstra et al., 2022). These include the medical illness itself, as well as predisposing, hospital-associated, and post-discharge factors. Predisposing factors that might increase susceptibility and diminish recovery capacity include older age, lower pre-illness function, comorbidities, and cognitive impairment. Hospital factors encompass undernutrition, treatment (e.g., repose, procedures, and medication), the institution (hospital and support staff availability), and the patient and medical staff's attitude towards encouragement of independence during hospitalization (Brown et al., 2007). Posthospital factors include the quality of the discharge planning, community support, and resources available (Covinsky et al., 2011).

Beyond individual risk factors, low levels of physical activity and poor mobility during hospitalization seem to play a major role in the negative health consequences associated with hospitalization (Agmon et al., 2017; Pavon et al., 2020; Zisberg et al., 2011). Indeed, hospitalized older adults often spend most of their time in bed even if they can ambulate independently (Brown et al., 2009), and nearly 75% of them do not walk at all during hospitalization (Callen et al., 2004). This leads to a significant loss of total lean mass in the lower limbs, and up to 5% of muscle strength loss per day (Coker et al., 2015; Zisberg et al., 2011).

On the other hand, the evolution of such physical decline is highly variable once the acute illness precipitating the hospitalization is reversed: between 30 and 60% of the older patients who experience disability during hospitalization recover their functional abilities at some point after discharge (D. E. Barnes et al., 2013; Boyd et al., 2008; X. Li et al., 2020). In addition, there is evidence to suggest that interdisciplinary exercise programs aimed at maintaining or improving the functional capacity of older patients during hospitalization have the potential to improve - or to attenuate the decline of - functional status and quality of life at discharge (Hartley et al., 2022; Valenzuela, Morales, et al., 2020).

1.1.2.2. Cognitive function

Optimal cognitive function is a crucial aspect of brain health and wellbeing. However, age is the major risk factor for cognitive decline (CD), a core and prodromal feature of mild cognitive impairment and dementia (World Health Organization, 2019). In fact, dementia describes a syndrome, usually of a chronic or progressive nature, that leads to deterioration of cognitive function beyond what might be expected from the usual consequences of biological ageing. Dementia affects language, memory, perception, personality, and cognitive skills, and of sufficient severity to impair daily life and independent function. The impairment in brain function is usually accompanied, and occasionally preceded, by changes in mood, emotional control, behavior, or motivation (World Health Organization, 2021). Dementia results from a variety of diseases and injuries that primarily or secondarily affect nerve cells and their connections in the brain, such as Alzheimer's disease or stroke. Indeed, the most common type of dementia is Alzheimer's disease, followed by vascular dementia and dementia with Lewy bodies.

Dementia is a rapidly growing public health problem affecting around 50 million people worldwide and entailing high socioeconomic costs. Every year, there are nearly 10 million new cases, and this figure is projected to triple by 2050 due to the global ageing population (World Health Organization, 2019). Moreover, dementia is a leading cause of disability and dependency among older people and a major cause of mortality worldwide (C. S. Liang et al., 2021; Tyrovolas et al., 2022). It accounts for 11.9% of the years lived with disability due to noncommunicable diseases.

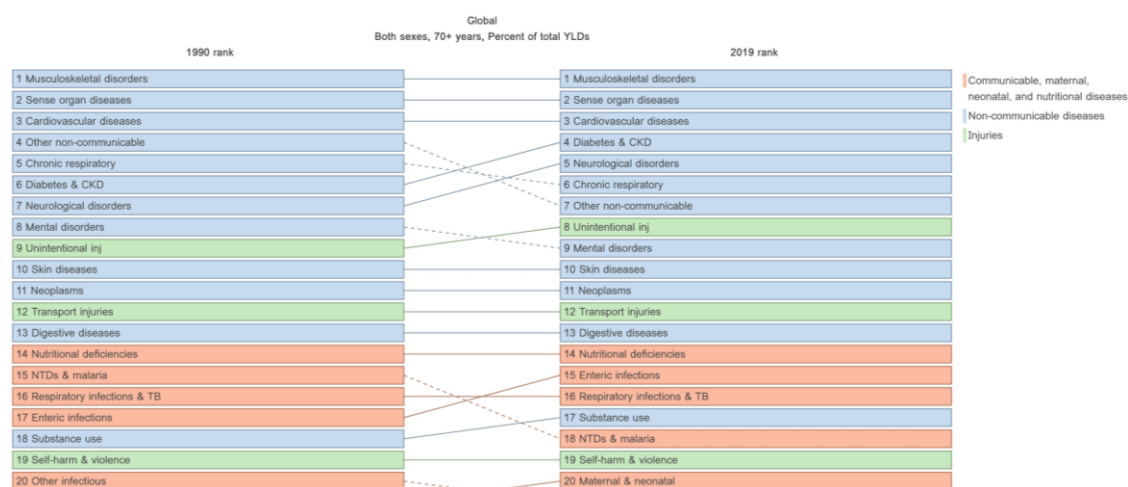
Several tests have been proposed to evaluate cognitive function, depending on the specific cognitive process being assessed. The Mini-Mental State Examination (MMSE) is a cognitive screening tool that provides a brief, objective measure of cognitive function. It can be used to screen for cognitive impairment and dementia, to estimate the severity of cognitive impairment, and to document cognitive change over time (Creavin et al., 2016; Folstein et al., 1975). The MMSE tests six cognitive domains for a maximal score of 30 points: time and place orientation, registration of words, verbal recall, attention and calculation, language, and visual construction. The Benton Visual Retention Test (BVRT) is a widely used test of visual memory, visual perception, and/or visual construction (Sivan, 1992). It is scored for the number of correct reproductions and for the number and types of errors. The Trail Making Test (TMT) is a timed test that involves visual scanning and working memory. The TMT has two parts, the TMT-A (rote

memory) and TMT-B (executive functioning) and it is scored by how long it takes to complete the test (with lower scores being better) (Retain, 1958). The average times to complete part A and B are 29 and 75 seconds, respectively. Finally, the Isaac's Set Test evaluates verbal fluency abilities and speed of verbal production by awarding each correct item offered, with a maximum of 10 in each set and a maximum total score of 40 (Isaacs & Kennie, 1973).

1.2. Healthy diet for healthy ageing

Despite the abovementioned age-related disorders, older populations don't have to be predominantly defined and restricted by poor health. Since most of the disease burden in older people is because of noncommunicable diseases (Figure 3), strategies designed for reducing their risk factors throughout life may enable the delay or even the prevention of these conditions (Chang et al., 2019; World Health Organization, 2015).

Figure 3. Global leading Level 2 causes of global YLDs and percentage of total YLDs (1990 and 2019), for both sexes combined for 70 years and older.



YLDs, years lived with disability. Source: The 2019 Global Burden of Disease (GBD) study.

This direction is aligned with the goal of healthy ageing proposed by the World Health Organization (WHO), which shift the focus from merely “absence of disease” to promote functional ability allowing older people to live fulfilling lives (World Health Organization, 2015).

Healthy ageing encompasses the ongoing process of developing and maintaining the functional ability that enables wellbeing in older age (World Health Organization, 2015). Functional ability (i.e., the health-related attributes that enable people to be and to do what they have reason to value) is determined by intrinsic capacity (i.e., the composite of all the physical and mental

capacities of an individual), the environment (i.e., all the factors in the extrinsic world that form the context of an individual's life), and the interactions between the two (Beard et al., 2016).

In this sense, there is growing evidence that healthy habits such as maintaining adequate nutrition and engaging in regular physical activity may play a pivotal role in preventing and managing chronic diseases, influencing intrinsic capacity, and thus improving wellbeing in older age (World Health Organization, 2015; Yeung et al., 2021).

Healthy dietary patterns have been defined as those with an appropriate caloric intake and largely consisting of the following components: (i) whole grains as the main source of carbohydrates, with low consumption of refined grains and added sugar; (ii) high intake of fruits and vegetables, not including starchy vegetables; (iii) protein sources primarily from legumes, nuts, fish and seafood, with a modest intake of lean meat, poultry, and eggs and no or low consumption of red meat and processed meat; (iv) moderate dairy consumption as an option; (v) unsaturated rather than saturated fats, especially from plant sources; (vi) and no or a low quantity of highly processed foods (Willett et al., 2019).

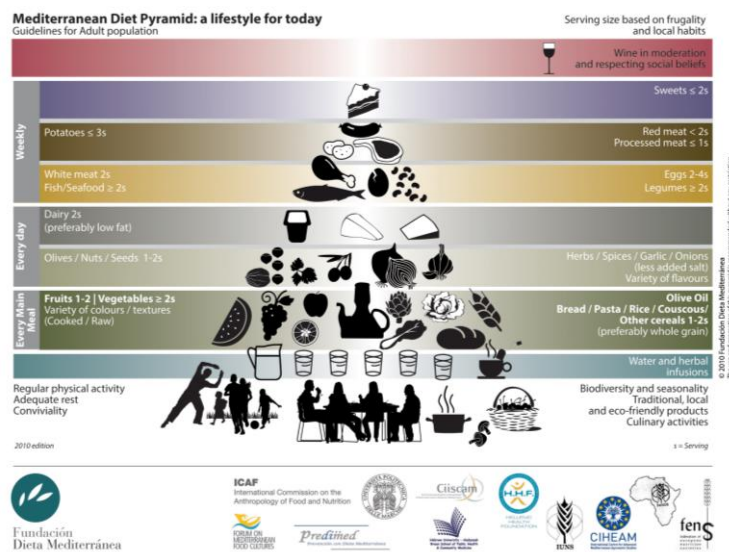
The human dietary intake is a complex, multicomponent, interacting exposure. Considering dietary patterns instead of individual nutrients, food components or foods provides an accurate approach to the individuals' actual intake as nutrients and foods are consumed in combination. Moreover, analyzing food consumption as dietary patterns accounts for the complex synergistic interaction between nutrients and the food matrix, thus offering a holistic and comprehensive effective method for assessing relationships and understanding the etiologic role between diet and healthy ageing (F. B. Hu, 2002; Jacobs & Steffen, 2003).

For instance, healthy dietary patterns such as the Mediterranean diet (MedDiet), the Dietary Approaches to Stop Hypertension (DASH), the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet, and other regional healthy dietary patterns have been associated - besides proven environmental sustainability (Willett et al., 2019) - with a reduced risk of major diet-related chronic diseases, including diabetes, cardiovascular disease (CVD), and some types of cancer (Afshin et al., 2019; Qiao et al., 2022). However, evidence for their roles in intrinsic capacity is still limited (Yeung et al., 2021).

1.2.1. The role of the Mediterranean diet in physical and cognitive performance

Within this context, the MedDiet has traditionally been characterized by a high intake of vegetables, legumes, fruits, nuts, seeds, whole grains, and olive oil, alongside a moderately high intake of fish, a low-to-moderate intake of dairy products, and a low intake of saturated lipid, meat, and poultry (Figure 4). Additionally, the MedDiet includes a regular but moderate intake of ethanol, primarily in the form of red wine and generally consumed during meals (Willett et al., 1995).

Figure 4. The Mediterranean diet pyramid.



Source: Bach-Faig et al., 2011.

This dietary pattern is of particular interest due to its associations with reduced risk of all-cause mortality and lower incidences of CVD, overall cancer, neurodegenerative diseases, and diabetes, as observed in multiple observational cohort studies and clinical trials (Dinu et al., 2018). A number of systematic reviews and meta-analyses of observational studies and randomized clinical trials have also inquired into the potential impact of the MedDiet on physical and cognitive function in old age, showing that high adherence to the MedDiet might be associated with greater physical and cognitive performance (Coelho-Júnior et al., 2021; Dominguez et al., 2023; Limongi et al., 2020; Poursalehi et al., 2023). Moreover, available evidence suggests that adherence to the MedDiet at midlife and among the elderly is associated with a higher likelihood of healthy ageing in later life (Critselis & Panagiotakos, 2020; Zhou et al., 2021).

Although the biological mechanism underlying these effects remains unclear, existing literature suggests that part of the observed protective effect could be derived from the antioxidant and

anti-inflammatory properties of certain compounds conferring the MedDiet, such as phytochemicals and unsaturated fatty acids (Tosti et al., 2018). These compounds are primarily found in plant-based food groups as well as in fish and seafood.

From plant-based food groups, and in the context of preserving intrinsic capacity among older individuals, it is interesting to emphasize pulses, which serve as an economical and sustainable source of high-quality protein (Willett et al., 2019). Legumes have a low glycemic load and are rich in fiber, phenolic acids, folate, vitamin B6, iron, phosphorous, and polyunsaturated (PUFAs) and monounsaturated fatty acids (MUFAs) including linoleic and oleic acids (Delgado et al., 2017). These components may collectively confer to legumes the potential to lower functional decline by reducing risk factors for CVD and diabetes, and decreasing risk of some cancers (e.g., colorectal adenoma) and ischemic heart disease (Bechthold et al., 2019; Martini et al., 2021; Schwingshackl et al., 2018). Furthermore, although research focusing on the effects of regular legume consumption on physical and cognitive function in old age is scarce, there is emerging evidence suggesting that legumes may exert a beneficial role in the preservation of physical and cognitive performance (Dalile et al., 2022; Kim et al., 2023; Ortolá et al., 2020).

From animal-based food groups, fish and seafood are of great interest as they are not only a protein source low in saturated fats and rich in vitamin D and selenium, but also a primary source of marine long-chain omega-3 PUFAs [specially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)]. Indeed, the beneficial effects of fish consumption have, to a large extent, been attributed to PUFAs (Rimm et al., 2018). There is a protective effect against CVD and cognitive dysfunction of elevated fish consumption (Bechthold et al., 2019; Kosti et al., 2022). Findings from longitudinal, cross-sectional and intervention studies indicate as well that intake of fish is associated with beneficial health effects in older adults, including healthier ageing (García-Esquinas et al., 2019), reduced risk of frailty (Konglevoll et al., 2023; Minamino et al., 2021) and improved muscle mass and function (Alhussain & Alshammari, 2021).

1.3. Nutritional metabolomics

Although diet is a modifiable risk factor for chronic diseases and healthy ageing, nutritional studies do not consistently support associations between specific dietary patterns, foods or nutrients, and health endpoints (L. L. Barnes et al., 2023; Huang et al., 2023). Part of these incongruities might be because most nutritional studies frequently rely on self-reported dietary assessment methods that are subject to recall bias and measurement error (Guertin et al., 2014).

1.3.1. Biomarkers of food intake

The limitations of the traditional dietary measurement tools have led to extensive efforts to develop more reliable dietary assessment techniques, in particular to the research on dietary biomarkers of food intake (BFIs). A BFI is defined as being a specific measurement in a biological specimen accurately reflecting the recent or average intake, selective and dose-dependent, of specific food groups, foods, or food components (Brouwer-Brolsma et al., 2017). They are assumed to be a more accurate measurement of dietary exposure than self-reported consumption as they also cover the bioavailability of dietary compounds and the food-food interactions. It is important to note that, to date, some BFIs can be used as generic biomarkers of a food group (e.g., flavonoids for fruit and vegetable intake), whereas others are well-established biomarkers for certain foods (e.g., proline betaine for citrus fruit and products consumption) (Gibbons et al., 2017).

The BFIs are part of the food metabolome, which is the set of compounds directly derived from the ingestion, digestion, absorption, and biotransformation of foods and their components by the host tissues or gut microbiota (Scalbert et al., 2014). In turn, the food metabolome is part of the human metabolome, which is also composed of the endogenous metabolome (consisting of chemicals related to cellular metabolism) and other xenobiotics derived from drugs, household chemicals, and pollutants. Like the food metabolome, part of the endogenous metabolome comes also from the gut microbiota biotransformation (Scalbert et al., 2014). Furthermore, the endogenous metabolome can be influenced by diet as well, since dietary components may interfere with multiple biochemical mechanisms and metabolic pathways (Wu et al., 2022). The human metabolome may be also influenced by genetic factors, body composition, lifestyle, and geographical location (Brouwer-Brolsma et al., 2017).

With this in mind, the integration of metabolomics with nutritional science - namely nutrimetabolomics - has emerged in the last decades in the field of dietary assessment through

the discovery and validation of BFIs thus providing a more objective snapshot of overall dietary intake (Ulaszewska et al., 2019). While metabolomics focuses on the comprehensive analysis, identification, and quantification of the set of molecules of low molecular weight derived from metabolism, including the human metabolome, nutrimentalomics studies the effect of diet on the metabolome (Ulaszewska et al., 2019).

Traditionally, two different approaches have been developed in the field of nutrimentalomics: untargeted and targeted metabolomics, which mainly differ in the identification of the molecules analyzed and the statistical data analysis (Rangel-Huerta, 2018). Untargeted (or discovery-based) analysis focuses on global detection and relative quantification of small molecules in a sample, aiming to gather as much data as possible using advanced chemometric techniques. However, the identification of many detected metabolites remains a challenge, requiring intensive post-analysis work for accurate biological interpretation. On the other hand, targeted (or validation-based) analysis involves identifying and quantifying known and pre-selected metabolites using pure standards and a methodology emphasizing precision and selectivity. Although the study coverage is limited, the main advantage of this approach lies in its clear biological interpretation, making it useful for hypothesis testing (Rangel-Huerta, 2018).

Part of this thesis has been developed using targeted metabolomics data, although participation in the analysis of legume composition as part of a postprandial intervention work using untargeted metabolomics is also embedded in it.

1.3.2. Biomarkers of dietary patterns intake

As discussed before, there has been an increased interest in nutrition research in examining dietary patterns, instead of evaluating the intake of single foods or nutrients, to capture the complex interconnections and synergy derived from the different combinations of foods. As a result, the ultimate goal of using dietary biomarkers within the context of dietary patterns is to classify individuals into certain patterns or to examine compliance to a certain predefined dietary pattern (score), including Mediterranean, Nordic, Western, prudent, and vegetarian diets, among others (Brennan & Hu, 2019; Guasch-Ferre et al., 2018).

Whereas, for foods, in most cases, single metabolites have been used to monitor their intake, this is not the case for dietary patterns. In such studies, researchers tend to provide multi-metabolite biomarker panels related to the overall dietary pattern that is under study (Garcia-Aloy & Andres-Lacueva, 2018). With this approach, there is a better estimation of dietary

assessment, in terms of both accuracy and precision, as the metabolites that are building the metabolite signature are providing complementary information (Garcia-Aloy & Andres-Lacueva, 2018).

1.3.3. Nutrimetabolomics and its applicability in ageing research

Nutrimetabolomics also focuses on the interactions between dietary components and the body metabolism and how diet influences metabolism and health (Ulaszewska et al., 2019). Since it accounts for metabolism and its variability - because of genetics, lifestyle, or diseases - by measuring downstream components or metabolic products of foods, it may provide a better proxy of real diet exposure (biomarkers of intake). Moreover, it may reveal associations between dietary patterns and certain metabolic pathways through the examination of relationship between dietary patterns and endogenous metabolites (biomarkers of effect), thus identifying potential mechanisms influenced by diet quality.

Additionally, nutrimetabolomics can help to study the intricate interplay between diet, gut microbiota, and host metabolism. Through the analysis of metabolites present in biological samples, the dietary components that are being metabolized by the gut microbiota can be identified, along with the metabolic pathways and the resultant end-products generated. This information contributes to the understanding of the mechanisms by which diet impacts metabolism and health, leading to the discovery of novel biomarkers or new therapeutic targets (Ulaszewska et al., 2019). On the other hand, the composition and activity of the gut microbiota are influenced by a variety of factors, including genetics, environment, and diet. The intestinal microbiota can, in turn, change constituents in food and make them available to themselves or the host for additional metabolism, thereby leading to shifts in host metabolism and potentially affecting several health outcomes (Guasch-Ferre et al., 2018).

These opportunities are of great relevance in the study of diet's impact on ageing. On the one hand, there is evidence that there is a large heterogeneity in health with ageing and that these differences might be reflected by the metabolome (Lowsky et al., 2014; Rutledge et al., 2022). Since health over the life course is determined by a multitude of risk factors to which people are exposed during their life, including diet, the identification and evaluation of both biomarkers of intake (exogenous biomarkers) and biomarkers of effect (endogenous biomarkers) may provide a closer and more inspiring grasp of biological ageing phenotypes and the mechanistic relationships by which diet influences them.

On the other hand, ageing is known to impair gastrointestinal physiology and function, which can impact the amount and types of nutrients and bioactive compounds digested and absorbed. This may, by that means, not only affect the physiology of other systems but also the gut microbiota composition and functionality (An et al., 2018). Additionally, the age-related alteration of immune function might impact host-microbe interactions, which can also contribute to changes in intestinal microbiota.

Altogether, nutrimetabolomics provide a strong potential for improving assessment of dietary intake in older adults accounting for the bioavailability of dietary compounds, evaluating individual metabolomic responses to different dietary components or patterns, and identifying and examining relationships between diet patterns and healthy ageing.

1.3.3.1. Biomarkers of polyphenol intake and cognitive function in older adults

Evidence about plasma and urine polyphenols and cognitive function in older adults is shown in the following book chapter, which has been prepared to be part of the book entitled “Frailty syndrome: a volume in the oxidative stress and disease book series” from the CRC Press publisher. The tables summarizing the characteristics and results of the papers included in this chapter (Table A.1 and Table A.2) are shown in the Appendices section of this thesis (Appendix 8.1).

Book chapter: Plasma and urine polyphenols and cognitive decline

14. Plasma and urine polyphenols and cognitive decline

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Abstract

Frailty syndrome has been associated with cognitive decline (CD) in older adults. Polyphenol intake has garnered significant attention as it may influence on cognitive function. However, current data mostly relies on self-reported questionnaires, which are prone to recall bias and do not account for polyphenol bioavailability. In this context, this chapter will attempt to provide evidence of the protective role of plasma and urine polyphenols, as objective measurements of their exposure, in cognitive function in older adults.

14.1. Introduction

Frailty in the elderly is defined as a clinical syndrome characterized by an increased risk of disability and vulnerability because of various factors, including loss of cognitive reserves. In turn, once frailty is established and progresses in its natural course, other geriatric syndromes like cognitive decline (CD) are more likely to emerge (1). Accumulating evidence from

observational studies supports an association between frailty, lower cognitive performance, mild cognitive impairment (MCI), and dementia (2–4).

Reduction of the prevalence and severity of frailty is likely to have large benefits for individuals, their families, and society overall. Given the bidirectional interplay between physical health and cognition, approaches targeting cognitive function across life might be of interest (5,6).

The trajectory of cognitive function is influenced by a complex interplay of genetic and environmental factors, including early-life exposure to cognitive stimulation, socioeconomic status, and educational background. Nutrition has garnered significant attention as a modifiable environmental factor, as it holds the potential to reduce the risk of age-related cognitive disorders through the diverse array of nutrients and food components that have been suggested to promote cognitive function (7).

Growing evidence from epidemiological and human intervention trials indicates that polyphenols may influence cognitive function given their antioxidant properties (7–9). Polyphenols are a large family of bioactive compounds derived from secondary plant metabolism and widely distributed in plant-based foods such as fruits, vegetables, whole grains, nuts, seeds, wine, tea, coffee, and cocoa (10). These compounds can be classified into four main groups, which are flavonoids, phenolic acids, lignans, and stilbenes. They can further be subdivided in numerous subcategories depending on the number of phenol units within their molecular structure, substituent groups, and/or the type of linkage between phenol units (11). The consumption of polyphenols considerably differs across the globe - ranging from approximately 300 mg/d in Brazil to 1700 in Denmark - as does their major food sources (12,13).

However, current epidemiological data on polyphenol intake mostly rely on self-reported questionnaires and food composition databases, which are subject to recall bias and measurement error (14). In addition, the protective effects of polyphenols on health and cognitive function depend not only on the quantity consumed but also on their bioavailability, which largely differs between compounds and is influenced by a large inter- and intra- subject variability (15,16). In this context, the evaluation of polyphenol metabolites in biological samples might be essential to provide an accurate assessment of polyphenol exposure and investigate their protective effects on cognition (17).

In this chapter, several observational studies and randomized clinical trials (RCTs) are reviewed in order to better understand the effect of biomarkers of polyphenol intake on cognitive

function in older adults. The research includes studies evaluating the association between biomarkers of polyphenol intake and cognitive function - assessed through cognitive tests - on the base of older human subjects.

Details of the design, subjects' characteristics, exposure or intervention, outcomes, and duration of the interventions or follow-up periods of the RCTs and the observational studies included in this review are shown in Table A.1 and Table A.2, respectively.

14.2. Observational studies and randomized clinical trials of biomarkers of polyphenol intake on cognitive function

14.2.1. Urinary total Polyphenols

Studies evaluating urinary total polyphenol concentrations showed a beneficial relationship between cognitive impairment (CI) and polyphenol excretion in older individuals. In fact, Rabassa et al. (2015) showed in their 3-year period study that higher urinary total polyphenol (UTP) levels were associated with lower risk of CD, according to Mini-Mental State Examination (MMSE) ($OR_{\text{extreme tertiles}} = 0.53$, 95% CI = 0.34–0.85; $p\text{-trend} = 0.008$) and with better performance on visual attention and processing speed ($OR_{\text{TMT-A}} = 0.52$, 95% CI = 0.28–0.96, $p\text{-trend} = 0.03$), but not on executive abilities [trail making test – part B (TMT-B)] (18). Urinary polyphenols derived from nuts intake were also associated with better cognitive function ($OR = 0.006$, 95% CI = 0.001–0.011; $p = 0.029$) and a lower risk of CD ($OR = 0.995$, 95% CI = 0.991–0.999; $p = 0.016$) (19). In the same line, a cross-sectional trial in a longitudinal cohort at high cardiovascular risk concluded that UTP were associated with better scores in immediate verbal memory ($\beta = 1.208$, 95% CI = 0.236–2.180; $p = 0.015$) but not in the MMSE test (20).

14.2.2. Flavonoids

The trials assessing flavonoids biomarkers reported mixed conclusions about the effects on cognitive performance.

Gleason et al. (2015) assessed isoflavones in patients with Alzheimer's disease (AD) and did not find any benefit in cognition but a significant correlation between changes in plasma isoflavone levels and changes in speeded dexterity and verbal fluency score ($p \leq 0.05$) (21).

However, in individuals with subjective cognitive complaints, reduced cognitive symptoms in everyday activities and improvements in recognition memory were found in the group that consumed a daily dose equivalent to 1 cup of whole blueberry fruit (22). Krikorian et al. (2020)

also reported cognition performance improvements [semantic access ($p = 0.01$), visual-spatial memory ($p = 0.05$), enhanced speed of processing ($p = 0.08$)] among patients with MCI who consumed blueberries supplementation. Moreover, urinary parent anthocyanins were correlated with neurocognitive benefit (23). Anthocyanin supplementation showed also positive changes in cognitive test scores for Consortium to Establish a Registry for AD (CERAD) learning ($p = 0.016$), recall ($p < 0.001$) and recognition ($p = 0.047$), and for STROOP test word ($p < 0.001$) and color ($p = 0.044$), in people with increased risk of dementia followed for 16 weeks (24).

In healthy participants, John et al. (2014) (25) reported that long-term changes in urinary isoflavonoids were not associated with global cognition or episodic memory [verbal episodic memory factors (i.e., list learning and logical memory) and visual episodic memory factor] although greater isoflavonoid exposure was associated with decrements in the general intelligence factor (25). Interestingly, this inverse association was of smaller magnitude in women who had experienced natural menopause rather than those who had undergone surgical menopause (25). The study of Igwe et al. (2017) did not also show a significant acute effect on various domains of cognitive function after 300 mL of anthocyanin-rich Japanese plum juice, regardless of its 2 dose-timing regimens of consumption (26). Likewise, neither verbal learning, short-term memory or executive function improved following daily consumption of 200 mL low-dose anthocyanin plum nectar in healthy older subjects (27).

On the other hand, Gleason et al. (2009) showed that consuming 100 mg/day soy isoflavones improved performance on tests of visual-spatial memory ($p < 0.01$) and construction ($p = 0.01$), verbal fluency ($p < 0.01$) and speeded dexterity ($p = 0.04$) whereas placebo-treated participants were significantly faster on executive function ($p < 0.05$) (28).

Considering flavonoid metabolites, total plasma equol was significantly correlated with speeded dexterity (Grooved Pegboard, $r_{\text{non-dominant hand}} = -0.33$, $p = 0.02$; $r_{\text{dominant hand}} = -0.29$, $p = 0.05$) as well as with verbal fluency (Phonemic Fluency $r = 0.29$, $p = 0.04$) in one study with AD patients (21). Bensalem et al. (2019) also revealed in their study that urinary concentrations of native and conjugated derivatives of (epi)catechins from a polyphenol-rich extract from grape and blueberry (PEGB) were strongly associated with episodic verbal recall memory improvement [$p = 0.016$ and $p = 0.010$ for native and conjugate derivatives of (epi)catechins, respectively] (29). Resembling associations in this outcome and in verbal recognition memory (VRM) delayed were found out when focusing only on cognitive decliners [$p = 0.036$ and $p = 0.053$ in episodic verbal recall memory for native and conjugate derivatives of (epi)catechins, respectively; $p = 0.032$, p

= 0.055 and $p = 0.032$ in VRM delayed for flavan-3-ols biomarkers of PEGB, native and conjugate derivatives of (epi)catechins, respectively] (29). The clinical trial of Krikorian et al. (2020) showed further positive correlations between effects on cognition, particularly semantic access and non-verbal memory, and urinary content of glycoside anthocyanin conjugates and total glycosidic anthocyanin forms (23). However, no significant association was found between the microbial metabolites of (epi)catechins (valerolactones, β -type procyanidins dimers or phenolic compounds) and memory improvement (29). Anthocyanin metabolites were also not associated with cognitive benefits in adults with cognitive complaints (22).

With regard to observational studies, no significant differences in plasma flavonoids concentrations were detected between adults with MCI and control subjects ($p > 0.05$) (30,31), although compared to controls, lower plasma total antioxidant capacity was detected in patients with MCI ($p < 0.05$) (30). Conversely, a cross-sectional study evaluating cognitive function according to equol-production status showed that, compared with equol non-producers, equol producers were less susceptible to suffer from MCI (31). In fact, regression analysis to assess the presence of MCI showed that being an equol non-producer was an independent risk factor for MCI (OR = 3.961, 95% CI = 1.048–14.969, $p = 0.042$) (31).

14.2.3. Phenolic acids

The study of Ochiai et al. (2019) examined the effect of chlorogenic acids beverage on older subjects with MCI (32). In the TMT-B test (attention and executive memory), participants had a significantly reduced number of errors when consuming the active beverage as compared with the placebo beverage ($p < 0.05$). There was still no significant correlation between change in blood chlorogenic acids levels and the number of TMT-B errors before and after intake of the active beverage ($r = 0.011$, $p = 0.571$) (32). In this sense, the authors concluded that effect of chlorogenic acids on cognitive function were unlikely related to chlorogenic acids levels in the blood. Scores in the MMSE, AD assessment scale-cognitive (ADAS-cog), and TMT-A tests did not differ significantly between groups (32).

Phenolic acids from a blueberry powder intervention showed conflicting results as change in plasma concentrations of ferulic acid glucuronide was negatively associated with change in switching errors ($\beta = -0.150$, $p < 0.01$) but positively associated with change in California Verbal Learning Test II (CVLT-II) repetition errors ($\beta = 0.044$, $p < 0.001$). Furthermore, change in the plasma concentrations of syringic acid was positively associated with change in CVLT-II

repetition errors ($\beta = 0.070$, $p < 0.001$) whereas change in hippuric acid was negatively associated with it ($\beta < -0.001$, $p < 0.001$) (33).

14.2.4. Other polyphenols

Experimental studies of other polyphenol classes and MCI concluded with mixed results. Bookheimer et al. (2013) evaluated the effects of drinking 236.59 mL of pomegranate juice in older adults with pre-existing mild memory complaints (34). As a result, the group who received the pomegranate juice intervention performed significantly better in the Buschke selective reminding test of verbal memory after 4 weeks (two-tailed $t = 2.8$, $p = 0.017$), in contrast to placebo group, which did not report any difference (two-tailed $t = 1.1$, $p > 0.3$). Between groups, the improvement in memory after the intervention was significantly greater in the pomegranate juice group compared to the placebo group, specifically on total recall measure (two-tailed $t = 2.3$, $p = 0.029$) as well as on consistent long-term retrieval (two-tailed $t = 2.4$, $p = 0.022$). A significant increase in plasma trolox-equivalent antioxidant capacity (TEAC) and urolithin A-glucuronide was also and only observed in the pomegranate group after intervention (34). Otherwise, in another intervention study evaluating curcumin and its metabolites (tetrahydrocurcumin, glucuronidated curcumin and glucuronidated tetrahydrocurcumin) efficacy on improving cognitive tasks in individuals with AD, investigators were not able to evidence differences between treatment groups (2 g/day and 4 g/day of curcumin) and placebo-treated subjects in the severity of cognitive and non-cognitive symptoms of dementia [ADAS-Cog, Neuropsychiatric Inventory (NPI), AD Cooperative Study Activities of Daily Living (ADCS-ADL) and MMSE tests] (35). When combining 2 g/day and 4 g/day curcumin groups, there was a trend for placebo-treated participants to perform better than intervention group on the MMSE ($p = 0.08$).

14.2.5. Summary of results

The present chapter examined the current evidence regarding the effect of biomarkers of polyphenol intake on CD in older people. The research included both observational and RCTs that used cognitive tests and measurements of polyphenols and their metabolites in biological samples. The results presented in this book chapter lead to mixed conclusions regarding the potential benefits of polyphenol consumption on CI, in line with previous systematic reviews (36). Flavonoids were tested in 6 studies with healthy elderly subjects and in 6 studies with old-age participants with some level of memory impairment, deriving inconsistent results. Total polyphenols, which were studied exclusively in healthy older participants in this chapter, also

reported heterogeneous findings, as well as phenolic acids in individuals with MCI (32). Among the results comprised in this chapter, only executive function has consistently presented a non-improvement associated with flavonoids and total polyphenol intake. On the other hand, positive results have been shown on executive function when consuming phenolic acids. Even though some negative results have been described, 13 out of the 18 studies included here reported inverse associations between consumption of polyphenols and at least one cognitive domain or screening test. Only flavonoids have evidenced a significantly beneficial effect on the psychomotor domain, regardless of subjects' cognitive status (21,23,28). On the contrary, flavonoids have not showed a significant inverse association in the attention domain (21,22,26).

14.3. Discussion

The studies included in this chapter analyzed different classes of polyphenols through different analysis techniques and assessed AD, dementia, MCI, and cognitive function using a diverse range of cognitive tests or tasks. This is especially important since some studies in the general literature have used biomarkers of AD or dementia, or the ApoE- ϵ 4 genotype, which tend to be more objective than cognitive tests (37). In fact, only six clinical trials evaluated cognitive function combining specific tests with biological markers. Ringman et al. used plasma levels of A β 1-40 and A β 1-42, and cerebrospinal fluid (CSF) levels of A β 1-42, total tau (t-tau), tau phosphorylated at threonine 181 (p-tau181) and F2-isoprostanes (35), while Igwe et al. (2020) analyzed plasmatic brain-derived neurotrophic factor (BDNF) (27). In patients with cognitive or cardiovascular dysfunctions, Gleason et al. (2015) (21), Mcnamara et al. (22) and Valls-Pedret (20) determined the ApoE- ϵ 4 genotype as well. Lastly, Bookheimer et al. (34) conducted functional magnetic resonance imaging (fMRI) while subjects were performing cognitive tasks to exclude subjects with atrophy indicative of dementia as well as to find significant functional brain activation differences. Moreover, there has been little consensus in human studies on the range of cognitive domains to be assessed or the specific tests to be employed, with some cognitive domains either under-represented or not explored at all (38).

Besides, only five studies (18,19,25,29,32) evaluated the association between polyphenols and CD focusing on urinary and/or plasma polyphenol concentrations, whereas the other studies used food frequency questionnaires and food composition databases. Assessment of urinary polyphenol biomarkers concentration is considered an objective estimation of total dietary polyphenol consumption (39,40) and a more accurate measurement method than food frequency questionnaires (FFQs). FFQs only consider polyphenols from the most representative

foods (prespecified food lists) and portion size and frequency estimation are subject to participants' perception and memory, potentially leading to misreporting and measurement errors (41–43). Therefore, it seems comprehensible that findings from the studies included in this chapter are mixed.

Urinary polyphenol concentration depends on their bioavailability and may be affected by biological traits, absorption, and metabolism, which largely differ between compounds and have a large intra- and inter- individual variability (17,44,45). In fact, ageing is known to impair gastrointestinal physiology and function, which may compromise the capacity for absorption and tolerance of polyphenols (46–49). For instance, one study concluded that the urinary phenolic acid profile varied between younger and older adults after a polyphenol-rich meal (50). However, these findings differed from those of Igwe et al. (2017), who concluded that urinary anthocyanin/metabolite profiles were similar between younger and older adults (26).

The aforementioned gastrointestinal disruptions can lead, in turn, to an increase of intestinal permeability (IP) (51), boosting the mucosal inflammation and the low-grade systemic inflammation detected in older adults (i.e., inflammaging process) (52). Increased IP has been suggested to participate in multiple neurodegenerative disorders, such as Parkinson's disease and AD (53,54). In this context, there is evidence pointing out the potential of polyphenols in regulating the gut barrier function and preventing the increase of IP (55).

Furthermore, one of the most common causes behind the increase of the IP is the changes in the gut microbiota composition (56). During aging, gut microbiota composition changes (57) and it is reduced in terms of species richness while instability and interindividual variability increases (58–60). Moreover, recent findings allow us to hypothesize that the reduction in the diversity of the gut microbiome (61–63) and the presence of a distinctive gut microbial signature in patients with dementia, CI, or AD is characterized by dysbiosis with differences in beta diversity, reduction of short-chain fatty acid-producing bacterium and overrepresentation of gram-negative bacteria (54,64). It is important to note that inconclusive results were observed regarding participants' age from the included studies in this chapter.

In fact, the specific role of the commensal microbial population in regulating immunity and inflammation beyond the gastrointestinal tract may represent a relevant influence on the neurodegeneration process: changes in the proteolytic processing of the amyloid precursor protein may result in the aggregation of the amyloid beta peptide, which might prompt an

immune response that leads to neuroinflammation and neurodegeneration in AD, specifically interfering with neuroplasticity and memory consolidation (65).

This is particularly relevant since there is growing evidence about the two-way interaction between (poly)phenols and gut microbiota (66). This is, the gut microbiota plays a key role in the metabolism and absorption of polyphenols, converting them into bioavailable and bioactive metabolites and contributing to the beneficial effect attributed to these compounds (45,67,68). At the same time, phenolic compounds may improve immune response modulation, inhibit the growth of detrimental gut species, and suppress the adhesion of gut pathogens to the human gut tract (69–71). Therefore, the colon microbiome is of great relevance for the interindividual variability in response to polyphenol consumption, leading to non-equally polyphenol effectiveness in all subjects. Results from an intervention study with polyphenol-rich foods supported that low-bioavailability of polyphenols in patients with increased IP could be attributed to alterations in the gut microbial metabolism. Moreover, it suggested that microbiota-derived metabolites could be largely responsible for the biological activity of dietary polyphenols against age-related disrupted IP (72). In this chapter, only Igwe et al. (2020) examined the effect of polyphenol intervention on microbial composition (27). They concluded that contrary to the extended evidence, there was no difference between intervention arms (i.e., low-dose anthocyanin QGP nectar and control) for the *Bifidobacterium* and *Clostridium* genera nor for the battery of cognitive tests performed and anti-inflammatory biomarkers (BDNF and hsCRP).

Despite the increasing scientific literature on the bioavailability of polyphenols in the digestive system, it remains unclear whether they can reach the brain (45). In experimental studies, several brain-available polyphenol metabolites have been identified, such as quercetin-3-O-glucuronide (73,74), malvidin-3-O-glucoside (74), 3-hydroxybenzoic acid, and 3-(3'-hydroxyphenyl) propionic acid (75), 3'-O-methyl-epicatechin-5-O- β -glucuronide (76), and urolithins (77). These metabolites are suggested to have potential benefits in relation to the onset and progression of AD-type pathophysiology. Nevertheless, special attention should be given to polyphenol interventions, as it is still unclear whether chronic consumption compared to acute consumption could influence the detected levels (78).

Thus, a nutritional biomarker of total dietary polyphenols and polyphenol metabolites is essential to have a rigorous evaluation of true polyphenol exposure to study their specific cognitive effects (39,52). Indeed, polyphenol-food metabolome and endogenous metabolites

modulated by polyphenol intake are being considered more and more given the health effects found behind them. One exploratory study identified more than 20 metabolites associated with age-related CD, six of which were food derived (79). Although no polyphenol was determined, hydroxycinnamate intake was significantly correlated with atractyligenin glucuronide and cyclo(leucyl-prolyl), two of the serum metabolites from the signature. Hydroxycinnamic acid intake was also correlated with an unidentified ion, which was not in the signature but had a univariate odds ratio for CD of 0.90. Atractyligenin glucuronide and cyclo(leucyl-prolyl) were also significantly correlated with coffee consumption, which is one of the main sources of polyphenols in European countries and Mexico (13,80). In 13 out of the 18 studies included in this chapter identified and/or quantified polyphenol metabolites, such as equol, dihydrogenistein, dihydrodaidzein, O-desmethylangolensin, peonidin-glycosides and -glucuronides, urolithin A-glucuronide and glucuronidated tetrahydrocurcumin, among others. This is particularly relevant since various studies have described that part of the beneficial effects of some polyphenols might be, in fact, due to their microbiota-derived metabolites (i.e., equol, urolithins, 3,4-DHPPA, 3/4-HPPA) (77), especially considering the usual low bioavailability of the parent compounds (68), such as anthocyanins (81,82). However, current evidence is ambiguous concerning whether the proportion of some metabolite producers changes with age (83–91).

The length of time needed to perceive polyphenol's effect on cognitive impairment is also not well established. The studies included in this chapter used a wide range of time periods, regardless of their design. The cause and consequence of such disagreement is the difficulty in recognizing whether the effect is led by parent polyphenols or by their metabolites (92). For instance, McNamara et al. (22) and Bergland et al. (24) discussed the influence of the very long-term retention of anthocyanins in the discrimination between their native and metabolized forms effect as well as the effect of having a substantial reservoir of anthocyanins from habitual consumption on absorption. This could explain the lack of favorable results in Igwe et al. (2017), where the time-period was punctual (26). The mechanism by which anthocyanins influence cognition is still unclear but seems to depend on the exposure period. Prior studies did not find a significant acute effect of fruit anthocyanins on cognitive processes (93).

Moreover, only two RCT (22,28) included an open-label extension to two and six months in order to evaluate the secondary absorption of isoflavones and anthocyanidins-derived metabolites in the gut, and therefore consider the influential role that these compounds may have in cognitive

performance. This is noteworthy as recent investigations demonstrate that non-extractable polyphenols metabolized by the gut microbiome led to a sustained release of bioactive metabolites with antioxidant and anti-inflammatory effects in preclinical models (94).

The vehicle for provision of polyphenols also warrants consideration since the presence or absence of food matrix influences polyphenol kinetics and absorption. Thus, the same dose of the same phenolic compound can result in misleading conclusions when it is ingested isolated or mixed with other polyphenols, nutrients, or phytochemicals. For example, anthocyanins have been shown to have a greater bioavailability when consumed together with other polyphenols (95–98). Four of the studies testing flavonoids in an intervention administered them in capsules (21,24,28,29) while four offered them as a powder (22,23,25,33). The remaining ones provided participants with juices or soy (26,27,31). Phenolic acids were administered in both beverages and powder forms, whereas total polyphenols were estimated from the participants' baseline diet. Interestingly, studies that included participants with CI and assessed polyphenols from fruits showed promising results (22–24,29,34).

Another important point to consider is the baseline diet, which was taken into account as exclusion criteria in Bookheimer et al. and Bensalem et al. trials (29,34). Seven RCTs (22,23,26,28,29,33,34) restricted polyphenol-rich foods and supplements during intervention. In the John et al. research, the consumption of isoflavone supplements during the preceding month of screening was ineligible (25). The investigations that included subjects with some memory impairment and controlled the background diet showed positive findings (22,23,29,34). Disregarding the background diet control, those investigations performed in Mediterranean countries (specifically in France, Italy, and Spain) and Japan had positive results (18–20,29,31,32). This is consistent with previous systematic reviews and meta-analyses (99–101), where the Mediterranean diet was the most investigated dietary pattern and with evidence supporting protection against CD among older adults. A positive effect of greater adherence to the Mediterranean diet on neurodegenerative diseases has been found, particularly on AD and dementia (99). A 2-point increase in adherence to the Mediterranean diet, characterized by a high consumption of plant-based food and poly- and mono-unsaturated fatty acids and a low consumption of processed foods, was associated with a significant 13% reduction in the incidence of neurodegenerative diseases (100). Characterized by a high intake of plant foods (soybean products, seaweeds, vegetables, and fruits) and fish and decreased intake of refined

carbohydrates and animal fats, the traditional Japanese diet could also evidence protective effects against CI (102,103).

However, the abovementioned inconclusive outcomes could be partly explained by the sizeable inter-individual variability in the host metabolism, absorption, and excretion of polyphenols (16). For instance, previous research has suggested that genetic polymorphisms of the drug transporters MRP2 and OATP1B1 may contribute to the high variability in catechins' pharmacokinetic parameters. This can, in turn, influence the real exposure to polyphenols and therefore partly explain the interindividual variation in beneficial effects of polyphenols (104). Miller et al. evidenced differences in small vessel tone according to COMT genotype after acute green tea extract (105), while in a population-based case-control study only those tea drinkers with the low-activity COMT enzyme showed a decreased risk of developing breast cancer compared to those who did not drink tea (106). In addition, Xing et al. reported that there was a synergistic effect between this polymorphism and ApoE-ε4 status on the susceptibility to AD (107). Nevertheless, the effect of polyphenol genetic polymorphism on CD has not been investigated to date, but there have been studies on the neurological effects resulting from genetic variants related to caffeine metabolism (108). In a recent clinical trial, caffeine enhanced CYP1A2 fast metabolizers' cognitive performance during exercise more than slow metabolizers (109), although evidence is still inconclusive and limited (108,110). On the other hand, the susceptibility to AD might be modulated by NAT2, an enzyme responsible for caffeine metabolism and elimination. Some research seems to link the expression of the NAT2 rapid acetylating phenotypes to potential pathogenesis of AD (111).

In this chapter, among the 18 studies we examined, only one publication considered an inter-individual variability factor in response to polyphenols, particularly the equol production capacity (31). This limitation complicates the understanding that genetic factors may play in individual response to polyphenol-rich food intake. Nonetheless, Rabassa et al. (2015, 2020) (18,19), Valls-Pedret et al. (20), Ochiai et al. (32) and Rutledge et al. (33) used plasma and urine polyphenols as a dependent variable, which accounts for the real bioavailability and may therefore be a more precise measure of true exposure as it considers polyphenol absorption and metabolism variability, besides the fact that it is an objective biomarker of intake that is not susceptible to the likelihood of bias or imprecise reporting.

14.3.1. Strengths and limitations

As far as we know, this chapter represents the first comprehensive examination of the effect of total polyphenol and polyphenol classes detected in plasma or urine on memory impairment. By encompassing both interventional and observational studies, we aimed to provide a thorough understanding of the existing evidence. Notably, 13 out of 18 articles included in this chapter were RCTs (21–29,32–35), which are considered the gold standard for providing internally valid and compelling evidence. Moreover, the investigation into the relationship between biomarkers of dietary polyphenols and CD includes both cognitively healthy subjects (18–20,25–28,31,33) and those experiencing memory difficulties (21–24,29,30,32,34,35).

This chapter has also identified the limitations of the current literature regarding biomarkers of polyphenol intake and their association with CD. Firstly, the conclusions are based on a limited number of studies ($n = 18$). Furthermore, only six studies (20–22,27,34,35) evaluated cognitive impairment through biomarkers of AD, which are generally considered more objective than cognitive tests (37). Thirdly, due to lack of consensus regarding the suitability of cognitive tests, more than six different tests were used to evaluate the same cognitive domain, resulting in a total of 60 different cognitive tests or tasks. This diversity in exposure and outcome definition and measurements has compromised the comparability of studies, making it challenging to interpret and generalize the results. Finally, the article search was conducted in three databases and non-English articles were excluded. Thus, not all the evidence may be represented, potentially introducing language bias (112).

14.3.2. Polyphenol intake recommendations

Given the overall scarcity of available evidence, it is not possible to make strong recommendations regarding polyphenol consumption in relation to AD and memory impairment until more robust and higher-quality evidence becomes available. Future research should focus on accurate measurement of all polyphenol-derived metabolites in blood and urine to gain a better understanding of the specific components responsible for polyphenols' potential cognitive protective effects. Additionally, a shift toward more physiologically and real-life-oriented experimental designs, such as realistic nutritional trials involving sustained but controlled polyphenol consumption, could provide a more reliable approach to exploring the intricate relationship between polyphenols and cognitive function (113). This, in turn, will enable healthcare professionals and public health authorities to make informed and prudent recommendations.

14.4. Conclusions

In this chapter, current evidence regarding the association between biomarkers of polyphenol intake and memory impairment in old age has been evaluated, in any of its stages and varieties. The relationship between polyphenol intake and CD is complex and likely influenced by factors such as the type of polyphenol, the background diet, and the subject's metabotype, among others. Studies investigating CD have yielded contradictory findings. As mentioned earlier, standardization of tools used to assess cognitive function is essential for evaluating the effectiveness of dietary factors in preventing CI. Moreover, it remains unclear whether the absence of demonstrable benefits is attributed to the bioavailability of specific formulation of certain polyphenol classes or to the inefficacy of these polyphenol classes as interventions for AD or memory dysfunction. Further research is needed in the field of metabolomics as there is a notable gap in the current knowledge.

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2. Hypothesis and Objectives

2.1. Hypothesis

This thesis presents the following hypothesis:

High adherence to a dietary pattern rich in phytochemicals and unsaturated fatty acids, such as the Mediterranean diet, may contribute to determining the physical and cognitive health status of older adults.

2.2. Objectives

2.2.1. Main objective

The main objective of this thesis is to study the effect of the Mediterranean diet and its components, evaluated through dietary assessment instruments and/or metabolomic tools, on physical and cognitive function in adults aged 65 years or over.

2.2.2. Specific objectives

In order to accomplish the main objective, the following specific objectives have been proposed:

Objective #1: To comprehend the discovery of bioactive compounds and biomarkers of food intake using metabolomics and their tools under the framework of legumes, a key food group of the Mediterranean diet.

- i. To describe and compare the qualitative profile of phytochemicals contained in legumes and identified through liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry methods.
- ii. To assess the metabolic fingerprint of urine and serum associated with the intake of the most widely consumed types of pulses in order to further identify their reliable biomarkers of food intake using a liquid chromatography-high-resolution-mass spectrometry untargeted metabolomics approach.

Objective #2: To investigate the relationship between Mediterranean diet adherence and response to an exercise and health education program to prevent hospitalization-associated disability in acutely hospitalized older subjects.

Objective #3: To investigate the association between fish intake, as a key food group of the Mediterranean diet, and cognitive decline in a large sample of community-dwelling older population.

Objective #4: To develop a Mediterranean diet metabolomic score and to investigate its association with cognitive decline in a large sample of community-dwelling older adults.

3. Materials and Methods

3.1. Study design

The objectives of this thesis have been developed within four studies described below: a postprandial intervention study, an intrahospital randomized clinical trial with an exercise and health education intervention, a prospective cohort study of community-dwelling older adults, and a nested case-control study of free-living older persons.

3.1.1. The Food Biomarker Alliance (FoodBALL) Project: a postprandial intervention study

The “Food Biomarker Alliance” (FoodBALL) Project, funded by the Joint Project Initiative ‘A Healthy Diet for a Healthy Life’ (JPI-HDHL), aimed at developing strategies for the exploration and validation of biomarker of major foods that are commonly consumed in Europe for a better dietary assessment (Brouwer-Brolsma et al., 2017).

In the FoodBALL Project, the identification and validation of BFIs were carried out through several research activities, including (i) the discovery of novel BFIs via extensive systematic literature reviews and the application of metabolomics in both acute intervention studies and observational studies; (ii) the development of a new validation scoring system for BFIs and its application to the newly discovered biomarkers; and (iii) the exploration of biological and health effects of BFIs (Brouwer-Brolsma et al., 2017). Additionally, to aid the harmonization of methodologies, new and advanced existing platforms for sharing knowledge and resources with the scientific community (e.g., FooDB, PhytoHub, Exposome-Explorer, and FoodComEx) were developed.

Given the limited knowledge about the composition of bioactive compounds of pulses and the lack of biomarkers of legume intake, this thesis particularly encompasses (i) a bibliographic description and comparison of the qualitative profile of phytochemicals contained in legumes and identified through LC-MS and GC-MS methods, and (ii) a standardized postprandial intervention study designed to discover new potential BFIs for legumes.

3.1.1.1. Search strategy design for the qualitative description and comparison of the profile of phytochemicals contained in legumes and identified through LC-MS and GC-MS

In order to collect all available information on phytochemicals in legumes, an extensive literature search was conducted in PubMed, Science Direct, Scopus and Google Scholar databases using combinations of the following search terms: legumes [All Fields], pulses [MeSH term or All Fields], fabaceae [MeSH term or All Fields], determination [All Fields], identification [All Fields], bioactive compounds [All Fields], phytochemicals [MeSH term or All Fields], HPLC-MS [MeSH term or All Fields], LC-MS [MeSH term or All Fields] and GC-MS [MeSH term or All Fields]. Additionally, other specific phytochemicals and seeds names had been used too (e.g., flavones, epicatechin, adzuki bean or lentils).

Firstly, titles and abstracts from 1975 until March 2017 were screened to remove duplicates references and determine whether they met the selection criteria. Proceeding articles, editorials, guidelines, conference and meeting abstracts, protocols, comments, letters, handbooks, reviews, and book chapters were dismissed. Publications with irrelevant focus or topic and in non-English language were further ruled out. Those papers analyzing legumes and identifying bioactive compounds were selected. A list of different varieties of legumes and their phytochemicals was obtained after revising the literature. Finally, both varieties of legumes and phytochemicals were grouped into major families.

3.1.1.2. Study design of a standardized postprandial intervention designed to discover new potential biomarkers of food intake for legumes

Healthy subjects aged 18–40 with a body mass index (BMI) of 18.5–30 kg/m² were selected for the study. The exclusion criteria were being a smoker, having a chronic or infectious disease, having anemia, use of medication (except oral contraceptive pills, which were allowed), intake of nutritional supplements, pregnancy, lactation, antibiotic treatment within the six months prior to the study, allergy or aversion to legumes or pasta, and blood donation during the previous two months.

Volunteers were screened via a telephone interview and then invited to a first visit, during which inclusion/exclusion criteria, health status, anthropometrics and dietary habits were evaluated. A total of 11 healthy subjects (4 males, 7 females) successfully underwent three postprandial treatment sessions consisting of consumption of lentils, chickpeas, and pasta (control), separated by at least one week of washout. In each session, participants were asked to consume

one of the study foods in a randomized order. Eight of them also participated in a fourth session, which comprised the consumption of white beans.

In the week leading up to each intervention, participants were requested to abstain from consuming any legumes or legume-based products. Furthermore, from two days before each intervention, volunteers were asked to follow a polyphenol-free diet, as well as to avoid consuming alcoholic beverages. The participants were provided with standardized meals from the evening before the intervention day until the last biological sample was collected. Throughout the study, volunteers were given dietary guidance to ensure compliance with the dietary restrictions. On the test day, participants ingested a single 300 g dose of the designated cooked food, warmed together with 7 g of butter, within a maximum time frame of 20 minutes. For the following 6 h, they remained at rest in the study center, during which time they consumed the standardized lunch.

Baseline urine and blood samples were collected before the ingestion of the test food. Then, urine samples were collected during the following time intervals after the food test consumption: 0–1 h, 1–2 h, 2–4 h, 4–6 h, 6–12 h, 12–24 h and 24–48 h.

Information about the study design, sampling process, and data collection has been detailed in a previous publication (Madrid-Gambin et al., 2018). The study was registered at the ISRCTN registry with the code ISRCTN17200423. The clinical intervention was approved by the Bioethical Committee of the University of Barcelona (ref: IRB00003099) and followed Helsinki Declaration guidelines. All participants provided written informed consent before starting their participation.

3.1.2. The Activity in GEriatric acute CARE Plus Health Education (AGECAR-PLUS)

Study: a physical exercise intervention

The Activity in GEriatric acute CARE Plus (AGECAR-PLUS) Health Education is a randomized clinical trial to assess the effectiveness of an intrahospital exercise and health education program in maintaining or improving the functional capacity of patients aged 75 years or older during short hospital stays and for three months after discharge.

Patients admitted to the acute care for older patient unit of the Hospital General Universitario Gregorio Marañón (Madrid, Spain) were considered eligible to participate in the study. The exclusion criteria were: non-ambulatory or dependent in all basic ADLs at baseline (i.e., two

weeks before admission, as assessed by retrospective interview); having an unstable CVD (or any other major medical condition contraindicating exercise), terminal illness, or severe dementia; expected length of hospitalization < three days; having a scheduled admission (which was usually associated with a length of hospitalization < three days) (Ortiz-Alonso et al., 2020); and being transferred from another hospital unit.

Eligible participants were randomized to a control group or intervention group. Participants allocated in the control group received standard hospital care. Participants allocated in the intervention group performed, during hospitalization, a multicomponent training program (30 minutes per session, twice a day on weekdays, lower limb strength training, balance training, walking, and inspiratory muscle training) and health education. Health education consisted of several informational activities. Each activity session taught the patient and caregiver how to perform the exercises to ensure continuity after discharge.

Subjects were included in the control or intervention group in a time-dependent manner (i.e., using 4-week blocks) to avoid the co-presence of patients from both groups in the unit, so that participants were blinded to actual group assignment.

Sociodemographic characteristics, medical information, anthropometric measurements, fasting blood samples and spot urine samples were collected at admission and at discharge. At admission, the validated 14-item Mediterranean Diet Adherence Screener (MEDAS) questionnaire was used to briefly assess adherence to a traditional MedDiet (Schröder et al., 2011).

For the work included in this thesis, a secondary analysis was conducted on 109 older participants with available MEDAS or urine samples upon admission (63 in the intervention group and 46 in the control group).

The Institutional Review Board approved the protocol (Hospital Universitario Gregorio Marañón, Madrid, Spain), and all participants provided written informed consent. When it was not possible to obtain the informed consent directly from a patient for medical reasons (e.g., impaired cognitive function), proxy consent was obtained from their relatives. All procedures were performed in accordance with the ethical standards from the Declaration of Helsinki and its later amendments.

3.1.3. The Invecchiare in Chianti (InCHIANTI) Study: a prospective cohort study

The Invecchiare in Chianti (InCHIANTI) Study is a prospective, population-based cohort study conducted in two towns (Greve in Chianti and Bagno a Ripoli) in the Chianti geographic area (Tuscany, Italy). Its aim is to evaluate the risk factors for mobility loss and age-related diseases in an older community-dwelling population (Ferrucci et al., 2000). Recruitment began in 1998 and was completed in 2000. To date, five follow-up assessments have been conducted every 3 years up to year 9 (2001–2003, 2004–2006, 2007–2009) and then two last assessments by year 15 (2013–2014) and 19 (2017–2018). A detailed description of the study design, sampling procedure, and data collection method has been previously published elsewhere (Ferrucci et al., 2000) and is available on-line at <https://www.nia.nih.gov/inchianti-study>.

Briefly, 1,260 persons aged 65 years or more were randomly selected from the population registry of the two cities, of whom 1,155 agreed to participate at baseline (age-range 65–102 years). Sociodemographic data, anthropometric measurements, lifestyle, and medical information were assessed using standardized questionnaires and hospital records. Participants also underwent 24-hour urine collection and fasting blood drawn at baseline, as well as medical imaging.

Subjects with missing data in dietary questionnaires ($n = 16$), those who died within the first 3 years of follow-up ($n = 123$), individuals with dementia at baseline ($n = 42$), and participants without two consecutive MMSE evaluations during the follow-up ($n = 91$) were excluded for the work included in this thesis. In total, 883 participants and 2,038 observations were included for the analyses.

All participants signed an informed consent form. The study protocol was approved by the Italian National Institute of Research and Care of Aging Institutional Review and Medstar Research Institute (Baltimore, MD, USA).

This thesis has been based on data collected at baseline and after the 3-year (2001–2003), 6-year (2004–2006), 9-year (2007–2009), and 15-year (2013–2014) follow-up periods.

3.1.4. *The Dietary modulators of Cognitive ageing and brain Plasticity (D-CogPlast)*

Project: a nested case-control study

The Dietary modulators of Cognitive ageing and brain Plasticity (D-CogPlast) Project is an observational study which involves nested case-control samples built among participants from the Three-City Study (3C Study), a population-based cohort on dementia that includes older persons (≥ 65 years) from three French cities (Bordeaux, Dijon, and Montpellier) (Alpérovitch et al., 2003). Sociodemographic and lifestyle characteristics, medical information, neuropsychological testing, blood pressure, anthropometric measurements and fasting blood samples were collected at baseline, and follow-up visits were then scheduled every 2-3 years for repeated health and neuropsychological assessment (Alpérovitch et al., 2003).

In the nested case-control study included in this thesis, two sample sets were built among participants from the centers of Bordeaux and Dijon of the 3C Study, with Bordeaux as the discovery set and Dijon as the set used for external validation (González-Domínguez et al., 2021; Low et al., 2019). From the entire Bordeaux and Dijon cohorts, eligible participants were selected if they were not diagnosed with dementia at baseline, had available serum samples, and had at least one repeated cognitive evaluation over the subsequent 12 years.

To build the case-control samples on CD, a composite score of global cognition at each follow-up visit was defined as the average Z-scores of five neuropsychological tests (MMSE, Benton Visual Retention Test, Isaac's Set Test, TMT part A, and TMT part B) (González-Domínguez et al., 2021; Low et al., 2019). Individual slopes of cognitive change were then estimated using linear mixed models, as previously detailed (Low et al., 2019). Cases were defined as the participants with the worst CD slopes. Then, each case was matched to a control (i.e., participant with a slope of cognitive change better than the population median) based on age at baseline, sex, and education level. Overall, 209 cases were individually matched to a control in Bordeaux, leading to a discovery sample of 418 subjects. In Dijon, 211 cases were successfully matched to 211 controls, leading to a validation sample of 422 subjects.

The study was performed in accordance with the principles contained in the Declaration of Helsinki. The Consultative Committee for the Protection of Persons participating in Biomedical Research at Kremlin-Bicêtre University Hospital (Paris, France) approved the 3C Study protocol, and all participants provided written consent.

3.2. Metabolomics analysis of legumes and biological samples

3.2.1. Untargeted metabolomics analysis

3.2.1.1. Analysis of food composition

The discovery and comprehensive understanding of food composition hold profound significance to subsequently be able to investigate and select potential biomarkers of their consumption. For this reason, the characterization of bioactive compounds from lentils, chickpeas, and white beans was carried out as an important complement of the postprandial intervention designed to discover new potential BFIs for legumes and detailed earlier.

The test foods used in this study were ready-to-use legumes from Spanish protected geographical indications (lentils “*Tierra de Campos*” and chickpeas “*Fuente saúco*”) or a protected designation of origin (white beans “*Mongetes del Ganxet*”). A total of 430 g of lentils, 441 g of chickpeas, and 564 g of white beans were ground and homogenized after washing them separately five times using Milli-Q water. The resulting paste was weighed, saved into amber containers, and stored for 24 h at -80 °C before the freeze-drying process. Subsequently, samples were placed in the freeze-dryer equipment (Telstar Cryodos, Spain) until dry. Then, each sample was placed in polyethylene bags and stored at -80 °C. A total of 20 mg of each sample were extracted with methanol/tert-butyl methyl ether and then with methanol/water. Aliquots of the eluates were then evaporated in a SpeedVac and derivatized by methoximation and trimethylsilylation. Untargeted analyses were performed using an LC-MS as described by Llorach et al. (Llorach et al., 2019) and by a comprehensive 2D gas chromatography-mass spectrometry (GC×GC-MS) system detailed elsewhere (Weinert et al., 2015).

Data processing was carried out utilizing the SquareDance workflow specifically designed for GC×GC-qMS datasets (Egert et al., 2015). Automated compound annotation was performed using an in-house spectral library with retention indices based on fatty acid methyl esters. Finally, the annotation was manually verified by matching against the National Institute of Standards and Technology v14 library, the Fiehn library, and the Golm Metabolome Database.

3.2.1.2. Analysis of biological samples

In the postprandial intervention study within the FoodBALL Project, untargeted metabolomics analyses were also carried out in accordance with previously validated methodologies (Garcia-

Aloy et al., 2020; Trošt et al., 2018) to analyze urine and serum samples and identify reliable BFIs of pulses.

For urine samples, 100 µL of urine were first diluted with 100 µL of internal standards (ISs) dissolved in methanol and filtered. The eluate was then mixed with 300 µL of external standard (ES) in Milli-Q water and transferred to amber vials to be analyzed (Garcia-Aloy et al., 2020). For serum samples, 50 µL of serum and 50 µL of ISs were placed on Ostro 96-well plates adding 150 µL of acetonitrile with 1% formic acid. After filtration, the elution step was repeated, and eluates were redissolved with 50 µL of ES and transferred to amber vials for analysis.

All samples were randomly distributed and analyzed using a Dionex UltiMate 3000 HPLC system coupled to a hybrid linear ion trap Fourier transform Orbitrap mass spectrometer (Thermo Fisher, Germany). The XCMS R package and Xcalibur 2.2 software (Thermo Fisher Scientific, France) were used for data processing.

Metabolite identification was performed through a multistep procedure using the features selected as discriminants. The levels of identification were reported in accordance with the guidelines provided by the Metabolomics Society (Sumner et al., 2007).

3.2.2. Targeted metabolomics analysis

In the D-CogPlast Project, targeted metabolomics analysis of serum samples was conducted using a multi-metabolite platform for the simultaneous detection and quantification of food-related metabolites, microbiota derivatives, and endogenous metabolites, following the validated methodology developed by González-Domínguez et al. (González-Domínguez, Jáuregui, et al., 2020).

3.2.2.1. Preparation of plasma samples

Protein precipitation was carried out by mixing 100 µL of each serum sample with 250 µL of methanol containing 0.1% formic acid (Low et al., 2019). The samples were then vortexed for 30 seconds and centrifuged at 10,000 × g for 10 min at 4 °C. After collecting the supernatant (250 µL), it was reconstituted with 250 µL acetonitrile and transferred to 96-well injection plates. Finally, a mixture of ISs was added to the samples for quantification purposes and quality control assessment (González-Domínguez, Jáuregui, et al., 2020).

3.2.2.2. LC-MS QTRAP analysis

The samples were randomly distributed for metabolomics analysis utilizing an Infinity UHPLC system (Agilent, USA) coupled to a QTRAP 6500 mass spectrometer equipped with an Ion Drive Turbo V ion source (Sciex, USA). Luna Omega Polar C18 column, 100 mm x 2.1 mm (i.d. 1.6 μ m) with a porous polar C18 security guard cartridge (Phenomenex, USA) was used to perform the chromatographic separation (González-Domínguez, Jáuregui, et al., 2020).

Mass spectrometry (MS) detection was performed under two detection ion modes (i.e., positive and negative ionization) in separate runs, in scheduled multiple reaction monitoring mode. Mobile phases employed under positive ion mode detection consisted of (a) water and (b) acetonitrile, both containing 0.5% formic acid, where acetonitrile was delivered as follows: 0–5 min, 5%–50%; 5–8 min, 50%–100%; 8–10 min, 100%; 10–10.1 min, 100%–5%; 10.1–12 min, 5%. The negative ion mode consisted of (a) water containing 0.1% formic acid and 10 mM ammonium formate and (b) acetonitrile, by applying the following gradient program with the acetonitrile: 0–8 min, 5%–20%; 8–10 min, 20%–100%; 10–12 min, 100%; 12–12.1 min, 100%–5%; 12.1–14 min, 5% (González-Domínguez, Jáuregui, et al., 2020). Quality controls, composed by a pool of samples of the study, were processed following the same procedures every 20 samples. Analyst 1.6.2 and Sciex OSQ software (ABSciex, USA) were used for data acquisition and data processing, respectively (González-Domínguez, Jáuregui, et al., 2020).

Figure 5. The UHPLC-MS equipment used in the D-CogPlast Project analyses.



Source: own preparation.

3.3. Dietary assessment

The evaluation of food, energy and nutrient intake, and dietary patterns provides comprehensive information on the nutritional status of individuals and populations. Accurate assessment of dietary intake enables the understanding of diet effects on human health and, consequently, the formulation of nutrition policies and dietary recommendations for individuals, population groups, and communities (Food and Agriculture Organization of the United Nations, 2018).

Various instruments have been developed to capture dietary intake, which fundamentally differ in the data collection method and the reference time frame. Each instrument has distinct features as well as inherent strengths and drawbacks, hence the selection criteria is ultimately dependent upon the scope of interest (e.g., the total diet or a limited number of dietary components), the study design, and research question. Lastly, reported dietary intakes are translated to energy and nutrients amounts through food composition databases (Yago Torregrosa et al., 2017).

In both InCHIANTI and D-CogPlast studies, dietary information was obtained through dietary surveys. Dietary surveys are direct and retrospective intake assessment methods (i.e., information provided directly by the respondents) where the unit of consumption is the individual. Specifically, the methods used were the 24-hour dietary recall (24HR) and the food frequency questionnaire (FFQ).

3.3.1. 24-hour dietary recall

The 24HR is a structured interview intended to capture detailed and comprehensive information about all foods, beverages, and dietary supplements consumed by the interviewee over the preceding 24 hours (i.e., from midnight to midnight of the previous day) (Food and Agriculture Organization of the United Nations, 2018). Detailed data about food preparation and cooking methods, ingredients, brand name of commercial products, time of the eating occasion, and portion size of each food and beverage are also captured. Twenty-four hours dietary recalls have traditionally been administered by a trained interviewer, although self-administered tools are also available (Bailey, 2021; National Institutes of Health. National Cancer Institute, n.d.).

3.3.2. *Food frequency questionnaire*

An FFQ consists of a close-ended list of foods or food groups, beverages, and dietary supplements with a frequency category section to assess the habitual consumption over a specified period of time (typically the past month or year) (Food and Agriculture Organization of the United Nations, 2018). Usual portion sizes can be asked separately for each food and beverage or combined with frequency information. FFQs are usually self-administered but interviewer-administration can be done when literacy challenges (Bailey, 2021; National Institutes of Health. National Cancer Institute, n.d.).

In the D-CogPlast Project, a 24HR and an FFQ were administered at home by a trained dietician (Féart et al., 2007; Samieri et al., 2008) at baseline. Data from these questionnaires were validated in an independent 3C subsample. The 24HR was used to estimate nutrient intake and total energy intake, while the frequency of consumption of 148 food and beverage items was recorded through the FFQ. The food items were converted into number of servings per week and then clustered into 20 food and beverage groups (Samieri et al., 2008). Concordance between responses to the FFQ and the 24HR was assessed for fatty acids, with acceptable correlations between number of weekly servings of foods obtained from the FFQ and the corresponding fatty acid intakes obtained from the dietary recall (Samieri et al., 2008).

In the InCHIANTI Study, a semi-quantitative FFQ developed and validated in the European Prospective Investigation into Cancer and Nutrition-Italy (EPIC-Italy) study (Pisani et al., 1997) was administered in interviews by trained dieticians at baseline and 3, 6 and 9 years after enrollment. Participants reported the frequency of consumption and usual portion size of 198 food and beverage items. Pictures to estimate portion size were included to minimize errors derived from personal interpretation of serving sizes. Daily consumption (grams per day) of food items was further calculated (Pisani et al., 1997). Finally, energy and nutrient data were estimated using the Food Composition Database for Epidemiological Studies in Italy (Salvini et al., 1998), a specific software created for the EPIC study (Bartali et al., 2006).

3.4. Evaluation of adherence to the Mediterranean diet

3.4.1. Quantification of urinary total polyphenols by the Folin-Ciocalteu method

In the AGECAR-PLUS clinical trial, the urinary total polyphenol (UTP) content was determined as it is a well-known objective biomarker of total dietary polyphenol exposure and a proxy measure of dietary fruit and vegetable intake (Urpi-Sarda et al., 2015). In fact, UTP has previously been positively correlated with adherence to the MedDiet (Ruiz et al., 2019). On the other hand, UTP depends on the bioavailability of polyphenols, which tends to decrease during ageing and may consequently impact their biological activity (Hidalgo-Liberona et al., 2020).

Total phenolic content in urine was analyzed using rapid Folin-Ciocalteu (F-C) assay after solid phase extraction (SPE). The F-C assay is based on an oxidation-reduction reaction, where the FC-reagent is reduced by the phenolic compounds at basic pH, giving rise to a blue coloration whose intensity is liable to be spectrophotometrically measured at 750 nm. On the other hand, SPE is considered the gold standard technique for the cleanup of complex biological samples as it reduces protein interaction, and it is useful for polyphenols extraction as well as for the targeted analysis of metabolites (Ajila et al., 2011).

Spot urine samples were first aliquoted, processed, and stored at -80 °C until analysis. SPE was then performed using a method described earlier (Urpi-Sarda et al., 2015). To carry out the F-C assay, 170 µL of Milli-Q water, 15 µL of urine extract, 12 µL of the F-C reagent, and 30 µL of 20% sodium carbonate were first mixed in a thermo microtiter 96-well plate. After 1 h of incubation at room temperature in the dark, 73 µL of Milli-Q water were added, and finally absorbance was measured at 765 nm using a UV/VIS Thermo Multiskan Spectrum spectrophotometer (Zamora-Ros et al., 2011). The mean absorbance and % relative standard deviation of the three replicates of each sample were calculated. A 100 mg/L gallic acid solution was used as a standard and the calibration curve was made with 6 concentrations at 0 (blank), 1, 2, 4, 6 and 8 mg/L to check the linearity. The absorbances were then plotted against the standard concentrations, and the results were multiplied by the urine output and expressed as mg of gallic acid equivalents (GAEs)/L. Finally, the total polyphenol results were normalized with reference to the urinary refractive index (OPTi Digital Handheld Refractometer, Bellingham + Stanley, UK) to account for interindividual differences in the hydration status and micturition frequency (Hidalgo-Liberona et al., 2020).

3.4.2. *Indexes of adherence to the Mediterranean diet*

The evaluation of adherence to the MedDiet is an essential element in nutritional studies that seek to establish associations between this dietary pattern and its beneficial effects on health. One of the most common tools used to measure adherence to a dietary pattern is through questionnaires that are presented as *a priori* diet quality indexes or scores (Zaragoza-Martí et al., 2018). These scores are composite constructs based on the current state of the art or scientific consensus.

In the case of the MedDiet, numerous studies have proposed various indexes adapted to different geographical areas, age groups, and populations with different underlying physiological states (Hernández Ruiz et al., 2015; Hutchins-Wiese et al., 2022). The differences between these indexes mostly rely on the scoring systems (e.g., 0–1, 0–2, 0–4, or 0–5), the cut points used (e.g., sex-specific medians, tertiles, quintiles, or fixed portions per day or week), and the components included (food, food groups, nutrients, ratios, and/or lifestyle factors) (Hernández Ruiz et al., 2015; Hutchins-Wiese et al., 2022). The validity, reliability, and content of MedDiet scoring systems for adherence have already been reviewed (Zaragoza-Martí et al., 2018).

In this thesis, two different scores have been used to assess adherence to the MedDiet. In the AGECAR-PLUS trial, the degree of agreement with MedDiet was measured through the MEDAS, while in both InCHIANTI and D-CogPlast studies, this evaluation was done through the Mediterranean diet score (MDS), developed by Trichopoulou et al. (Trichopoulou et al., 2003).

3.4.2.1. *The Mediterranean diet score (MDS)*

The first and most widely used score of adherence to the MedDiet was created by Trichopoulou et al in 1995 to evaluate the association between adherence to this dietary pattern and overall mortality risk in an older population (Trichopoulou et al., 1995). This index consisted of eight components: seven food groups [moderate ethanol intake, high consumption of legumes, cereals (including bread and potatoes), fruits and nuts, and vegetables; low consumption of meat and meat products, milk and dairy products] and one ratio to account for the quality of fat (high MUFA:SFA ratio). This score was later modified by the same authors including high consumption of fish and excluding potatoes intake, and a higher adherence to this index was inversely associated with overall and cause-specific (i.e., coronary heart disease and cancer) mortality risk in adults (Trichopoulou et al., 2003).

The scoring system consists in assigning one point when the intake (grams per day) of beneficial components (i.e., legumes, cereals, fruits and nuts, vegetables, fish, and MUFA:SFA ratio) is at or above the sex-specific-sample population median, or when the consumption of foods presumed to be detrimental (i.e., meat and meat products, milk and dairy products) is below the median, and zero in the opposite situations. For ethanol, a value of 1 is assigned to men who consumed between 10 and 50 g/day and to women who consumed between 5 and 25 g/day. Therefore, the MDS usually ranged from 0 (minimal adherence) to 9 (maximum adherence) if the index had nine components (Trichopoulou et al., 2003).

Since then, various scores have been published to assess adherence to the MedDiet. Some of them significantly differ from its definition, while others are based on minor modifications of the initially proposed scores. This is the case of the index suggested by Féart et al. in the 3C Study, which identified the food groups considered to be part of a MedDiet as legumes, cereals (including bread, pasta, and rice - whole and refined grains), vegetables, fruits, fish, meat, dairy products, and alcohol (Féart et al., 2009). For the last item, cutoffs were established at 10-20 g/day for men and 1.4-5.7 g/day for women, according to the second quartile of the distribution and representing mild-to-moderate consumption (Féart et al., 2009).

3.4.2.2. The Mediterranean Diet Adherence Screener (MEDAS)

Another index proposed to quantify adherence to the MedDiet is the 14-point MEDAS, a brief tool developed to promptly monitor for compliance with the dietary intervention of the Prevención con Dieta Mediterránea (PREDIMED) study (Schröder et al., 2011), a multicenter clinical trial aimed at assessing the effects of the traditional MedDiet on the primary prevention of CVD (Estruch et al., 2006, 2018). This questionnaire reports the habitual frequency of consumption or amount consumed of 12 main components of the MedDiet and 2 food intake habits considered characteristic of the Spanish MedDiet. Each of the 14 items is scored 1 or 0, depending on whether patients meet each MedDiet condition. One point is assigned for using olive oil as the principal source of fat for cooking; for preferring white meat over red meat; for consuming ≥ 4 tablespoons of olive oil/day (including that used in frying, salads, meals eaten away from home, etc.); ≥ 2 servings of vegetables/day; ≥ 3 servings of fruit/day (including freshly squeezed juice); < 1 serving of red meat, hamburger, or sausages/day; < 1 serving of animal fat/day (i.e., butter, margarine, or cream); < 1 cup of sugar-sweetened and/or carbonated beverage/day; ≥ 7 cups of red wine/week; ≥ 3 servings of pulses/week; ≥ 3 servings of fish or seafood/week; < 2 servings of commercial pastry/week; ≥ 3 servings of nuts/week; and ≥ 2

servings/week of a dish with *sofrito* (*sofregit*, in Catalan), a traditional sauce of tomatoes, garlic, onion, or leeks sautéed in olive oil (Schröder et al., 2011). Therefore, the sum score for this index ranges from 0 to 14 points, and levels of adherence to the MedDiet have been proposed as low (< 9) and high (≥ 9) (E. A. Hu et al., 2013).

Recently, an updated version of this questionnaire has been validated, with the inclusion of 14 items on food consumption and 3 items on eating behaviors (Schröder et al., 2021). These modifications aimed to capture an energy-restricted MedDiet in order to determine its effect on CVD (Martínez-González et al., 2019).

3.4.3. The Mediterranean diet metabolomic score (MDMS)

In the D-CogPlast study, and in line with the MDS defined by Trichopoulou et al. (Trichopoulou et al., 2003), a MedDiet metabolomic score (MDMS) was developed de novo using a 14-point linear scale that incorporated two putative dietary metabolomic biomarkers of seven key components of the MedDiet (vegetables, legumes, fruits, cereals, dairy, fats, and fish) (Table 1) (Féart et al., 2009; Trichopoulou et al., 2003).

Table 1. Metabolites included in the MDMS as biomarkers of key MedDiet food groups.

MDMS metabolite	metabolite class	food	references
EL	enterolignans (microbial metabolites of lignans)	fiber-rich fruits and vegetables, grains, nuts, and legumes	(González-Domínguez, Urpi-Sarda, et al., 2020; Landete, 2012)
HA	hydroxybenzoic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, coffee, cocoa, grains, nuts, legumes, olive oil, and wine	(Angelino et al., 2017; Bolling et al., 2011; Boskou et al., 2005; Crozier et al., 2010; Del Rio et al., 2010; González-Domínguez, Urpi-Sarda, et al., 2020; Michielsen et al., 2018; Rothwell et al., 2018; Singh et al., 2017)

Table 1. (cont'd)

MDMS metabolite	metabolite class	food	references
proline betaine	proline derivatives	citrus fruits (mainly), Chinese artichoke, and rye and wheat bran	(González-Domínguez, Urpi-Sarda, et al., 2020; Kärkkäinen et al., 2018; Lang et al., 2017; Pekkinen et al., 2015)
trigonelline	pyridinium derivatives	coffee, legumes, and rye and wheat bran	(Ashihara et al., 2015; González-Domínguez, Urpi-Sarda, et al., 2020; Kärkkäinen et al., 2018; Pekkinen et al., 2015)
UroA-G	urolithins (microbial metabolites of ellagitannins)	berries, wine, pomegranate, and nuts	(García-Villalba et al., 2016; González-Domínguez, Urpi-Sarda, et al., 2020)
3,4-DHBA	hydroxybenzoic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, coffee, cocoa, grains, nuts, legumes, olive oil, and wine	(Angelino et al., 2017; Bolling et al., 2011; Boskou et al., 2005; Crozier et al., 2010; Del Rio et al., 2010; González-Domínguez, Urpi-Sarda, et al., 2020; Michielsen et al., 2018; Rothwell et al., 2018; Singh et al., 2017)
3,4-DHPAA-S	hydroxyphenylacetic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, cocoa, olives and olive oil, and wine	(Boskou et al., 2005; Crozier et al., 2010; Del Rio et al., 2010; González-Domínguez, Urpi-Sarda, et al., 2020; Michielsen et al., 2018; Rothwell et al., 2018)

Table 1. (cont'd)

MDMS metabolite	metabolite class	food	references
3,4-DHPV-S	hydroxyphenylvalerolactones (microbial metabolites of flavan-3-ols)	procyanidin-rich foods (berries, apple, tea, cocoa, wine, nuts, and legumes)	(Bolling et al., 2011; Del Rio et al., 2010; González-Domínguez, Urpi-Sarda, et al., 2020; Michielsen et al., 2018; Rana & Bhushan, 2016; Rothwell et al., 2018; Singh et al., 2017)
DHA	n-3 PUFAs	total fish and shellfish	(Cuparencu et al., 2019)
EPA	n-3 PUFAs	total fish and shellfish	(Cuparencu et al., 2019)
margaric acid	LCFAs	dairy products	(Münger et al., 2018)
pentadecanoic acid	LCFAs	dairy products	(Münger et al., 2018; Smedman et al., 1999)
oleic acid	LCFAs	dietary MUFAs	(Garcia-Aloy et al., 2019; Palomer et al., 2018)
palmitic acid	LCFAs	dietary SFAs	(Palomer et al., 2018)

DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. HA, hippuric acid. LCFAs, long-chain fatty acids. MDMS, Mediterranean diet metabolomic score. MedDiet, Mediterranean diet. MUFAs, monounsaturated fatty acids. PUFAs, polyunsaturated fatty acids. SFAs, saturated fatty acids. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate.

The metabolites for the MDMS were selected based on three criteria: a) metabolites reported as putative biomarkers of key MedDiet food groups (Angelino et al., 2017; Ashihara et al., 2015; Crozier et al., 2010; Cuparencu et al., 2019; Garcia-Aloy et al., 2019, 2020; Landberg et al., 2019; Landete, 2012; Lang et al., 2017; Münger et al., 2018; Palomer et al., 2018; Smedman et al., 1999; Ulaszewska et al., 2018, 2020; Vázquez-Manjarrez et al., 2020), b) metabolites available in the serum metabolomic panel described previously (González-Domínguez et al., 2021; González-Domínguez, Jáuregui, et al., 2020; González-Domínguez, Urpi-Sarda, et al., 2020) and c) metabolites with described health-related properties (Table 2).

In Table 2, information about the health-related properties described in the plant-derived metabolites of the MDMS is detailed.

Table 2. Health-related properties described in the plant-derived metabolites of the MDMS.

MDMS metabolite	metabolism	health effects	references
EL	microbiome-generated metabolite	• longer time in circulation → long-term effect on health • antiproliferative and anti-inflammatory activities <i>in vitro</i> • exhibits blood-brain barrier permeability • protects murine microglia against inflammation • associated with favorable cardiometabolic risk factors	(Espín et al., 2017; Frankenfeld, 2017; Johnson et al., 2019; Kawabata et al., 2019)
HA	microbiome-generated metabolite	• longer time in circulation → long-term effect on health • myoprotective properties <i>in vitro</i> • contribution to a better glycemic control and β-cell function • reduce the risk of developing age-related cognitive disorders	(de Mello et al., 2017; Kawabata et al., 2019; Ticinesi et al., 2023)
proline betaine	betaine derivative	• negatively correlated with glucose and insulin concentrations, blood lipid profiles, BMI and hsCRP • inversely associated with blood pressure	(Kärkkäinen et al., 2018; Loo et al., 2018; Noerman et al., 2021)

Table 2. (cont'd)

MDMS metabolite	metabolism	health effects	references
trigonelline	betaine derivative	• neuroprotective and antidiabetic effects • anti-inflammatory and antioxidant proprieties • lower risk of type 2 diabetes • urine molecular signature in the metabolic syndrome	(Anwar et al., 2018; Bruzzone et al., 2021; Farid et al., 2020; Y. Liang et al., 2023; Surma et al., 2023; Wang et al., 2022)
UroA-G	microbiome-generated metabolite	• longer time in circulation → long-term effect on health • most common urolithin • variable production and found only in approximately 40% of the elderly population → restricted positive impacts on health conditions (e.g., mitochondrial, cardiometabolic and muscle health)	(Andreux et al., 2019; Cerdá et al., 2005; Cortés-Martín et al., 2018; D'Amico et al., 2021; González-Sarrías et al., 2017; Kawabata et al., 2019; Romo-Vaquero et al., 2019)
3,4-DHBA	• microbiome decarboxylation of caffeic acid or demethylation of vanillic acid • easily conversion from anthocyanins at physiological pH • abundantly formed and absorbed in the large intestine	• longer time in circulation → long-term effect on health • higher antioxidant capacity than monohydroxylated metabolites • 3,4- isomer position shows inhibitory effects, as well as antioxidant, anti-inflammatory, antihyperglycemic and neuroprotective activities • bioactive properties that promote mitochondrial health and cerebrovascular microcapillary function	(Juurlink et al., 2014; Kawabata et al., 2019; Koksai et al., 2016; Masella et al., 2012; Melrose, 2023; Monagas et al., 2009)

Table 2. (cont'd)

MDMS metabolite	metabolism	health effects	references
3,4-DHPAA-S	microbiome-generated metabolite	• longer time in circulation → long-term effect on health • higher antioxidant capacity than monohydroxylated metabolites • decrease in the formation of amyloid fibrils, protection from cytotoxicity and oxidative stress, regulation of inflammatory pathways <i>in vitro</i> • reduction in hsCRP in patients with type 2 diabetes	(Álvarez-Cilleros et al., 2018; Fongaro et al., 2022; Kawabata et al., 2019; Liu et al., 2017; Lofft et al., 2022; Monagas et al., 2009; Moreno Uclés et al., 2021)
3,4-DHPV-S	microbiome degradation product of (epi)catechin (pyrogallol C-ring opening)	• longer time in circulation → long-term effect on health • higher antioxidant capacity than monohydroxylated metabolites • preserve the antioxidant and anti-inflammatory activity of the native flavonoid • bioactive properties that promote mitochondrial health and cerebrovascular microcapillary function	(Kawabata et al., 2019; Melrose, 2023; Monagas et al., 2009)

BMI, body mass index. EL, enterolactone. HA, hippuric acid. hsCRP, high-sensitivity C-reactive protein. MDMS, Mediterranean diet metabolomic score. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate.

In this MDMS score, the meat item was excluded from the MDS as it is not possible to establish a set of metabolites that can reliably discriminate between meat intake, products of human muscle catabolism, and other protein-rich foods (e.g., fish and seafood), as well as between

different meat sources (e.g., red meat vs. poultry) (Cuparencu et al., 2019). An alcohol-related item was not either included since low levels of alcohol have shown slight reductions in some chronic diseases (i.e., ischemic heart disease and diabetes in very specific populations) but an increased risk of other health-related conditions, including cirrhosis, infectious diseases, early dementia, and cancer (Griswold et al., 2018; Topiwala & Ebmeier, 2018).

Therefore, the MDMS consisted of a total of $n = 14$ serum biomarker components. Four categories of foods (vegetables, fruits, legumes, and cereals) were decided to be grouped as a plant-based food group since some of the biomarkers described could be ubiquitously present among all these food groups. Enterolactone (EL), hippuric acid (HA), proline betaine; trigonelline; 3,8-dihydroxy-urolithin glucuronide (UroA-G), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3',4'-dihydroxyphenylacetic acid sulfate (3,4-DHPAA-S), and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate (3,4-DHPV-S,) represented the plant-based food group. DHA and EPA depicted the fish group, while pentadecanoic acid and margaric acid represented the dairy products group. Finally, the dietary intake of MUFAs and saturated fatty acids (SFAs) were depicted by oleic acid and palmitic acid, respectively.

Following the same design used by Trichopoulou et al. (Trichopoulou et al., 2003), a value of 0 or 1 was assigned to each dietary biomarker with the use of the sex-specific median as the cutoff. For dietary biomarkers of beneficial components (vegetables, legumes, fruits, cereals, and fish), subjects whose circulating level was below the median were assigned a value of 0, and individuals whose level was at or above the median were assigned a value of 1. According to the MDS defined by Trichopoulou et al. (Féart et al., 2009; Trichopoulou et al., 2003), dairy products are presumed to be detrimental. For this reason, the scoring for dietary biomarkers of dairy products was inverted (Féart et al., 2009; Trichopoulou et al., 2003). Finally, for the relation between MUFAs and SFAs, a value of 1 was assigned to those individuals whose oleic acid concentration was at or above the median and a value of 0 to those whose concentrations were below it. The scoring was inverted for palmitic acid concentrations. Oleic acid and palmitic acid are the most abundant dietary and plasma FAs (Abdelmagid et al., 2015; Palomer et al., 2018). Moreover, oleic acid is a biomarker of olive oil intake, the main source of MUFAs in the MedDiet context (Garcia-Aloy et al., 2019; Trichopoulou et al., 2003).

Table 3. Concentrations of MDMS dietary biomarkers (µg/L) by sex-specific medians in the 3C discovery (Bordeaux, n = 418) and validation (Dijon, n = 422) cohorts.

MDMS metabolite	p50 (µg/L)	Bordeaux cohort (n = 418)		Dijon cohort (n = 422)	
		males (n = 142)	females (n = 276)	males (n = 156)	females (n = 266)
EL	< p50	5.2 (1.2)	5.0 (1.2)	6.1 (3.0)	4.9 (2.8)
	≥ p50	7.1 (0.7)	7.3 (0.8)	15.3 (3.6)	14.3 (3.7)
HA	< p50	866.0 (395.6)	800.7 (381.3)	546.7 (192.6)	589.3 (249.7)
	≥ p50	3,229.6 (2,617.6)	3,105.1 (2,792.0)	1,912.3 (1,330.5)	2,518.7 (1,866.2)
proline betaine	< p50	66.0 (29.1)	85.8 (38.7)	94.5 (40.0)	102.0 (47.4)
	≥ p50	357.1 (239.6)	451.4 (233.5)	415.9 (262.5)	539.5 (290.7)
trigonelline	< p50	4.7 (2.5)	4.8 (3.0)	3.4 (2.1)	3.9 (2.2)
	≥ p50	18.3 (9.3)	25.3 (19.8)	14.4 (9.8)	19.1 (13.7)
UroA-G	< p50	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	≥ p50	5.1 (3.0)	8.8 (7.6)	6.0 (3.3)	5.7 (4.7)
3,4-DHBA	< p50	19.9 (17.5)	1.2 (4.1)	4.1 (6.9)	1.7 (4.0)
	≥ p50	97.2 (81.8)	68.9 (109.8)	47.5 (28.8)	57.1 (125.3)
3,4-DHPAA-S	< p50	0.0 (0.0)	845.3 (1,116.0)	423.3 (955.3)	1,408.8 (1,480.3)
	≥ p50	13,366.9 (20,301.1)	19,975.7 (36,555.3)	11,009.7 (22,301.8)	20,090.8 (33,765)
3,4-DHPV-S	< p50	1.9 (1.3)	1.0 (0.9)	0.9 (0.8)	0.9 (0.7)
	≥ p50	13.6 (14.0)	10.9 (13.8)	15.7 (15.6)	16.2 (22.7)

Table 3. (cont'd)

MDMS metabolite	p50 (µg/L)	Bordeaux cohort (n = 418)		Dijon cohort (n = 422)	
		males	females	males	females
		(n = 142)	(n = 276)	(n = 156)	(n = 266)
DHA	< p50	5,058.3 (802.3)	4,927.0 (643.5)	4,817.4 (334.8)	4,668.9 (323.0)
	≥ p50	7,999.1 (1,890.9)	8,014.8 (2,765.6)	6,124.8 (874.9)	5,983.9 (741.2)
EPA	< p50	2,601.1 (158.2)	2,612.4 (116.2)	2,949.4 (122.0)	2,896.4 (128.1)
	≥ p50	3,449.9 (832.7)	3,361.8 (1,036.1)	3,597.2 (665.6)	3,493.6 (403.3)
margaric acid	< p50	3,874.4 (1,014.5)	3,842.2 (781.5)	4,272.8 (169.3)	4,248.1 (160.0)
	≥ p50	6,989.3 (1,441.5)	7,405.0 (2,168.3)	4,967.1 (384.1)	4,955.9 (344.6)
pentadecanoic acid	< p50	3,243.3 (967.4)	3,852.2 (845.9)	3,672.6 (314.6)	3,679.4 (296.5)
	≥ p50	6,573.1 (2,101.6)	6,991.3 (1,529.4)	4,572.2 (422.2)	4,850.0 (530.9)
oleic acid	< p50	45,939.9 (11,711.6)	47,191.4 (12,749.5)	56,327.0 (12,694.6)	54,107.8 (12,206.9)
	≥ p50	83,203.0 (16,105.8)	92,450.0 (22,296.8)	98,210.2 (15,334.8)	96,632.8 (20,054.6)
palmitic acid	< p50	25,222.8 (5,963.9)	24,828.0 (5,043.9)	14,774.5 (2,448.1)	14,392.4 (2,316.8)
	≥ p50	43,709.5 (8,755.8)	46,357.9 (13,344.3)	23,282.6 (2,917.5)	22,176.2 (4,098.4)

Values are mean (SD). DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. HA, hippuric acid. MDMS, Mediterranean diet metabolomic score. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 3C, Three-City. 3,4-

DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate.

Thus, the total MDMS ranged from 0 (minimal adherence) to 14 (maximal adherence). The MDMS was secondarily categorized, using cutoff values based on the sex- and cohort-specific medians, as low (< 7 points) and high (≥ 7 points) adherence for both sexes and cohorts.

3.5. Statistical analyses

3.5.1. *Data pre-processing of targeted metabolomics data*

3.5.1.1. *Targeted metabolomics data*

In targeted metabolomic studies, the data is often presented in a "raw" data matrix once the metabolites have been quantified, and generally requires to be processed before data analysis. This step, known as preprocessing, entails various operations, such as imputation of missing values, sample normalization, and transformation of metabolomic variables.

In the D-CogPlast Project, metabolomics data were pre-processed using a standardized protocol developed in the research group. First, data from exogenous metabolites containing values under the limit of quantification were considered as zeros (i.e., they cannot be reliably quantified in the plasma sample). On the other hand, data from endogenous metabolites containing values under the limit of quantification were considered missing values (i.e., assuming they should always be in the sample, at least in very low concentrations). These missing values were imputed using the k-nearest neighbors algorithm ($k = 10$) (Armitage et al., 2015), after metabolites of the data with more than 20% of missing values in all the study groups were removed. Finally, data was normalized by applying log transformation and Pareto scaling (González-Domínguez et al., 2022).

3.5.1.2. *Other data*

In the AGECAR-PLUS study, normal distribution of UTP was tested by using QQ plots. Since it was not normally distributed, it was thus normalized using a Box-Cox transformation ($\alpha = 0.00001$ and $\lambda = 0.25$) (Urpi-Sarda et al., 2015).

3.5.2. Data analyses

3.5.2.1. Univariate and bivariate analyses

Normal distribution of baseline characteristics was tested by using QQ plots, Boxplots and by checking skewness and kurtosis statistic values.

Baseline characteristics of the participants were expressed as means and standard deviations (SDs) for numerical variables with normal distribution, as medians and interquartile ranges (IQRs) for numerical variables with asymmetric distribution, and as number of subjects and proportions for categorical variables.

Differences in baseline characteristics between groups were analyzed using different tests. For numerical variables, Student's t-test, Mann-Whitney U test, Kruskal-Wallis H test or one-way analysis of variance (ANOVA) were used as appropriate. Pearson chi square (χ^2) test was performed for categorical variables. In the D-CogPlast Project, comparisons were tested employing conditional logistic regression models.

Correlation analysis was performed to examine the direction and magnitude of the association between two quantitative variables. Pearson coefficient or Spearman coefficient were calculated depending on whether variables were normally distributed or not, respectively.

3.5.2.2. Multivariate analyses

3.5.2.2.1. Data visualization

In this thesis, visualization tools were used to evidence the state of the art of the qualitative profile of phytochemicals contained in pulses, as well as to compare the intensities of discriminant metabolites and their distribution among legumes. Specifically, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were carried out through the *MetaboAnalyst 3.0* platform, and the R software and its related packages.

(I) Cluster analysis

In (agglomerative) HCA, each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster (Trygg et al., 2007). Euclidean distance was applied as a similarity measure, which is one of the two parameters needed to be considered when performing hierarchical clustering. The other one is clustering algorithms, and Ward's linkage (clustering to minimize the sum of squares of any two clusters) was employed here.

In the qualitative reviewed analysis of legumes, HCA was performed from a qualitative perspective (i.e., absence or presence of a certain compound) and was further displayed by dendrograms plotted on the corresponding sides of a heatmap. The heatmap depicted the actual data into a corresponding 2-D color map using two colors allowing to easily visualize changing patterns in phytochemical presence across types of legumes.

In the postprandial intervention study designed to discover new potential BFIs for legumes, data visualization was performed also through heatmaps of HCA to visualize the discriminatory ability of significant metabolites deciphered by the statistical analyses after intake of legumes. The graph was produced taking into consideration the peak area of the most intensive feature per identified metabolite.

(II) Principal Component Analysis

PCA is an unsupervised method of clustering and classification for dimension reduction of the data by performing linear combinations of the original variables (correlated with each other) into smaller new sets of variables (uncorrelated with each other). These new combinations are called principal components (PC) and are sorted in descending order according to the percentage of the total data variance they explain (Cozzolino et al., 2019). Thus, the first PC (PC1) direction of the data is that along which the observations vary the most, while in the following components (PC2, PC3, etc.) the percentage of variance described decreases.

In the qualitative reviewed analysis of legumes, PCA analyses were applied to the data to highlight qualitative differences and similarities between legumes. In the postprandial intervention with legumes, PCA analyses were performed to check data quality (i.e., potential batch effect due to signal drift, sensitivity loss, etc.), to detect the presence of outliers, and to assess potential associations among samples (i.e., urinary and serum samples).

Results were visualized using a score plot, which provides an intuitive summary of the sample clustering patterns by projecting high-dimensional metabolomics and phytochemical data into two in a way that explains the maximal variance. The score plots showed the samples (i.e., legumes) distributed in a coordinate matrix so that the variation could be interpreted in terms of similarities or dissimilarities of the inherent fingerprints between the samples.

3.5.2.2.2. Multivariate models

In this thesis, multivariate analyses have been used in an attempt to explain specific age-related phenomena considering several potentially influencing variables simultaneously. All multivariate analysis models were adjusted for sex, age at baseline, BMI and other characteristics that are potential risk factors of the specific age-related health outcomes, and might be associated with particular dietary factors, thus acting as potential confounders (i.e., distorting the true association between these variables).

On the one hand, one-factor analysis of variance (ANOVA) for repeated measures was performed to test differences on functional status over hospitalization. Bonferroni post hoc test was used to compare changes in functional status in response to the intervention groups (i.e., exercise + health education program and control groups) and levels of MedDiet adherence (low and high levels of MEDAS and concentration of UTP at admission). The effect of the interaction of two factors was studied: time as a within-participant factor (admission and discharge) and groups.

To assess the effects of individual factors, the differences between discharge and admission values for BI were finally calculated and then a one-factor analysis of covariance (ANCOVA) test was applied adjusting for the above-mentioned variables and for functional status at admission.

Furthermore, distinct multivariate regression models were performed to determine the effect of the dietary variables on CD considering other confounding variables. Based on the dependent variable features and the study design, the following models have been carried out:

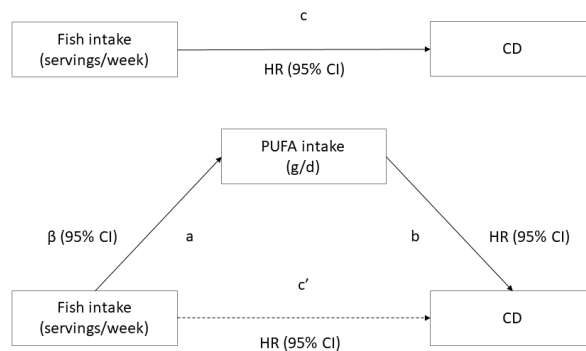
- a) In the InCHIANTI Study, time-dependent Cox models were used for the association between fish intake and CD risk. Time-to-event was defined as the time elapsed from baseline to the one at which CD was first detected.
- b) In the D-CogPlast Project, the association between MDMS and CD was studied using logistic regression models conditioned on matching variables (i.e., age at baseline, sex, and education level).

In order to assess whether the effect of the independent variables from these studies on CD depended on the value of other factors, effect modification by these factors was assessed by adding product terms in the fully adjusted regression models. Multiple testing correction was

carried out utilizing Benjamini-Hochberg false discovery rate (FDR) and Bonferroni correction in the InCHIANTI study and the D-CogPlast work, respectively.

In the InCHIANTI study, mediation analysis was performed to clarify the role of PUFA intake in the association between fish intake and CD. This analysis was based on the standardized three-step criteria proposed by Baron and Kenny (Baron & Kenny, 1986). The following models were fitted: (i) a time-dependent Cox regression model assessing the association between fish intake and CD excluding the mediator (PUFA intake) from the model to check that there is an effect that may be mediated (Figure 6, path c); (ii) a linear mixed-effects regression model assessing the association between fish intake and PUFA intake (Figure 6, path a); and (iii) a time-dependent Cox regression model assessing the association between PUFA intake and CD, with additional adjustment for fish intake (Figure 6, path b). The same procedure was conducted to explore the potential mediation effect of circulating EPA and DHA. Analogously, the potential mediation pathway of higher fish intake and lower CD risk, through a lower CVD risk, was evaluated.

Figure 6. Mediation diagram of PUFA intake as a mediatory in the association between fish intake and CD.



CD, cognitive decline. CI, confidence interval. HR, Hazard ratio. PUFA, polyunsaturated fatty acid.

Path *a* indicates the effect of fish intake (exposure) on PUFA intake (mediator); path *b* indicates the effect of PUFA intake (mediator) on CD (outcome); path *c* indicates the total effect of fish intake (exposure) on CD (outcome), ignoring PUFA intake (mediator); path *c'* indicates the direct effect of fish intake (exposure) on CD (outcome) while considering PUFA intake (mediator)

In the AGE-CAR-PLUS study, missing data of covariates was not imputed, and thus only available data were used for analysis. In both InCHIANTI and D-CogPlast studies, the reference category

(for categorical covariables) and the baseline median value (for continuous covariables) were assigned to missing values.

All statistical analyses were performed using the SPSS (SPSS 25.0, IBM, NY; SPSS 27.0, IBM, NY) and the R 4.2.3 (R foundation, Vienna) software and its related packages. A significance level of $p < 0.05$ was considered statistically significant.

4. Results

4.1. Description and comparison of the qualitative profile of phytochemicals in legumes: A qualitative reviewed analysis

In the context of the FoodBall Project (JPI-HDHL), the research group has been engaged in the identification and comparison of metabolites resulting from legume consumption, with the aim of assessing their potential candidacy as biomarkers of legume intake.

For this purpose, a comprehensive review has been first conducted to describe the phytochemical content in order to expand knowledge of the phytochemical profile of legumes. Metabolomics data analysis tools have been used to visualize qualitative differences between legumes.

Publication 1: Alba Tor-Roca, Mar Garcia-Aloy, Fulvio Mattivi, Rafael Llorach, Cristina Andres-Lacueva, and Mireia Urpi-Sarda. Phytochemicals in Legumes: A Qualitative Reviewed Analysis. J. Agric. Food Chem. 2020;68(47):13486–13496.

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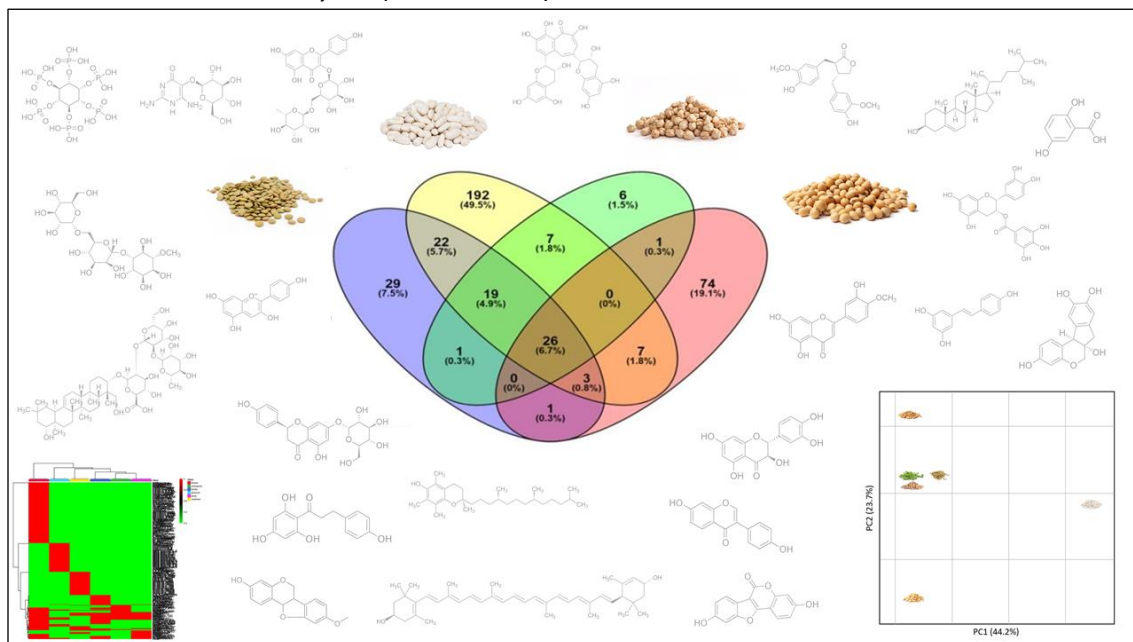
Abstract

The aim of this review was to describe and compare the qualitative profile of phytochemicals contained in legumes and identified through liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) methods. Among the 478 phytochemicals reported in 52 varieties of legumes, phenolic compounds were by far the most frequently described (n = 405, 85%). Metabolomics data analysis tools were used to visualize the qualitative differences, showing beans to be the most widely analyzed legumes and those with the highest number of discriminant phytochemicals (n = 180, 38%). A Venn diagram showed that lentils, beans, soybeans, and chickpeas shared only 7% of their compounds. This work highlighted the huge chemical diversity among legumes and identified the need for further research in this field and the use of metabolomics as a promising tool to achieve it.

Resum

L'objectiu d'aquesta revisió va ser descriure i comparar el perfil qualitatiu dels fitoquímics presents en els llegums i identificats a través dels mètodes de cromatografia líquida-espectrometria de masses (LC-MS) i cromatografia de gasos-espectrometria de masses (GC-MS). Entre els 478 fitoquímics reportats en 52 varietats de llegums, els compostos fenòlics van ser, amb diferència, els més descrits ($n = 405$, 85%). Es van usar eines d'anàlisi de dades metabolòmiques per visualitzar les diferències qualitatives, i es va demostrar que les mongetes eren els llegums més analitzats i amb el nombre més alt de fitoquímics discriminants ($n = 180$, 38%). El diagrama de Venn va mostrar que les lleties, les mongetes, la soia i els cigrons compartien només el 7% dels seus components. Aquest treball va destacar l'enorme diversitat química entre els llegums i va identificar la necessitat de més recerca en aquest camp i l'ús de la metabolòmica com una eina prometedora per aconseguir-ho.

Figure 7. Graphical abstract of the manuscript entitled “Phytochemicals in Legumes: A Qualitative Reviewed Analysis” (Publication 1).



Phytochemicals in Legumes: A Qualitative Reviewed Analysis

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ABSTRACT: Legumes are an excellent source of nutrients and phytochemicals. They have been recognized for their contributions to health, sustainability, and the economy. Although legumes comprise several species and varieties, little is known about the differences in their phytochemical composition and the magnitude of these. Therefore, the aim of this review is to describe and compare the qualitative profile of phytochemicals contained in legumes and identified through LC-MS and GC-MS methods. Among the 478 phytochemicals reported in 52 varieties of legumes, phenolic compounds were by far the most frequently described ($n = 405$, 85%). Metabolomics data analysis tools were used to visualize the qualitative differences, showing beans to be the most widely analyzed legumes and those with the highest number of discriminant phytochemicals ($n = 180$, 38%). A Venn diagram showed that lentils, beans, soybeans, and chickpeas shared only 7% of their compounds. This work highlighted the huge chemical diversity among legumes and identified the need for further research in this field and the use of metabolomics as a promising tool to achieve it.

KEYWORDS: phytochemicals, legumes, qualitative analysis, nutrimentalomics, polyphenols

■ INTRODUCTION

Legumes have been shown to promote health and reduce the risk of cardiovascular disease (CVD) as well as that of some cancers, such as colon cancer, among other pathological conditions.^{1,2} They also have particular relevance for sustainability and local economies, as they reduce greenhouse gas emissions, help decrease animal-based protein consumption, and are rooted in communal gastronomies.³ In the last few decades, legumes have gained popularity worldwide as being a good source of phytochemical compounds. Phytochemicals are non-nutrient, plant-based minor components that differ substantially in their biochemistry, source distribution, and physiological effects. Their biological activities, such as antioxidant,⁴ anti-inflammatory⁵ and antimicrobial,⁶ have also been described, and it has been suggested that they offer significant, meaningful benefits to human health.⁴ The vast majority of studies conducted to describe the phytochemical composition of legumes have focused on only one type of legume^{7–9} or on a small number of phytochemicals. The comparison of legumes from a phytochemical profile viewpoint is essential for distinguishing properties and potential applications of legumes as well as for enhancing the state of the art and promoting their production, consumption, and use. Therefore, the aim of this review is to describe and compare the qualitative profile of bioactive compounds contained in legumes for human consumption that have been identified through LC-MS and GC-MS methods.

In this review, we first described the qualitative profile of phytochemicals contained in legumes for human consumption which had been identified through LC-MS and GC-MS methods. We followed this description by conducting a comparative analysis between the different groups of legumes using statistical dichotomous techniques. Finally, a description

of bioactive compounds that could discriminate between legumes was discussed.

■ VARIETIES AND NUTRITION OF LEGUMES

Definition and Types of Pulses and Legumes.

Legumes are the edible seeds of the *Fabaceae* or *Leguminosae* family, the third-largest group of plants (more than 20 000 species and 700 genera). They produce between 1 and 12 grains of various sizes, shapes, and colors within a pod. Having been spread across the world for about 90 million years, even in regions with extreme weather, their seeds have been used for at least 10 000 years for both human and animal consumption.¹⁰

The Food and Agricultural Organization (FAO) considered pulses a subgroup of legumes that are defined as “Leguminosae crops harvested exclusively for their grain, including chickpeas, dry beans, peas and lentils”. This definition excludes legumes harvested for oil extraction, such as soybeans and peanuts, and those used as vegetables, like green beans and green peas.¹⁰ Specifically, the FAO recognizes the following 11 primary pulses: dry beans (kidney, pinto, navy, azuki, mung, black gram, scarlet runner, rice bean, moth, and tepary beans), dry broad beans (horse, broad, and field bean), dry peas (garden and field pea), chickpeas, dry cowpeas (cowpea and black-eyed pea/bean), pigeon peas (pigeon pea and cajan pea), lentils, Bambara beans (Bambara groundnut and earth pea), vetch

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Table 1. Potential Health Effects of Legumes Consumption

disease	legumes consumption effect	mechanism of action
CVD ^a	RR (95% CI) = 0.92 (0.85–0.99) ¹³ <i>Positive effect on CVD risk</i>	Relation with specific nutrients and components found in legumes: ↑ K ²³ ↑ Mg ²⁴ ↑ fiber ²⁵ cholesterol-free Relation with various cardiometabolic risk factors found in nonsoy legumes: ↓ LDL cholesterol ^{14,15} ↓ total cholesterol ¹⁵ ↓ blood pressure ¹³ ↑ weight management ^{16,17} ↓ glycemic index ^{18,19} ↓ metabolic syndrome risk factors
CHD ^b	RR (95% CI) = 0.90 (0.83–0.99) ¹³ <i>Positive effect on CHD risk</i>	Relation with specific nutrients and components found in legumes: ↑ K ²³ Relation with various cardiometabolic risk factors found in nonsoy legumes: ↓ glycemic index ^{18,19}
prostate cancer	RR (95% CI) = 0.85 (0.75–0.96) ²⁶ <i>Positive effect on prostate cancer risk</i>	Relation with specific nutrients and components found in legumes: ↑ phytoestrogens ²⁷
colorectal cancer	RR (95% CI) = 0.91 (0.84–0.98) ³⁶ colorectal cancer: RR (95% CI) = 1.00 (0.95–1.06) ³⁷ colon cancer: RR (95% CI) = 0.97 (0.83–1.15) ³⁷ rectal cancer: RR (95% CI) = 0.99 (0.78–1.25) ³⁷ <i>Controversial effects on colorectal cancer risk</i>	Relation with specific nutrients and components found in legumes: ↑ fiber ²⁵
stroke	RR (95% CI) = 0.98 (0.86–1.11) ¹³ <i>No effects on stroke risk but possible effects on markers of stroke risk</i>	Relation with specific nutrients and components found in legumes: ↑ K ²³ Relation with various cardiometabolic risk factors found in nonsoy legumes: ↓ glycemic load ³⁹
diabetes	RR (95% CI) = 0.93 (0.83–1.05) ¹³ <i>No effects on diabetes risk but possible effects on markers of diabetes</i>	Replacement animal protein for vegetable protein improves glycemic control of diabetes ¹⁹ Relation with various markers of glycemic control: HbA _{1c} ^c and fructosamine ¹⁸
satiety and food intake	RoM ^d (95% CI) = 1.31 (1.09–1.58) ³⁸ <i>Positive effects on acute satiety</i>	Relation with various diabetes risk factors found in nonsoy legumes: ↓ metabolic syndrome risk factors ²² Relation with specific nutrients and components found in legumes: ↓ glycemic index ³⁸ ↓ fat content ³⁸ ↑ fiber ³⁸ Relation with various risk factors found in nonsoy legumes: blood sugar and insulin levels stabilization ¹²

^aCVD: cardiovascular disease. ^bCHD: coronary heart disease. ^cHbA_{1c}: glycated hemoglobin. ^dRoM: ratio of means.

(spring/common vetch), lupins, and other “minor” pulses (hyacinth or lablab, jack or sword, winged, velvet, guar and yam beans).¹¹ In order to homogenize the concepts, we will use in this review the more generic term “legumes” to refer to both pulses and legumes for human consumption: soybeans, peanuts, chickpeas, lentils, beans, and peas (Table S1).

Legumes are considered an essential superfood, not only thanks to their desirable nutritional profile and health properties but also to their great influence and power in two other key human aspects: the environment and society.

Nutritional Composition and Health Effects. Recent studies have brought to light the relationship between a regular consumption of legumes and the prevention of some chronic diseases (Table 1). A daily intake of legumes is associated with a decreased risk of CVD, especially coronary heart disease (CHD).¹³ In fact, legumes consumption leads to the reduction of various CVD risk factors, such as LDL cholesterol,^{14,15} total cholesterol,¹⁵ blood pressure,¹³ body weight,^{16,17} glycemic index (GI),^{18,19} insulin resistance,²⁰ and C-reactive protein,²¹ among other metabolic syndrome risk factors.²² This is due to several compositional traits of legumes, specifically their amount of potassium,²³ magnesium,²⁴ and soluble fiber,²⁵ along with their cholesterol-free condition¹ (Table 1).

Findings also suggest an inverse association between the intake of legumes and the risk of prostate cancer.²⁶ One possible explanation for this could be their phytoestrogen content.²⁷ Additionally, maternal consumption of legumes during pregnancy may have a protective effect on acute lymphoblastic leukemia in children.²⁸ Furthermore, it has been suggested that consuming legumes could reduce the risk of breast cancer,²⁹ due mainly to their flavonol, flavone, and

isoflavone content,^{30–33} although evidence is still limited.³⁴ Regarding endometrial cancer, findings of a meta-analysis suggest a weak inverse association between consumption of isoflavones from soy products and legumes and endometrial cancer risk.³⁵ Although previous meta-analysis and systematic reviews³⁶ supported the notion that a high intake of legumes was also associated with a low incidence of colorectal cancer due to their high fiber content,²⁵ an update of the evidence of the World Cancer Research Fund–American Institute for Cancer Research (WCRF-AICR) Continuous Update Project states that legume intake is not associated with colorectal cancer risk³⁷ (Table 1).

Finally, having a low GI and fat content, as well as a high fiber content, increases satiety³⁸ and helps stabilize blood sugar and insulin levels. This makes legumes ideal for weight management.^{16,17}

On the other hand, it seems that legumes consumption has no effect either on stroke risk or diabetes risk.¹³ Despite this, legumes can possibly have effects on their biomarkers. In the case of stroke risk, this can be explained by the amount of potassium they contain²³ and their capacity to reduce glycemic load,³⁹ whereas in the case of diabetes risk, this seems to be due to the replacement of animal protein for vegetable protein effect,¹⁹ as well as for the improvement of longer-term glycemic control markers and the reduction of metabolic syndrome risk factors.^{18,22}

This beneficial role of legumes could be explained by their desirable nutritional and non-nutritional profile. Legumes have a high amount of complex carbohydrates and fiber, thereby offering an average low-energy density of 1.3 kcal/g.¹ They are also known for being poor in sodium and rich in other

Table 2. Number of Phytochemicals (and Percentage) of Each Group of Legumes in Relation to Their Family

phytochemical classes	total (%)	lentils (%)	beans (%)	chickpeas (%)	peas (%)	soybeans (%)	peanuts (%)	ref
phytic acids	1 (0.2)	1 (1)	1 (0.4)	1 (2)	1 (2)	1 (1)	1 (1)	41, 61, 64, 89–94
RFO - galactosides	5 (1)	4 (4)	5 (2)	4 (7)	4 (6)	4 (4)	0 (0.0)	41, 59, 64, 100–105
saponins	33 (7)	4 (4)	18 (7)	2 (3)	9 (14)	71 (63)	2 (2)	96–98, 106
phytosterols	6 (1)	5 (5)	5 (2)	6 (10)	5 (8)	4 (4)	3 (6)	71–74, 77, 78
tocopherols	3 (0.6)	3 (3)	3 (1)	3 (5)	3 (5)	3 (3)	2 (2)	52, 69, 71–76
carotenoids	11 (2)	8 (8)	1 (0.4)	3 (5)	0 (0.0)	1 (1)	0 (0.0)	52, 69, 74, 90
alkaloids	12 (3)	0 (0.0)	12 (4)	0 (0.0)	1 (2)	0 (0.0)	0 (0.0)	7, 58–61
polyphenols: stilbenoids	6 (1)	2 (2)	2 (1)	1 (2)	1 (2)	0 (0.0)	2 (2)	7, 8, 63, 72, 107
nonflavonoids: lignans	2 (1)	2 (2)	2 (1)	2 (3)	1 (2)	1 (0.9)	2 (2)	79, 95
coumestans	1 (0.2)	1 (1)	1 (0.4)	1 (2)	0 (0.0)	1 (0.9)	1 (0.8)	79, 95
phenolic acids	63 (13)	18 (18)	48 (17)	11 (18)	13 (20)	1 (0.9)	15 (13)	7, 8, 72, 84, 90, 107–113, 9, 114, 52–54, 56, 62, 63, 67
hydrolyzable tannins	1 (0.2)	0 (0.0)	1 (0.4)	0 (0.0)	1 (2)	0 (0.0)	0 (0.0)	64, 65
polyphenols: isoflavones	64 (13)	10 (10)	11 (4)	13 (22)	7 (11)	9 (8)	6 (5)	7, 8, 63, 68, 72, 79–82, 115–117
flavonoids: dihydrochalcones	4 (0.8)	0 (0.0)	4 (1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7, 54
pterocarpans	2 (0.4)	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7, 54
flavones	31 (6)	9 (9)	19 (7)	3 (5)	6 (9)	0 (0.0)	6 (5)	7, 8, 53, 54, 56, 62, 63, 72, 85, 95, 107, 111, 113, 118
flavonols	79 (17)	13 (13)	64 (23)	8 (13)	5 (8)	8 (7)	6 (5)	7–9, 52–57, 62, 67–69, 72, 84, 85, 95, 107, 110, 111, 113, 115, 118–120
flavanones	20 (4)	1 (1)	12 (4)	0 (0.0)	3 (5)	4 (4)	3 (3)	7, 8, 53, 54, 68, 110, 113, 118
flavanonols	4 (0.8)	0 (0.0)	3 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2)	7, 8, 54, 62, 63
flavanols	17 (4)	7 (7)	16 (6)	1 (2)	5 (8)	1 (0.9)	3 (3)	7–9, 52, 54, 63, 69, 72, 83–88, 110, 113
condensed tannins	90 (19)	10 (10)	23 (8)	0 (0.0)	0 (0.0)	1 (0.9)	65 (54)	7–9, 52, 61, 64, 85, 86, 88, 107, 112, 121–124
anthocyanidins	21 (4)	2 (2)	21 (8)	0 (0.0)	0 (0.0)	2 (2)	0 (0.0)	52–56, 67, 84, 85
brazilin	1 (0.2)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7, 8
squalene	1 (0.2)	1 (1)	1 (0.4)	1 (1.7)	1 (1.5)	0 (0.0)	1 (0.8)	7, 8
TOTAL	478 (100)	101 (100)	276 (100)	60 (100)	66 (100)	112 (100)	120 (100)	

minerals, such as potassium, zinc, calcium, and iron.^{1,40} Legumes are an excellent source of protein (20–30% of their energy value), relatively low in tryptophan and sulfur-containing amino acids, such as methionine and cysteine, but rich in lysine.^{1,40} Moreover, they do not contain gluten. Their predominant fatty acid is linoleic acid,⁴⁰ and vitamins A, E, and B are notably abundant.¹ Legumes also contain a high variety of phytochemicals and other minor components that have significant meaningful benefits for human health: α -galactosides, phytosterols, tocopherols, saponins, alkaloids, and phytic acid, as well as carotenoids and (poly)phenolic compounds.^{1,41–47} Recent literature has shown that canning and household cooking significantly differ in their effects on nutritional composition and bioactive content of legumes, and even have contrary effects on different types of legumes.⁴⁸ Agronomic, storage, processing, food formulation, as well as bioaccessibility and bioavailability of the phytochemicals are also factors involved in the ultimate health outcomes of consumption of leguminous foods.

Despite the many health benefits of legumes, their cultivation and intake have been cautiously handled due to the presence of some bioactive compounds, such as phytic acid, convicine, protease and amylase inhibitors, as they can act as antinutrients. Antinutritional factors may cause toxicity or interfere with the digestion and absorption of certain dietary components, causing adverse physiological effects (for example, flatulence, favism, lathyrism, small intestine damage,

and growth depression).⁴¹ This is especially important since several legumes cause concern, particularly in areas characterized by poverty and malnutrition where a single type of legume can be ingested in high amounts. However, most antinutrients can still be reduced or removed by thermal processing (boiling, steaming, roasting, autoclaving, dry heating), storage, irradiation, soaking, dehulling, milling, fermentation, and germination.^{41,49}

■ QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN LEGUMES

The qualitative profile of a large variety of legumes was obtained by applying the systematic search referred to in the [Supporting Information](#). A list of different varieties of legumes and their phytochemicals was obtained after revising the literature.

Types of Legumes. All the legumes reported on in this review are shown in [Table S1](#). The biggest group of legumes is beans, where 16 varieties belonged to *Phaseolus vulgaris*: black, brown, cream, white, red, small red, cranberry, yellow, black Jamapa, red and white kidney, pinto, brown string, yellow wax, pink and common beans. Two more kinds of beans of the same genus were runner (*P. coccineus*) and butter bean (*P. lunatus*) ([Table S1](#)). Moreover, from the *Vigna* genus, nine different beans could be considered: black-eyed bean and red cowpea (*V. unguiculata*), mungo bean (*V. mungo*), rice bean (*V. umbellata*), moth bean (*V. acutifolia*), black and green mung

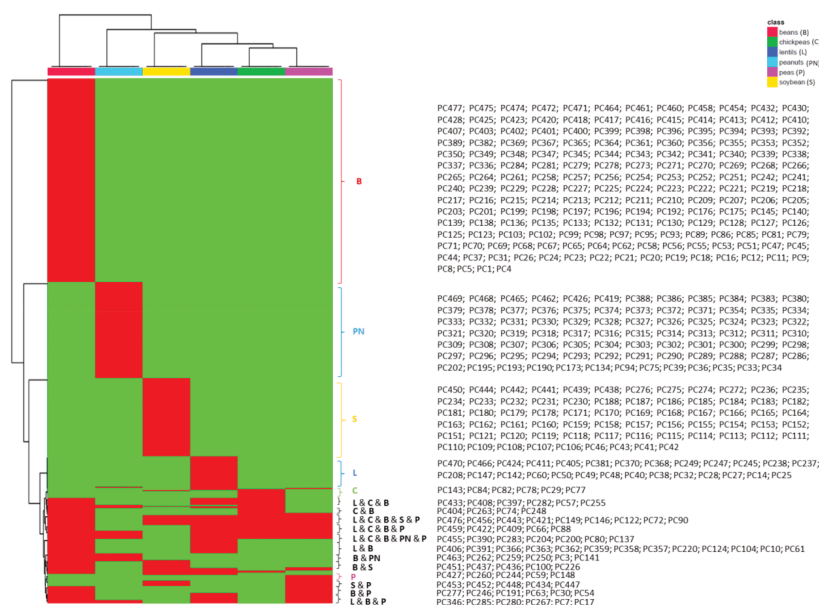


Figure 1. Qualitative heatmap of the phytochemicals distributed in lentils, chickpeas, beans, peas, soybeans, and peanuts. Overview (red: presence; green: absence). For a detailed version, please see Figure S1. Note: The phytochemical name of each PC-number code is shown in Table S2. Detailed information is also stated on Table S3 and Table S4. Legume groupings with less than four common compounds are not displayed in this figure.

bean (*V. radiata*), and green and red adzuki bean (*V. angularis*). Finally, broad bean (*Vicia faba*), horse gram (*Macrotyloma uniflorum*), hyacinth bean (*Lablab purpureus*), black and red sword bean (*Canavalia gladiata*), jack bean (*Canavalia ensiformis*), white lupin bean (*Lupinus albus*), blue lupin bean (*Lupinus angustifolius*), yellow lupin bean (*Lupinus luteus*) and morama bean (*Tylosema esculentum*) are also part of this group (Table S1). From *Lens culinaris*, commonly known as lentils, seven varieties were considered. They were beluga or black, brown, green, yellow, gray, red and tan lentils. Also, yellow and black chickpeas (*Cicer arietinum*), green, black, and yellow soybeans (*Glycine max*), and peanuts (*Arachis hypogaea*) have been reported. Peas were grouped in three different species: green and yellow pea (*Pisum sativum*), snowpea (*Pisum sativum var. saccharatum*), and pigeon pea (*Cajanus cajan*).

Phytochemical Foodprint of Legumes. The search gave a total of 478 phytochemicals described in legumes. The biggest group was condensed tannins ($n = 90$), which represented 19%, followed by flavonols ($n = 79$), isoflavones ($n = 64$) and phenolic acids ($n = 63$), which represented 17%, 13%, and 13%, respectively. The following have also been reported: 5 α -galactosides, 33 saponins, 6 phytosterols, 2 lignans, 1 coumestan, 3 tocopherols, 12 alkaloids, 6 stilbenes, 4 dihydrochalcones, 2 pterocarpanes, 31 flavones, 20 flavanones, 4 flavanols, 17 flavanols, 1 hydrolyzable tannin, 21 anthocyanidins, 11 carotenoids, and 3 other compounds (Table 2 and Table S3). Therefore, phenolic compounds were the major

family of phytochemicals described in legumes, with 405 of them (85%). The second-biggest family was saponins, with 33 constituents (7%) (Table 2 and Table S3).

In regard to phytochemical families and legume groups, the literature showed that phytic acid, saponins, phytosterols, tocopherols, lignans, phenolic acids, isoflavones, flavonols and flavanols have been identified in all kinds of legumes, whereas carotenoids were found in lentils, chickpeas, beans, and soybeans but not in peas or peanuts (Table 2 and Table S3). Ciceritol, an α -galactoside, has been determined in all legumes except peanuts,^{41,50} while anthocyanidins were found in lentils, beans, and soybeans.^{51–57} Moreover, alkaloids were only identified in beans and peas,^{7,58–61} but flavanols were found in beans and peanuts.^{7,54,62,63} Since dihydrochalcones, pterocarpanes, hydrolyzable tannins, and brazilin had only previously been described in beans,^{7,54,64,65} to the best of our knowledge no raffinose family of oligosaccharides (RFO) in peanuts and no coumestans in peas have been determined before. Finally, soybeans had no stilbenes, flavones, or squalene, and chickpeas lacked flavanones and condensed tannins.

In addition, Table 2 also shows the particular composition of each group of legumes, with beans being the group with the most compounds identified ($n = 276$, 58%), followed by peanuts, soybeans, and lentils ($n = 120$, 112, and 101, respectively), while only 60 and 66 chemicals were reported in chickpeas and peas, respectively.

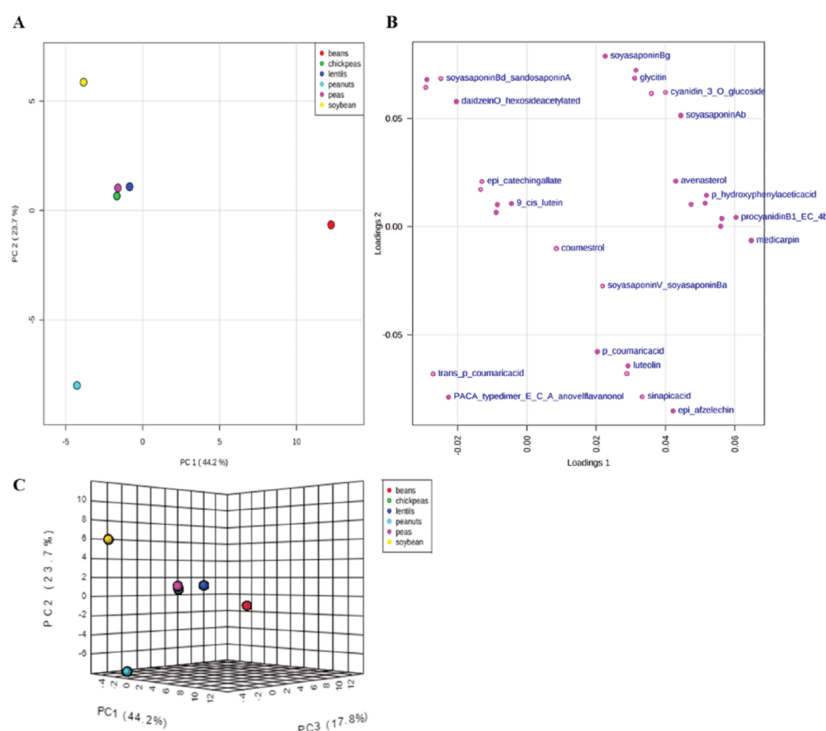


Figure 2. PCA score plot 2D (A), loading plot (B), and PCA score plot 3D (C).

Qualitative Cluster Analysis of Legumes. In this review, we decided to use visualization tools to evidence the state of the art of the phytochemicals in legumes. A heatmap analysis was constructed by using *MetaboAnalyst 3.0*⁶⁶ employing the qualitative data (Figure 1, Figure S1, Figure S2, Table S3). This heatmap shows the distribution of bioactive substances, according to each legume, that were or were not determined in them. In parallel with the heatmap, Table S4 shows the 372 discriminant compounds, marked in red in Figure 1, that were determined in the specific legumes. The group with the highest number of discriminant compounds ($n = 180$) was beans, representing around 38% of all compounds. Flavonols were the main phytochemicals that allowed beans to be discriminated from other legumes (e.g., quercetin 3-*O*-xylosylglucoside⁵⁷ and faralatoside⁵⁴). The next groups of legumes in terms of proportion were peanuts and soybeans, where 18% and 14% ($n = 85$ and $n = 69$), respectively, of their compounds allowed them to be discriminated from the other legumes. Peanuts stand out for condensed tannins (e.g., prorobinetidin and prodelphinidin⁸) and soybeans for isoflavones (e.g., daidzein *O*-dihexoside and genistein *O*-hexoside⁶⁸). There were 27 specific compounds from lentils (Table S4), with carotenoids (e.g., 9-*cis*-lutein and 9-*cis*-zeaxanthin⁶⁹) being the most

prevalent, amounting to 6% of the total. Otherwise, chickpeas and peas had only a very few unique compounds, with each amounting to only 1% of all specific compounds ($n = 6$ and $n = 5$, respectively).

Therefore, given that some phytochemicals have only been determined or identified in one type of legume, such as canthoxanthine in chickpeas, glycitin and derivatives in soybeans, several alkaloids such as lupanine and angustifoline in beans and morin in peas (Table S4), future analytical needs should be oriented toward validating whether these compounds are exclusive to these legumes or whether legumes have not been fully analyzed. For instance, although the literature is extensive in this field, recently other works have revealed the presence of some phytochemicals in different legumes by applying untargeted metabolomic approaches to foodprint them,⁷⁰ indicating that this is a niche area to study.

Additionally, in our qualitative review, we identified a total of 14 phytochemicals that were distributed among all groups of legumes. These compounds were α -tocopherol,^{69,71–75} β + γ -tocopherol,^{52,69,71–76} β -sitosterol,^{71–74,77,78} campesterol,^{71–74,77,78} stigmasterol,^{71,73,74,77,78} biochanin A,^{8,63,79–81} formononetin,^{7,63,79,80} daidzein,^{63,79–81} genistein,^{7,68,72,79–81} genistin,^{80–82} epi-catechin,^{8,9,52,63,72,83–88} and phytic

acid,^{41,61,64,89–94} secoisolariciresinol,^{79,95} and soyasaponin Bb,^{96–98} which are basically phytosterols and isoflavones. Consequently, none of them are shown in the heatmap.

Principal Component Analysis of Phytochemical Composition of Legumes. Principal component analysis (PCA) was applied to the data to highlight qualitative differences and similarities between legumes (Figure 2). Principal component 1 (PC1) was responsible for 44.2% of the variance, whereas PC2 and PC3 explained 23.7% and 17.8%, respectively. The most qualitative difference was obtained between beans and the other legumes (PC1). In addition, peanuts and soybeans also had quite a different profile from the other legumes. They are positioned in the bottom-left and top-left corner, respectively, of the PCA score plot. In fact, 65% of the compounds determined in beans were exclusively identified in them, while in the case of soybeans and peanuts, the proportion was 62% and 71%, respectively. On the other hand, chickpeas, lentils, and peas have quite a central position in the PCA score plot (Figure 2). Only 27% of the compounds reported in lentils were specifically identified in them, while in the case of chickpeas and peas, the proportion was 10% and 8%, respectively. The PCA score plot revealed a close cluster between lentils, chickpeas, and peas, where 35 compounds were shared only among them (Figure 2B), representing 32% of the chemicals found in peas, 35% of the compounds reported in chickpeas, and 21% of the phytochemicals identified in lentils (Table S5). Most of them were phenolic acids, isoflavones, and flavonols. Additional score plot 3D showed that lentils were separated from chickpeas and peas in the PC3 (Figure 2C).

Venn Diagram Analysis. A Venn diagram of the four groups has been created with the most well-known, widely considered,¹¹ and popular legumes (lentils, beans, chickpeas, and soybeans) for better interpretation and visualization of the data. The obtained diagram⁹⁹ allows us to visualize the proportion of the number of phytochemicals by group (Figure 3). Lentils, soybeans, beans, and chickpeas had 26 shared compounds (7%), mainly isoflavones. Lentils and soybeans were the only ones in which soyasaponin Bd (isoflavone) was

identified, while sinapic, chlorogenic, and cinnamic acids (phenolic acids), luteolin 8-C-glucoside (flavone), myricetin 3-O-rhamnoside, quercetin 3-O-galactoside (flavonols), and β -carotene were identified in beans and chickpeas. Both chickpeas and lentils contained gentisic acid (phenolic acid). There was no specific compound shared between soybeans, beans, and chickpeas or between soybeans, lentils, and chickpeas either.

CONCLUSIONS AND FUTURE PERSPECTIVES

In the present work, a phytochemical foodprint of 478 phytochemicals from 52 varieties of legumes has been extracted from literature. This foodprint includes 405 phenolic compounds (which constitute the main group of phytochemicals), 33 saponins, 12 alkaloids, 11 carotenoids, 6 phytosterols, 5 α -galactosides, 3 tocopherols, phytic acid, brazilin, and squalene. Metabolomic techniques have been used for the first time for visualizing qualitative differences between legumes. Beans are the most widely analyzed legumes globally and have the highest number of their own phytochemicals ($n = 180$, 38% of the total), followed by peanuts ($n = 85$, 18% of the total), soybeans ($n = 69$, 14% of the total), and lentils ($n = 27$, 6% of the total), with the proportion being 1% for chickpeas and peas. The qualitative PCA suggested that beans had the most differentiated profile, while lentils, chickpeas, and peas revealed a central and close position with a high number of shared compounds. In addition, the Venn diagram showed that lentils, chickpeas, soybeans, and beans shared only 7% of their determined compounds.

This work has allowed us to identify several niches to be developed in this field. In particular, future research directions should be aimed at establishing an exhaustive approach to uncovering the whole profile of some legumes, since our review indicates that there are meaningful differences between legumes. This is the case for peas and chickpeas, whose phytochemical profile is numerically far below the other legumes' foodprint.

It is recommended that future research should work toward increasing our knowledge about the underrepresented groups of phytochemicals, such as tocopherols and other non-polyphenolic compounds, in order to obtain more complete phytochemical profiles of legumes. Identification of these phytochemical profiles will enable their synergistic effect on bioavailability to be studied, along with the mechanism of action and biological function, and finally enhance our understanding of the health benefits and suitability for human consumption of each type of legume. This new knowledge will also be useful for quantifying these phytochemicals and for obtaining biomarkers of compliance, as well as enabling better quality control of legume-based foods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c04387>.

Research and visualization methodology, binomial and common name of each legume reported in the review (Table S1), legend of phytochemicals (PC) numbers with their corresponding names of phytochemicals (Table S2), families and phytochemicals described in legumes (Table S3), potential discriminant phytochem-

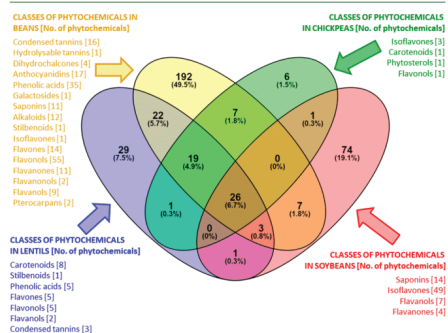


Figure 3. Venn diagram analysis of qualitative data in beans, chickpeas, lentils, and soybeans. Note: Bold numbers mean the total amount of phytochemicals on beans, chickpeas, lentils, and soybeans and phytochemicals shared by each legume combination. In brackets, the percentage of phytochemicals over total number of phytochemicals.

icals of each group of legumes according to the bibliographic search (Table S4), phytochemicals shared between legumes (Table S5), qualitative heatmap of the phytochemicals distributed in legumes (detailed version; Figure S1) and original overview of the qualitative heatmap file from *MetaboAnalyst 3.0* (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CHD, coronary heart disease; CVD, cardiovascular diseases; FAO, Food and Agriculture Organization; GI, glycemic index; PCA, principal component analysis; RoM, ratio of means; RFO, raffinose family of oligosaccharides; WCRF-AICR, World Cancer Research Fund–American Institute for Cancer Research

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Supporting Information

Phytochemicals in Legumes: A Qualitative Reviewed Analysis

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Methods

Systematic search. In order to identify the papers on phytochemicals in all legumes, we carried out an extensive literature search through PubMed, Science Direct, Scopus and Google Scholar databases. The bibliography was searched from 1975 until March 2017. All the articles reviewed were based on LC-MS and GC-MS techniques.

The words employed to the search were: legumes [All Fields], pulses [MeSH term or All Fields], fabaceae [MeSH term or All Fields], determination [All Fields], identification [All Fields], bioactive compounds [All Fields], phytochemicals [MeSH term or All Fields], HPLC-MS [MeSH term or All Fields], LC-MS [MeSH term or All Fields] and GC-MS [MeSH term or All Fields]. Additionally, other specific phytochemicals and seeds names had been used too (e.g. flavones, epicatechin, adzuki bean or lentils).

Use of Visualization Tools. *MetaboAnalyst 3.0* was used to visualize the qualitative data of phytochemicals in legumes through hierarchical cluster analysis and principal component analysis. In (agglomerative) hierarchical cluster analysis, each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster. Euclidean distance was applied as similarity measure, which is one of the two parameters needed to be considered when performing hierarchical clustering. The other one is clustering algorithms, and Ward's linkage (clustering to minimize the sum of squares of any two clusters) was employed in that case.

A Venn diagram was created in order to plot which phytochemicals from lentils, beans, chickpeas and soybeans identified in the literature overlapped between them. The diagram was created by a free on-line tool from Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. Available at: <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.

Table S1. Binomial name and common name of each legume reported in this review.

Legumes	Binomial name	Common name
Beans	<i>Canavalia ensiformis</i>	Jack bean
	<i>Canavalia gladiata</i>	Sword bean
	<i>Lablab purpureus</i>	Hyacinth bean
	<i>Lupinus albus</i>	Lupin bean, white
	<i>Lupinus angustifolius</i>	Lupin bean, blue
	<i>Lupinus luteus</i>	Lupin bean, yellow
	<i>Macrotyloma uniflorum</i>	Horse gram
	<i>Phaseolus vulgaris</i>	Bean, black (= turtle bean, black); bean, yellow; bean, brown; Jamapa bean, black; bean, cream; kidney bean, red; kidney bean, white; bean, white (= Great Northern bean); pinto bean; bean, red; small bean, red; string bean, brown; wax bean, yellow; bean, pink; cranberry bean; common bean (= field bean = haricot bean = bean, green = navy bean)
	<i>Phaseolus coccineus</i>	Runner bean
	<i>Phaseolus lunatus</i>	Butter bean (= Lima bean)
	<i>Vicia faba</i>	Broad bean = fava bean = field bean
	<i>Vigna acontifolia</i>	Moth bean
	<i>Vigna angularis</i>	Adzuki bean
	<i>Vigna mungo</i>	Cow gram, black (= urd bean = mungo bean)
	<i>Vigna radiata</i>	Mung bean
	<i>Vigna umbellata</i>	Rice bean
	<i>Vigna unguiculata</i> = <i>Vigna sinensis</i>	Black-eyed bean/pea (= cowpea = string bean = Hakmeitau bean); cowpea
	<i>Tylosema esculentum</i>	Morama bean
Lentils	<i>Lens culinaris</i>	Lentil, beluga or black; lentil, green; lentil, red; lentil, brown; lentil, yellow; lentil, grey; lentil, tan
Chickpeas	<i>Cicer arietinum</i>	Chickpea, black; chickpea, yellow
Peanuts	<i>Arachis hypogaea</i>	
	<i>Cajanus cajan</i>	Pigeon pea
Peas	<i>Pisum sativum</i>	Pea, green; pea, yellow
	<i>Pisum sativum</i> var. <i>saccharatum</i>	Snowpea
Soybeans	<i>Glycine max</i>	Soybean, black; soybean, yellow; soybean, green

PC number	Phytochemical name	PC number	Phytochemical name
PC1	theaflavin	PC265	myricetin hexose = myricetin 3-O-glucoside
PC2	(β + γ)-tocopherol	PC266	myricetin hexose deoxyhexose
PC3	(epi)afzelechin	PC267	naringenin
PC4	(epi)afzelechin-3-O-glucoside	PC268	naringenin 6-C- β -D-glucoside (hemipholin)
PC5	(epi)afzelechin-4'-O-glucoside	PC269	naringenin 7-glucoside (prunin)
PC6	(epi)catechin	PC270	naringenin 7-methyl ether = sakuranetin
PC7	(epi)catechin-(4 α ->2)-phloroglucinol	PC271	naringenin 7-rutinoside
PC8	(epi)catechin-(epi)afzelechin dimer diglucoside	PC272	naringenin O-hexoside malonylated I
PC9	(epi)catechin-(epi)gallocatechin	PC273	naringenin di-C-hexoside
PC10	(epi)catechin-3-O-glucoside	PC274	naringenin O-hexoside malonylated II
PC11	(epi)catechin-7-O-glucoside	PC275	naringenin O-hexoside malonylated III
PC12	(epi)catechin-di-C-glucoside	PC276	naringenin O-hexoside malonylated IV
PC13	(epi)catechin gallate	PC277	naringin
PC14	(epi)catechin glucoside	PC278	neohesperidin di-hydrochalcone
PC15	(epi)gallocatechin	PC279	noricitrin hexoside
PC16	(epi)gallocatechin-[(epi)catechin] $\times$ 3	PC280	o-coumaric acid = 2-hydroxycinnamic acid
PC17	(epi)gallocatechin-(4 α ->2)-phloroglucinol	PC281	o-methylpiscic acid
PC18	(epi)gallocatechin-(epi)catechin	PC282	ononin
PC19	(epi)gallocatechin-(epi)gallocatechin	PC283	p-coumaric acid
PC20	(epi)gallocatechin-dihexoside	PC284	p-coumaryl aldaric acid
PC21	(epi)gallocatechin hexoside	PC285	p-hydroxyphenylacetic acid
PC22	3,4-dihydroxybenzoic acid	PC286	PAC A-type decamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC23	4-hydroxybenzoic acid = p-hydroxybenzoic acid	PC287	PAC A-type dimer [(E)C C --> A --> (E)Azf --> phloroglucinol]
PC24	11,12-deidelupanine	PC288	PAC A-type dimer [(E)C C --> A --> (E)Azf --> phloroglucinol] - C6H6O3
PC25	13-cis-lutein	PC289	PAC A-type dimer [(E)C --> A --> (E)C]
PC26	13- α -hydroxylupanine	PC290	PAC A-type dimer [(E)C C --> A --> (E)C --> phloroglucinol]
PC27	13'-cis-lutein	PC291	PAC A-type dimer [(E)C C --> A --> (E)C --> phloroglucinol] - C2H2O1
PC28	15-cis-lutein	PC292	PAC A-type dimer [(E)C C --> A --> (E)C --> phloroglucinol] - C8H8O3
PC29	2"-apiosylastragalin	PC293	PAC A-type dimer [(E)C C --> A --> (E)C --> phloroglucinol] - C8H8O4
PC30	2'-hydroxygenistein	PC294	PAC A-type dimer [(E)C --> A --> a novel flavanonol]
PC31	2x[(Epi)gallocatechin]-(epi)catechin	PC295	PAC A-type dodecamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC32	3-(1H-Indol-3-yl)-2-propenoic acid	PC296	PAC A-type heptamer [(E)C --> (E)C --> (E)C --> (E)C --> (E)C]
PC33	3',4',5,6-tetrahydroxy-methoxyflavone	PC297	PAC A-type heptamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC34	3',4',5'-trimethylrobinetin	PC298	PAC A-type heptamer [(E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC35	3-(4-hydroxyphenyl)lactic acid	PC299	PAC A-type hexamer [(E)C --> (E)C --> (E)C --> (E)C --> (E)C]
PC36	3',5,6-trihydroxy-4',7-dimethoxyflavone		
PC37	3'-O-methyl(3',4'-dihydroxybenzyl tartaric acid) (3'-O-methylfukic acid)		
PC38	3,4,5-trihydroxybenzaldehyde		
PC39	3,4-dihydroxyphenylacetic acid (DOPAC)		
PC40	3-hydroxycinnamic acid		
PC41	3-O-[R-L-rhamnopyranosyl(1f2)- β -D-galactopyranosyl(1f2)]- β -Dglucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)- β -D-glucopyranosyl(1f2)]- β -D-trihydroxyolean-12-ene		
PC42	3-O-[R-L-rhamnopyranosyl(1f2)- β -D-galactopyranosyl(1f2)]- β -Dglucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)-R-L-		

PC43	arabinopyranosyl]-3 β , 22 β , 24-trihydroxyolean-12-ene	PC300	PAC A-type hexamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
	3-O-{[R-L-rhamnopyranosyl(1f2)]- β -D-glucopyranosyl-(1f3)]- β -D-galactopyranosyl(1f2)]- β -D-glucuronopyranosyl]-22-O-[[β -D-glucopyranosyl(1f2)-R-L-arabinopyranosyl]-3 β , 22 β , 24-trihydroxyolean-12-ene	PC301	PAC A-type hexamer [(E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC44	4'- or 5'-O-Methyl-(epi)catechin hexoside	PC302	PAC A-type hexamer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC45	4'- or 5'-O-Methyl-(epi)catechin I	PC303	PAC A-type nonamer [(E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C]
PC46	6"-O-acetylglucitin	PC304	PAC A-type nonamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC47	7-rutinoside = 7-neohesperidoside of hesperetin	PC305	PAC A-type octamer [(E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C --> (E)C]
PC48	9-cis lutein	PC306	PAC A-type octamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC49	9-cis-zeaxanthin	PC307	PAC A-type pentamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC50	9'-cis lutein	PC308	PAC A-type pentamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC51	α -isolupanine	PC309	PAC A-type pentamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC52	α -tocopherol	PC310	PAC A-type pentamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC53	ajugose	PC311	PAC A-type pentamer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC54	alkaloids	PC312	PAC A-type pentamer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC55	anagiryne	PC313	PAC A-type pentamer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC56	angustifoline	PC314	PAC A-type pentamer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC57	apigenin	PC315	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC58	apigenin-6,8-di-C-glucoside (Vicenin-2)	PC316	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC59	apigenin 6-C-glucoside	PC317	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC60	apigenin 7-apioglucoside (apiin)	PC318	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC61	apigenin 7-O-glucoside	PC319	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC62	apigenin 7-O-neohesperoside	PC320	PAC A-type tetramer [(E)C --> A --> (E)C --> A --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC63	apigenin 8-C-glucoside	PC321	PAC A-type tetramer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC64	apigenin 8- or 6-C-glucoside (vitexin or isovitexin)	PC322	PAC A-type tetramer [(E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC65	apigenin disaccharide	PC323	PAC A-type tetramer [1B, 2A]
PC66	Δ^5 -avenasterol	PC324	PAC A-type trimer [(E)C --> A --> (E)C --> A --> (E)C --> A --> (E)C --> A --> (E)C]
PC67	AzII		
PC68	AzIV		
PC69	azukisaponin II		
PC70	azukisaponin IV		
PC71	azukisaponin VI		
PC72	β -sitostanol = stigmastanol		
PC73	β -sitosterol		
PC74	β -carotene		
PC75	biflavonoid [eriodictyol --> C-methyl robinetinidol]		
PC76	biochanin A		
PC77	biochanin A-7-glucoside-6-malonate		
PC78	biochanin A hexoside		
PC79	brazilin		
PC80	caffeic acid		
PC81	caffeoyl hexose		
PC82	campestanol		
PC83	campesterol		
PC84	canthoxanthine		
PC85	catechin-kaempferol dimer		
PC86	chikusetsusaponin IVa		
PC87	chlorogenic acid		
PC88	chrysin		
PC89	chrysoeriol 7- β -D-glucopyranoside		

PC90	ciceritol	PC325	A --> (E)C]
PC91	cinnamic acid		PAC A-type trimer [(E)C --> A --> (E)C -->
PC92	condensed tannins		A --> (E)CG]
PC93	convicine	PC326	PAC A-type trimer [(E)C --> A --> (E)C --> B
PC94	coumaric acid		--> (E)C]
PC95	coumaroyl hexose	PC327	PAC A-type trimer [(E)C --> A --> (E)C --> B
PC96	coumestrol		--> (E)C --> G]
PC97	coutaric acid = phaseolic acid	PC328	PAC A-type trimer [(E)C --> A --> (E)C --> B
PC98	cutaric acid hexoside		--> (E)CG]
PC99	cyanidin	PC329	PAC A-type trimer [(E)C --> B --> (E)C --> A
PC100	cyanidin-3-O-galactoside (= ideain)		--> (E)C]
PC101	cyanidin-3-O-glucoside	PC330	PAC A-type trimer [(E)C --> B --> (E)C --> B
PC102	cyanidin 3-O-(6"-malonyl)glucoside		--> (E)C]
PC103	cytisine	PC331	PAC A-type undecamer [(E)C --> A --> (E)C
PC104	D-malic acid p-coumarate = trans p-coumaroyl-malic acid		--> A --> (E)C --> B --> (E)C --> B --> (E)C -
			--> B --> (E)C --> B --> (E)C --> B --> (E)C --
			> B --> (E)C --> B --> (E)C --> B --> (E)C]
PC105	daidzein	PC332	PAC B-type dimer [(E)C --> B --> (E)C]
PC106	daidzein O-di-(hexoside malonylated)	PC333	PAC B-type dimer [(E) C --> B --> (E)C -->
PC107	daidzein O-di-hexoside I		phloroglucinol]
PC108	daidzein O-di-hexoside II	PC334	PAC B-type dimer [(E) C --> B --> (E)C -->
PC109	daidzein O-di-hexoside malonylated I		phloroglucinol] - C8H8O3
PC110	daidzein O-di-hexoside malonylated II	PC335	pelargonidin
PC111	daidzein O-di-hexoside malonylated III	PC336	pelargonidin-3,5-diglucoside
PC112	daidzein O-di-hexoside O-rhamnoside	PC337	pelargonidin 3-O-(6"-malonyl)glucoside
PC113	daidzein O-hexoside	PC338	pelargonidin 3-O-glucoside
PC114	daidzein O-hexoside acetylated	PC339	peonidin 3-O-glucoside
PC115	daidzein O-hexoside malonylated I	PC340	peonidin 3-O-malonylglucoside
PC116	daidzein O-hexoside malonylated II	PC341	petunidin
PC117	daidzein O-hexoside malonylated III	PC342	petunidin 3-O-glucoside
PC118	daidzein O-hexoside malonylated IV		petunidin dyglucoside
PC119	daidzein O-hexoside malonylated V	PC343	phaseoside I
PC120	daidzein O-hexosyl-pentoside I	PC344	phloreic acid = 3-(4-
PC121	daidzein O-hexosyl-pentoside II		Hydroxyphenyl)propionic acid
PC122	daidzin	PC345	phloretin
PC123	delphinidin	PC346	phloretin 3',5'-di-C-glucoside
PC124	delphinidin 3,5-diglucoside	PC347	phloretin di-C-hexoside
PC125	delphinidin 3-O-glucoside	PC348	phloretin glucoside
PC126	di-O-feruloylsucrose	PC349	phytic acid
PC127	digalloyl hexoside	PC350	piscidic acid
PC128	dihydrochrysin (pinocembrin)	PC351	pleargonidin rutinoside
PC129	dihydromyricetin (ampelopsin) 3'-O-B-D-glucopyranoside	PC352	pratensein
		PC353	primeveroside salicylic acid
PC130	dihydroxyl-flavone	PC354	proanthocyanidin
PC131	dimethoxy-pterocarpin-hexo-acetate	PC355	procyanidin B1 (EC-(4b,8)-C) = (epi)catechin-
PC132	elpilupinine		(epi)catechin I
PC133	eriodictyol-di-C-dihexoside	PC356	procyanidin B2 (EC-(4b,8)-EC) =
PC134	ethyl protococatechuate		(epi)catechin-(epi)catechin II
PC135	eugenol malonylglucoside (citrusin C malonate)	PC357	procyanidin B3 (C-(4a,8)-C) = (epi)catechin-
			(epi)catechin III
PC136	faralatoside	PC358	procyanidin B4 (C-(4a,8)-EC)
PC137	ferulic acid	PC359	procyanidin B5 (EC-(4b,6)-EC)
PC138	ferulic acid hexoside	PC360	procyanidin B6 (C-(4a,6)-C)
PC139	feruloyl-malic acid	PC361	procyanidin B7 (EC-(4,6)-C)
PC140	ferulyl aldaric acid	PC362	procyanidin B8 (C-(4,6)-EC)
PC141	fisetin = 5-desoxyquercetin	PC363	procyanidin C1 (EC-(4b,8)-EC-(4b,8)-EC)
PC142	flavone	PC364	procyanidin C2 (C-(4,8)-C-(4,8)-C)
PC143	formononetin-7-glucoside-6-malonate	PC365	procyanidin C3 = [(Epi)catechin]x3 II
PC144	formononetin (formonetin)	PC366	procyanidin D
PC145	fukiic acid	PC367	procyanidin dimer-7-O-diglucoside-7-O-
PC146	δ-tocopherol		

PC147	galactopinitol	PC368	glucoside
PC148	galangin	PC369	procyanidin T
PC149	gallic acid		prodelphinidin dimer [(E)C --> B --> methyl
PC150	genistein		(E) GC]
PC151	genistein O-di-(hexoside malonylated)	PC370	prodelphinidin dimer [(E)GC --> A --> (E) GC]
PC152	genistein O-di-hexoside I	PC371	prodelphinidin A-type dimer [(E) C --> A -->
PC153	genistein O-di-hexoside II		(E) GC]
PC154	genistein O-di-hexoside III	PC372	prodelphinidin A-type dimer [(E) C --> B -->
PC155	genistein O-di-hexoside malonylated I		(E) GC --> A --> luteolin or kampferol]
PC156	genistein O-di-hexoside malonylated II	PC373	prodelphinidin A-type dimer [(E) GC --> A --
PC157	genistein O-di-hexoside malonylated III		> (E) C]
PC158	genistein O-di-hexoside malonylated IV	PC374	prodelphinidin A-type dimer [(E) GC --> A --
PC159	genistein O-di-hexoside malonylated V		> (E) GC]
PC160	genistein O-di-hexoside O-rhamnoside	PC375	prodelphinidin A-type dimer [(E) C --> A -->
PC161	genistein O-hexoside		5-O-methyl (E)GC]
PC162	genistein O-hexoside acetylated	PC376	prodelphinidin A-type tetramer [(E) C --> A -
PC163	genistein O-hexoside malonylated I		--> (E)GC --> A --> (E) C--> A --> (E) C]
PC164	genistein O-hexoside malonylated II	PC377	prodelphinidin A-type trimer [(E) GC --> A --
PC165	genistein O-hexoside malonylated III		> (E) C --> A --> (E) C]
PC166	genistein O-hexoside malonylated IV	PC378	prodelphinidin B-type dimer [(E) GC --> B --
PC167	genistein O-hexoside malonylated V		> (E) C]
PC168	genistein O-hexoside malonylated VI	PC379	prodelphinidin B3
PC169	genistein O-hexoside malonylated VII	PC380	prodelphinidin B4
PC170	genistein O-hexosyl-pentoside I	PC381	propelargonidin A-type dimer [(E)Afz --> A--
PC171	genistein O-hexosyl-pentoside II		> (E)C --> A --> (E)C]
PC172	genistin	PC382	propelargonidin A-type dimer [(E)C --> A-->
PC173	genkwanin		(E)Afz]
PC174	gentisic acid = 2,5-dihydroxybenzoic acid	PC383	propelargonidin A-type dimer [(E)C--> A-->
			(E)Afz--> B -->(E)C]
PC175	geraldone	PC384	prorobinetidin A-type dimer [(E)C --> A -->
PC176	glucopyranosyl rutin		C-methyl (epi)robinetinidol]
PC177	glycitein	PC385	protocatechuic acid
PC178	glycitein O-di-hexoside	PC386	protocatechuic acid derivat
PC179	glycitein O-di-hexoside malonylated I	PC387	protocatechuic acid hexoside
PC180	glycitein O-di-hexoside malonylated II	PC388	quercetin
PC181	glycitein O-di-hexoside malonylated III	PC389	quercetin-3-O-glucosylglucoside (baimaside)
PC182	glycitein O-hexoside I		= quercetin-3-O-diglucoside
PC183	glycitein O-hexoside II	PC390	quercetin 7-O-glucoside = quercetin hexose
PC184	glycitein O-hexoside malonylated I	PC391	quercetin-acetyl-rhamnoside
PC185	glycitein O-hexoside malonylated II	PC392	quercetin-rhamnosyl acetyl-hexoside-
PC186	glycitein O-hexoside malonylated III		rhamnoside
PC187	glycitein O-hexoside malonylated IV	PC393	quercetin 3-(6''-acetyl-galactoside)-7-
PC188	glycitein O-hexoside malonylated V		rhamnoside
PC189	glycitin	PC394	quercetin 3(2G-rhamnosylrutinoside)
PC190	hesperetin	PC395	quercetin 3,7-di-O-rhamnoside = quercetin-3-
PC191	hesperidin		rhamnoside-7-rhamnoside = quercetin-3-O-
PC192	hexosyl rutin		rhamnoside-7-O-rhamnoside
PC193	homoeiodictyol	PC396	quercetin 3- α -L-arabinoside (Avicularin) =
PC194	homoeiodictyol-(hydroxybutanoyl)	PC397	quercetin-arabinofuranoside
	hexosyl-hexoside	PC398	quercetin 3-glycoside
PC195	homovanillic acid	PC399	quercetin 3-O-(2-glucosile)-glucoside-7-
PC196	homovanillic acid hexoside		rhamnoside
PC197	hydrolysisable tannins	PC400	quercetin 3-O-(6''-O-malonyl)glucoside
PC198	hydroxybenzyl-malic acid = eucomic acid	PC401	quercetin 3-O-6''-acetylglucoside
		PC402	quercetin 3-O- β -xylopyransyl-(1 $\rightarrow$ 2)-O- α -
PC199	hydroxyeucomic acid		rhamnopyranoside
PC200	hydroxyl benzoic acid = salicylic acid	PC403	quercetin 3-O-galactoside
PC201	hydroxytrimesic acid trimethyl ester	PC404	quercetin 3-O-glucopyranoside
PC202	isosakuranetin = 4'-Methylnaringenin	PC405	quercetin 3-O-glucoside = quercetin hexose
PC203	isoschaftoside	PC406	quercetin 3-O-glucoside acetate

PC204	kaempferol	PC407	quercetin 3-O-rhamnoside = quercitrin
PC205	kaempferol 3-rutinoside-7-glucoside	PC408	quercetin 3-O-rutinoside (=rutin)
PC206	kaempferol-3,7-diglucoside	PC409	quercetin 3-O-xylosyloglucoside
PC207	kaempferol-3-O-rhamnoside (afzelin)	PC410	quercetin 3-xyloside
PC208	kaempferol-3-robinoside-7-rhamnoside	PC411	quercetin deoxyhexose
PC209	kaempferol-3-rutinoside (= rhamnoglucoside)	PC412	quercetin deoxyhexose hexose
PC210	kaempferol-acetyl-glucoside-rhamnoside	PC413	quercetin derivate
PC211	kaempferol-acetyl-hexoside	PC414	quercetin di-deoxyhexose hexose
PC212	kaempferol-arabinosyl-rhamnoside	PC415	quercetin di-hexoside
PC213	kaempferol-hexoside-rhamnoside	PC416	quercetin di-rhamnoside
PC214	kaempferol-xylo-pyranosyl-rhamnoside-rhamnoside	PC417	quercetin hexose deoxyhexose
PC215	kaempferol 3-(2G-xylosylrutinoside)	PC418	quercetin methyl ether = isorhamnetin
PC216	kaempferol 3-O-(6''-O-malonyl)glucoside	PC419	quercetin pentose deoxyhexose
PC217	kaempferol 3-O-(6-rhamnosyl)-galactoside-7-rhamnoside	PC420	raffinose
PC218	kaempferol 3-O-(malonyl)glucoside	PC421	resveratrol
PC219	kaempferol 3-O- α -L-arabinopyranosyl-7-O- α -L-rhamnopyranoside	PC422	resveratrol 3-O- β -D-glucoside
PC220	kaempferol 3-O-glucoside	PC423	resveratrol 5-O-glucoside
PC221	kaempferol 3-O-xylosyloglucoside	PC424	rhoifolin glucoside
PC222	kaempferol 3-rhamnosyl-acetyl-galactoside-rhamnoside	PC425	robinetin = 5-deoxymyricetin
PC223	kaempferol 3-sambubioside	PC426	rosmaniric acid
PC224	kaempferol 7-O-glucoside	PC427	salicylic acid O-glucoside
PC225	kaempferol deoxyhexose	PC428	secoisolariciresinol
PC226	kaempferol di-hexoside	PC429	sibiricose A3
PC227	kaempferol di-rhamnoside	PC430	sinapic acid
PC228	kaempferol glycoside	PC431	sinapoyl hexose
PC229	kaempferol hexose deoxyhexose	PC432	sissotrin
PC230	kaempferol O-di-hexoside malonylated I	PC433	soyasaponin A1
PC231	kaempferol O-di-hexoside malonylated II	PC434	soyasaponin A2
PC232	kaempferol O-hexoside, O-hexosyl-rhamnoside	PC435	soyasaponin Ab
PC233	kaempferol O-hexoside malonylated	PC436	soyasaponin α g
PC234	kaempferol O-hexosyl-rhamnoside I	PC437	soyasaponin β a
PC235	kaempferol O-hexosyl-rhamnoside II	PC438	soyasaponin Bc' = soyasaponin Bx
PC236	kaempferol O-rhamnoside, O-hexosyl-rhamnoside hexoside	PC439	soyasaponin Bd = sandosaponin A
PC237	kaempferol tetraglycoside	PC440	soyasaponin Be = dehidrosoyasaponin
PC238	kaempferol triglycoside	PC441	soyasaponin Bf
PC239	lablaboside D	PC442	soyasaponin VI = soyasaponin β g
PC240	lablaboside E	PC443	soyasaponin Bh
PC241	lablaboside F	PC444	soyasaponin I = soyasaponin Bb
PC242	lupanine	PC445	soyasaponin II = soyasaponin Bc
PC243	luteolin	PC446	soyasaponin III = soyasaponin Bb'
PC244	luteolin-3,7-diglucoside	PC447	soyasaponin IV
PC245	luteolin 4-glucoside	PC448	soyasaponin V = soyasaponin Ba
PC246	luteolin 6-glucoside (isoorientin)	PC449	soyasaponin γ a
PC247	luteolin 7-glucoside	PC450	soyasaponin γ g
PC248	luteolin 8-C-glucoside = orientin	PC451	soyasaspogenol A
PC249	luteolin glycoside	PC452	soyasaspogenol B
PC250	luteolin methyl ether = diosmetin	PC453	sparteine
PC251	malvidin	PC454	squalene
		PC455	stachyose
		PC456	stigmaterol
		PC457	synapil aldaric acid
		PC458	syringic acid
		PC459	syringic acid hexoside
		PC460	taraxafolin B
		PC461	taxifolin
		PC462	taxifolin-3-O-rhamnoside (=dihydroquercetin)
		PC463	taxifolin hexoside
		PC464	trans-3,3',5,5'-tetrahydroxy-4'-methoxystilbene
		PC465	trans-lutein
		PC466	

PC252	malvidin 3-O-acetylglucoside	PC467	trans-p-coumaric acid
PC253	malvidin 3-O-glucoside	PC468	trans-piceatannol
PC254	malvidin dyglicoside	PC469	trans-resveratrol
PC255	matairesinol	PC470	trans-zeaxantin
PC256	medicarpin	PC471	trihydroxyacetophenone-di-C-hexoside
PC257	methylferulic acid	PC472	trihydroxyl-flavone
PC258	methylfukiic acid	PC473	vanillic acid
PC259	methyl gallate	PC474	vanillic acid β -glucoside
PC260	morin	PC475	vanillin hexoside
PC261	myricitrin I	PC476	verbascose
PC262	myricetin	PC477	vicine
PC263	myricetin 3-O-rhamnoside	PC478	xanthophyll = lutein
PC264	myricetin di-O-hexose = myricetin di-hexoside		

Table S3. Families and phytochemicals described in legumes.

Phytochemical group	Compounds
Phytic acid	phytic acid (PC351)
RFOs	ajugose (PC53); ciceritol (PC90); raffinose (PC421); stachyose (PC456); verbascose (PC476)
Saponins	3-O-[R-L-rhamnopyranosyl(1f2)-β-D-galactopyranosyl(1f2)]-β-D-glucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)-R-L-arabinopyranosyl]-3β, 22β,24-trihydroxyolean-12-ene (PC42); 3-O-[R-L-rhamnopyranosyl(1f2)-β-D-galactopyranosyl(1f2)]-β-D-glucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)-β-D-glucopyranosyl]-3β, 22β,24-trihydroxyolean-12-ene (PC41); 3-O-[R-L-rhamnopyranosyl(1f2)-[β-D-glucopyranosyl(1f3)-β-D-galactopyranosyl(1f2)]-β-D-glucuronopyranosyl]-22-O-[β-D-glucopyranosyl(1f2)-R-L-arabinopyranosyl]-3β,22β,24-trihydroxyolean-12-ene (PC43); AzII (PC67); AzIV (PC68); azukisaponin II (PC69); azukisaponin IV (PC70); azukisaponin VI (PC71); chikusetsusaponin Iva (PC86); lablaboside D (PC239); lablaboside E (PC240); lablaboside F (PC241); phaseoside I (PC345); soyasaponin A1 (PC434); soyasaponin A2 (PC435); soyasapogenol B (PC453); soyasaponin Ab (PC436); soyasaponin I = soyasaponin Bb (PC445); soyasaponin II = soyasaponin Bc (PC446); soyasaponin III = soyasaponin Bb' (PC447); soyasaponin IV (PC448); soyasaponin V = soyasaponin Ba (PC449); soyasaponin αg (PC437); soyasaponin βa (PC438); soyasaponin Bc' = soyasaponin Bx (PC439); soyasaponin Bd = sandosaponin A (PC440); soyasaponin Be = dehidrosoyasaponin (PC441); soyasaponin Bf (PC442); soyasaponin VI = soyasaponin βg (PC443); soyasaponin Bh (PC444); soyasaponin γa (PC450); soyasaponin γg (PC451); soyasapogenol A (PC452)
Phytosterols	campestanol (PC82); campesterol (PC83); stigmasterol (PC457); β-sitostanol = stigmastanol (PC72); β-sitosterol (PC73); Δ5-avenasterol (PC66)
Tocopherols	α-tocopherol (PC52); (β+γ)-tocopherol (PC2); δ-tocopherol (PC146)
Alkaloids	alkaloids (PC54); angustifoline (PC56); anagiryne (PC55); convicine (PC93); cytosine (PC103); elpilupinine (PC132); lupanine (PC242); sparteine (PC454); vicine (PC477); α-isolupanine (PC51); 11,12-deidelupanine (PC24); 13-α-hydroxylupanine (PC26)
Carotenoids	β-carotene (PC74); xanthophyll = lutein (PC478); 13'-cis-lutein (PC27); 13-cis-lutein (PC25); 15-cis-lutein (PC28); 9-cis-lutein (PC48); 9'-cis-lutein (PC50); 9-cis-zeaxanthin (PC49); canthoxanthine (PC84); trans-lutein (PC466); trans-zeaxanthin (PC470)
Lignans	matairesinol (PC255); secoisolariciresinol (PC429)
Coumestans	coumestrol (PC96)
Stilbenes	resveratrol (PC422); resveratrol-3-O-β-D-glucoside (PC423); resveratrol-5-O-glucoside (PC424); trans-3,3',5,5'-tetrahydroxy-4'-methoxystilbene (PC465); trans-piceatannol (PC468); trans-resveratrol (PC469)
Dihydrochalcones	phloretin (PC347); phloretin glucoside (PC350); phloretin 3',5'-di-C-glucoside (PC348); phloretin di-C-hexoside (PC349)
Pterocarpan	dimethoxy-pterocarpan-hexo-acetate (PC131); medicarpin (PC256)
Phenolic acids	3-(1H-Indol-3-yl)-2-propenoic acid (PC32); 3-(4-hydroxyphenyl)lactic acid (PC35); 3,4,5-trihydroxybenzaldehyde (PC38); 3,4-dihydroxyphenylacetic acid (DOPAC) (PC39); 3,4-dihydroxybenzoic acid (PC22); 4-hydroxybenzoic acid = p-hydroxybenzoic acid (PC23); 3'-O-methyl(3',4'-dihydroxybenzyl tartaric acid) (3'-O-methylfukiic acid) (PC37); 3-hydroxycinnamic acid (PC40); coumaric acid = phaseolic acid (PC97); coumaric acid hexoside (PC98); digalloyl hexoside (PC127); di-O-feruloylsucrose (PC126); D-malic acid p-coumarate = trans p-coumaroyl-malic acid (PC104); ethyl protocatechuate (PC134); eugenol

	malonylglucoside (citrusin C malonate) (PC135); gentisic acid = 2,5-dihydroxybenzoic acid (PC174); o-coumaric acid = 2-hydroxycinnamic acid (PC280); p-hydroxyphenylacetic acid (PC285); primeveroside salicylic acid (PC355); p-coumaric acid (PC283); caffeic acid (PC80); caffeoyl hexose (PC81); chlorogenic acid (PC87); cinnamic acid (PC91); coumaric acid (PC94); coumaroyl hexose (PC95); ferulic acid (PC137); ferulic acid hexoside (PC138); feruloyl malic acid (PC139); ferulyl aldaric acid (PC140); fukiic acid (PC145); galactopinitol (PC147); gallic acid (PC149); homovanillic acid (PC195); homovanillic acid hexoside (PC196); hydroxybenzyl-malic acid = eucomic acid (PC198); hydroxyeucomic acid (PC199); hydroxyl benzoic acid = salicylic acid (PC200); hydroxytrimesic acid trimethyl ester (PC201); methyl gallate (PC259); methylferulic acid (PC257); methylfukiic acid (PC258); o-methylpiscidic acid (PC281); vanillin hexoside (PC475); piscidic acid (PC352); protocatechuic acid (PC387); protocatechuic acid derivate (PC388); p-coumaroyl aldaric acid (PC284); phloretic acid = 3-(4-hydroxyphenyl)propionic acid (PC346); protocatechuic acid hexoside (PC389); rosmarinic acid (PC427); salicylic acid O-glucoside (PC428); sibiricose A3 (PC430); sinapic acid (PC431); synapil aldaric acid (PC458); sinapoyl hexose (PC432); syringic acid (PC459); syringic acid hexoside (PC460); taraxafolin B (PC461); trans-p-coumaric acid (PC467); trihydroxyacetophenone-di-C-hexoside (PC471); vanillic acid (PC473); vanillic acid β -glucoside (PC474)
Isoflavones	biochanin A-7-glucoside-6-malonate (PC77); daidzein O-di-(hexoside malonylated) (PC106); daidzein O-di-hexoside malonylated I (PC109); 6"-O-acetylglucitin (PC46); 2'-hydroxygenistein (PC30); biochanin A (PC76); biochanin A hexoside (PC78); daidzein (PC105); daidzein O-hexoside (PC113); daidzein O-di-hexoside I (PC107); daidzein O-di-hexoside II (PC108); daidzein O-hexosyl-pentoside I (PC120); daidzein O-hexosyl-pentoside II (PC121); daidzein O-hexoside acetylated (PC114); daidzin (PC122); formononetin = formononetin (PC144); formononetin-7-glucoside-6-malonate (PC143); genistein (PC150); genistein O-di-hexoside I (PC152); genistein O-di-hexoside II (PC153); genistein O-di-hexoside III (PC154); genistein O-hexoside (PC161); genistein O-hexoside acetylated (PC162); genistein O-hexosyl-pentoside I (PC170); genistein O-hexosyl-pentoside II (PC171); genistin (PC172); glycitein (PC177); glycitein O-di-hexoside (PC178); glycitein O-hexoside I (PC182); glycitein O-hexoside II (PC183); glycitin (PC189); daidzein O-di-hexoside malonylated II (PC110); daidzein O-di-hexoside malonylated III (PC111); daidzein O-di-hexoside O-rhamnoside (PC112); daidzein O-hexoside malonylated I (PC115); daidzein O-hexoside malonylated II (PC116); daidzein O-hexoside malonylated III (PC117); daidzein O-hexoside malonylated IV (PC118); daidzein O-hexoside malonylated V (PC119); genistein O-di-(hexoside malonylated) (PC151); genistein O-di-hexoside malonylated I (PC155); genistein O-di-hexoside malonylated II (PC156); genistein O-di-hexoside malonylated III (PC157); genistein O-di-hexoside malonylated IV (PC158); genistein O-di-hexoside malonylated V (PC159); genistein O-di-hexoside O-rhamnoside (PC160); genistein O-hexoside malonylated I (PC163); genistein O-hexoside malonylated II (PC164); genistein O-hexoside malonylated III (PC165); genistein O-hexoside malonylated IV (PC166); genistein O-hexoside malonylated V (PC167); genistein O-hexoside malonylated VI (PC168); genistein O-hexoside malonylated VII (PC169); glycitein O-di-hexoside malonylated I (PC179); glycitein O-di-hexoside malonylated II (PC180); glycitein O-di-hexoside malonylated III (PC181); glycitein O-hexoside malonylated I (PC184); glycitein O-hexoside malonylated II (PC185); glycitein O-hexoside malonylated III (PC186); glycitein O-hexoside malonylated IV (PC187); glycitein O-hexoside malonylated V (PC188); ononin (PC282); pratensein (PC354); sissotrin (PC433)
Flavones	3',4',5,6-tetrahydroxy-methoxyflavone (PC33); 3',4',5'-trimethylrobinetin (PC34); 3',5,6-trihydroxy-4',7-dimethoxyflavone (PC36); apigenin (PC57); apigenin 6-C-glucoside (PC59); apigenin-7-apioglucoside = apiin (PC60); apigenin-7-O-neohesperoside (PC62); apigenin 8- or 6-C-glucoside = vitexin = isovitexin (PC64); apigenin-6,8-di-C-glucoside = vicianin-2 (PC58); chrysoeriol 7- β -D-glucopyranoside (PC89); luteolin 6-glucoside = isoorientin (PC246); luteolin 8-C-glucoside = orientin (PC248); luteolin methyl ether = diosmetin = chrysoeriol (PC250); luteolin-3,7-diglucoside (PC244); noricarinin hexoside (PC279); apigenin 8-C-glucoside (PC63); apigenin disaccharide (PC65); apigenin-7-O-glucoside (PC61); chrysin (PC88); dihydroxyl-flavone (PC130); eriodictyol-di-C-dihexoside (PC133); flavone (PC142); genkwanin (PC173); geraldone (PC175); isoschaftoside (PC203); luteolin (PC243); luteolin 4-glucoside (PC245); luteolin-7-glucoside (PC247); luteolin glycoside

	(PC249); trihydroxyl-flavone (PC472); rhoifolin glucoside (PC425)
Flavanones	7-rutinoside = 7-neohesperidoside of hesperetin (PC47); dihydrochrysin = pinocembrin (PC128); homoeriodictyol (PC193); homoeriodictyol-(hydroxybutanoyl) hexosyl-hexoside (PC194); isosakuranetin = 4'-methylnaringenin (PC202); naringenin 6-C-β-D-glucoside = hemipholin (PC268); naringenin-7-methyl eter = sakuranetin (PC270); naringenin O-hexoside malonylated I (PC272); naringenin O-hexoside malonylated II (PC274); naringenin O-hexoside malonylated III (PC275); naringenin O-hexoside malonylated IV (PC276); hesperetin (PC190); hesperidin (PC191); myricitrin I (PC261); naringenin (PC267); naringenin-7-glucoside = prunin (PC269); naringenin-7-rutinoside (PC271); naringenin di-C-hexoside (PC273); naringin (PC277); neohesperidin di-hydrochalcone (PC278)
Flavanonols	dihydromyricetin (ampelopsin) 3'-O-B-D-glucopyranoside (PC129); taxifolin-3-O-rhamnoside = dihydroquercetin (PC463); taxifolin (PC462); taxifolin hexoside (PC464)
Flavonols	kaempferol-3-(2G-xylosylrutinoside) (PC215); kaempferol-3-O-(6''-O-malonyl)glucoside (PC216); kaempferol-3-O-(6-rhamnosyl)-galactoside-7-rhamnoside (PC217); kaempferol-3-O-(malonyl)glucoside (PC218); kaempferol-3-O-rhamnoside = afzelin (PC207); kaempferol-3-O-xylosylglucoside (PC221); kaempferol-3-O-α-L-arabinopyranosyl-7-O-α-L-rhamnopyranoside (PC219); kaempferol-3-rhamnosyl-acetyl-galactoside-rhamnoside (PC222); kaempferol hexose deoxyhexose (PC229); kaempferol O-di-hexoside malonylated I (PC230); kaempferol O-di-hexoside malonylated II (PC231); kaempferol O-hexoside malonylated (PC233); kaempferol O-hexoside, O-hexosyl-rhamnoside (PC232); kaempferol O-hexosyl-rhamnoside I (PC234); kaempferol O-hexosyl-rhamnoside II (PC235); kaempferol O-rhamnoside, O-hexosyl-rhamnoside hexoside (PC236); kaempferol-3-rutinoside-7-glucoside (PC205); kaempferol-3-robinoside-7-rhamnoside (PC208); kaempferol-acetyl-glucoside-rhamnoside (PC210); kaempferol-acetyl-hexoside (PC211); kaempferol-arabinosyl-rhamnoside (PC212); kaempferol-hexoside-rhamnoside (PC213); kaempferol-xylo-pyranosyl-rhamnoside-rhamnoside (PC214); myricetin hexose deoxyhexose (PC266); quercetin-3-(2G-rhamnosylrutinoside) (PC396); quercetin-3-(6''-acetyl)galactoside-7-rhamnoside (PC395); quercetin-3,7-di-O-rhamnoside = quercetin-3-rhamnoside-7-rhamnoside = quercetin-3-O-rhamnoside-7-O-rhamnoside (PC397); quercetin-3-O-(2-glucosyl)-glucoside-7-rhamnoside (PC400); quercetin-3-O-(6''-O-malonyl)glucoside (PC401); quercetin-3-O-6''-acetylglucoside (PC402); quercetin-3-O-glucopyranoside (PC405); quercetin-3-O-glucoside acetate (PC407); quercetin-3-O-xylosylglucoside (PC410); quercetin-3-O-β-xylopyransyl-(1→2)-O-α-rhamnopyranoside (PC403); quercetin-3-α-L-arabinoside (avicularin) = quercetin-arabinofuranoside (PC398); quercetin deoxyhexose hexose (PC413); quercetin di-deoxyhexose hexose (PC415); quercetin hexose deoxyhexose (PC418); quercetin pentose deoxyhexose (PC420); quercetin-3-O-glucosylglucoside = baimaside = quercetin-3-O-diglucoside (PC391); quercetin-rhamnosyl acetyl-hexoside-rhamnoside (PC394); 2''-apiosylastragalin (PC29); faralatoside (PC136); fisetin = 5-desoxyquercetin (PC141); galangin (PC148); glucopyranosyl rutin (PC176); hexosyl rutin (PC192); kaempferol (PC204); kaempferol glycoside (PC228); kaempferol-3-O-glucoside (PC220); kaempferol-3-rutinoside = rhamnoglucoside (PC209); kaempferol-3-sambubioside (PC223); kaempferol-7-O-glucoside (PC224); quercetin-acetyl-rhamnoside (PC393); kaempferol deoxyhexose (PC225); kaempferol di-hexoside (PC226); kaempferol di-rhamnoside (PC227); kaempferol tetraglycoside (PC237); kaempferol triglycoside (PC238); kaempferol-3,7-diglucoside (PC206); morin (PC260); myricetin (PC262); myricetin 3-O-rhamnoside (PC263); myricetin di-O-hexose = myricetin di-hexoside (PC264); myricetin hexose = myricetin-3-O-glucoside (PC265); quercetin (PC390); quercetin-3-glycoside (PC399); quercetin-3-O-galactoside (PC404); quercetin-3-O-glucoside = quercetin hexose (PC406); quercetin-3-O-rhamnoside = quercitrin (PC408); quercetin-3-O-rutinoside = rutin (PC409); quercetin-3-xyloside (PC411); quercetin-7-O-glucoside = quercetin hexose (PC392); quercetin deoxyhexose (PC412); quercetin derivate (PC414); quercetin di-hexoside (PC416); quercetin di-rhamnoside (PC417); quercetin methyl ether = isorhamnetin (PC419); robinetin = 5-deoxymyricetin (PC426)
Flavanols (flavan-3-ols)	4'- or 5'-O-methyl-(epi)catechin hexoside (PC44); 4'- or 5'-O-methyl-(epi)catechin I (PC45); (epi)afzelechin (PC3); (epi)afzelechin-3-O-glucoside (PC4); (epi)afzelechin-4'-O-glucoside (PC5); (epi)catechin (PC6); (epi)catechin gallate (PC13); (epi)catechin glucoside (PC14);

	(epi)catechin-(4 α →2)-phloroglucinol (PC7); (epi)catechin-3-O-glucoside (PC10); (epi)catechin-7-O-glucoside (PC11); (epi)catechin-di-C-glucoside (PC12); (epi)gallo catechin (PC15); (epi)gallo catechin-dihexoside (PC20); (epi)gallo catechin hexoside (PC21); (epi)gallo catechin-(4 α →2)-phloroglucinol (PC17); catechin-kaempferol dimer (PC85)
Anthocyanidins	cyanidin-3-O-(6"-malonyl)glucoside (PC102); cyanidin-3-O-galactoside = ideain (PC100); malvidin-3-O-acetylglucoside (PC252); delphinidin-3,5-diglucoside (PC124); pelargonidin-3-O-glucoside (PC339); pelargonidin-3,5-diglucoside (PC337); peonidin-3-O-malonylglucoside (PC341); pelargonidin-3-O-(6"-malonyl)glucoside (PC338); delphinidin (PC123); malvidin (PC251); cyanidin (PC99); pelargonidin (PC336); petunidin (PC342); cyanidin-3-O-glucoside (PC101); malvidin-3-O-glucoside (PC253); malvidin dyglicoside (PC254); delphinidin-3-O-glucoside (PC125); pelargonidin rutinoside (PC353); peonidin-3-O-glucoside (PC340); petunidin-3-O-glucoside (PC343); petunidin dyglicoside (PC344)
Hydrolysable tannins	hydrolysable tannins (PC197)
Condensed tannins	(-)-theaflavin (PC1); (epi)catechin-(epi)afzelechin dimer diglucoside (PC8); (epi)catechin-(epi)gallo catechin (PC9); (epi)gallo catechin-(epi)catechin (PC18); (epi)gallo catechin-(epi)gallo catechin (PC19); (epi)gallo catechin-[(epi)catechin]×3 (PC16); 2×[(epi)gallo catechin]-(epi)catechin (PC31); biflavonoid [eriodictyol→C-methyl robinetinidol] (PC75); condensed tannins (PC92); PAC A-type decamer (PC286); PAC A-type dimer [8 types] (PC287-294); PAC A-type dodecamer (PC295); PAC A-type heptamer [3 types] (PC296-298); PAC A-type hexamer [4 types] (PC299-302); PAC A-type nonamer [2 types] (PC303-304); procyanidin B1 (EC-(4b,8)-C) (PC357); procyanidin B2 (EC-(4b,8)-EC) (PC358); procyanidin B3 (C-(4a,8)-C) (PC359); procyanidin B4 (C-(4a,8)-EC) (PC360); procyanidin B5 (EC-(4b,6)-EC) (PC361); procyanidin B6 (C-(4a,6)-C) (PC362); procyanidin B7 (EC-(4,6)-C) (PC363); procyanidin B8 (C-(4,6)-EC) (PC364); procyanidin C1 (EC-(4b,8)-EC-(4b,8)-EC) (PC365); procyanidin C2 (C-(4,8)-C-(4,8)-C) (PC366); procyanidin C3 = [(Epi)catechin]×3 II (PC367); procyanidin D (PC368); procyanidin dimer-7-O-diglucoside-7-O-glucoside (PC369); procyanidin T (PC370); prodelfphinidin dimer [2 types] (PC371-372); prodelfphinidin A-type dimer [5 types] (PC373-377); prodelfphinidin A-type tetramer (PC378); PAC A-type octamer [2 types] (PC305-306); PAC A-type pentamer [9 types] (PC307-315); PAC A-type tetramer [9 types] (PC316-324); PAC A-type trimer [7 types] (PC325-331); PAC A-type undecamer (PC332); PAC B-type dimer [3 types] (PC333-335); proanthocyanidin (PC356); prodelfphinidin A-type trimer (PC379); prodelfphinidin B3 (PC381); prodelfphinidin B4 (PC382); prodelfphinidin B-type dimer (PC380); propelargonidin A-type dimer [3 types] (PC383-385); prorobinetidin A-type dimer (PC386)
Others	brazilin (PC79)
	squalene (PC455)

PAC: proanthocyanidins

Table S4. Potential discriminant phytochemicals of each group of legumes according to the bibliographic search.

[illegible]

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	3β,22β,24-trihydroxyolean-12-ene; 3-O-[R-L-rhamnopyranosyl(1f2)-β-D-galactopyranosyl(1f2)]-β-D-glucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)-R-L-arabinopyranosyl]-3β, 22β,24-trihydroxyolean-12-ene; 3-O-[R-L-rhamnopyranosyl(1f2)-β-D-galactopyranosyl(1f2)]-β-D-glucuronopyranosyl-22-O-[R-L-rhamnopyranosyl(1f2)-β-D-glucopyranosyl]-3β, 22β,24-trihydroxyolean-12-ene	
Beans	sparteine; lupanine; elpilupinine; cytosine; convicine; angustifoline; anagiryne; α-isolupanine; 13-α-hydroxylupanine; 11,12-deidrelupanine; vicine; petunidin dyglicoside; petunidin-3-O-glucoside; petunidin; peonidin-3-O-malonylglycoside; peonidin-3-O-glucoside; pelargonidin-3-O-glucoside; pelargonidin-3-O-(6"-malonyl)glucoside; pelargonidin-3,5-diglucoside; pelargonidin; cyanidin; malvidin dyglicoside; malvidin-3-O-glucoside; malvidin-3-O-acetylglycoside; malvidin; delphinidin-3-O-glucoside; delphinidin; cyanidin-3-O-(6"-malonyl)glucoside; prodelphinidin B4; procyanidin dimer-7-O-diglucoside-7-O-glucoside; procyanidin C3 = [(epi)catechin]×3 II; procyanidin C1 (EC-(4b,8)-EC-(4b,8)-EC); procyanidin B8 (C-(4,6)-EC); procyanidin B5 (EC-(4b,6)-EC); procyanidin B4 (C-(4a,8)-EC); proanthocyanidins; primeveroside salicylic acid; 2×[(epi)galocatechin]-(epi)catechin; (epi)galocatechin-dihexoside; (epi)galocatechin-(epi)galocatechin; (epi)galocatechin-(epi)catechin; (epi)galocatechin-[(epi)catechin]×3; (epi)catechin-(epi)afzelechin dimer diglucoside; (-)-theaflavin; phloretin glucoside; phloretin di-C-hexoside; phloretin 3',5'-di-C-glucoside; phloretin; catechin-kaempferol dimer; 4'- or 5'-O-methyl-(epi)catechin I; 4'- or 5'-O-methyl-(epi)catechin hexoside; (epi)galocatechin hexoside; (epi)catechin-di-C-glucoside; (epi)catechin-7-O-glucoside; (epi)catechin-(epi)galocatechin; (epi)afzelechin-3-O-glucoside; (epi)afzelechin-4'-O-glucoside; neohesperidin di-hydrochalcone; naringenin di-C-hexoside; naringenin-7-rutinoside; naringenin-7-methyl eter (= sakuranetin); naringenin-7-glucoside (= prunin); naringenin 6-C-β-D-glucoside (= hemipholin); homoeriodictyol-(hydroxybutanoyl) hexosyl-hexoside; 7-rutinoside (= 7-neohesperidoside of hesperetin); taxifolin hexoside; trihydroxyl-flavone; rhoifolin glucoside; noricarinin hexoside; isoschaftoside; geraldone; eriodictyol-di-C-dihexoside; dihydroxyflavone; chrysoeriol 7-β-D-glucopyranoside; apigenin disaccharide; apigenin 8- or 6-C-glucoside (= vitexin or isovitexin); apigenin-7-O-neohesperoside; apigenin-6,8-di-C-glucoside (vicenin-2); quercetin pentose deoxyhexose; quercetin hexose deoxyhexose; quercetin di-rhamnoside; quercetin di-hexoside; quercetin di-deoxyhexose hexose; quercetin derivate; quercetin deoxyhexose; quercetin deoxyhexose hexose; quercetin-3-O-xylosyloglucoside; quercetin-3-O-glucoside; quercetin-3-O-β-xylopyranosyl-(1→2)-O-α-rhamnopyranoside; quercetin-3-O-6"-acetylglycoside; quercetin-3-O-(6"-malonyl)glucoside; quercetin-3-O-(2-glucosile)-glucoside-7-rhamnoside; quercetin-3-glycoside; quercetin-3-α-L-arabinoside (= avicularin = quercetin-arabinofuranoside); quercetin-3-(2G-rhamnosylrutinoside); quercetin-3-O-6"-acetylglycoside; quercetin-rhamnosyl acetyl-hexoside-rhamnoside; quercetin-acetyl-rhamnoside; quercetin-7-O-glucoside (= quercetin hexose); myricetin hexose deoxyhexose; myricetin hexose (= myricetin 3-O-glucoside); myricetin di-O-hexose (= myricetin di-hexoside); myricitrin I; kaempferol hexose deoxyhexose; kaempferol glycoside; kaempferol di-rhamnoside; kaempferol deoxyhexose; kaempferol-7-O-glucoside; kaempferol-3-sambubioside; kaempferol-3-rhamnosyl-acetyl-galactoside-rhamnoside; kaempferol-3-O-xylosyloglucoside; kaempferol-3-O-α-L-arabinopyranosyl-7-O-α-L-rhamnopyranoside; kaempferol-3-O-(malonyl)glucoside; kaempferol-3-O-(6-rhamnosyl)-galactoside-7-rhamnoside; kaempferol-3-O-(6"-O-malonyl)glucoside; kaempferol-3-(2G-xylosylrutinoside); kaempferol-xylo-pyranosyl-rhamnoside-rhamnoside; kaempferol-hexoside-rhamnoside; kaempferol-arabinosyl-rhamnoside; kaempferol-acetyl-hexoside; kaempferol-acetyl-glucoside-rhamnoside; kaempferol-3-rutinoside-7-glucoside; kaempferol-3-O-rhamnoside (= afzelin); kaempferol-3,7-diglucoside; kaempferol-3-rutinoside (= rhamnoglucoside); hexosyl rutin; glucopyranosyl rutin; faralatoside; dihydromyricetin (ampelopsin) 3'-O-B-D-glucopyranoside; dihydrochrysin (= pinocembrin); hydrolysable tannins; vanillin hexoside; vanillic acid β-glucoside; trihydroxyacetophenone-di-C-hexoside; taraxafolin B; syringic acid hexoside; synapil aldaric acid; sinapoyl hexose; sibiricoseA3; salicylic acid O-glucoside;	6,11,25– 34,15,35– 37,18–24

	protocatechuic acid hexoside; pelargonidin rutinoside; piscidic acid; p-coumaroyl aldaric acid; o-methylpiscidic acid; methylfukiic acid; methylferulic acid; hydroxytrimesic acid trimethyl ester; hydroxyeucomic acid; hydroxybenzyl-malic acid (= eucomic acid); homovanillic acid hexoside; fukiic acid; feruloyl aldaric acid; feruloyl-malic acid; ferulic acid hexoside; eugenol malonylglucoside (= citrulin C malonate); digalloyl hexoside; di-O-feruloylsucrose; cutaric acid hexoside; coumaric acid (= phaseolic acid); coumaroyl hexose; caffeoyl hexose; 3'-O-methyl(3',4'-dihydroxybenzyl tartaric acid) (= 3'-O-methylfukiic acid); 4-hydroxybenzoic acid (= p-hydroxybenzoic acid); 3,4-dihydroxybenzoic acid; medicarpin; dimethoxy-pterocarpan-hexo-acetate; ajugose; phaseoside I; lablaboside F; lablaboside E; lablaboside D; chikusetsusaponin IVa; brazilin; azukisaponin VI; azukisaponin IV; azukisaponin II; AzIV; AzII; resveratrol-3-O-β-D-glucoside	
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PAC: proanthocyanidins

Table S5. Phytochemicals shared between legumes.

Legumes	Phytochemical name	Family
Beans & Peanuts	taxifolin-3-O-rhamnoside (=dihydroquercetin)	flavanonols
	myricetin	flavonols
	methyl gallate	phenolic acids
	luteolin methyl ether (= diosmetin =chrysoeriol)	flavanones
	(epi)afzelechin	flavanols
	fisetin (= 5-desoxyquercetin)	flavonols
Beans & Soybeans	soyasaponin γ g	saponins
	soyasaponin α g	saponins
	soyasaponin Ab	saponins
	cyanidin-3-O-galactoside (= ideain)	condensed tannins
	kaempferol di-hexoside	flavonols
Beans & Peanuts & Lentils & Peas	protocatechuic acid	phenolic acids
	(epi)gallocatechin	flavanols
	luteolin	flavones
Beans & Lentils & Chickpeas	sissostrin	isoflavones
	quercetin-3-O-rhamnoside (= quercitrin)	flavonols
	quercetin-3,7-di-O-rhamnoside = quercetin-3-rhamnoside-7-rhamnoside = quercetin-3-O-rhamnoside-7-O-rhamnoside	flavonols
	ononin	isoflavones
	apigenin	flavones
	matairesinol	lignans
Beans & Peanuts & Chickpeas	sinapic acid	phenolic acids
Beans & Lentils & Peas	phloretic acid = 3-(4-hydroxyphenyl)propionic acid	phenolic acids
	p-hydroxyphenylacetic acid	phenolic acids
	o-coumaric acid = 2-hydroxycinnamic acid	phenolic acids
	naringenin	flavanones
	(epi)catechin-(4 α →2)-phloroglucinol	flavanols
	(epi)gallocatechin-(4 α →2)-phloroglucinol	condensed tannins
Beans & Chickpeas & Peas	chlorogenic acid	phenolic acids

	cinnamic acid	phenolic acids
Beans & Peanuts & Soybeans	soyasaponin V (= soyasaponin Ba)	saponins
Beans & Soybeans & Peas	soyasaponin A2	saponins
Peanuts & Lentils	trans-p-coumaric acid	phenolic acids
Lentils & Peas	(epi)catechin gallate	flavanols
Lentils & Chickpeas	gentisic acid (= 2,5-dihydroxybenzoic acid)	phenolic acids
Soybeans & Chickpeas	xanthophyll (= lutein)	carotenoids
Soybeans & Lentils	soyasaponin Bd = sandosaponin A	saponins
Soybeans & Peas	soyasaspogenol B	saponins
	soyasaspogenol A	saponins
	soyasaponin IV	saponins
	soyasaponin A1	saponins
	soyasaponin III (=soyasaponin Bb')	Saponins
Beans & Peas	naringin	flavanones
	luteolin 6-glucoside (= isoorientin)	flavones
	hesperidin	flavanones
	apigenin 8-C-glucoside	flavones
	2'-hydroxygenistein	isoflavones
	alkaloids	alkaloids
Beans & Chickpeas	quercetin-3-O-galactoside	flavonols
	myricetin 3-O-rhamnoside	flavonols
	β -carotene	carotenoids
	luteolin 8-C-glucoside (= orientin)	flavones
Beans & Lentils	quercetin-3-O-glucoside (= quercetin hexose)	flavonols
	quercetin-3-O-glucosylglucoside (= baimaside = quercetin-3-O-diglucoside)	flavonols
	procyanidin C2 (C-(4,8)-C-(4,8)-C)	condensed tannins
	procyanidin B7 (EC-(4,6)-C)	condensed tannins
	procyanidin B6 (C-(4a,6)-C)	condensed tannins
	procyanidin B3 (C-(4a,8)-C) [= (epi)catechin-(epi)catechin III]	condensed tannins
	procyanidin B2 (EC-(4b,8)-EC) [= (epi)catechin-(epi)catechin II]	condensed tannins

	procyanidin B1 (EC-(4b,8)-C) [= (epi)catechin-(epi)catechin I]	condensed tannins
	kaempferol-3-O-glucoside	flavonols
	delphinidin-3,5-diglucoside	anthocyanidins
	D-malic acid p-coumarate (= trans p-coumaroyl-malic acid)	phenolic acids
	(epi)catechin 3-O-glucoside	flavanols
	apigenin-7-O-glucoside	flavones
Beans & Peanuts & Lentils & Peas & Soybeans	α -tocopherol	tocopherols
	β + γ -tocopherol	tocopherols
	β -sitosterol	phytosterols
	campesterol	phytosterols
	stigmasterol	phytosterols
	biochanin A	isoflavones
	formononetin	isoflavones
	daidzein	isoflavones
	genistein	isoflavones
	genistin	isoflavones
	epi-catechin	flavanols
	phytic acid	phytic acid
	secoisolariciresinol	lignans
	soyasaponin Bb	saponins

Note. There are no compounds exclusively shared between lentils & peas & chickpeas

Figure S1. Qualitative heatmap of the phytochemicals distributed in lentils, chickpeas, beans, peas, soybeans and peanuts. Detailed version (red: presence; green: absence).

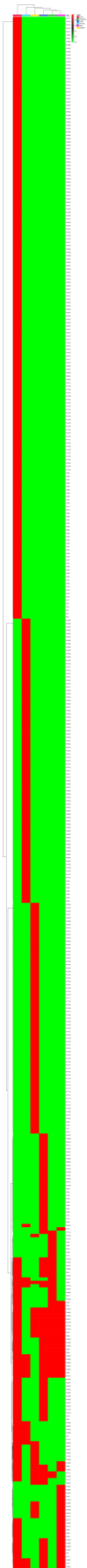
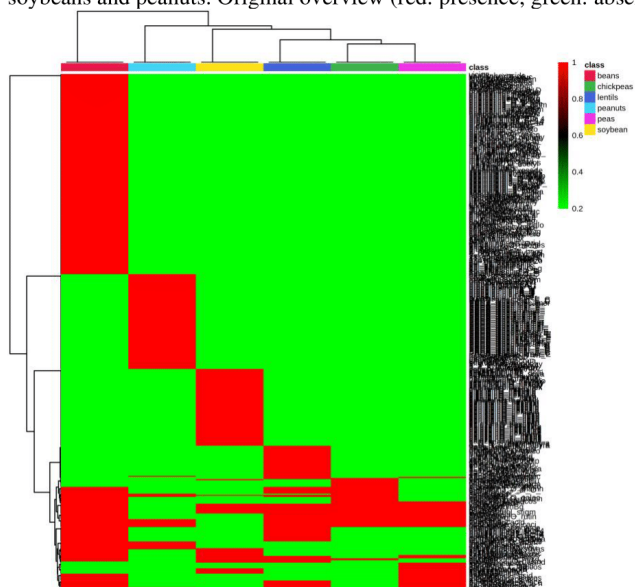


Figure S2. Qualitative heatmap of the phytochemicals distributed in lentils, chickpeas, beans, peas, soybeans and peanuts. Original overview (red: presence; green: absence) from *MetaboAnalyst 3.0*.



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4.2. Application of nutrimetabolomics in the discovery of bioactive compounds of legumes and biomarkers of their consumption

In line with the abovementioned objective within the FoodBall Project, an intervention study has been conducted to find metabolites associated with the consumption of some of the most widely consumed types of legumes via untargeted metabolomic techniques. To properly achieve it, this work also encompasses the characterization of the bioactive compound composition of the legumes used.

Publication 2: Mar Garcia-Aloy, Marynka Ulaszewska, Pietro Franceschi, Sheila Estruel-Amades, Christoph H Weinert, Alba Tor-Roca, Mireia Urpi-Sarda, Fulvio Mattivi, Cristina Andres-Lacueva. Discovery of Intake Biomarkers of Lentils, Chickpeas, and White Beans by Untargeted LC-MS Metabolomics in Serum and Urine. Mol Nutr Food Res. 2020;64(13):e1901137.

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Abstract

The aim of this study was to identify reliable biomarkers of food intake (BFIs) of pulses. A randomized crossover postprandial intervention study was conducted on 11 volunteers who consumed lentils, chickpeas, and white beans. Urine and serum samples were collected at distinct postprandial time points up to 48 h and analyzed by liquid chromatography-high-resolution-mass spectrometry (LC-HR-MS) untargeted metabolomics. Hypaphorine, trigonelline, several small peptides, and polyphenol-derived metabolites proved to be the most discriminating urinary metabolites. Two arginine-related compounds, dopamine sulfate and epicatechin metabolites, with their microbial derivatives, were identified only after intake of lentils, whereas protocatechuic acid was identified only after consumption of chickpeas. Urinary hydroxy-jasmonic and hydroxydihydro-jasmonic acids, as well as serum pipercolic acid and methylcysteine, were found after white bean consumption. Most of the metabolites identified in the postprandial study were replicated as discriminants in 24 h urine samples, demonstrating

that, in this case, the use of a single, noninvasive sample is suitable for revealing the consumption of pulses. The results of the present untargeted metabolomics work revealed a broad list of metabolites that are candidates for use as biomarkers of pulse intake. Further studies are needed to validate these BFIs and to find the best combinations of them to boost their specificity.

Resum

L'objectiu d'aquest estudi va ser identificar els biomarcadors dietètics (BFI) fiables de llegums. Es va realitzar un estudi aleatoritzat d'intervenció postprandial amb 11 voluntaris que consumien llenties, cigrons i mongetes blanques. Les mostres d'orina i sèrum es van recollir en diferents moments postprandials fins a 48 h i es van analitzar per metabolòmica no dirigida de cromatografia líquida-espectrometria de masses d'alta resolució (LC-HR-MS). La hipaforina, la trigonel·lina, diversos pèptids petits i metabòlits derivats dels polifenols van resultar ser els metabòlits urinaris més discriminants. Dos compostos relacionats amb l'arginina, el sulfat de dopamina i els metabòlits de l'epicatequina, amb els seus derivats microbians, només es van identificar després de la ingesta de llenties, mentre que l'àcid protocatecuic només es va identificar després del consum de cigrons. Els àcids hidroxijasmònic i hidroxidihidro-jasmònic urinaris, així com l'àcid pipecòlic i la metilcisteïna sèrics, es van trobar després del consum de mongetes blanques. La majoria dels metabòlits identificats en l'estudi postprandial van ser replicats com a discriminants en mostres d'orina de 24 h, demostrant que, en aquest cas, l'ús d'una sola mostra no invasiva és adequada per revelar el consum de llegums. Els resultats de l'actual treball de metabolòmica no dirigida van revelar un ampli llistat de metabòlits candidats per al seu ús com a biomarcadors de consum de llegums. Es necessiten més estudis per validar aquests BFIs i trobar les millors combinacions d'aquests per incrementar la seva especificitat.



RESEARCH ARTICLE

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Discovery of Intake Biomarkers of Lentils, Chickpeas, and White Beans by Untargeted LC–MS Metabolomics in Serum and Urine

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Scope: To identify reliable biomarkers of food intake (BFIs) of pulses.

Methods and results: A randomized crossover postprandial intervention study is conducted on 11 volunteers who consumed lentils, chickpeas, and white beans. Urine and serum samples are collected at distinct postprandial time points up to 48 h, and analyzed by LC–HR–MS untargeted metabolomics. Hypaphorine, trigonelline, several small peptides, and polyphenol-derived metabolites prove to be the most discriminating urinary metabolites. Two arginine-related compounds, dopamine sulfate and epicatechin metabolites, with their microbial derivatives, are identified only after intake of lentils, whereas protocatechuic acid is identified only after consumption of chickpeas. Urinary hydroxyjasmonic and hydroxydihydrojasmonic acids, as well as serum pipercolic acid and methylcysteine, are found after white bean consumption. Most of the metabolites identified in the postprandial study are replicated as discriminants in 24 h urine samples, demonstrating that in this case the use of a single, noninvasive sample is suitable for revealing the consumption of pulses. **Conclusions:** The results of the present untargeted metabolomics work reveals a broad list of metabolites that are candidates for use as biomarkers of pulse intake. Further studies are needed to validate these BFIs and to find the best combinations of them to boost their specificity.

1. Introduction

Pulses refer to the edible non-oil dried seeds of legumes, such as lentils, chickpeas, dry beans, and dry peas. They present a unique nutritional value characterized by a high content of protein, fiber, and a variety of phytochemicals, as well as by a low glycemic index. Consumption of pulses has been associated with beneficial effects on human health. In fact, regular pulse consumption has been associated with lower body weight,^[1] blood pressure,^[2] and LDL cholesterol,^[3] as well as with an improvement in markers of glycemic control.^[4] In recent years the promotion of their consumption has been prioritized by a wide range of public and private stakeholders culminating in the declaration of the year 2016 as the International Year of Pulses by the General Assembly of the United Nations, which was coordinated by the Food and Agriculture Organization of the United Nations.

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To study in depth the effects of these foods and to gain more insight into the molecular mechanisms by which they act, a precise measurement of food intake is required. Here, biomarkers of food intake (BFIs) have emerged as a promising tool for correctly assessing food intake.^[5] To date, only a limited number of foods have been associated with validated BFIs, but pulses are not among them, since only a few studies dealing with this topic have been recently published.^[6–11] The first of them was focused on the quantification of kaempferol in urine after bean consumption.^[6] However, this compound is present in a wide range of vegetables.^[12] Results from another human study suggested serum pipercolic acid and S-methylcysteine as biomarkers of dry bean consumption.^[7] Animal studies reported an increase in the urinary excretion of trigonelline, homoeriodictyol chalcone, and two peptides after a bean-based diet in dogs,^[8] and increased urinary excretions of different species of dipeptides, as well as arginine-related metabolites in hypertensive rats fed with lentils.^[9] More recently our research group published two human studies applying untargeted NMR experiments, one in an observational framework^[10] while the other was a postprandial controlled intervention study.^[11] The first study showed that pulse consumers had higher urinary excretions of different metabolites related to choline, protein, and energy metabolism,^[10] whereas the results of the second study suggested trigonelline, 3-methylhistidine, dimethylglycine, TMA, glutamine, choline, lysine, and histidine as candidate biomarkers of pulse intake.^[11] However, as the authors pointed out, these metabolites are not highly specific to pulse consumption.

In light of this scarcity of knowledge regarding biomarkers of pulse consumption, the aim of the present study was to assess the metabolic fingerprint of urine and serum associated with their intake in order to further identify reliable BFIs of the most commonly consumed types of pulses (i.e., lentils, chickpeas, and dry beans) using an LC–MS untargeted metabolomics approach. This technique enables the detection of a wide range of metabolites even at low concentrations while biosamples selected for analysis usually carry a large amount of dietary information. This research was developed within the same context of the NMR study mentioned before.^[11] In addition to analyzing the nutrinetic profiles in urine and serum, in this study we aimed to go one step further and develop a complementary experiment with pooled urinary samples. This was focused on a comparison of the data provided by the nutrinetic profile with the 6 and 24 h pooled urine samples to check the overlapping information. The aforementioned experiment was carried out in order 1) to get an overview of the postprandial impact of the evaluated foods without the interference of any additional intake (6 h pools), and 2) to evaluate the information provided by a simpler, less expensive, and more easily available type of sample such as the 24 h urine pool.

2. Experimental Section

2.1. Study Design

The design of this study (Figure 1) had been defined within the frame of the European JPI-funded project “Food Biomarker Alliance” (FoodBALL).^[13] In fact, this was one of the seven stan-

dardized postprandial intervention studies designed to discover new potential food intake biomarkers for a broad range of common foods. The study was focused on different types of the most commonly consumed kinds of pulses, such as, lentils, chickpeas, and white beans, using white pasta as the control food. The study was registered at the ISRCTN registry with the code ISRCTN17200423. Details of this randomized, controlled, crossover study had been reported previously^[11] and were listed in the Supporting Information. The clinical intervention was approved by the Bioethical Committee of the University of Barcelona (ref: IRB00003099) and followed Helsinki Declaration guidelines.

A total of 84 subjects expressed their interest in participating in this study, of whom 31 were screened. Twenty-six of them fulfilled the inclusion criteria and 14 were enrolled since they were the ones that finally fully agreed to participate in the study, with 11 (4 men and 7 women) completing the study in a crossover design with lentils, chickpeas, and pasta (Figure S1, Supporting Information). A subgroup of eight participants underwent the fourth intervention with white beans, which was scheduled later. The subjects were on average 28 ± 6 years old (range: 19–37) and had a BMI of 23.8 ± 3.6 kg m⁻² (range: 18.6–28.9).

2.2. Analysis of Food Samples

Test foods were freeze-dried in the study center, shipped to the analytical lab on dry ice, and stored at -80 °C before further preparation. 20 mg of each sample were extracted twice with methanol/tert-butyl methyl ether first and methanol/water afterward. Aliquots of the supernatants were then evaporated in a SpeedVac and subsequently derivatized (methoximated and trimethylsilylated). Untargeted analyses were performed by LC–MS (results previously published by Llorach et al.^[14]) and also using a comprehensive 2D gas chromatography mass spectrometry (GC×GC–MS) system described elsewhere.^[15] Data processing was done using the SquareDance workflow developed for GC×GC–qMS data sets.^[16] Further details of sample preparation and the analytical procedure are provided in the Supporting Information. Automated compound annotation was performed using an in-house spectral library with fatty acid methyl esters-based retention indices. Finally, the annotation was manually verified by matching against the National Institute of Standards and Technology v14 library, the Fiehn library, and the Golm Metabolome Database. The compounds identified in the four food products through the analyses with comprehensive 2D gas chromatography mass spectrometry are listed in Table S2, Supporting Information.

2.3. Untargeted Metabolomics Experiments on Biological Samples

Untargeted metabolomics analyses were performed in accordance with previously validated methodologies.^[17] Briefly, urine samples were diluted with a series of internal and external standards in pure methanol and in Milli-Q water, whereas serum samples were prepared using Ostro plates. Afterward, all samples were subjected to high-throughput metabolomics analysis

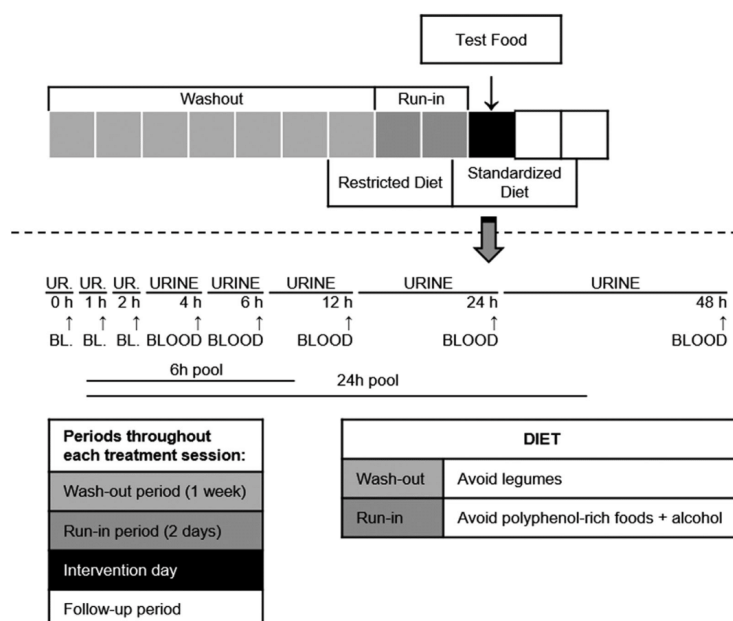


Figure 1. Schematic representation of the design of the randomized, controlled, and crossover study. This scheme was repeated in each treatment session. The top panel (above the dashed line) refers to the days that each of the interventions lasted, while the one below includes the information from the samples collected on the day of the intervention. Participants were assigned randomly to the test food in a crossover design. Urine samples were collected before the ingestion of the test product and during the indicated time intervals, whereas blood samples were also collected before the administration of the assigned meal and at the specified time points. After a 1 week washout period, volunteers followed a 2-day restricted diet before the intervention day. Standardized meals were provided to the volunteers from the dinner of the day before the intervention day, as well as from 6 h up to 48 h after the administration of the test food.

using a reversed-phase liquid chromatography coupled to a high-resolution Orbitrap mass spectrometer. Later, acquired data were processed using the XCMS package in the R platform. A detailed description of these procedures, together with an exhaustive explanation about statistical analysis and metabolite identification, is given in Supporting Information (section "Un-targeted Metabolomics Experiments on Biological Samples").

3. Results

3.1. Identification of Metabolites Associated with Legume Consumption in the Nutrikinetic Study

Data acquired in this study showed good analytical performance. In-depth information about the quality of acquired data, as well as verification of data processing, is provided in the section "Data Acquisition Quality" in Supporting Information. After the processing of the raw MS data, further filtration steps were applied to the obtained data sets in order to reduce the amount of noisy and/or irrelevant features. The number of features in both the

original and filtered data sets can be found in Supporting Information ("Data Filtering" section). Finally, before proceeding with the feature selection, principal component analysis was applied on each data set to explore trends in the data. A complete description of these results and their interpretation is provided in the section "Unsupervised Multivariate Analysis" in Supporting Information.

Among all the features retained after data filtering, 320 met the first selection requirement, based on their nutrikinetic curve behavior (see Supporting Information for further details). Of these, 265 were found to be statistically significant (adjusted p -value < 0.05 of AUCs), 151 of which were assigned to one of the 54 identified metabolites (Table 1), whereas the rest remained unknown. Out of the 54 identified metabolites, 12 were identified at level I, 29 at level II, and 13 at level III. Identified compounds were categorized into several groups taking into consideration information on the chemical class of their precursors (metabolites) or their common pathways of origin (Figure S5, Supporting Information): small peptides, flavan-3-ol-derived metabolites, other polyphenol-derived metabolites, fatty acids, other food-derived metabolites, and endogenous metabolites. A detailed description

Table 1. Identified metabolites that were significantly different in the nutrkinetics experiment after intake of lentils, chickpeas, white beans, or control.

M	Metabolite	Formula (m/z)	RT	Annotations	LI (ref)	Behavior	Fluid: time	p-adjusted
M01	Asp-Ala	C ₇ H ₁₂ N ₂ O ₅ (204.07461)	0.92	205.0819 [M + H] ⁺	2 (MyCompoundD)	(L & P & B) > C	U: 02-12 h	4.47 × 10 ⁻⁵
M02	Asp-Gly / Gly-Asp	C ₆ H ₁₀ N ₂ O ₅ (190.05896)	0.90	191.0662 [M + H] ⁺	1 (sid)	(L & P & B) > C	U: 04-24 h	7.97 × 10 ⁻⁵
M03	Asp-Leu	C ₁₀ H ₁₈ N ₂ O ₆ (246.12156)	U: 2.98 S: 3.03	U: 247.1289 [M + H] ⁺ ; 248.1321 ¹³ C [M + H] ⁺ ; 245.1145 [M - H] ⁻ ; 343.0913 [M - H + C ₂ H ₃ O ₂ N] ⁻ ; 227.1039 [M - H - H ₂ O] ⁻ ; S: 247.1287 [M + H] ⁺ ; 245.1142 [M - H] ⁻	1 (sid)	(L & P) > B > C	U: 04-12 h S: 02-06 h	U: 1.96 × 10 ⁻⁵ S: 1.09 × 10 ⁻⁵
M04	Asp-Met	C ₉ H ₁₆ N ₂ O ₅ (264.07798)	1.43	265.0853 [M + H] ⁺	2 (sid)	P > (L & B & C)	U: 02-12 h	7.60 × 10 ⁻⁴
M05	Asp-Phe	C ₁₃ H ₁₆ N ₂ O ₅ (280.10591)	3.77	281.1132 [M + H] ⁺ ; 282.1165 ¹³ C [M + H] ⁺ ; 279.0987 [M - H] ⁻	2 (sid)	(L & P & B) > C	U: 02-06 h	1.12 × 10 ⁻⁵
M06	Asp-Ser / Ser-Asp	C ₇ H ₁₂ N ₂ O ₆ (220.06952)	0.91	221.0769 [M + H] ⁺	3	(L & P & B) > C	U: 04-12 h	5.15 × 10 ⁻⁵
M07	Asp-Tyr	C ₁₃ H ₁₆ N ₂ O ₆ (296.10082)	U: 1.86 S: 1.67	U: 297.1082 [M + H] ⁺ ; 279.0976 [M + H - H ₂ O] ⁺ ; S: 297.1081 [M + H] ⁺	2 (sid)	(L & P & B) > C	U: 02-06 h S: 02-06 h	U: 1.09 × 10 ⁻⁵ S: 1.91 × 10 ⁻⁵
M08	Asp-Val	C ₉ H ₁₆ N ₂ O ₅ (232.10591)	U: 1.33 S: 1.35	U: 233.1132 [M + H] ⁺	2 (sid)	(L & P & B) > C	U: 02-12 h S: 04-06 h	U: 1.48 × 10 ⁻⁵ S: 4.66 × 10 ⁻⁴
M09	Asp-(l)Leu-(l)Leu / (l)Leu-Asp-(l)Leu	C ₁₆ H ₂₈ N ₄ O ₆ (359.20561)	5.46	360.2127 [M + H] ⁺	2 (MyCompoundD)	(L & P) > B > C	U: 02-06 h S: 02-06 h	9.81 × 10 ⁻⁶
M10	Asp-(l)Leu-Pro / (l)Leu-Asp-Pro	C ₁₅ H ₂₂ N ₃ O ₆ (343.17432)	4.38	344.1818 [M + H] ⁺	3	P > L > (B & C)	U: 06-12 h	9.55 × 10 ⁻⁶
M11	Asp-Ala-(l)Leu / Ala-Asp-(l)Leu	C ₁₃ H ₂₀ N ₃ O ₆ (317.15869)	3.64	318.1660 [M + H] ⁺	2 (MyCompoundD)	P > (L & B & C)	U: 04-12 h	5.52 × 10 ⁻⁵
M12	Asp-Asn-Val / Asn-Asp-Val	C ₁₃ H ₂₂ N ₄ O ₇ (346.14883)	1.31	347.1563 [M + H] ⁺	1 (sid)	(L & P) > B > C	U: 02-12 h	1.03 × 10 ⁻⁵
M13	Asp-Gly-(l)Leu	C ₁₂ H ₁₇ N ₃ O ₆ (303.14302)	U: 3.36 S: 3.57	U: 304.1504 [M + H] ⁺ ; 302.1359 [M - H] ⁻ ; S: 304.1503 [M + H] ⁺	1 (sid)	(L & P & B) > C	U: 04-06 h S: 02-06 h	U: 4.28 × 10 ⁻⁴ S: 1.81 × 10 ⁻⁵
M14	Asp-Gly-Tyr / Gly-Asp-Tyr	C ₁₅ H ₁₈ N ₂ O ₇ (353.12230)	1.86	354.1297 [M + H] ⁺	3	P > L > (B & C)	U: 02-12 h	1.85 × 10 ⁻⁵
M15	Asp-Gly-Val	C ₁₁ H ₁₆ N ₂ O ₆ (289.12737)	1.50	291.1380 ¹³ C [M + H] ⁺	1 (sid)	(L & P) > (B & C)	U: 04-06 h	2.03 × 10 ⁻²
M16	Asp-Thr-Pro / Thr-Asp-Pro	C ₁₃ H ₂₂ N ₂ O ₆ (331.13793)	1.23	332.1452 [M + H] ⁺	3	P > L > (B & C)	U: 04-24 h	8.52 × 10 ⁻⁶
M17	Pro-HPro-Gly / HPro-Pro-Gly	C ₁₃ H ₁₆ N ₂ O ₆ (285.13245)	3.74	286.1398 [M + H] ⁺	2 (Metlin)	(L & P & B) > C	U: 04-06 h	1.71 × 10 ⁻²
M18	Cyclo((l)Leu-Phe)	C ₁₅ H ₂₀ N ₂ O ₅ (260.15246)	6.75	261.1596 [M + H] ⁺	2 (Yamamoto 2016/MetFrag)	P > (L & B & C)	S: 01-06 h	4.47 × 10 ⁻⁵
M19	Cyclo(His-Pro)	C ₁₁ H ₁₆ N ₂ O ₅ (234.11166)	1.17	235.1191 [M + H] ⁺	2 (Yamamoto 2016/MetFrag)	(L & P) > B > C	U: 01-04 h	3.60 × 10 ⁻³
M20	(l)Leucine derivative (I)	C ₁₀ H ₁₈ N ₂ O ₅ (246.12156)	3.64	247.1288 [M + H] ⁺	3	(L & P) > B > C	S: 02-06 h	2.70 × 10 ⁻⁵
M21	(l)Leucine derivative (II)	C ₁₂ H ₂₀ N ₂ O ₆ (288.13212)	U: 4.64 S: 4.89	U: 289.1394 [M + H] ⁺ ; S: 289.1392 [M + H] ⁺	3	(L & P & B) > C	U: 06-12 h S: 04-06 h	U: 1.96 × 10 ⁻⁴ S: 2.45 × 10 ⁻⁴
M22	Glycine derivative	C ₉ H ₁₇ N ₃ O ₄ (231.12191)	1.40	232.1292 [M + H] ⁺ ; 215.1026 [M + H - NH ₃] ⁺	3	(L & B) > (P & C)	U: 02-06 h	1.09 × 10 ⁻³

(Continued)

Table 1. Continued.

M	Metabolite	Formula (<i>m/z</i>)	RT	Annotations	LI (ref)	Behavior	Fluid. time	<i>p</i> -adjusted
M23	Phenylalanine derivative	C ₉ H ₉ N ₃ O ₅ (399.17940)	5.34	400.1867 [M + H] ⁺	3	(P & B) > (L & C)	U: 01–12 h	1.03 × 10 ⁻⁵
M24	Proline derivative	C ₁₀ H ₁₀ N ₂ O ₄ (228.11099)	U: 3.59 S: 3.98	U: 229.1183 [M + H] ⁺ S: 229.1181 [M + H] ⁺	3	(L & P & B) > C	U: 04–06 h S: 02–06 h	U: 4.39 × 10 ⁻² S: 5.15 × 10 ⁻³
M25	Hydroxyarginine	C ₆ H ₁₄ N ₄ O ₃ (190.10658)	0.80	191.1139 [M + H] ⁺	2 (MetFrag)	L > (P & B & C)	U: 01–48 h	6.28 × 10 ⁻⁵
M26	Oxarginine	C ₆ H ₁₁ N ₃ O ₃ (173.08003)	0.93	174.0872 [M + H] ⁺	2 (MetFrag)	L > (P & B & C)	U: 02–48 h	1.61 × 10 ⁻⁴
M27	(Ep)catechin sulfate	C ₁₅ H ₁₆ O ₆ S (370.03584)	4.33	369.0287 [M – H] ⁻	2 (van der Hoof 2012)	L > (P & B & C)	U: 01–24 h	4.37 × 10 ⁻⁵
M28	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulfate	C ₁₁ H ₁₂ O ₆ S (306.04092)	4.23	305.0337 [M – H] ⁻	2 (van der Hoof 2012)	L > (P & B & C)	U: 06–12 h	3.37 × 10 ⁻⁵
M29	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-3-O-sulfate	C ₁₁ H ₁₂ O ₇ S (288.03036)	4.70	306.0643 [M + NH ₄] ⁺ ; 287.0232 [M – H] ⁻ ; 288.0665 ¹³ C [M – H] ⁻ ; 207.0664 [M – H – sulf] ⁻	2 (van der Hoof 2012)	L > (P & B & C)	U: 04–48 h	7.31 × 10 ⁻⁵
M30	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-O-glucuronide	C ₁₇ H ₂₀ O ₁₀ (384.10563)	4.58	385.1129 [M + H] ⁺ ; 402.1395 [M + NH ₄] ⁺ ; 209.0808 [M + H – gluc] ⁺	2 (van der Hoof 2012)	L > (P & B & C)	U: 04–24 h	1.63 × 10 ⁻⁵
M31	5-(3',5'-Dihydroxyphenyl)-γ-valerolactone-methyl-glucuronide	C ₁₈ H ₂₂ O ₁₀ (398.12128)	4.76	416.1551 [M + NH ₄] ⁺ ; 223.0964 [M + H – gluc] ⁺ ; 397.1141 [M – H] ⁻	2 (van der Hoof 2012)	L > (P & B & C)	U: 04–48 h	8.09 × 10 ⁻⁶
M32	Phloroglucinol glucuronide	C ₁₂ H ₁₄ O ₆ (302.06377)	U: 1.85 S: 1.79	U: 303.0711 [M + H] ⁺ ; 304.0745 ¹³ C [M + H] ⁺ ; 325.0531 [M + Na] ⁺ ; 341.027 [M + K] ⁺ ; 468.1498 [H ₂]; 301.0566 [M – H] ⁻ ; 302.0597 ¹³ C [M – H] ⁻ ; 399.0336 [M – H + C ₂ H ₃ O ₂ K] ⁻ ; 303.0710 [M + H] ⁺	2 (std)	(L & P & B) > C	U: 01–12 h S: 01–04 h	U: 4.50 × 10 ⁻⁵ S: 4.24 × 10 ⁻⁴
M33	Phloroglucinol sulfate	C ₉ H ₈ O ₆ S (205.98850)	U: 1.35 S: 1.43	U: 206.9954 [M + H] ⁺ ; 224.0228 [M + NH ₄] ⁺ ; 244.9517 [M + K] ⁺ ; 127.0388 [M + H – sulf] ⁻ ; 204.9814 [M – H] ⁻ ; 205.9847 ¹³ C [M – H] ⁻ ; 206.9772 ²³ C [M – H] ⁻ ; 302.9583 [M – H + C ₂ H ₃ O ₂ K] ⁻ ; 125.0248 [M – H – sulf] ⁻ ; S: 204.9814 [M – H] ⁻	1 (std)	(L & P & B) > C	U: 01–12 h S: 01–04 h	U: 7.31 × 10 ⁻⁵ S: 3.37 × 10 ⁻⁵
M34	2-Hydroxyhippuric acid	C ₉ H ₈ NO ₄ (195.05315)	5.28	196.0604 [M + H] ⁺ ; 241.1048 [M + C ₂ H ₃ N] ⁺ ; 239.0925 [M + C ₂ H ₃ N] ⁻	1 (std)	B > (L & P & C)	U: 01–06 h	8.72 × 10 ⁻³
M35	Aminosalicilic acid	C ₉ H ₁₀ N ₂ O ₄ (210.06405)	1.78	211.0713 [M + H] ⁺ ; 209.0569 [M – H] ⁻	3 (in silico)	(L & P & B) > C	U: 02–24 h	6.37 × 10 ⁻⁶
M36	Mucic acid	C ₆ H ₈ O ₄ (142.02660)	1.64	141.0197 [M – H] ⁻	2 (mzCloud)	(L & P & B) > C	U: 01–24 h	5.52 × 10 ⁻⁵
M37	Protocatechuic acid glucoside	C ₁₃ H ₁₆ O ₆ (316.07942)	2.62	315.0722 [M – H] ⁻ ; 413.0493 [M + C ₂ H ₃ O ₂ K] ⁺	2 (Cuparencu 2016/mzCloud)	P > L > (B & C)	U: 02–04 h	6.37 × 10 ⁻⁶
M38	Vanillic acid sulfate	C ₉ H ₈ O ₇ S (247.99906)	2.91	246.9920 [M – H] ⁻	2 (mzCloud)	L > (P & B & C)	U: 04–12 h	7.49 × 10 ⁻⁴

(Continued)

Table 1. Continued.

M	Metabolite	Formula (m/z)	RT	Annotations	LI (ref)	Behavior	Fluid: time	p-adjusted
M39	Hydroxyjasmonic acid	C ₁₂ H ₁₈ O ₄ (226.12049)	5.18	227.1278 [M + H] ⁺ ; 225.1134 [M - H] ⁻	2 (Valente 2018)	B > (L & P & C)	U: 01-24 h	6.96 × 10 ⁻⁴
M40	Hydroxyhydrojasmonic acid (I)	C ₁₂ H ₁₈ O ₄ (228.13614)	4.81	229.1434 [M + H] ⁺ ; 211.1328 [M + H - H ₂ O] ⁺ ; 193.1223 [M + H - 2(H ₂ O)] ⁺ ; 227.1290 [M - H] ⁻	3 (in silico)	B > (L & P & C)	U: 01-12 h	3.98 × 10 ⁻⁴
M41	Hydroxyhydrojasmonic acid (II)	C ₁₂ H ₁₈ O ₄ (228.13614)	5.37	211.1328 [M + H - H ₂ O] ⁺ ; 227.1290 [M - H] ⁻	3 (in silico)	B > (L & P & C)	U: 01-48 h	8.99 × 10 ⁻⁴
M42	Ascorbic acid	C ₆ H ₈ O ₆ (176.03208)	0.95	177.0993 [M + H] ⁺ ; 175.0251 [M - H] ⁻ ; 115.0041 [M - H - C ₂ H ₄ O ₂] ⁻	1 (std)	P > (L & B & C)	U: 04-06 h	1.23 × 10 ⁻³
M43	Dopamine sulfate	C ₈ H ₁₁ NO ₃ S (233.03578)	1.31	234.0430 [M + H] ⁺ ; 232.0287 [M - H] ⁻	2 (std)	L > (P & B & C)	U: 01-12 h	1.20 × 10 ⁻⁴
M44	Hypaphorine	C ₁₄ H ₁₈ N ₂ O ₂ (246.13681)	U: 4.07 S: 4.36	U: 247.1441 [M + H] ⁺ ; 188.0706 [M + H - (CH ₃) ₂ N] ⁺ S: 247.1440 [M + H] ⁺ ; 248.1473 ¹³ C [M + H] ⁺ ; 188.0704 [M + H - (CH ₃) ₂ N] ⁺ ; 189.0737 ¹³ C [M + H - (CH ₃) ₂ N] ⁺ ; 146.0598 [M + H - (CH ₃) ₂ N - C ₂ H ₄ O] ⁺ ; 144.0805 [M + H - C ₂ H ₄ N - CO ₂] ⁺ ; 245.1295 [M - H] ⁻ ; 246.1328 ¹³ C [M - H] ⁻ ; 291.1349 [M + FA - H] ⁻ ; 292.1382 ¹³ C [M + FA - H] ⁻ ; 186.0561 [M - H - (CH ₃) ₂ N] ⁻	1 (std)	(L & P) > (B & C)	U: 02-48 h S: 01-48 h	U: 2.42 × 10 ⁻⁵ S: 1.02 × 10 ⁻⁵
M45	Methylcysteine	C ₄ H ₉ NO ₂ S (135.03539)	1.10	136.0425 [M + H] ⁺	1 (std)	B > (L & P & C)	S: 01-48 h	4.86 × 10 ⁻⁴
M46	Pipecolic acid	C ₆ H ₁₁ NO ₂ (129.07897)	1.12	130.0861 [M + H] ⁺ ; 131.0894 ¹³ C [M + H] ⁺ ; 152.0681 [M + Na] ⁺	1 (std)	B > (L & P & C)	S: 01-24 h	4.51 × 10 ⁻⁴
M47	Trigonelline	C ₇ H ₇ NO ₂ (137.04767)	0.88	138.0549 [M + H] ⁺ ; 139.0582 ¹³ C [M + H] ⁺ ; 94.0650 [M + H - CO ₂] ⁺	1 (std)	B > P > L > C	U: 02-06 h	6.37 × 10 ⁻⁶
M48	γ-CEHC-glucose	C ₂₁ H ₃₀ O ₆ (426.18896)	6.28	444.2225 [M + NH ₄] ⁺	2 (std)	(L & P & B) > C	U: 06-12 h	8.46 × 10 ⁻⁴
M49	γ-CEHC-glucuronide	C ₂₁ H ₃₀ O ₁₀ (440.16822)	6.39	441.1753 [M + H] ⁺ ; 458.2016 [M + NH ₄] ⁺ ; 459.2051 ¹³ C [M + NH ₄] ⁺ ; 463.1569 [M + Na] ⁺ ; 501.2073 [M + H + C ₂ H ₄ O ₂] ⁺ ; 265.1434 [M + H - gluc] ⁺ ; 266.1467 ¹³ C [M + H - gluc] ⁺ ; 151.0752 [M + H - gluc - H ₂ O - C ₄ H ₆ - COCH ₃] ⁺ ; 537.1381 [M - H + C ₂ H ₃ O ₂ K] ⁻	2 (std)	(L & P & B) > C	U: 04-12 h	1.89 × 10 ⁻⁴
M50	Dihydroxydecanoic acid glucuronide	C ₁₆ H ₂₈ O ₁₀ (380.16822)	U: 5.52 S: 5.60	U: 381.1755 [M + H] ⁺ ; 398.2020 [M + NH ₄] ⁺ ; 379.1611 [M - H] ⁻ S: 379.1608 [M - H] ⁻	2 (in silico)	C > (L & P) > B	U: 04-06 h S: 02-06 h	U: 1.34 × 10 ⁻² S: 3.35 × 10 ⁻⁴

(Continued)

Table 1. Continued.

M	Metabolite	Formula (m/z)	RT	Annotations	LI (ref)	Behavior	Fluid: time	p-adjusted
M51	Dodecanedioic acid glucuronide	$C_{18}H_{32}O_{10}$ (406.18387)	U: 6.14 S: 6.11	U: 407.1915 [M + H] ⁺ ; 424.2175 [M + NH ₄] ⁺ ; 425.2211 ¹³ C [M + NH ₄] ⁺ ; 429.1737 [M + Na] ⁺ ; 467.2230 [M + H + C ₃ H ₄ O ₂] ⁺ ; 389.1805 [M + H - H ₂ O] ⁺ ; 390.1839 ¹³ C [M + H - H ₂ O] ⁺ ; 231.1591 [M + H - gluc] ⁺ ; 405.1767 [M - H] ⁻ ; 406.1802 ¹³ C [M - H] ⁻ ; 427.1588 [M + Na] ⁺ ; 503.1559 [M - H + C ₃ H ₄ O ₂] ⁻ S: 405.1765 [M - H] ⁻	2 (std)	C > (L & P) > B	U: 02-06 h S: 01-06 h	U: 4.26 × 10 ⁻³ S: 2.11 × 10 ⁻⁴
M52	Hydroxydodecanedioic acid glucuronide	$C_{18}H_{32}O_9$ (388.17331)	7.07	U: 195.1379 [M + H - gluc - H ₂ O] ⁺ ; 387.1661 [M - H] ⁻	2 (Metlin)	C > (L & P & B)	U: 02-06 h	1.43 × 10 ⁻²
M53	Tetradecanedioic acid glucuronide	$C_{20}H_{34}O_{10}$ (434.21517)	U: 6.89 S: 6.73	U: 435.2222 [M + H] ⁺ ; 457.2039 [M + Na] ⁺ ; 417.2118 [M + H - H ₂ O] ⁺ ; 233.1692 [M + H - gluc - 2(H ₂ O)] ⁺ ; 433.2080 [M - H] ⁻ ; 434.2114 ¹³ C [M - H] ⁻ S: 455.1903 [M + Na] ⁺ S: 433.2077 [M - H] ⁻	2 (in silico)	C > (L & P) > B	U: 04-06 h S: 01-06 h	U: 2.39 × 10 ⁻² S: 4.20 × 10 ⁻⁴
M54	Dicarboxylic fatty acid C12:1, dihydroxy	$C_{12}H_{22}O_8$ (260.12597)	5.05	U: 261.1333 [M + H] ⁺	3	C > (L & P) > B	U: 02-12 h	2.38 × 10 ⁻²

C, control; B, white beans; gluc, glucuronide; L, lentils; LI, level of identification; M, metabolite; P, chickpeas; ref, reference; RT, retention time; S, serum; sulf, sulfate; U, urine.

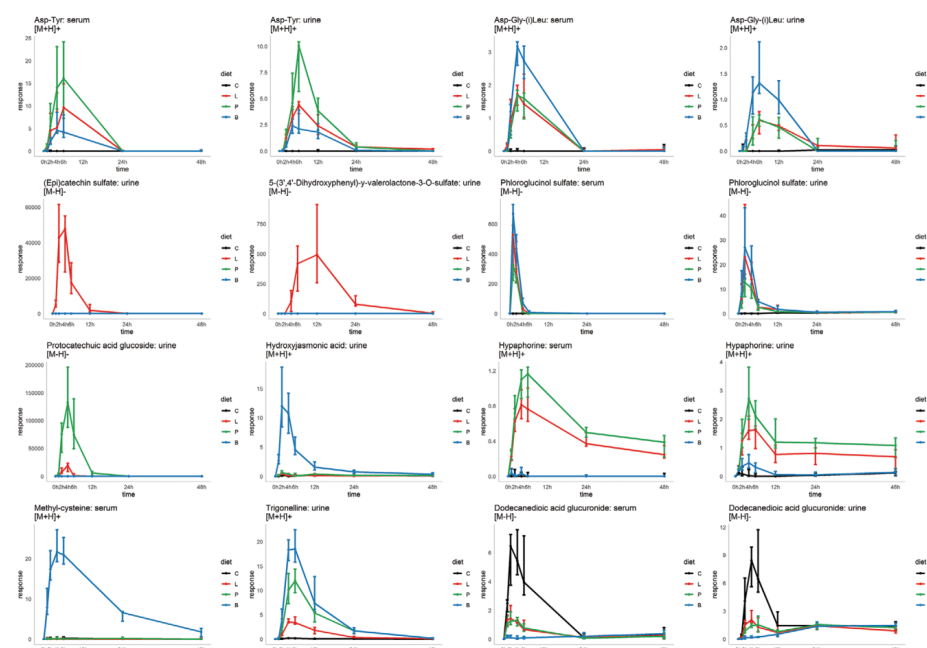


Figure 2. Selected metabolite kinetic curves in urine and serum samples. X axis: time point; Y axis: peak intensity (MS response). Lines represent medians and bars IQR range. C, control; B, white beans; L, lentils; P, chickpeas.

of the identification of discriminant metabolites is provided in the section “Identification of Discriminating Metabolites” in the Supporting Information, and information regarding their fragmentation spectra is given in Table S5, Supporting Information. The responses of a selected group of characteristic metabolites are depicted in **Figure 2**, while **Figure S6**, Supporting Information includes the kinetic curves of all identified metabolites. All plots were constructed with the MS response (peak area) of the most intensive feature of each metabolite.

3.2. Experiment on Pooled Samples

In order to compare the data provided by nutrikinetic profiles and a simpler type of sample such as 24 h urine, an additional experiment was performed. This was done with the aim of checking the overlapping information between these types of samples. Additionally, 6 h urine pools were also analyzed since during the first 6 h volunteers only ingested the test meal (i.e., this pool included samples collected 1, 2, 4, and 6 h after consuming the study food, **Figure 1**). In both cases, most of the identified urinary metabolites in the nutrikinetic experiments were also observed as being discriminant in both types of pooled samples (Table S6 and **Figure S7**, Supporting Information). This

demonstrates that in this case using a simpler type of sample (in that it is not required for the study volunteer to collect the samples in different containers) is also useful for observing the BFIs of pulse consumption. This has important connotations since 24 h urinary samples are usually collected in long intervention and epidemiological studies (although unfortunately some of them only have available spot urine instead of 24 h urine samples). Furthermore, the HCA (**Figure 3**) demonstrated that, although not all statistically significant features were annotated, the information on those already assigned to one of the identified metabolites allowed a good sample clustering performance.

4. Discussion

In this work, several metabolites were found to be associated with the intake of some of the most widely consumed types of pulses. They were identified using liquid chromatography coupled to high-resolution mass spectrometry. Most of these metabolites are candidates as BFIs for this food group since they showed increased urinary and/or serum concentrations after the consumption of at least one type of pulse, while the values observed after the consumption of the control food (pasta) were negligible. It was difficult to find biomarkers exclusively for one type of pulse,

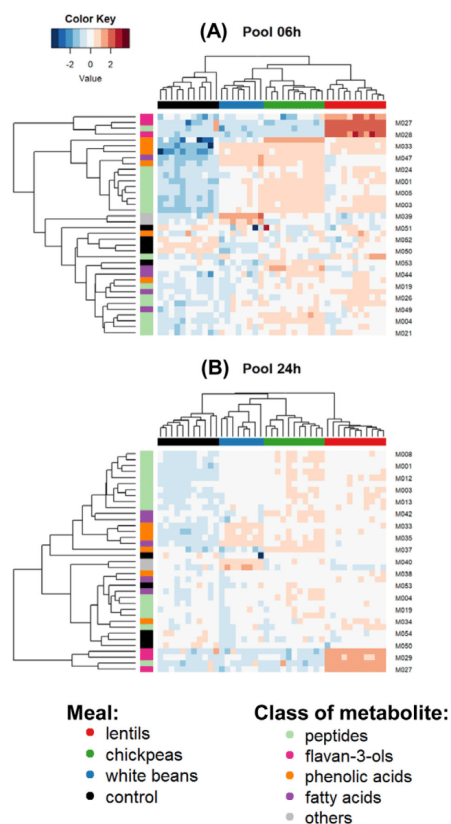


Figure 3. Heatmaps of identified metabolites after intake of lentils (L), chickpeas (P), beans (B), or control (C) in (A) 6 h pools and (B) 24 h pools. Blue and orange cells correspond to low- and high-metabolite levels, respectively. Columns are samples, and rows are metabolites colored by treatment and class of compound, respectively.

as most of them were common to at least two or three pulse types. The exceptions were flavan-3-ol-derived metabolites (M27–M31), two arginine-related compounds (M25 and M26), and dopamine sulfate (M43), which appeared to be specific for lentil intake in urine samples, whereas protocatechuic acid glucoside (M37) and ascorbic acid (M42) were elevated after the consumption of chickpeas. Hydrojasmonic (M39) and two hydroxydihydrojasmonic (M40 and M41) acids were characteristic of white bean intake. In serum samples, methylcysteine (M45) and pipecolic acid (M46) also resulted in particular from white bean consumption, and in both types of biosamples hypaphorine (M44) was discriminant for chickpeas and lentils. Two of the identified BFIs (i.e., methylcysteine and pipecolic acid) were detected as such in the test food to which they were assigned as markers (Table S2, Supporting In-

formation). This observation indicates that they were most likely nonmetabolized or not fully metabolized, suggesting that they would not be subject to substantial interindividual differences.

4.1. BFI for Lentils

The results for flavan-3-ol-derived metabolites are consistent with the analyses of test foods, which showed that catechins were particular to lentils (Table S2, Supporting Information). They were observed only in urine and their presence was due to the metabolism of catechins and proanthocyanidins by host and by intestinal microbiota. The urine integrated intensity–time curves clearly show two distinct nutrkinetic patterns, as already reported in another independent postprandial dietary intervention.^[17] On the one hand, (epi)catechin sulfate (M27) reached its maximum urinary levels within the first 2–4 h post-consumption followed by a rapid decrease within the next few hours. This behavior suggests that this compound is probably absorbed in the upper part of the gastrointestinal tract with little or no contribution from the microbiota. On the other hand, the four valerolactone-derived metabolites (M28–M31) were characterized by a delayed appearance in urine. Their levels started to increase 4 h after consumption of lentils, reaching the maximum levels after 12 h, still being significantly higher after 24 h and reaching the baseline values within 48 h. This performance suggests a prolonged metabolism throughout the large intestine with the involvement of the microbiota. These profiles are in good agreement with previous studies reporting the presence of (epi)catechin and valerolactone conjugates in urine.^[17] This suggests that the former may only be useful as a BFI when 24 h urine is available, whereas the others could be more useful as BFIs when only spot urines are accessible. However, increase in urinary excretions after the intake of other catechin- and proanthocyanidin-rich foods such as tea, cocoa, and grape products has also been observed in previous studies,^[18] thereby limiting their specificity as candidate BFIs of lentils. Additionally, hydroxy- and oxoarginine (M25 and M26) urinary levels also increased with the intake of lentils. Maximum excretions of these compounds were observed 4 and 6 h, respectively, after intake of lentils, although differences were observed from the first few hours until 48 h post-consumption (Table 1). Hydroxyarginine is a free nonprotein amino acid particular to lentils,^[9] and oxoarginine is a metabolite of the arginine pathway recently associated with lentil consumption.^[9] Therefore, they are interesting indicators of lentil intake. Additionally, it has been suggested that they could contribute to the blood pressure-lowering effects associated with the consumption of lentils through an increase in the production of nitric oxide.^[9] Due to the well-known degradation of the guanidino group during trimethylsilylation prior to GC×GC–MS analysis, the presence (or abundance) of arginine and related compounds in the test foods could not be determined.^[19] An increased urinary excretion of dopamine sulfate (M43, the predominant form of dopamine in the circulation) was also particularly noticeable after the consumption of lentils. It has already been reported that it is largely affected by meal consumption, coming from the intake of dopamine, dopamine sulfate, or 1-dihydroxyphenylalanine (DOPA), the conversion of dietary tyramine to dopamine, the action of tyrosinase to produce

L-DOPA in the gastrointestinal lumen, or an increased release and metabolism of endogenous dopamine in gastrointestinal lining cells.^[20] Very recently, dopamine sulfate was found to be among the BFIs best predicting the intake of banana.^[21]

4.2. BFI for Chickpeas

Ascorbic acid (M42) appeared as a discriminant compound for chickpea consumption during the period 4–6 h post-intake. In line with that, its oxidation product, dehydroascorbic acid, was only detected in high amounts in chickpeas (Table S2, Supporting Information). However, ascorbic acid cannot be considered a compound from chickpeas per se, since it is employed as a food additive. Furthermore, since it is widely used in the food industry as a stabilizing agent, it lacks the specificity to be used as a BFI and was not further investigated.

4.3. BFI for White Beans

Methylcysteine (M45) and pipecolic acid (M46) appeared in serum samples particularly after white bean intake. Their levels were already significantly increased 1 h post-consumption and remained as such until the period 24–48 h (Table 1, Figure S7a, Supporting Information). In accordance with this observation, the serum levels of pipecolic acid and methylcysteine have already been proposed as potential biomarkers of dry bean consumption.^[7] They are common nonprotein nitrogen components of the *Phaseolus vulgaris* species.^[22] Specifically, whereas methylcysteine is not influenced by microbial metabolism, pipecolic acid has been reported to be a product of microbial metabolism derived from lysine and has been described as a precursor of microbial compounds with anti-inflammatory, antitumor, and antibiotic properties.^[7] On the other hand, three jasmonic derivatives (M39–M41) appeared to be particular to white bean intake. They are lipid-derived phytohormones generated in plants as a response to stress and their presence has been described in different varieties and cultivars of *Vicia faba* beans.^[23]

4.4. BFI for Legumes

As legumes are an important food source of proteins, amino acid-derived metabolites could be indicators of their consumption. Indeed, we observed higher serum and/or urinary levels of several species of di- and tripeptides (M01–M17). When differences among the different types of legumes were observed, in most cases chickpeas were among the types that resulted in the highest concentrations. This could have been related to the fact that chickpeas could have a higher amount of protein in their composition, but this did not turn out to be the case when we consulted the nutritional composition of the foods used.^[11] These compounds may indicate that they are directly excreted in urine after pulse intake, and/or may represent proteolytic breakdown products of larger proteins present in these food sources. Most of the peptides were aspartyl-containing compounds. Interestingly, in another study developed within the FoodBALL project but focused on meat biomarkers, a series of small peptides were also identified as BFIs. However, in that case these were hydroxyproline-containing di- and tripeptides^[24] and none of them were in line with the present study where a completely different protein-based food was consumed.

Another interesting discriminant compound was hypaphorine (M44), which is also referred to as tryptophan betaine or lenticin. This last nomenclature was assigned since it is found in lentils,^[25,26] although it has also been reported in chickpeas.^[27] It is an indole alkaloid composed of tryptophan and three methyls. In the present study, it has been observed as being discriminant for the consumption of chickpeas and lentils both in serum and urine biofluids. In serum it has been observed as discriminant in the first hour after consumption, peaking 4–6 h after consumption and resulting in a prolonged disappearance in both biofluids, indicating a slow excretion or metabolism. However, the complete clearance of its metabolism could not be estimated since this metabolite was still detected during the 24–48 h period, when the last sample was collected. It has been correlated with nut intake in different cross-sectional studies,^[28–31] and it has also been associated with the Mediterranean diet^[32] and the Dietary Approaches to Stop Hypertension^[33] dietary patterns. Additionally, a study focused on peanut consumption also reported its presence in the breast milk of lactating women.^[34] Curiously, it has been positively associated with homocysteine levels after a Mediterranean-based dietary treatment in subjects with metabolic syndrome features.^[32] Although it has been shown to be a compound with neurological and glucose-lowering effects in rodents, its potential functional role in humans remains to be addressed.

In parallel and in agreement with the results observed in our NMR experiments,^[11] urinary levels of trigonelline (M47) were also increased after intake of the three different types of pulses. In this case, urinary trigonelline achieved higher levels after the intake of white beans followed by chickpeas and lentils, reflecting the different amounts observed in their composition^[11] and, therefore, suggesting a possible dose–response connection. Interestingly, this was also described after bean consumption in the two independent untargeted studies previously published.^[7,8] However, this alkaloid has also been proposed as a BFI of coffee intake.^[35] Therefore, this lack of specificity could limit its use as a single BFI, but we hypothesize that it could be considered within a multi-metabolite biomarker panel together with some of the other discriminating metabolites also observed in this study.^[18] For example, combined with flavan-3-ol-derived metabolites, arginine-related compounds and hypaphorine increase the specificity for monitoring lentil consumption.

Other metabolites whose amounts were increased in urine after pulse intake were tentatively identified as phloroglucinol glucuronide (M32) and phloroglucinol sulfate (M33). Both are metabolites containing benzoyl groups, probably generated by microbial degradation of polyphenols contained in pulses, and therefore reflecting polyphenol metabolite cleavage products of gut microbiota action.

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4.5. Endogenous Metabolites

Major increases in glucuronidated forms of dicarboxylic fatty acids (i.e., dodecanedioic acid glucuronide, M51, and

tetradecanedioic acid glucuronide, M53) and hydroxy fatty acids (i.e., dihydroxydecanoic acid glucuronide, M50, and hydroxydodecadienoic acid glucuronide, M52) were observed after pasta intake (used as control food) when compared with the consumption of the other types of pulses. Given the type of metabolites (glucuronidated medium-chain fatty acids), these metabolites probably reflect a differential endogenous response rather than being a metabolite from an exogenous food compound (also bearing in mind that pasta was not the food with the highest fat content^[11]). Therefore, this could indicate a different response related to lipid metabolism. For example, in the case of dicarboxylic fatty acids, they probably reflect a shift from a reduced β -oxidation toward an enhanced ω -oxidation of fatty acids since dicarboxylic fatty acids are enhanced when there is a deficiency in fatty acid oxidation or when β -oxidation is overwhelmed.^[36] Additionally, dihydroxydecanoic acid is an intermediate from the ω -oxidation of hydroxydecanoic acid to hydroxydecanedioic acid,^[37] and 4-hydroxydodecadienoic acid has also been previously detected in human urine and measured in higher amounts in situations characterized by the presence of oxidative stress such as that due to aging and diabetes.^[38] Given that insulin resistance is mediated by a dysregulation of fatty acid metabolism^[39] and that pulse consumption has been associated with both better glycemic control^[4] and healthier blood lipid levels,^[3] the differential urinary levels of these metabolites could be related to an alteration in the corresponding pathways manifested early postprandially that could mediate these beneficial effects already observed in the aforementioned clinical outputs. However, given the design of the current study, this is only a hypothesis of the underlying mechanisms, which should be verified in further studies designed specifically for this purpose.

4.6. Strengths and Weaknesses

Most of the BFIs identified in urinary samples from the kinetic study were replicated as discriminant metabolites in 24 h pooled urine. The collection of this type of sample is much simpler than the methodology that has to be used for performing a nutrkinetic study. Additionally, in several epidemiological and intervention studies this type of sample is the one collected by the researchers. Therefore, our observation has important connotations for the future, since it demonstrates that with this type of sample we can reach the same conclusions. Additionally, further analyses of 24 h urine samples available in biobanks from previous studies with legumes will be able to be carried out in order to see which of these candidate BFIs for legumes are replicated in independent studies.

Given that in the already available scientific bibliography none of the identified metabolites were highly specific, further research focused on their combination is required in order to evaluate their robustness and usefulness as candidate BFIs of these foods.

The crossover design of the present study allowed comparisons between the different meals on a within-participant basis. This allowed a better evaluation of the treatment effect since each participant served as its own control.^[40] However, it was not the case with white beans, which were administered in the last phase for all volunteers. Further, an additional untargeted analysis of

the study foods helped us to find out whether the detected marker candidates were original constituents of the test foods or resulted from host or microbial metabolism.

The study also has some additional weaknesses. First, a full-confidence identification level could not be reached for most of the differential metabolites as the corresponding chemical standards were commercially unavailable. However, identifications at levels II and III have also provided interesting information that has allowed us to infer a biological interpretation of the observed results of the present study. On the other hand, the highly controlled environment in which the participants were involved throughout the study together with the high quantity of food provided would have favored the observation of significant candidate BFIs. Therefore, some of the candidate BFIs deciphered in the current study could not be replicated in other studies with lower amounts of ingested food and/or where the background diet is not as controlled as in our case.^[40] Therefore, further studies are needed to validate the results deciphered by the present analyses. Lastly, in this case, pasta was chosen as a control food since it is a low-polyphenol food product, although other foods could also have been chosen instead of it.

4.7. Concluding Remarks

In conclusion, the results of the present untargeted metabolomics work revealed a long list of metabolites that are candidates to be used as biomarkers of pulse intake. Flavan-3-ol-derived metabolites and arginine-related compounds were found to be specific for lentils, whereas protocatechuic acid glucoside was particular to chickpeas; methylcysteine and pipelicolic, hydroxyjasmonic and hydroxydihydrojasmonic acids to white beans; and hypaphorine to chickpeas and lentils. On the other hand, a wide range of peptides, trigonelline, and some polyphenol-derived metabolites appeared to be more generic indicators of pulse consumption. Most of the BFIs identified in urinary samples were replicated as discriminant metabolites in 24 h pooled urine, demonstrating that with 24 h urine pools we can reach almost the same conclusions. The next step would be to develop and apply a quantitative method for the compounds identified in this untargeted metabolomics analysis. Next, biological validation will be required to test these candidate biomarkers in other populations with more individuals and under different experimental conditions and even under free-living conditions.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The authors' responsibilities were as follows: M.G.-A., C.A.-L., and F.M. designed the research; M.G.-A., S.E.-A., M.U.-S., and C.A.-L. designed and conducted the clinical trial; M.G.-A. and A.T.-R. ran the food sample preparation; C.H.W. conducted the GC×GC-MS metabolomics analyses; M.G.-A., M.U., and F.M. conducted the LC-MS metabolomics analyses; M.G.-A., M.U., P.F., and F.M. conducted data processing and statistical analysis; M.G.-A. drafted the manuscript; M.G.-A., C.A.-L., and F.M. have primary responsibility for the final content of the manuscript; and all authors provided critical intellectual input, and revised and approved the final manuscript.

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biomarkers, dietary assessment, legumes, metabolomics, nutrimentalomics

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Supporting Information

Discovery of intake biomarkers of lentils, chickpeas and white beans by untargeted LC-MS metabolomics in serum and urine

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Materials and Methods

Study design

Healthy subjects aged between 18 and 40 with a BMI of between 18.5 and 30 kg/m² were selected for the study. Those volunteers that presented any of the following characteristics were excluded: being a smoker, presence of a chronic or infectious disease, presence of anemia, use of medication (except oral contraceptive pills, which were allowed), intake of nutritional supplements, pregnancy, lactation, antibiotic treatment within the 6 months prior to the study, allergy or aversion to legumes or pasta, or blood donation during the previous 2 months. Volunteers were screened via a telephone interview and invited to make a first visit, during which inclusion/exclusion criteria, health status, anthropometrics and dietary habits were assessed. All volunteers signed an informed consent form before starting their participation. Eleven subjects (four males, seven females) completed three postprandial treatment sessions consisting of consumption of lentils, chickpeas and pasta (control), which were separated by at least 1 week. In each session, each subject was requested to consume one of the study foods in a randomized order. Eight of them also participated in a fourth session, which comprised the consumption of white beans (Figure S1). This sample size has been previously proved to have acceptable power to detect statistically significant postprandial changes associated with specific dietary interventions in an untargeted metabolomics setting. The randomization scheme was generated with the use of the Second Generator from www.randomization.com before the study began. Participants were assigned to the randomization scheme in the order of their inclusion. The study investigators generated the randomization scheme, enrolled participants and assigned participants to interventions.

During the week before starting each intervention, volunteers were asked not to consume any type of legume or legume-based product. Additionally, from 2 days before each intervention, volunteers were asked to follow a polyphenol-free diet, as well as to avoid the ingestion of alcohol. Standardized meals were provided to the volunteers from the evening prior to the day of the intervention until the collection of the last biological sample. Throughout the study, volunteers received dietary advice to ensure the application of the dietary restrictions. On the test day, participants ingested in a maximum time frame of 20 minutes a single 300 g dose of the assigned food, which was warmed together with 7 g of

butter, and they remained at rest in the study center for the following 6 h, during which time they consumed the standardized lunch in the same place. The legumes used in this study were ready-to-use legumes from Spanish protected geographical indications (lentils “Tierra de Campos” and chickpeas “Fuentesaúco”) or a protected designation of origin (white beans “Mongetes del Ganxet”).

Baseline urine and blood samples were collected before the ingestion of the test food. Then, urine samples were collected during the following time intervals: 0–1 h, 1–2 h, 2–4 h, 4–6 h, 6–12 h, 12–24 h and 24–48 h. They were stored at 4 °C until being centrifuged at 1800 g for 10 minutes at 4 °C, aliquoted and stored at -80 °C until the day of the analysis. Urinary 6 h and 24 h pools were prepared later in the laboratory taking into account the weight of the urine excreted in each collection interval. Additionally, further blood samples were taken 1 h, 2 h, 4 h, 6 h, 24 h and 48 h after the ingestion of the test food using Vacutainer tubes. These samples were stored on ice for 30 minutes and then centrifuged at 1600 g for 15 minutes. Afterwards, the supernatants were aliquoted and stored at -80 °C until the day of the analysis.

Untargeted GC×GC-MS metabolome analysis of food samples

Sample extraction, evaporation and derivatization

Work step	Action
<i>Extraction</i>	
1	Thaw study samples and QC sample aliquots on ice
2	Add 750 µL of methanol/tert-butyl methyl ether (70:30)
3	Add 20 µL of a mixture of internal standards containing hexylamine (2 mM), 1- <i>O</i> -methyl-2-desoxy-D-ribose (3.8 mM), 2-chlorophenylacetic acid (1.9 mM), 5-chlorosalicylic acid (3.9 mM), <i>p</i> -chloro-L-phenylalanine (2 mM), 5-bromo-2,4-dihydroxybenzoic acid (2.5 mM), 2-(4-chlorophenyl)ethylamine (2 mM) and phenyl-D-glucopyranoside (1.2 mM) in ethanol/water
4	Mix samples vigorously
5	Incubate samples for 10 min at 35 °C and 1,400 rpm in a shaker
6	Remove insoluble matter by centrifugation using a mini centrifuge
7	Transfer 700 µL of the supernatants into new 2 mL reaction tubes
8	Add 750 µL of methanol/water (50:50) and repeat steps 4–6
9	Combine and mix both supernatants
10	Sediment insoluble matter by centrifugation for 5 min at 4 °C and 16100 × g
11	Transfer 60 µL of the combined supernatants to screw-capped glass vials with 300 µL micro inserts
<i>Evaporation</i>	
12	Dry samples in a speedvac for 1 h and 40 °C and p < 1 mbar
13	Redissolve samples in 15 µL of methanol
14	Dry samples again to remove traces of water (30 min, 40 °C, p < 1 mbar)
<i>Derivatization</i>	
15	Add 40 µL of methoxylamine-hydrochloride in dried pyridine (20 mg/mL)
16	Incubate for 1 h at 40 °C and 1,000 rpm in a shaker
17	Add 100 µL of MSTFA with 1% TMCS
18	Incubate for 1.5 h at 75 °C without shaking
19	Add 10 µL of retention index marker mixture only to daily blank

GC×GC-MS system

Instrument component	Name	Manufacturer
Gas chromatograph (single oven)	GC-2010	Shimadzu Corp, Kyoto, Japan
Single quadrupole mass spectrometer	QP2010 Ultra	Shimadzu Corp, Kyoto, Japan
Autosampler	AOC-5000	Shimadzu Corp, Kyoto, Japan
PTV injector	OPTIC-4	GL Sciences, Eindhoven, The Netherlands
Loop-type cryogenic modulator	ZX2	ZOEX Corp., Houston, USA
Roughing pump	RV-3	Edwards, Crawley, UK

GC×GC method and consumables

Carrier gas	Helium 5.0
GC mode	Constant velocity
Purge flow	3 mL/min
Initial column head pressure	165.9 kPa
Liner type	Deactivated, fritted split liner with quartz wool
Primary column	Rxi-5SilMS, $^1L = 30$ m plus 7.5 m of an integrated pre-column, $^1d_c = 0.25$ mm, $^1d_f = 0.25$ μ m (Restek, Bellefont, USA)
Secondary column	BPX50, $^2L_{total} = 2.2$ m, including a “separation segment” of $^2L_{sep} = 0.7$ m, $^2d_c = 0.15$ mm, $^2d_f = 0.15$ μ m (Trajan Scientific, Ringwood Victoria, Australia)
Column connector	SilTite MiniUnion (Trajan Scientific, Ringwood Victoria, Australia)
GC temperature ramp	90 °C $\rightarrow$ 2 °C/min $\rightarrow$ 320 °C $\rightarrow$ 20 °C/min $\rightarrow$ 340 °C (hold 10.67 min)
Runtime	90 min
Injection mode	Cold split
PTV temperature program	90 °C $\rightarrow$ 60 °C/s $\rightarrow$ 220 °C (hold 1 min) $\rightarrow$ 60 °C/s $\rightarrow$ 300 °C (hold until end of run)
Split ratio program	1:5 (hold 2 min) $\rightarrow$ 1:30 (hold until end of run)
Injection volume	1.2 μ L
Injector syringe	10 μ L, with PTFE-tipped plunger (Trajan Scientific, Ringwood Victoria, Australia)
Syringe wash solvent 1	Heptane
Syringe wash solvent 2	Acetone
Interface temperature	320 °C

Modulator settings

Modulation period (P_M)	2.4 s
Cold jet temperature	-90 °C
Hot jet temperature program	200 °C (hold until 20 min) $\rightarrow$ 250 °C (hold until 40 min) $\rightarrow$ 300 °C (hold until 55 min) $\rightarrow$ 360 °C (hold until 88 min) $\rightarrow$ 200 °C (hold until end of run)
Hot jet pulse duration	250 ms

qMS settings

Interface temperature	280 °C
Ion source temperature	200 °C
Ionization mode	EI (70 eV)
MS mode	Scan
Scan speed	20,000 u/s
Scan range	m/z 60–550
Event time	30 ms
Data acquisition frequency	33 s ⁻¹

Untargeted metabolomics experiments on biological samples

Chemicals and reagents

HPLC-grade methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Milli-Q system from Millipore (Bedford, MA, USA) was used for purifying demineralized water. The 96-well plates with 0.45 µm PVDF filters used were also from Millipore. Ostro plates were purchased from Waters (USA). Labeled-with-deuterium tryptophan-d5, hippuric acid-d5, trans-cinnamic acid-d5, glycocholic acid-d4, cholic acid-d4 and deoxycholic acid-d4 were obtained from CDN ISOTOPES, Inc. (Pointe-Claire, Quebec, Canada). The labeled standards were used for additional control of retention time (RT) and MS signal stability, as well as extraction efficiency. All the standards used for the process of identifying the discriminant metabolites were purchased from Sigma, except for small peptides that were obtained from Spectra 2000 (Rome, Italy). Valerolactone metabolites were chemically synthesized by Del Rio's Laboratory in Parma (Italy).

Sample preparation

All samples were thawed at 4 °C. Quality control (QC) samples were prepared by mixing equal volumes of each sample.

For the nutrikynamic study, given the high number of samples, they were prepared and analyzed in four different batches per sample type (i.e. separately for urine and serum). All samples from the same subject were included in the same batch. Batches were equilibrated by gender (i.e. males/females) and the number of received interventions (i.e. three/four). Samples were located randomly within each batch.

For the pooled samples experiment, two pools were prepared for each subject and food by combining urine samples for up to 6 hours (6 h pool) and 24 hours (24 h pool). For pooling, volumes were mixed proportionally to the weights of each sample. All samples were prepared and analyzed within a single batch in a randomized order.

Internal standards were prepared in pure methanol and external standards in Milli-Q water.

For urine samples, 100 µL of urine diluted with 100 µL of internal standard dissolved in methanol (d5 labeled tryptophan and d5 labeled hippuric acid, 5 ppm final concentration) were placed on 96-well plates from Millipore and filtered. Eluate was further diluted with 300 µL of external standard in Milli-Q water (d5 labeled trans-cinnamic acid, 0.8 ppm final

concentration). After shaking, the mixture was transferred to amber vials to be analyzed on the LC-MS instrument.

For serum samples, 50 μ L of serum were placed on Ostro 96-well plates followed by 50 μ L of internal standard (d5 labeled tryptophan 5 ppm, d5 labeled hippuric acid 5 ppm and d4 deoxycholic acid 2 ppm). Subsequently 150 μ L of cold acetonitrile with 1% formic acid were added to the Ostro plates. The mixture was shaken for 2 minutes and filtered using a positive pressure-96 manifold (Waters, USA). Additionally, the filtering plate was further eluted with a second amount of 150 μ L of cold acetonitrile with 1% formic acid, vortexed and filtered again using the same procedure. Eluates were evaporated with a gentle stream of nitrogen to dryness using a Techne Dri-Block DB 3D heater at room temperature and redissolved with 50 μ L of Milli-Q water and 50 μ L of external standard (d5 labeled trans-cinnamic acid 2 ppm, d4 labeled glycocholic acid 2 ppm and d4 labeled cholic acid 1 ppm). All samples were transferred to amber vials to be analyzed on the LC-MS instrument.

Sample analysis

All samples were analyzed using a Dionex UltiMate 3000 HPLC system coupled to a hybrid linear ion trap Fourier transform (LTQ FT) Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany). The chromatograph was equipped with an autosampler and a quaternary gradient HPLC pump. The sample volumes injected were 10 μ L for urine and 5 μ L for serum. The column used was a Kinetex C18 (150 mm x 2.1 mm I.D., particle size 2.6 μ m, Phenomenex Torrance, CA, USA), together with a 4.0 mm x 2.0 mm I.D. pre-column, which were maintained at a temperature of 40 °C. The flow rates were 350 μ L/min and 300 μ L/min for urine and serum samples, respectively. The mobile phases used were Milli-Q water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). For chromatographic analysis of urine samples, the following gradient was adopted: 0–1 min 5% B; 1–7 min linear gradient 5–45 % B; 7–8.5 min linear gradient 45–80 % B; 8.5–10.5 min maintaining 80% B; 10.5–11 min linear gradient from 80% B to 5% B; 11–12 min maintaining 5% B. Meanwhile, the elution gradient used for the analysis of serum samples was the following: 0–1 min 5% B; 1–12.5 min linear gradient from 5% to 100% B; 12.5–14 min maintaining 100% B; 14–14.3 min linear gradient 100–5 % B; 14.3–15.3 min 5% B.

The Orbitrap LTQ was equipped with an electrospray ionization (ESI) probe and sequentially operated in positive and negative ionization modes, as well as using both the full scan (FS) and the data-dependent acquisition (DDA) modes (in total four injections for each sample). The conditions in ESI positive (and negative) mode were: source voltage 5.0 kV (3.5 kV), heated capillary temperature 320 °C, capillary voltage 30 V (−30 V) and tube lens 110 V (−110 V). In the LTQ component of the instrument, nitrogen was used as both the sheath gas (70 U) and the auxiliary gas (30 U), and helium was used as the damping gas. All measurements were done using the automatic gain control of the LTQ to adjust the number of ions entering the trap. Mass calibration was performed just prior to starting the batch by using flow injection of the manufacturer's calibration standards mixture allowing for mass accuracies of < 5 ppm in external calibration mode. In the FS method the mass range was from 80 to 800 Da at a mass resolution of 30,000 FWHM (m/z 400) in centroid mode. In the DDA method, during the chromatographic run both full scan and MS/MS spectra of the three most intense ions of each full scan were acquired. The resolving power for MS² scans was 7,500. Product ions were generated in the LTQ trap at a collision energy of 35 eV using an isolation width of 1 Da. These double injections with two different mass spectrometry acquisition methods enabled a collection of high-quality full scan data in the first injection with the FS mode dedicated to further statistical analysis, while the second injection with DDA modality allowed for an automatic collection of a high number of MS² and pseudo-MSn spectra. Whenever necessary, additional MSn experiments were performed for selected ions showing statistically significant differences between meals. Three samples were used for each ion of interest and samples were chosen based on maximal intensity.

One solvent sample (methanol/water 1:1) followed by two QC samples were injected every 10 samples to evaluate the stability and performance of the analytical system in terms of retention time drift, mass accuracy, signal intensities and carryover phenomena, and to detect possible analytical variations.

In order to detect any unexpected event in acquisition during the analytical runs, raw data files were examined manually using the Qual Browser module of Xcalibur 2.2 software (Thermo Fisher Scientific, Les Ulis, France).

Data processing

Raw files were converted into mzXML format using the MSconvert module of ProteoWizard software. The obtained data files were further processed using the XCMS package in the R platform. XCMS parameters (Table S1) were optimized separately for each acquired data set (i.e. for each experiment, biofluid and ionization mode) using a series of analyses. This was evaluated through correlations between manual integration of selected peaks (IS, ES and endogenous compounds present in samples) and the corresponding features obtained after data processing, as well as correlations between different features from the same compound (i.e. molecular ion, ^{13}C isotopes, ion-source fragments and adducts). The manual peak integration was performed using the Qual Browser module of Xcalibur 2.2 software (Thermo Fisher Scientific, Les Ulis, France) for the most intensive ion of internal and external standards, as well as for a series of endogenous compounds. Data processing provided a two-dimensional data set, where each feature was defined by a specific RT and mass-to-charge ratio value (m/z). The signal intensities of the features for each sample were represented by peak area.

Prior to data analysis, each data set was filtered in order to discard noisy and irrelevant features. Firstly, those features whose first decimal place took a value between 4 and 8 were removed from each data set. Subsequently, the signals that showed a higher variation (according to the coefficient of variation) within QC samples than within study samples were also excluded. In order to ensure the selection of features with suitable MS/MS fragmentation spectra recording, only features with a median intensity above 2,000,000 or 200,000 spectra counts in urine or serum samples, respectively, in at least one group (defined according to time and meal) were retained in the data set. Finally, a “20% rule” was applied, excluding those features not present in at least 20% of the samples of at least one group. This threshold was adopted given the fact that this study is focused on biomarkers of food intake, and because of the potential presence of different metabotypes among volunteers, who may respond differently to food intake due to different microbiota composition and/or activity (e.g. urolithin B metabolites are only produced by 10–50 % of the population after ingesting ellagitannin-rich foods) [1]. All zero values were replaced by a random number between 1 and 500. These thresholds were fixed empirically according to study data, and correspond to the intensity level representing noise on the chromatogram.

Statistical analysis

All the analyses were performed using the R software employing code written in-house. Firstly, principal component analysis (PCA) on log-transformed and Pareto-scaled data was applied to check data quality (i.e. possible batch effect), to detect the presence of outliers and to assess potential associations among samples. The subsequent data analysis strategy was designed differently for the nutrikinetic data and for the pooled sample experiments.

Nutrikinetic data

Since initial data exploration PCAs showed that batch differences had the greatest source of variance for each data set (see Results section), a batch correction technique was applied. This was based on transforming the data in order that the median of each feature in each batch was equal to 1. Additionally, data sets corresponding to urine samples were further normalized by the corresponding feature of creatinine. The effectiveness of batch correction was assessed with additional PCAs.

The abundances of each feature of baseline samples were subtracted from the samples corresponding to the same subject and food intervention and subsequently negative values were replaced by 0. Discriminant features were selected using a two-step procedure on the basis of their nutrikinetic curve behavior, as well as considering their statistical significance. For that purpose, we first selected those features where at a minimum of two consecutive time points the lowest values of one group were greater than the highest values of the other groups. The 25th and 75th percentiles of feature intensities were used as a measure of the lowest and highest values, respectively. The same procedure was applied when two or more groups presented the same behavior, which differed from that of the other groups. Then, the area under the curve (AUC) of each selected feature was calculated between time 0 h and time 48 h using the “pracma” R package. Differences of AUCs among diets were tested using the Kruskal-Wallis test and the obtained p-values were corrected using the Benjamini-Hochberg (BH) method. Adjusted p-values < 0.05 were considered statistically significant.

Pooled sample experiments

In order to correct data for an individual subject, the intensity of each feature corresponding to the pooled sample after intake of the control food was subtracted from the other samples from the same volunteer. Next, Kruskal-Wallis analysis on corrected data was applied to select discriminant features, correcting for multiple testing using the BH procedure and a p-value threshold of < 0.05 for defining statistical significance.

Data visualization was performed through boxplots on transformed data to see the intensities of discriminant metabolites and their distribution among groups; and through heatmaps of hierarchical clustering analysis (HCA) on Pareto-scaled data using Euclidean distance to further visualize the discriminatory ability of significant metabolites deciphered by the statistical analyses. Both types of graphs were produced taking into consideration the MS response (peak area) of the most intensive feature per identified metabolite.

Metabolite identification

Metabolite identification was performed through a multistep procedure using the features selected as discriminants. Firstly, correlations between features with a very close RT were used in order to make clusters of features originating from the same metabolite (i.e. molecular ions, ¹³C isotopes, adducts and in-source fragments), all characterized by presenting a very close RT and high correlations between them. This step was complemented by the use of the R package “cliqueMS” [2]. Cytoscape’s (v.2.8.3) plug-in MetaNetter [3] was used to infer the assignation of each feature within each metabolite using an in-house database of the expected adducts and neutral losses. Once the molecular ion of each metabolite had been deciphered, its monoisotopic molecular weight and LC-MS/MS spectra were used to obtain the molecular formula. The chemical formula for parent ions was calculated from accurate mass (with a mass accuracy < 5 ppm) using a mass decomposition web tool (<http://predret.org/tools/mass-decomposition/>). Later, the R package “CluMSID” [4] was used to access the data from DDA experiments to get the MS2 and pseudo-MSn spectra of ions of interest in order to perform the structural elucidation through the evaluation of fragment ions and neutral losses.

The levels of identification were reported taking into consideration the recommendations of the Metabolomics Society [5]. Briefly, level I was assigned to those metabolites that were confirmed by matching their retention time, mass accuracy and fragmentation pattern with the corresponding commercial standard; level II was associated with those metabolites for which no reference standard was available but their exact mass and fragmentation spectra matched one provided by a database library (such as mzCloud, HMDB, METLIN, MassBank, LipidMaps and/or MyCompoundID) and/or scientific bibliography; and level III was used for those putatively identified metabolites for which there was a theoretical matching of the exact mass and fragmentation pattern, although to the best of our knowledge no fragmentation spectra were available.

Results

Data acquisition quality

Analytical variability within QC samples

Quality control samples were firstly used to manually verify the quality of raw data and analytical performance of the analyses in terms of variations in RT and mass accuracy and in signal intensities (peak area) of internal and external standards and for selected native metabolites (Table S3). Analytical performance was mainly related to the total number of samples analyzed. In the experiment with pooled samples ($n = 150$ consecutive injections), retention time shifts (≤ 5 sec), mass accuracy deviations (≤ 3 ppm) and variations in signal intensities ($CV \leq 15\%$) for all monitored compounds satisfied the criteria usually accepted in untargeted metabolomics studies (Table S3-B). Experiments with the entire nutrikine data set ($n = 476$ and $n = 438$ consecutive injections for urine and serum samples, respectively) suffered from higher deviations of peak area for internal and external standards, while retention time shift and mass accuracy were within acceptable limits (Table S3-A). The reason for such a drop in intensity was the long queue of ca 100 hours for each biofluid.

Verification of data processing

Additionally, manual peak integration of data with Qual Browser was used for verification of automatic XCMS data processing. As shown in Figure S2, all monitored metabolites showed high correlations between their manual integration and the corresponding feature from XCMS extraction. Furthermore, the correlations between the different features of the same compound showed high correlations among them (Figure S3).

Data filtering

The total number of detected features, as well as the number of features considered for the statistical analyses after feature reduction, are specified in Table S4. After data filtering, the data sets contained 13–28 % of the original features. All filtered data sets included redundant mass signals (i.e. molecular ions, ^{13}C isotopes, adducts and in-source fragments).

Unsupervised multivariate analysis

As already mentioned in the methodology section, PCA analyses were performed to check data quality (i.e. potential batch effect due to signal drift, sensitivity loss, etc.). Due to the different number of analyzed samples in both experiments (i.e. kinetics and pools), the output of these initial analyses was clearly different. Therefore, they are going to be discussed in separate subsections.

Nutrikinetic experiment

Figure S4-A depicts the PCA score plots for the raw data using both study samples and QC samples. When the samples are colored according to the batch, the drift effect on the variances is clear. Therefore, before proceeding with the following analyses to select the discriminant features according to the meal, data have been normalized according to the batch. Additionally, data from urine samples were further normalized by the feature corresponding to creatinine. Once normalized, the batch effect became less clear, at least as the main source of variability evaluated with the first two components of the PCA. Another usual utility of PCAs in untargeted metabolomics is the capability to detect the presence of potential outliers. In this case, none were recognized.

From the PCA score plots of normalized data in urine study samples (Figure S4-B1), when samples are colored according to time point a time course trajectory can be seen through PC1. Specifically, it can be clearly seen that samples collected from 02 h up to 06 h after meal consumption are clustered on the left side of the plots, suggesting that the postprandial state probably plays an important role in urinary metabolome. On the other hand, in these samples an important interindividual factor can also be seen since samples from the same subject tend to be clustered together. Both time course and subject factors seem to be the factors with the highest influence on the variability of urinary metabolome, with this being even higher than the potential impact of the meal ingested.

In the case of serum samples, at least when looking at the first two components of the PCA, which cover the higher variability (up to 15% approx.), in data from positive ionization mode the postprandial time point is reflected in the second component, whereas in data from negative ionization mode the subject factor seems to have the highest influence, while postprandial time points are not that visible anymore (Figure S4-B2).

Pooled samples experiment

For pooled samples, the PCAs were first performed by including all study samples together with QC samples, and showed that QC samples (green points) clustered together (Figure S4-C), and that in this case no major analytical drifts were observed between the first (yellow) and the second (blue) half of the analyzed samples. Furthermore, these graphs also indicated a low technical variability during the analyses, as well as the absence of any trend potentially related to sequence order, thereby corroborating the robustness of the procedure.

Again, additional PCAs were performed including only study samples to verify the presence of potential associations among them. These PCAs revealed that the main sources of variability were the time of sample collection (06 h vs. 24 h pool) followed by the subject factor (i.e. samples from the same volunteer clustered together). It was also difficult to observe clustering due to the type of consumed food using the whole data set, suggesting once more that despite being in a postprandial state, interindividual variability has a larger impact on the whole metabolome than that of ingested food.

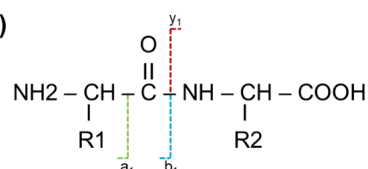
Identification of discriminating metabolites

Small peptides (M01–M26)

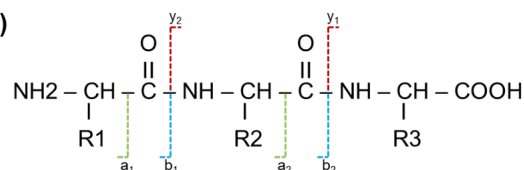
Dipeptides (M01–M08) were annotated taking into consideration both the accurate mass and the backbone fragmentation since the cleavage of the amide bond of protonated dipeptides leads to formation of the y1 ion, which corresponds to the protonated amino acid that was originally located in the C-terminal position of the dipeptide [6]. Additionally, for some of these dipeptides the immonium ions were also observed at the low-mass end of the spectrum. Therefore, the observation of the masses of the amino acid residues along with the masses of their immonium ions led us to assign level II to their identification when the commercial standard was not available. Additional fragments were observed at the high-mass end of the spectrum, such as loss of neutral molecules like H₂O, HCOOH, CO₂ and NH₃ [7].

Peptide fragmentation nomenclature

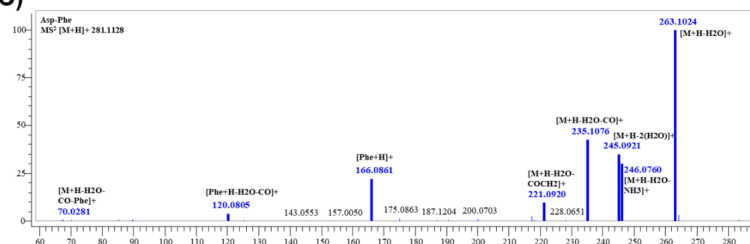
(A)



(B)



(C)



Therefore, the protonated molecular ions at m/z 247.1289, m/z 265.0853, m/z 281.1132, m/z 297.1082 and m/z 233.1132 were assigned, respectively, to the dipeptides Asp-Leu, Asp-Met, Asp-Phe, Asp-Tyr and Asp-Val. The corresponding MS/MS data suggested that the C-terminal dipeptide moieties were leucine, methionine, phenylalanine, tyrosine and valine, respectively, since the MS² spectra contained their protonated ions (m/z 132.1017, m/z 150.0579, m/z 166.0860, m/z 182.0813 and m/z 118.0854, respectively). Additionally, their MS/MS mass spectra also contained the immonium ions of Leu, Met, Phe, Tyr and Val, respectively. These spectra also contained m/z features resulting mainly from the loss of H₂O (-18 u) and HCOOH (-46 u), among others. When their commercial standards were spiked in urine samples, a small shift in the RT was observed, suggesting that the injected standard was probably an isomer of the compound found in the biological sample. It is for this reason that the identification level assigned to these compounds was II instead of I (Table S5).

Tripeptides (M09–M17) were also annotated according to the fragmentation pattern of small peptides under low collision energy [7]. This fragmentation pattern typically consists of series of a, b and y fragment ions, as described in Figure 5b. The information provided by these ions reflects the amino acid sequence. The exact mass together with the presence of some of these diagnostic ions in their MS/MS spectra allowed specific annotation for most of the tripeptides, although for some of them the exact amino acid sequence was not possible due to the insufficient information on the MS/MS spectra. Therefore, the protonated molecular ions at m/z 360.2127, 318.166 and 304.1504 were assigned to the tripeptides containing the amino acids Asp-(i)Leu-(i)Leu, Asp-Ala-(i)Leu and Asp-Gly-(i)Leu, respectively. The product ions (b₂) at m/z 229.1181 (+), m/z 187.0713 (+) and m/z 173.0559 (+), respectively, indicated the presence of a leucine (m/z 131.09462) at the end of the molecule [7, 8]. Additionally, the identification of the tripeptides Asp-Gly-Val, Asp-Asn-Val and Asp-Gly-Leu was further confirmed by their analytical standards.

Moreover, two cyclic dipeptides were also identified (M18 and M19). This was done based on the molecular formula, as well as one of their main fragments that agreed with the one indicated by Yamamoto et al. (2016) [9]. Finally, five compounds (M20–M24) were assigned to this class of small peptides (level III) since their MS² spectra showed the protonated ion of an amino acid or the loss of an exact mass corresponding to an amino acid.

However, the specific structure was not deciphered due to the lack of sufficient diagnostic ions in MS/MS spectra.

On the other hand, the amino acids hydroxyarginine (M25) and oxoarginine (M26) appeared in urine only after the intake of lentils. Both compounds presented mass accuracies of < 0.5 ppm with values of the ring and double bond equivalent (RDB) consistent with the corresponding molecular formulas (1.5 and 2.5, respectively). Additionally, hydroxyarginine was further annotated with the information on the MS/MS mass spectra. It resulted in the loss of up to two ammonia groups together with a loss of a CO moiety. Different possible structural isomers and stereoisomers of hydroxyarginine have already been described, according to the position of the hydroxyl group and the structure of the amino terminal. Since the observed MS/MS mass spectra did not match the fragmentation pattern of the N(omega)-hydroxyarginine commercial standard, we were not able to elucidate the isomeric form of the molecule. Therefore, this metabolite has been generically identified as hydroxyarginine.

Flavan-3-ol-derived metabolites (M27–M31)

Four metabolites were putatively annotated as different flavan-3-ol-derived metabolites based on fragments already reported in the scientific bibliography [10]. One of them was assigned to a phase 2 metabolite of (epi)catechin, namely (epi)catechin sulfate (M27, level II), for which the main fragments corresponded to the sulfate moiety loss [m/z 289.0717 (-)]. Other additional fragments already reported by van der Hooft et al. [10] were also present in MS² (m/z 245.0819 and m/z 205.0506) and pseudo-MS³ (m/z 245.0817, m/z 205.0507, m/z 179.0351 and m/z 231.0300) spectra (Table S3).

For the two 5-(3',4'-dihydroxyphenyl)- γ -valerolactone conjugates (level II), the most abundant fragment was m/z 207.0663 (-), attributed to the neutral loss of sulfate (M29) and glucuronide (M30), and the additional fragments agreed with the ones specified by van der Hooft et al. [10]. In both cases the isomer 5-(3',5'-dihydroxyphenyl)- γ -valerolactone was discarded based on the comparison with the information provided by van der Hooft et al. [10] and with the authentic standard of the metabolites 5-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'-O-glucuronide, 5-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'-O-sulfate and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone-3'-O-sulfate. For the glucuronide, the isomer 5-(3',5'-dihydroxyphenyl)- γ -valerolactone was discarded since the retention time of the

standard was appreciably different (3.76 min) and also because this standard did not show the fragment 163.0766 corresponding to the assignation [M-H-glucuronide-CO₂]-, which van der Hooft et al. [10] attributed specifically to the isomer 5-(3',4'-dihydroxyphenyl)- γ -valerolactone. A similar situation occurred with the sulfate conjugate. In that case, the isomer 5-(3',5'-dihydroxyphenyl)- γ -valerolactone was also discarded due to a considerable difference in retention time (4.04 min) and also because this standard presented the MS² fragments m/z 223.0618 (-) and m/z 179.07221 (-) and the pseudo-MS³ 207.0669 (-) fragments m/z 123.0457, 189.0560 and 145.0662 [10], whereas the isomer 5-(3',4'-dihydroxyphenyl)- γ -valerolactone-4'-O-sulfate was discarded despite an equal MS² fragmentation pattern between the standard and the discriminant metabolite due to a slight difference in retention time (4.53 min), which is in line with the coelution reported by van der Hooft et al. between 3'-O and 4'-O-sulfate isomers [10].

Another valerolactone-derived metabolite was identified as 5-(3',5'-dihydroxyphenyl)- γ -valerolactone-O-glucuronide-O-methyl (M31), based on the presence of the fragments m/z 221.0796 (-), m/z 175.0251 (-) and m/z 113.0243 (-) [10]. In regard to 4-hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulfate (M28, level II), it was identified on the basis of its molecular ion [m/z 305.0337 (-)], its main fragment m/z 225.0771 corresponding to the loss of sulfate moiety and the additional fragments m/z 207.0665, m/z 163.0768, m/z 123.0455, m/z 101.0248, and m/z 179.0721 [10].

Other polyphenol-based metabolites (M32–M38)

The two phloroglucinol phase 2 metabolites (M32 and M33) were initially identified according to the information provided by van der Hooft et al. [10]. In both cases, the MS² spectra in negative mode showed the daughter ion at m/z 125.0246, which corresponds to the deprotonated ion of phloroglucinol, indicating the loss of the sulfate and glucuronide moieties. Other fragments found in MS² spectra of phloroglucinol glucuronide are included in Table S3 and are in line with the literature [10]. Additionally, the sulfate conjugate was further confirmed with the injection of the corresponding standard.

Protocatechuic glucoside (M37) was tentatively identified at level II based on the MS² spectra, since the parent ion [m/z 315.0721 (-)] showed a series of fragments (m/z 153.01964, 152.01186, 109.02981, 108.02195) that corresponded to the fragmentation of

protocatechuic acid [11]. This observation is in line with the composition of test foods, since the amounts of protocatechuic glucoside were higher in chickpeas than in lentils and white beans [12].

Vanillic acid sulfate (M38) was assigned after observing that one of its fragments (m/z 123.002) had the m/z value corresponding to the loss of a sulfate moiety and the corresponding pseudo-MS³ fragmentation pattern was in line with the one observed in the database mzCloud for vanillic acid. Neither vanillic acid nor protocatechuic acid were detected in the legumes in the unconjugated form in the GCxGC-MS analyses.

Jasmonic acids (M39–M41)

The identification of hydrojasmonic (M39) and two hydroxydihydrojasmonic isomer (M40 and M41) acids was done on the basis of their accurate mass and was supported by comparison of their MS/MS spectra with those of jasmonic acid standard. All of them showed losses of H₂O and CH₃CO₂H moieties in the positive ionization mode. Additionally, the fragmentation pattern of hydrojasmonic acid in negative ionization mode was in line with the information reported in the literature [13], which has stated that the fragmentation pattern of parent ions in negative mode is characterized by the loss of –CO₂ (m/z 181.1233), –CH₃CO₂H (m/z 165.0921), –H₂O (m/z 207.1026) and –CO₂-H₂O (m/z 163.1129). Additionally, the main fragments of both hydroxydihydrojasmonic isomers were those corresponding to the loss of –H₂O (m/z 209.1182) and –CO₂-H₂O (m/z 165.1284). Jasmonic acid was not detectable in the test foods in the GCxGC-MS analyses.

Other food-derived metabolites (M42–M49)

Trigonelline (M47, level I) was identified using the information from the positive ionization mode on the basis of the comparison with its authentic retention time standard, together with the accurate mass of its molecular ion at m/z 138.0549 and its characteristic losses of CO₂ (m/z 94.0650), HCOOH (m/z 92.0492) and CO (m/z 110.0598). The same procedure was followed for the identification of ascorbic acid, methyleysteine, pipecolic acid and hypaphorine (Table S5). The fragmentation pattern observed in the MS² spectra of ascorbic acid (M42, level I) in negative ionization mode included the fragment ions at m/z 115.0041 (loss of C₂H₄O₂), 87.0096 (additional loss of CO) and 59.0143 (additional loss of

two CO). On the other hand, methylocysteine (M45, level I) was characterized by the loss of NH₃ (m/z 119.0158) and its daughter ion 73.0280 in the positive ionization mode. Pipecolic acid (M46, level I) was characterized by the loss of H₂O and CO (m/z 84.0804) in the positive ionization mode. Finally, hypaphorine (M44, level I) was annotated after matching its retention time, exact mass and fragmentation pattern in the positive ionization mode with those obtained after injection of the commercial standard under the same analytic conditions. In this case, the main fragment was (+) 188.0702, which at the same time had as its principal ion daughter (+) 146.0600. Charged compounds like trigonelline and hypaphorine are not GC compatible and were thus not covered by the GCxGC-MS food analysis.

The ions at m/z 234.0430 (+) and at m/z 232.0287 (-) were assigned to dopamine sulfate (M43). The MS² experiments in both ionization modes showed a neutral loss of 80 Da (79.9582) producing the fragment ions at m/z 154.0858 and m/z 152.0719 in positive and negative ionization modes, respectively. The pseudo-MS³ fragmentations of the main daughter ions of conjugated metabolites produced a series of ions that matched the fragmentation pattern observed for the dopamine unconjugated commercial standard (Table S5). Unconjugated dopamine was not detectable in the legumes.

The feature at m/z 441.1753 (+) was initially assigned to a phase 2 metabolite since its main fragment was assigned to the ion corresponding to the loss of the glucuronide moiety. The mass of the aglycone form matched for γ -carboxyethyl hydroxychroman (γ -CEHC) and abscisic acid. Injection of corresponding standards showed that both compounds had in common the losses up to two molecules of water (i.e. m/z 247.1329 and m/z 229.1223 in positive ionization mode). Specific ions for each molecule were also observed. In particular, abscisic acid showed daughter ions at m/z 191.0699, m/z 187.1140 and m/z 163.0751, whereas γ -CEHC presented ions at m/z 203.1068 and m/z 151.0754. Since the MS_n of our aglycone showed the fragments at m/z 203.1068 and m/z 151.0754, we concluded that this metabolite was γ -CEHC glucuronide (M49), with a level of annotation set to II. The same procedure was followed for γ -CEHC glucose (M48).

Endogenous metabolites (M50-M54)

Several fatty acids conjugated to glucuronide moiety were found as statistically significant features, namely: dihydroxydecanoic, dodecanedioic, hydroxydodecanedioic and tetradecanedioic acids. The features at m/z 407.1915 (+) and 405.1766 (-) were annotated as dodecanedioic acid glucuronide (M51) since both the exact mass and the fragmentation pattern of the aglycone form ion fitted with the one observed when injecting the standard of dodecanedioic acid. In other words, MS/MS fragmentation of the protonated aglycone ion at m/z 231.15907 resulted in the sequential losses of water moieties (i.e. m/z 213.1489, m/z 195.1381, m/z 177.1277 and m/z 159.1173), followed by the additional loss of the carbon monoxide group (i.e. m/z 167.1442 and m/z 149.1326) and CH_2 groups (m/z 121.1010, m/z 107.0852 and m/z 93.0695). On the other hand, the MS/MS spectrum of the deprotonated aglycone ion at m/z 229.1444 resulted in a main ion corresponding to the loss of a water and CO_2 moiety (i.e. m/z 167.1442). The same fragmentation scheme was observed for the other metabolites of this group.

Figures

Figure S1. CONSORT Flow Diagram

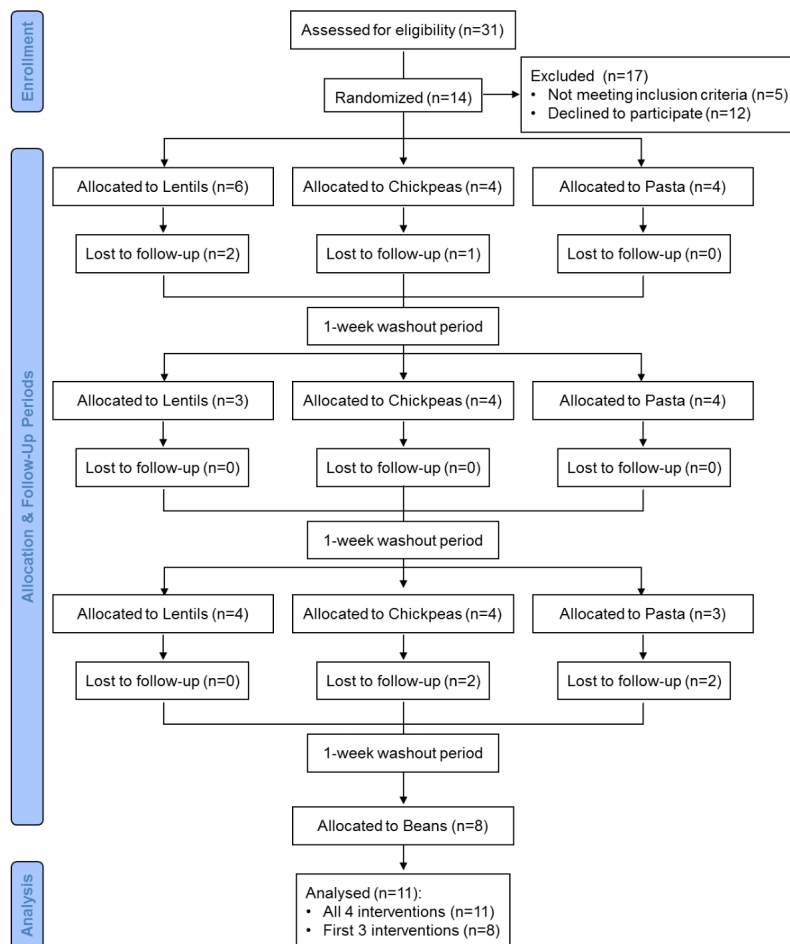
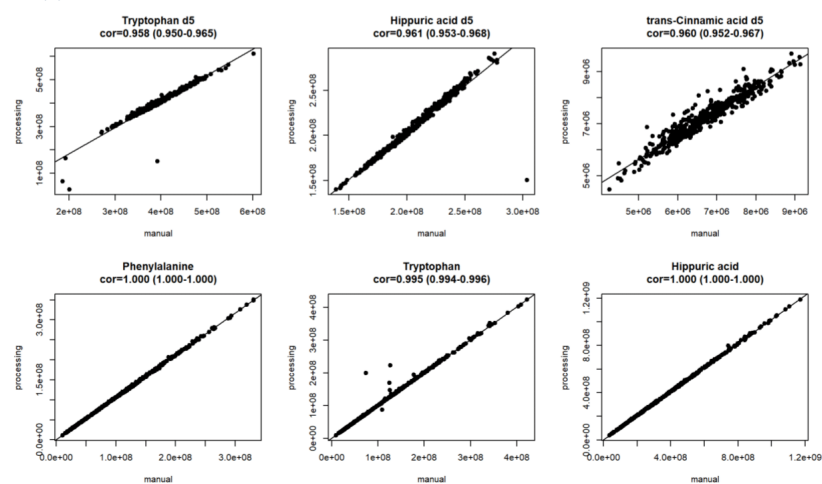


Figure S2. Correlation plots comparing data from manual integration with XCMS automated integration

Figure S2-A. Nutrikinetic experiment

Urine samples

ESI(+)



ESI(-)

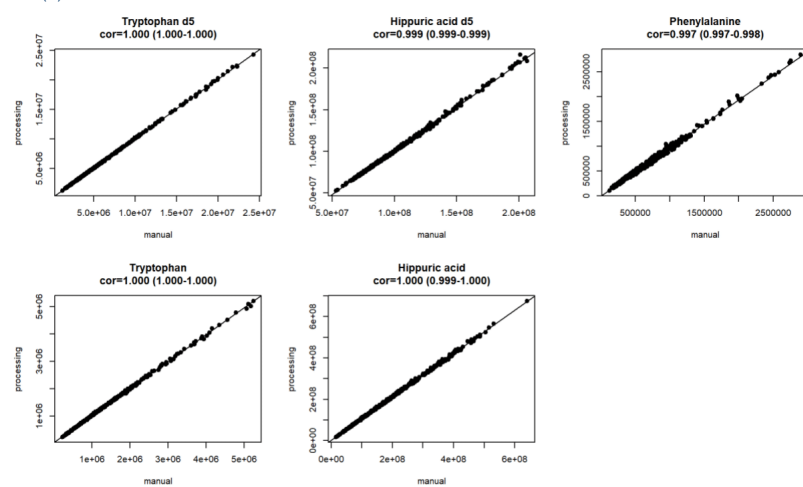


Figure S2-A. Nutrikinetic experiment (continuation)

Serum samples

ESI(+)

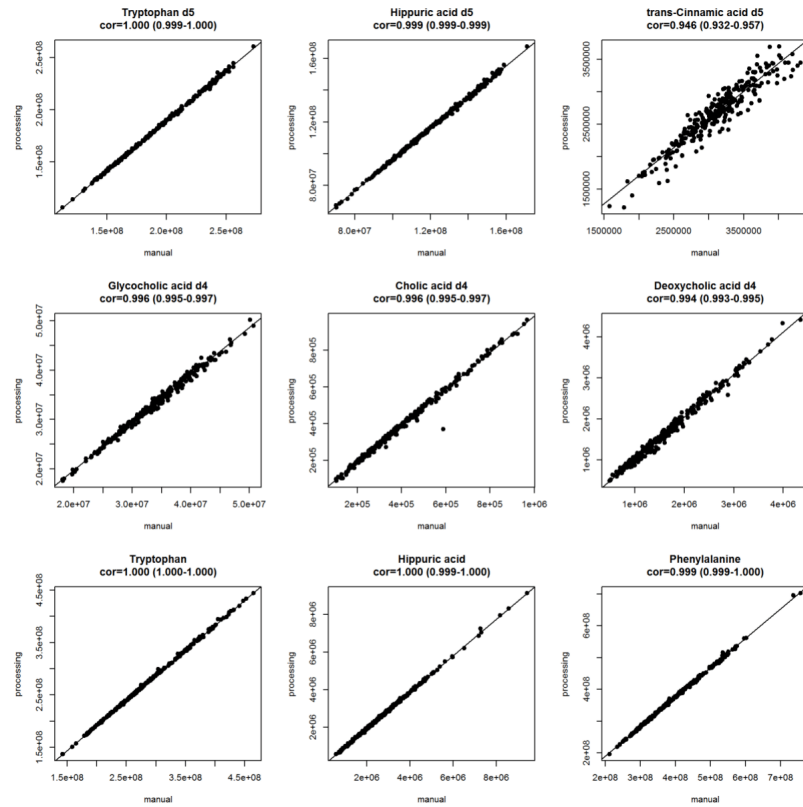


Figure S2-A. Nutrikinetic experiment: Serum samples (continuation)

ESI(-)

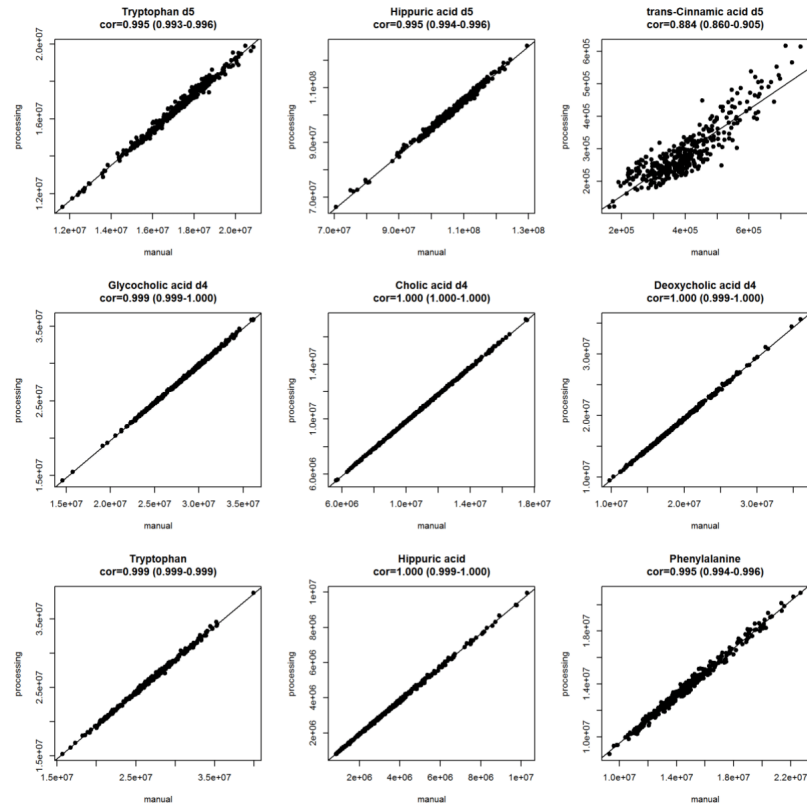
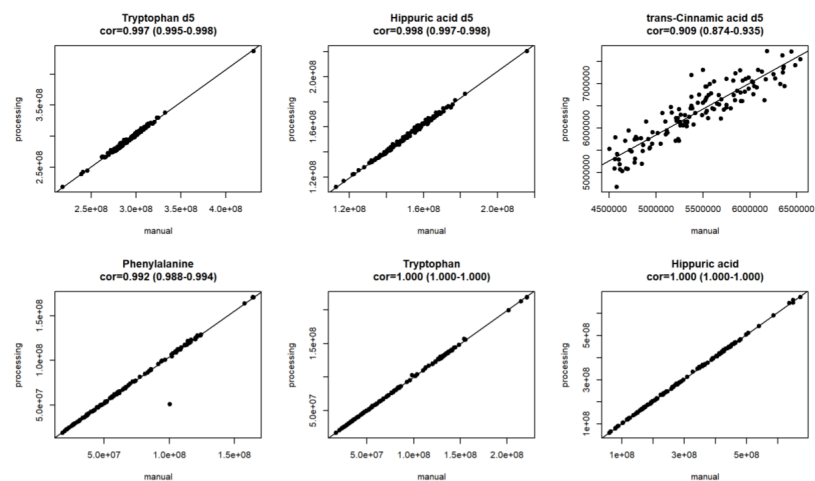


Figure S2-B. Pooled samples experiment
ESI(+)



ESI(-)

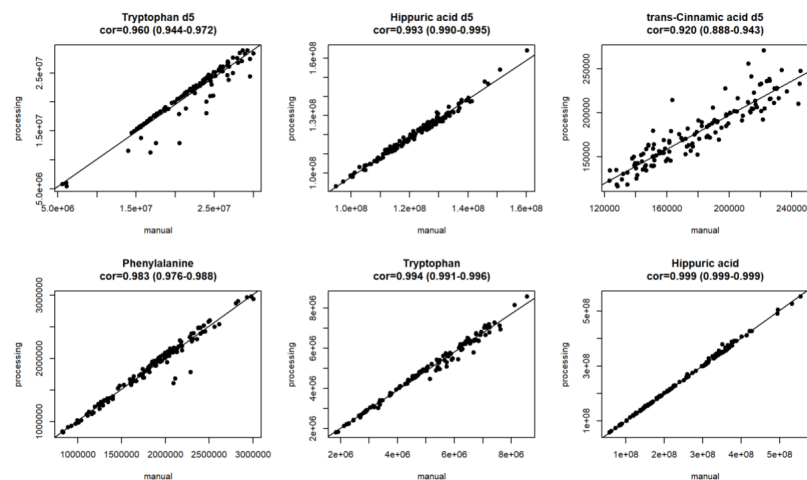
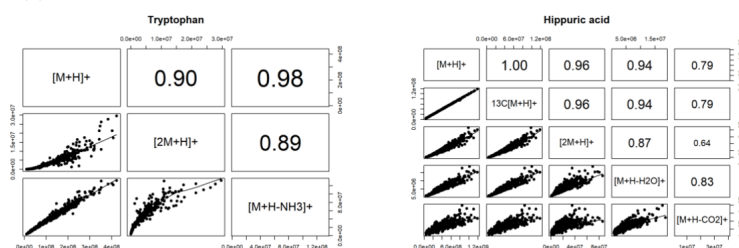


Figure S3. Correlations between the different features from the same metabolite

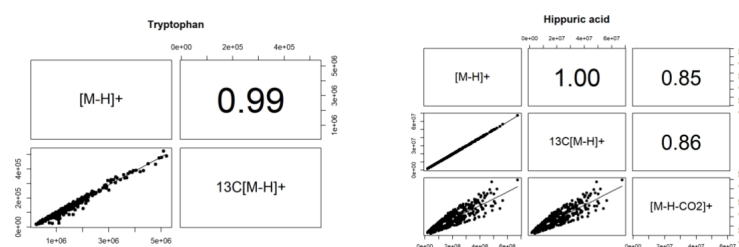
Figure S3-A. Nutrikinetic experiment

Urine samples

ESI(+)

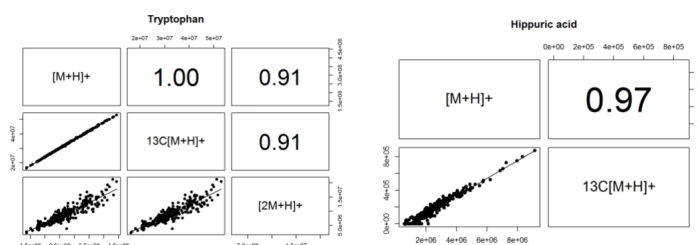


ESI(-)



Serum samples

ESI(+)



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Figure S3-A. Nutrikinetic experiment: Serum samples (continuation)

ESI(-)

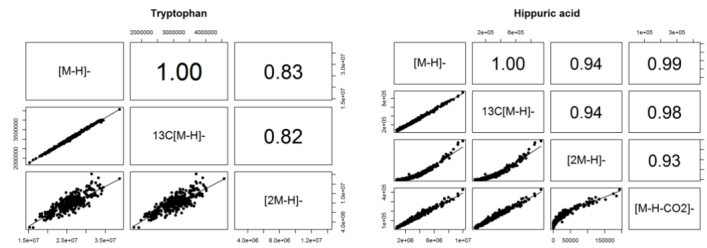
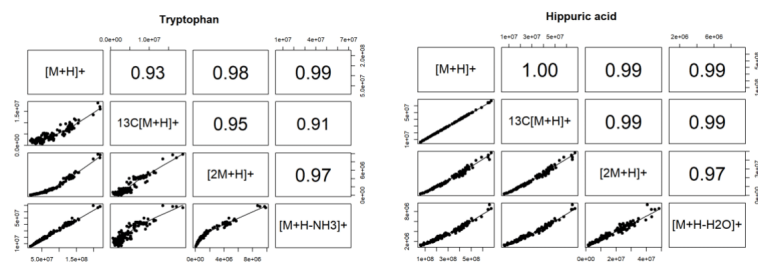


Figure S3-B. Pooled samples experiment

ESI(+)



ESI(-)

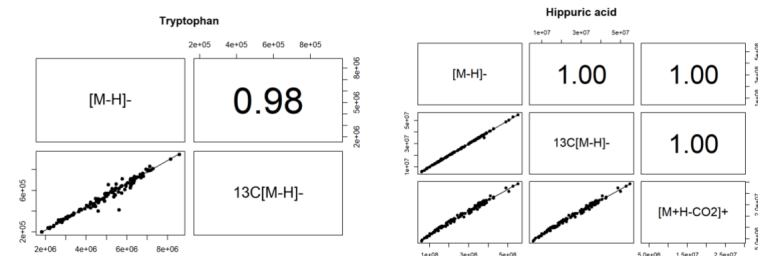
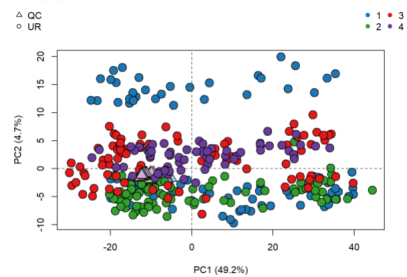


Figure S4. Score plots of the principal component analysis with QC and all study samples

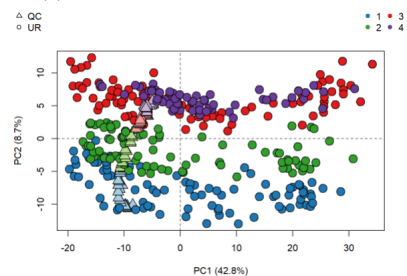
Figure S4-A. Nutrikinetic experiment – Raw data

Urine samples

ESI(+)

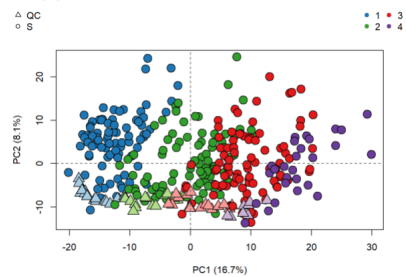


ESI(-)



Serum samples

ESI(+)



ESI(-)

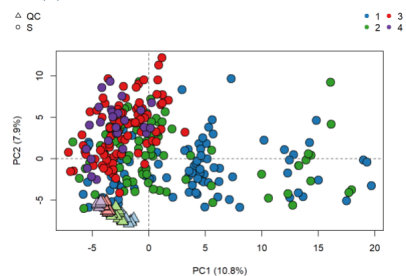
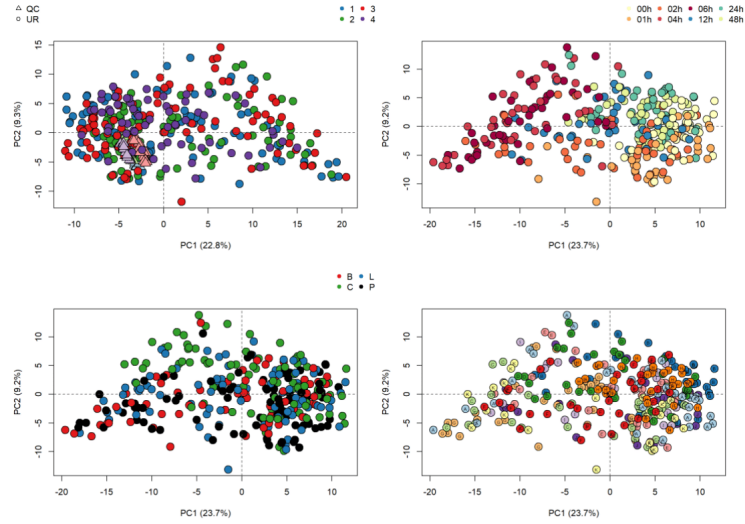


Figure S4-B. Nutrikinetic experiment – Normalized data

Figure S4-B1. Urine samples

ESI(+)



ESI(-)

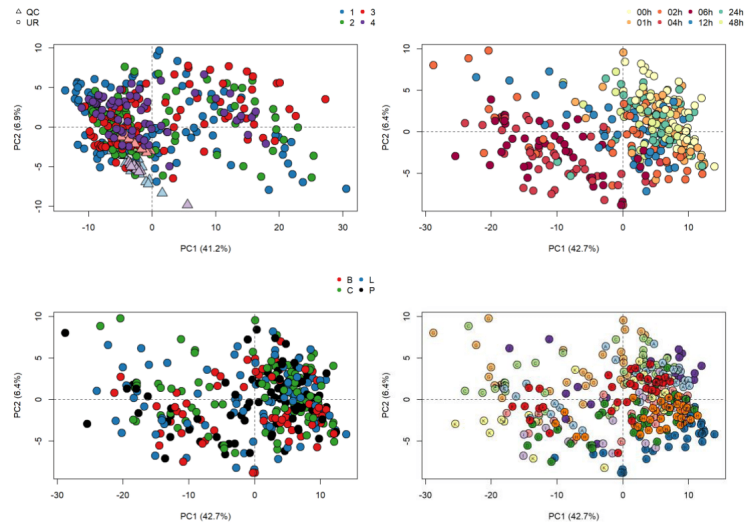
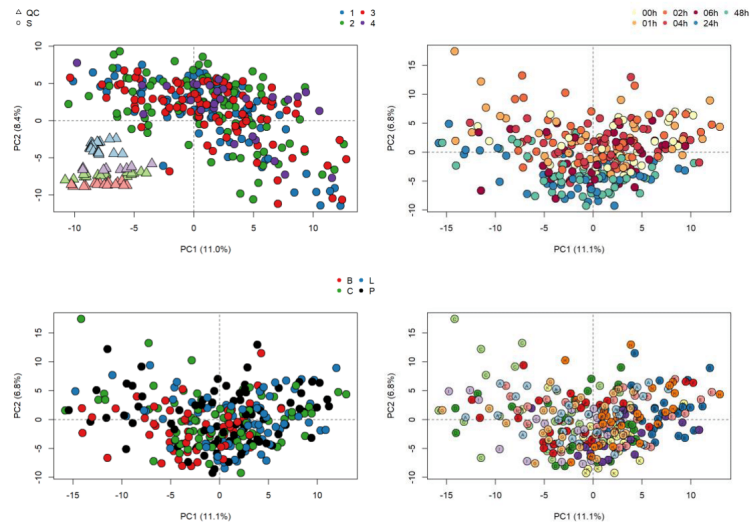


Figure S4-B2. Serum samples

ESI(+)



ESI(-)

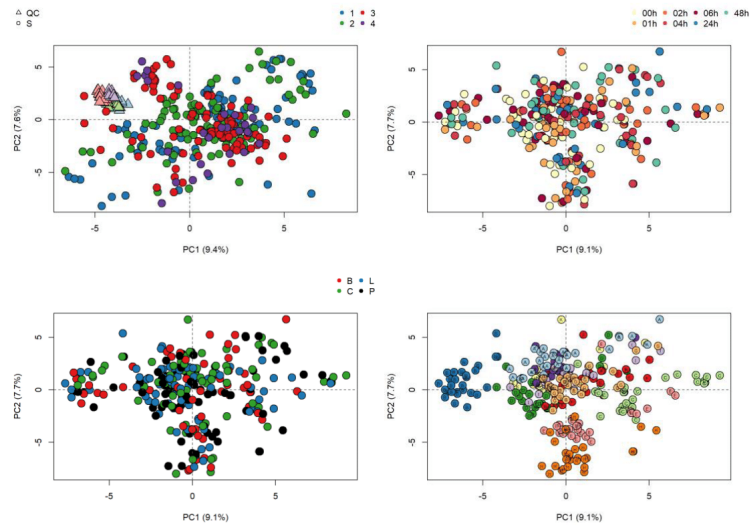
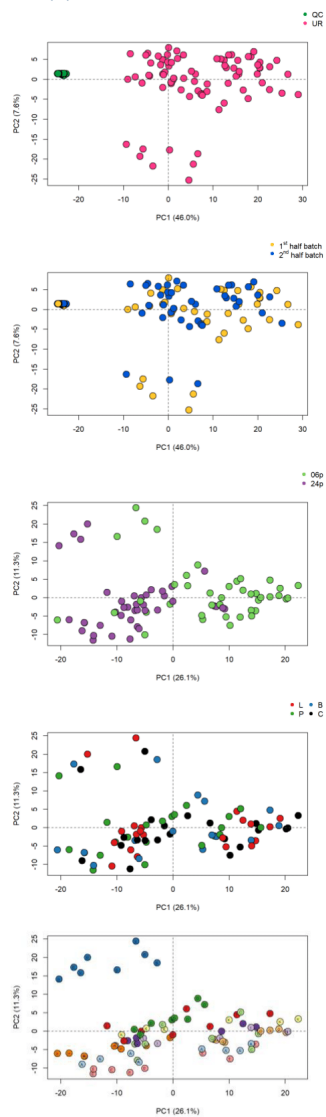


Figure S4-C. Pooled samples experiment

ESI(+)



ESI(-)

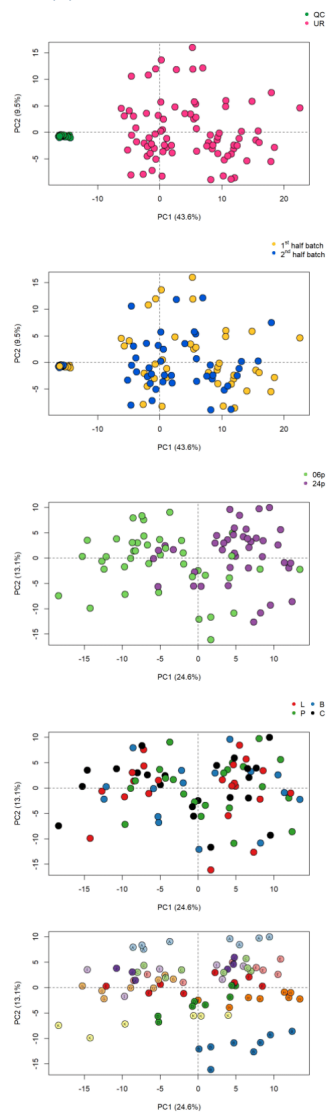


Figure S5. Categories of identified metabolites

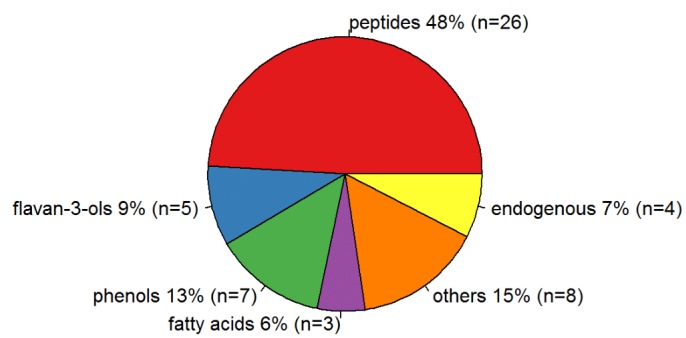


Figure S6. Urine and serum kinetic curves for metabolites that were statistically significant

Urine samples

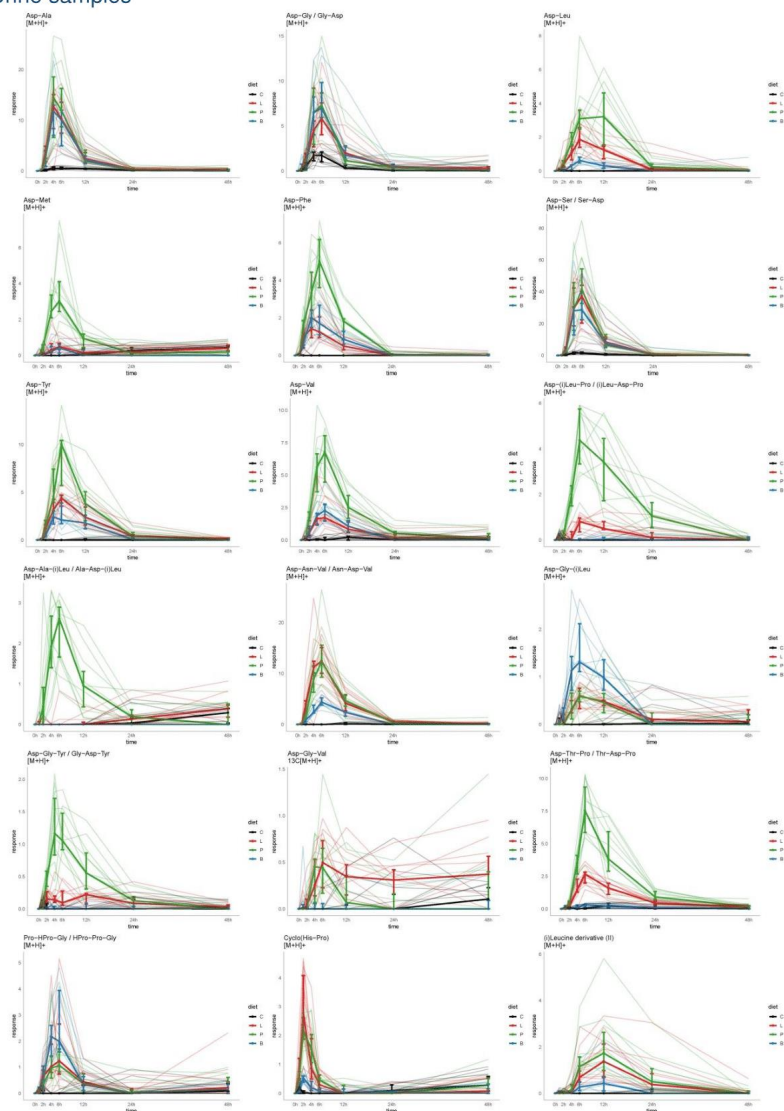


Figure S6. Urine samples (continuation)

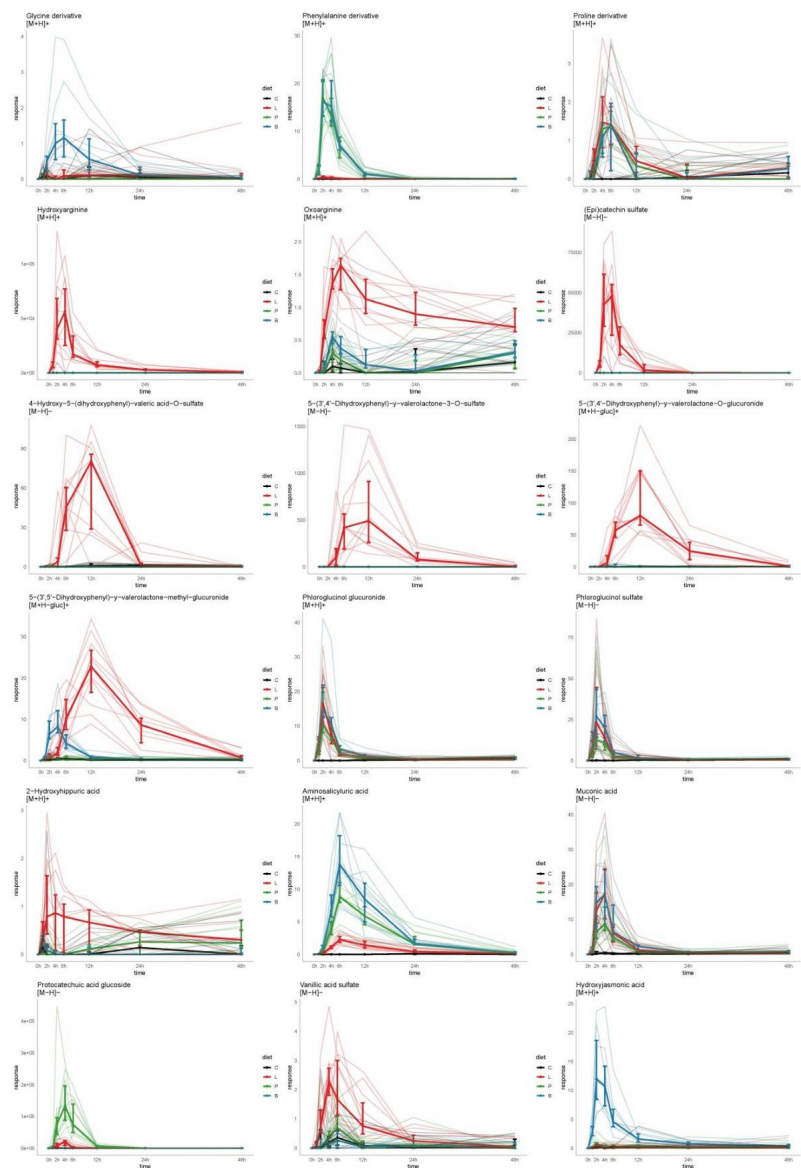
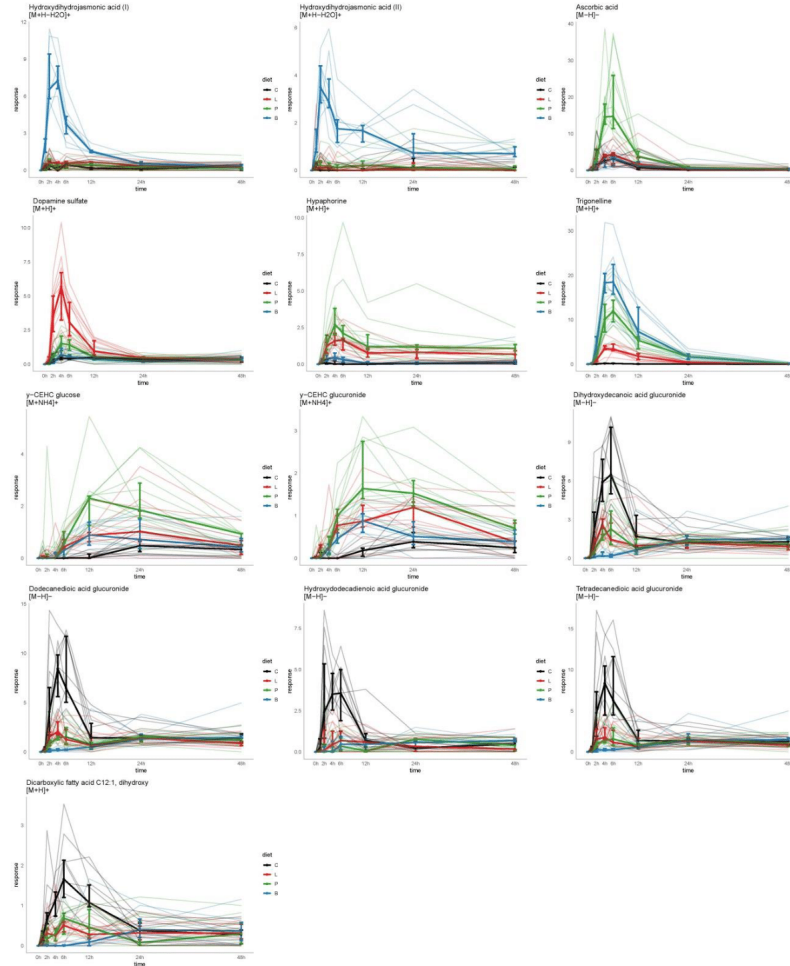


Figure S6. Urine samples (continuation)

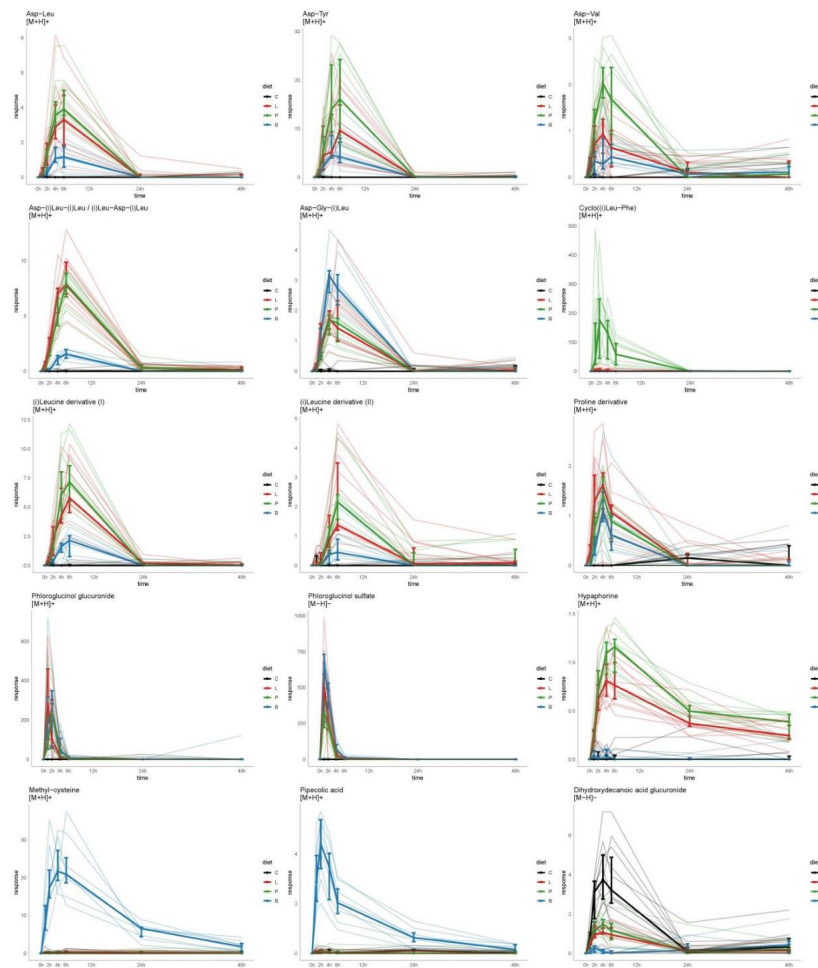


X axis: time point; Y axis: peak intensity (MS response). Bold lines represent medians and bars IQR range, and thin lines are individual values.

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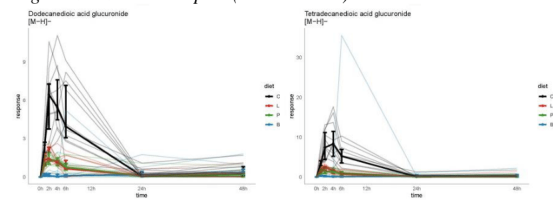
Serum samples



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Figure S6. Serum samples (continuation)



X axis: time point; Y axis: peak intensity (MS response). Bold lines represent medians and bars IQR range, and thin lines are individual values.

Figure S7. Boxplots of the metabolites that were significantly different in 6 h and/or 24 h pools after intake of lentils (L), chickpeas (P), white beans (B) or control (C)

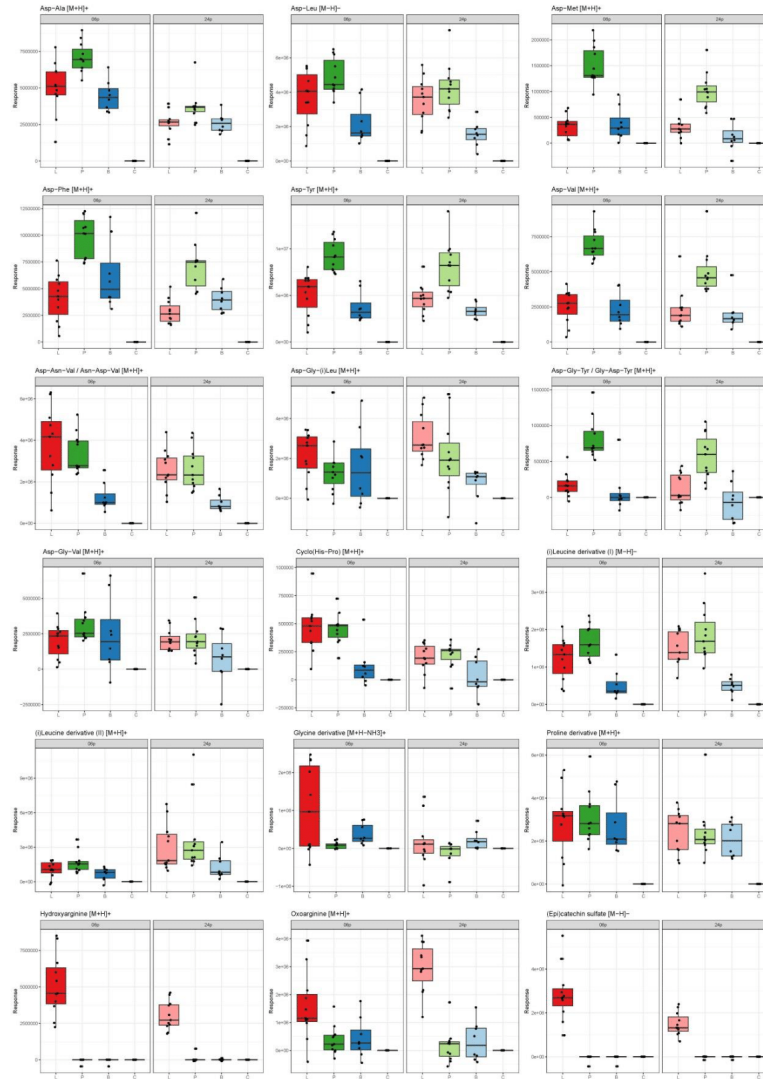
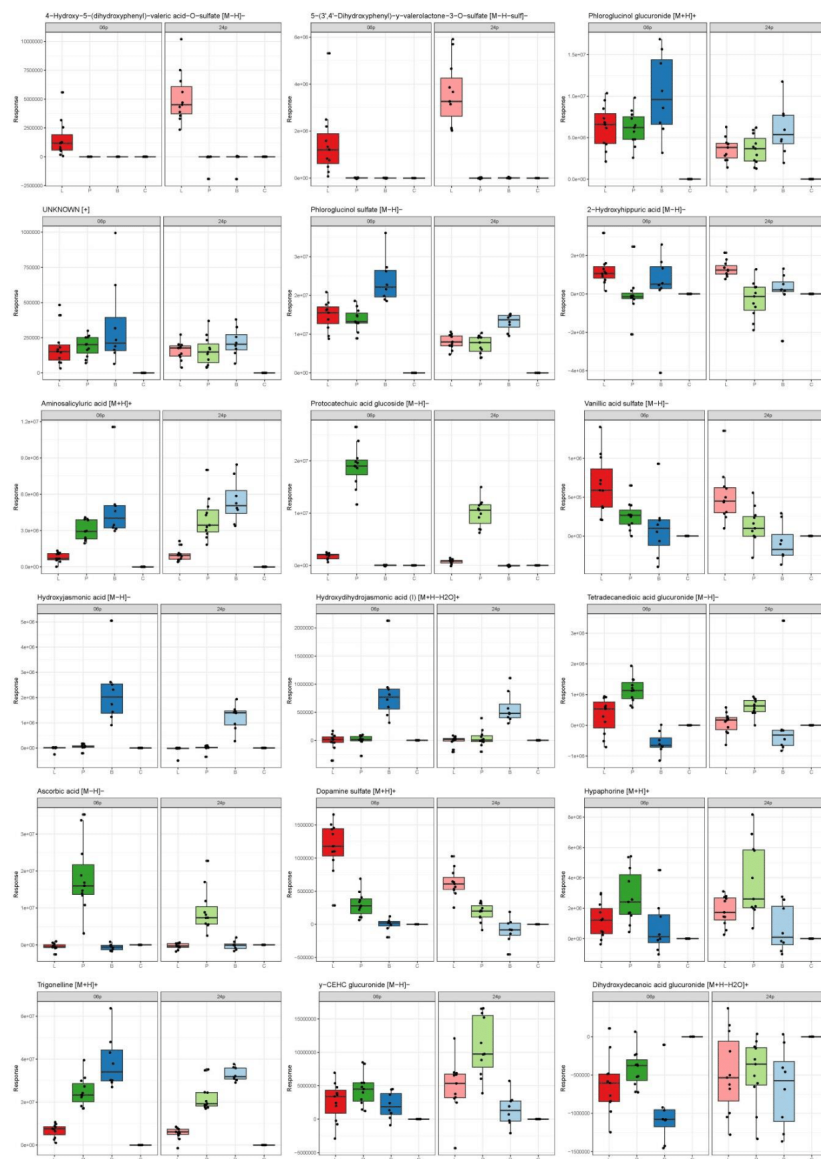


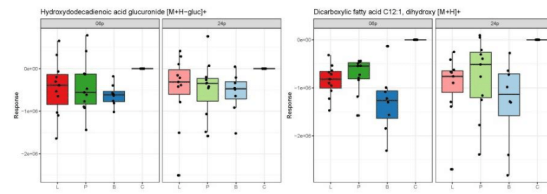
Figure S7 (continuation)



Supporting Information

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Figure S7 (continuation)



Tables

Table S1. Parameters used for data processing with XCMS

Nutrikinetic experiment

Step	Parameter	URINE		SERUM	
		ESI(+)	ESI(-)	ESI(+)	ESI(-)
Peak picking	Method	centWave			
	Signal-to-noise threshold	5			
	Relative deviation in the m/z dimension (ppm)	20			
	Binning width in the profile matrix	0.005			
	Peak width (range in seconds)	(7, 30)	(8, 20)	(5, 10)	
	Prefilter (scans, intensity)	(3, 25000)		(3, 5000)	
Peak grouping	Min. fraction of samples a peak should be found in	0.25			
	m/z width	0.007			
	Bandwidth of peaks	20/10			
Retention time correction	Method	Loess			
	Degree of smoothing for local polynomial regression fitting	0.6/0.2			
	N° of missing samples to allow in RT correction groups	300/200			
	N° of extra peaks to allow in RT correction groups	150/100			
Filling missing values	Retention time (expand, minimum width)	(1.5, 5)			
	m/z (expand, minimum width)	(1, 0.007)			

Pooled samples experiment

Step	Parameter	ESI(+)	ESI(-)
Peak picking	Method	centWave	
	Signal-to-noise threshold	5	
	Relative deviation in the m/z dimension (ppm)	20	
	Binning width in the profile matrix	0.005	
	Peak width (range in seconds)	(5, 30)	(3, 30)
	Prefilter (scans, intensity)	(3, 25000)	
Peak grouping	Minimum fraction of samples a peak should be found in	0.25	
	m/z width	0.007	
	Bandwidth of peaks	20/10	
Retention time correction	Method	Loess	
	Degree of smoothing for local polynomial regression fitting	0.6/0.2	
	N° of missing samples to allow in RT correction groups	100/80	
	N° of extra peaks to allow in retention time correction groups	50/40	
Filling missing values	Retention time (expand, minimum width)	(1.5, 5)	
	m/z (expand, minimum width)	(1, 0.007)	

Table S2. Compounds identified in the cooked food samples using two-dimensional gas chromatography mass spectrometry (GC×GC-MS)

Class	Compound	Signal intensity			
		Pasta	Lentils	Chickpeas	White beans
Amino acids	Alanine 2TMS	1,008,831	2,808,505	541,221	6,631,730
	beta-Alanine 3TMS	ND	ND	ND	1,413,831
	Glycine 3TMS	594,808	1,179,246	1,398,776	4,382,747
	Valine 2TMS	307,117	547,476	570,340	2,532,969
	Phenylalanine TMS	ND	ND	808,517	805,913
	Phenylalanine 2TMS	ND	55,502	334,494	1,003,235
	Tryptophan 2TMS	ND	ND	ND	870,711
	Tyrosine 2TMS	ND	ND	321,292	257,243
	Tyrosine 3TMS	ND	ND	421,447	501,106
	Aspartic acid 3TMS	643,027	560,904	131,589	612,695
	Glutamic acid 3TMS	ND	1,008,866	1,476,774	2,843,178
	Serine 2TMS	ND	ND	133,522	352,826
	Serine 3TMS	ND	ND	ND	252,007
	Threonine 3TMS	ND	289,812	279,904	1,311,338
	Asparagine 3TMS	ND	ND	ND	957,895
	N-Methylisoleucine TMS	ND	ND	ND	234,284
	Oxoproline 2TMS	1,056,019	5,951,721	9,448,122	23,212,720
	4-Hydroxynorvaline 3TMS	ND	ND	ND	525,011
	Similar to threonine 2TMS	ND	ND	146,327	ND
	S-Methylcysteine TMS	ND	ND	ND	7,088,736
	S-Methylcysteine 2TMS	ND	ND	ND	8,491,174
	Pipecolic acid TMS	ND	ND	ND	27,797,025
Phenolic acids	Catechin 5TMS	ND	1,361,323	ND	ND
	Epicatechin 5TMS	ND	320,665	ND	ND
	p-Cumaric acid 2TMS	ND	ND	ND	197,509
	Arbutin 5TMS or similar	ND	245,718	ND	ND
Carboxylic acids	Pyruvic acid MEOX-TMS	66,944	422,534	327,214	1,191,587
	cis-Aconitic acid 3TMS	ND	ND	303,660	583,763
	Citric acid 4TMS	2,002,890	33,754,193	89,472,429	166,332,551
	Citric acid derivative	ND	453,253	ND	ND
	alpha-Ketoglutaric acid MEOX-2TMS	ND	ND	1,194,076	500,518
	Succinic acid 2TMS	2,731,957	3,458,823	3,264,279	7,799,013
	2-Methylsuccinic acid 2TMS	ND	310,949	112,646	ND
	Fumaric acid 2TMS	1,232,978	156,176	102,228	202,953
	Malic acid 3TMS	20,408,776	7,190,177	10,177,775	17,767,457
	Malonic acid 2TMS	ND	551,979	828,709	559,758
	2-Hydroxyglutaric acid 3TMS	108,817	239,051	501,157	1,290,084
	C5 trihydroxydicarboxylic acid or similar	ND	121,788	190,211	ND

Table S2 (continuation)

Class	Compound	Pasta	Lentils	Chickpeas	White beans
Fatty acids	Oleic acid derivative	ND	828,875	1,068,532	ND
	1-Monoolein 2TMS or similar	1,449,421	ND	ND	ND
	Monolinolein or similar	ND	212,099	865,744	ND
	Palmitic acid derivative	185,294	1,523,163	1,169,605	332,708
	Glycidyl palmitate	ND	1,038,387	602,035	ND
	alpha-Hydroxybutyric acid 2TMS	85,588	169,851	156,731	104,926
	beta-Hydroxybutyric acid 2TMS	94,721	ND	ND	ND
	2-Hydroxy-3-methylbutyric acid 2TMS	ND	ND	71,780	89,220
	2-O-Methylhexonic acid 5TMS or similar	ND	255,926	254,836	684,478
Sugars	Glucose MEOX-5TMS derivative2	4,376,782	6,016,770	13,285,587	8,730,297
	Fructose MEOX-5TMS	28,234,618	18,752,084	77,505,069	78,178,084
	Galactinol 9TMS	7,046,340	25,119,842	68,731,631	34,552,431
	Galactinol-like (I)	ND	282,457	3,404,059	ND
	Galactinol-like (II)	ND	804,291	3,065,730	135,530
	Galactinol-like (III)	ND	851,552	1,797,272	570,468
	Galactinol-like (IV)	ND	ND	293,593	88,399
	Galactinol-like (V)	ND	ND	566,653	268,584
	Tagatose MEOX 5TMS derivative1	ND	280,651	326,320	ND
	Sucrose 8TMS	128,954,228	182,556,937	303,707,978	340,882,374
	Maltose MEOX-8TMS derivative1	268,613,894	1,092,954	2,262,288	1,365,927
	Similar to Maltose (< Sucrose)	5,883,968	ND	ND	ND
	Melibiose MEOX-8TMS derivative1	281,645	ND	2,139,578	865,015
	Melibiose MEOX-8TMS derivative2	ND	ND	632,577	164,971
	beta-Gentiobiose MEOX-8TMS derivative2	ND	ND	399,490	ND
	Disaccharide	ND	36,594,631	93,891,519	ND
	Disaccharide (< Sucrose) (I)	119,251	270,221	953,994	444,632
	Disaccharide (< Sucrose) (II)	ND	1,437,359	ND	ND
	Disaccharide (< Sucrose) (III)	ND	119,642	639,376	357,222
	Disaccharide (> Sucrose) (I)	ND	ND	ND	441,975
	Disaccharide (> Sucrose) (I)	10,597,372	ND	ND	ND
	Disaccharide (> Sucrose) (II)	ND	ND	ND	185,316
	Disaccharide (> Sucrose) (II)	ND	9,524,931	18,011,562	ND
	Disaccharide-like (< Sucrose) (I)	1,009,903	ND	ND	ND
	Disaccharide-like (< Sucrose) (II)	2,292,961	ND	ND	ND
	Disaccharide-like (> Sucrose)	11,513,148	ND	ND	ND
	Disaccharide-like (> Sucrose)	ND	ND	ND	420,028
	Sugar (like disaccharide)	355,997	ND	ND	ND

Table S2 (continuation)

Class	Compound	Pasta	Lentils	Chickpeas	White beans
Sugars	Trisaccharide	ND	838,591	1,249,610	ND
	Trisaccharide (raffinose 11TMS or similar)	ND	ND	393,873	117,228
	Sugar (< trisaccharide)	470,561	3,738,860	8,762,881	5,635,818
	Sugar (> trisaccharide)	ND	ND	2,610,252	2,687,708
	Minor trisaccharide	ND	778,830	ND	ND
	Arabinose MEOX-4TMS or isomer	1,072,042	1,045,770	1,993,467	1,453,999
	Xylose MEOX-4TMS derivative2	408,406	398,206	413,346	518,205
	Similar to ribonolactone 3TMS	ND	988,589	1,587,547	ND
	Glucaric acid 6TMS	ND	146,839	ND	41,145,523
	Glucaric acid lactone1 4TMS	ND	ND	ND	1,233,596
	Glucaric acid lactone2 4TMS	ND	ND	ND	751,968
	Galactaric acid 6TMS	ND	ND	120,583	2,920,641
	Galacturonic acid MEOX-5TMS derivative1	113,270	ND	ND	ND
	delta-Gluconolactone 4TMS	2,128,144	364,563	537,697	2,799,116
	Sugar (< C6)	ND	138,651	1,145,060	117,569
	Sugar (< Sucrose)	ND	302,814	1,137,685	ND
	Sugar/sugar-like	ND	539,553	1,066,372	ND
	Sugar/sugar-like (ca. C4)	ND	ND	ND	525,549
	Sugar/sugar-like (C6)	ND	1,236,189	2,372,375	820,859
	Sugar/sugar-like (> C6)	ND	ND	828,324	ND
	Sugar/sugar-like (> C6)	ND	ND	151,771	ND
	Sugar/glycoside (< dissaccharide) (I)	ND	ND	ND	718,334
	Sugar/glycoside (< dissaccharide) (II)	ND	ND	394,602	564,266
	Sugar/glycoside (< dissaccharide) (III)	ND	ND	ND	515,667
	Sugar/glycoside (< dissaccharide) (IV)	ND	ND	ND	1,194,382
	Sugar/glycoside (< dissaccharide) (V)	117,343	ND	ND	1,026,373
	Sorbitol 6TMS	3,218,521	955,329	1,575,922	8,562,541
	Mannitol 6TMS	430,642	ND	328,934	2,341,019
	Glycerol 3TMS	6,805,273	3,090,167	4,668,944	5,683,875
	Threitol	ND	ND	252,217	ND
	Xylitol 5TMS	204,887	ND	285,571	566,126
	meso-Erythritol	131,545	95,528	289,320	ND
	chiro-Inositol 6TMS	ND	763,292	3,159,500	ND
	myo-Inositol phosphate 7TMS	116,587	1,636,411	2,451,827	1,563,052
	Inositol derivative	274,931	3,741,241	4,568,067	2,114,795
	Sugar/sugar alcohol (C4) (I)	ND	ND	937,534	ND
	Sugar/sugar alcohol (C4) (II)	ND	ND	1,215,061	ND
	C5 sugar alcohol	ND	163,552	186,302	436,629

Table S2 (continuation)

Class	Compound	Pasta	Lentils	Chickpeas	White beans
Sugars	Threonic acid 4TMS	ND	2,633,278	9,365,513	423,599
	Ribonic acid 5TMS or similar	ND	5,338,050	2,849,233	86,338
	Gluconic acid 6TMS	1,665,437	2,044,321	2,571,539	12,279,587
	Similar to/isomer of gluconic acid (I)	ND	ND	175,908	2,463,966
	Similar to/isomer of gluconic acid (II)	ND	ND	ND	425,822
	Like gluconid acid 6TMS	ND	ND	112,266	1,151,733
	Similar to lactobionic acid (> sucrose)	38,999,244	2,482,803	9,660,360	715,478
	Glyceric acid 3TMS	95,138	487,759	539,004	1,196,945
	Tartaric acid 4TMS	ND	ND	ND	180,821
	Sugar acid (C6)	ND	ND	ND	1,486,998
	Similar to phenylglucopyranoside 4TMS	ND	103,037	571,961	525,009
Others	Lactic acid 2TMS	5,175,326	5,290,571	2,102,463	19,701,352
	Glycolic acid 2TMS	514,833	1,541,433	682,915	660,765
	Dehydroascorbic acid derivative 1	ND	ND	21,128,949	ND
	Dehydroascorbic acid derivative 2	ND	ND	2,646,525	ND
	Dehydroascorbic acid derivative 3	ND	ND	1,023,232	ND
	Dehydroascorbic acid derivative 4	ND	ND	2,800,449	ND
	Dehydroascorbic acid derivative 5	ND	ND	526,151	ND
	Dehydroascorbic acid derivative 6	ND	ND	595,113	ND
	Similar to dehydroascorbic acid deriv 2	ND	ND	795,760	ND
	Pinitol 6TMS	ND	26,915,695	61,279,020	ND
	Probably quinic acid derivative (> C6)	337,867	ND	ND	146,860
	Quinic acid 5TMS	143,255	ND	103,988	ND
	Phloroglucinol 3TMS	ND	533,797	ND	ND
	Ethanolamine 3TMS	207,609	351,772	1,044,450	2,400,848
	Adenosine 4TMS	129,270	320,521	941,098	1,141,819
	Hypoxanthine 2TMS	ND	296,971	272,789	299,262
	2-Hydroxypyridine TMS	ND	ND	1,152,821	ND
	Nicotinic acid TMS	ND	136,409	151,204	74,878
	Nicotinic acid isomer	ND	165,043	285,934	374,232
	Uracil 2TMS	158,875	172,770	133,895	149,410
	2-Pyrrolidinone TMS	40,071	ND	ND	303,613
	Campesterol TMS	ND	948,448	2,199,025	237,066
	Stigmasterol	ND	1,040,306	1,138,758	2,983,463
	beta-Sitosterol TMS	2,606,696	8,867,611	14,865,839	4,998,335
	alpha/beta-Amyrin or similar	ND	775,606	1,562,514	111,149
	Triterpene derivative	ND	ND	ND	1,015,055
	gamma-Tocopherol TMS	ND	974,447	2,723,863	420,642
	Similar to erythroneolactone 3TMS (I)	ND	ND	2,063,888	ND
	Urea 2TMS	ND	ND	251,284	2,820,945

Table S2 (continuation)

Class	Compound	Pasta	Lentils	Chickpeas	White beans
Others	Phosphate 3TMS	15,926,575	28,390,148	45,557,457	71,088,004
Unknowns	Unknown01	ND	ND	1,527,967	ND
	Unknown02	ND	ND	ND	1,677,759
	Unknown03	ND	ND	ND	60,759
	Unknown04	ND	ND	ND	397,056
	Unknown05	ND	ND	ND	126,365
	Unknown06	ND	ND	ND	940,099
	Unknown07	ND	350,589	472,824	129,685
	Unknown08	ND	432,740	ND	ND
	Unknown09	ND	294,957	ND	ND
	Unknown10	ND	354,545	ND	ND
	Unknown11	ND	ND	1,206,633	ND
	Unknown12	1,045,515	ND	102,887	1,592,311
	Unknown13	ND	ND	662,648	ND
	Unknown14	ND	ND	1,467,182	ND
	Unknown15	ND	ND	ND	279,340
	Unknown16	ND	ND	ND	74,607
	Unknown17	ND	ND	ND	181,176
	Unknown18	ND	ND	492,110	ND

Table S3. Monitoring of analytical variability across the runs in QC samples distributed sequentially along the injections

Table S3-A. Nutrikinetic experiment

Urine samples

Compound	RT (min)		Experimental mass (m/z)		Area (counts)	
	mean	Δ	mean	max. error (ppm)	mean	CV (%)
ESI(+)						
Tryptophan d ₅ (IS)	3.33	0.08	210.1284	3.0	71,008,155	5
Hippuric acid d ₅ (IS)	4.35	0.03	185.0968	2.7	49,950,683	6
<i>trans</i> -Cinnamic acid d ₅ (ES)	7.37	0.03	154.0909	3.2	2,594,318	10
Phenylalanine	1.85	0.04	166.0861	2.7	31,662,881	6
Tryptophan	3.41	0.06	205.0971	2.6	141,294,317	5
Hippuric acid	4.40	0.03	180.0654	3.4	457,938,857	6
ESI(-)						
Tryptophan d ₅ (IS)	3.45	0.06	208.1141	2.5	4,930,973	23
Hippuric acid d ₅ (IS)	4.45	0.06	183.0825	3.0	88,228,518	14
<i>trans</i> -Cinnamic acid d ₅ (ES)	8.00	0.43	152.0767	3.0	114,395	45
Phenylalanine	1.93	0.07	164.0720	3.7	687,810	18
Tryptophan	3.51	0.06	203.0828	2.5	1,469,942	29
Hippuric acid	4.49	0.05	178.0512	3.0	214,938,428	13

Abbreviations: CV, coefficient of variation; ES, external standard; IS, internal standard; RT, retention time

Serum samples

Compound	RT (min)		Experimental mass (m/z)		Area (counts)	
	mean	Δ	mean	max. error (ppm)	mean	CV (%)
ESI(+)						
Tryptophan d ₅ (IS)	3.61	0.07	210.1283	2.5	190,991,891	17
Hippuric acid d ₅ (IS)	4.69	0.03	185.0966	2.3	113,078,630	17
Deoxycholic acid d ₄ (IS)	10.17	0.03	361.3031	3.6	20,856,521	8
<i>trans</i> -Cinnamic acid d ₅ (ES)	7.30	0.03	154.0909	2.7	2,377,110	21
Glycocholic acid d ₄ (ES)	7.88	0.04	470.3407	2.7	29,467,085	16
Cholic acid d ₄ (ES)	8.72	0.03	430.3459	2.7	13,844,962	24
Phenylalanine	1.93	0.07	166.0860	2.8	388,798,550	21
Tryptophan	3.69	0.06	205.0970	2.1	301,373,163	18
Hippuric acid	4.72	0.03	180.0654	1.7	2,766,320	14
ESI(-)						
Tryptophan d ₅ (IS)	3.60	0.08	208.1140	2.0	16,702,932	6
Hippuric acid d ₅ (IS)	4.69	0.02	183.0824	1.9	97,798,038	4
Deoxycholic acid d ₄ (IS)	10.17	0.03	395.3105	1.7	18,955,349	16
<i>trans</i> -Cinnamic acid d ₅ (ES)	7.30	0.02	152.0767	2.4	257,301	27
Glycocholic acid d ₄ (ES)	7.87	0.04	468.3267	2.0	27,234,728	10
Cholic acid d ₄ (ES)	8.72	0.03	411.3054	1.9	11,042,743	19
Phenylalanine	1.93	0.07	164.0718	2.5	14,228,365	12
Tryptophan	3.68	0.07	203.0827	2.0	27,563,950	6
Hippuric acid	4.73	0.02	178.0512	3.0	3,761,611	3

Abbreviations: CV, coefficient of variation; ES, external standard; IS, internal standard; RT, retention time

Table S3-B. Pooled samples experiment

Compound	RT (min)		Experimental mass (m/z)		Area (counts)	
	mean	Δ	mean	max. error (ppm)	mean	CV (%)
ESI(+)						
Tryptophan d ₅ (IS)	3.34	0.05	210.1286	1.7	293,211,619	4
Hippuric acid d ₅ (IS)	4.36	0.03	185.0970	1.6	144,099,621	4
<i>trans</i> -Cinnamic acid d ₅ (ES)	7.36	0.02	154.0911	1.9	5,059,538	9
Phenylalanine	1.84	0.03	166.0863	1.5	116,439,757	6
Tryptophan	3.42	0.03	205.0973	1.8	22,199,471	4
Hippuric acid	4.40	0.02	180.0656	1.7	455,059,144	5
ESI(-)						
Tryptophan d ₅ (IS)	3.34	0.07	208.1140	1.0	16,707,966	6
Hippuric acid d ₅ (IS)	4.34	0.03	183.0822	1.4	112,501,449	5
<i>trans</i> -Cinnamic acid d ₅ (ES)	7.36	0.03	152.0767	1.7	141,744	13
Phenylalanine	1.84	0.05	164.0720	2.5	2,306,945	9
Tryptophan	3.39	0.03	203.0827	1.0	6,374,752	9
Hippuric acid	4.39	0.03	178.0510	0.8	357,428,614	5

Table S4. Data filtering of processed data sets

Nutrikinetic experiment

Filter	Condition	URINE		SERUM	
		ESI(+)	ESI(-)	ESI(+)	ESI(-)
Original data set		10,678	4,206	13,894	7,340
Rule 20%	Presence in < 20% of samples in all groups	10,678	4,206	13,894	7,340
CV	CV(QC) > CV(samples)	10,206	4,075	12,394	6,755
Exact mass (m/z)	1 st decimal ≥ 0.4 and ≤ 0.8	9,539	3,894	8,899	5,528
Intensity (counts)	Median intensity < 2,000,000 (urine) or < 200,000 (serum) in all groups	1,509	873	3,201	1,307

Pooled samples experiment

Filter	Condition for exclusion	ESI(+)	ESI(-)
Original data set		10,517	2,530
Rule 20%	Presence in < 20% of samples in all groups	10,507	2,529
CV	CV(QC) > CV(samples)	9,759	2,426
Exact mass (m/z)	1 st decimal ≥ 0.4 and ≤ 0.8	8,894	2,379
Intensity (counts)	Median intensity < 2,000,000 in all groups	1,355	715

Abbreviations: CV, coefficient of variation; ESI, electrospray ionization; QC, quality control.

Table S5. Mass spectrometry spectra of discriminant metabolites

M	Metabolite	Ion mode	MS ² and pseudo-MS ⁿ spectra
M01	Asp-Ala	POS	MS ² 205.0820 [M+H] ⁺ : 159.0763 (100), 187.0712 (30), 85.0282 (17), 145.0494 (9), 70.0285 (8), 90.0547 (8)
M02	Asp-Gly / Gly-Asp	POS	N.A.
M03	Asp-Leu	POS	MS ² 247.1293 [M+H] ⁺ : 201.1233 (100), 229.1181 (17), 132.1017 (5), 86.0962 (3), 183.1123 (2)
M04	Asp-Met	POS	MS ² 265.0852 [M+H] ⁺ : 219.0795 (100), 171.0763 (52), 247.0742 (39), 229.0638 (30), 150.0579 (18), 104.0527 (8), 217.0828 (8)
M05	Asp-Phe	POS	MS ² 281.1130 [M+H] ⁺ : 263.1023 (100), 235.1074 (39), 245.0918 (31), 246.0759 (27), 166.0860 (19), 221.0918 (8), 120.0804 (3)
M06	Asp-Ser / Ser-Asp	POS	MS ² 221.0769 [M+H] ⁺ : 203.0658 (100) [M+H-H ₂ O] ⁺
M07	Asp-Tyr	POS	MS ² 297.1088 [M+H] ⁺ : 251.1030 (100), 182.0813 (13), 279.0979 (9), 136.0757 (5), 165.0549 (3), 261.0869 (2)
M08	Asp-Val	POS	MS ² 233.1132 [M+H] ⁺ : 187.1073 (100), 215.1022 (18), 118.0854 (3), 72.0804 (2)
M09	Asp-(i)Leu / (i)Leu-Asp-(i)Leu	POS	MS ² 360.2127 [M+H] ⁺ : 229.1181 (100), 201.1233 (57), 342.2029 (33), 283.6823 (13)
M10	Asp-(i)Leu-Pro / (i)Leu-Asp-Pro	POS	MS ² 344.18 [M+H] ⁺ : 116.0702 (100), 229.1178 (38), 326.1703 (23), 201.1222 (19)
M11	Asp-Ala-(i)Leu / Ala-Asp-(i)Leu	POS	MS ² 318.1668 [M+H] ⁺ : 187.0713 (100), 159.0765 (69), 300.1555 (65), 282.1448 (15)
M12	Asp-Asn-Val / Asn-Asp-Val	POS	MS ² 347.1561 [M+H] ⁺ : 329.1454 (100), 312.1190 (64), 230.0771 (41), 330.1297 (19), 311.1353 (8), 301.1508 (4), 212.0666 (3)
M13	Asp-Gly-(i)Leu	POS	MS ² 304.1508 [M+H] ⁺ : 286.1400 (100), 268.1296 (37), 240.1343 (29), 173.0559 (15), 258.1449 (15), 132.1020 (11), 145.0609 (6), 189.1235 (3), 191.0657 (3)
M14	Asp-Gly-Tyr / Gly-Asp-Tyr	POS	MS ² 354.1295 [M+H] ⁺ : 336.1196 (100), 182.0814 (46), 290.1141 (29), 318.1089 (25), 165.0548 (9), 136.0766 (6), 308.1246 (5)
M15	Asp-Gly-Val	POS	MS ² 290.1348 [M+H] ⁺ : 272.1240 (100), 254.1135 (31), 226.1186 (22), 173.0556 (21), 244.1289 (13), 118.0861 (6), 145.0606 (6), 191.0661 (3), 227.1029 (3), 175.1078 (2)
M16	Asp-Thr-Pro / Thr-Asp-Pro	POS	MS ² 332.1500 [M+H] ⁺ : 314.1340 (100), 116.0700 (13)
M17	Pro-HPro-Gly / HPro-Pro-Gly	POS	MS ² 286.1400 [M+H] ⁺ : 211.1074 (100), 240.1338 (34), 268.1294 (27)
M18	Cyclo(i)Leu-Phe	POS	MS ² 261.1595 [M+H] ⁺ : 233.1635 (100), 120.0806 (89), 216.1364 (49)
M19	Cyclo(His-Pro)	POS	MS ² 235.1200 [M+H] ⁺ : 110.0710 (100), 162.1023 (87), 166.0608 (68), 207.1238 (41), 217.1081 (22), 190.0973 (20)
M20	(i)Leucine derivative (I)	POS	MS ² 247.1300 [M+H] ⁺ : 201.1231 (100), 229.1178 (26), 132.1016 (6), 86.0961 (4)
M21	(i)Leucine derivative (II)	POS	MS ² 289.1398 [M+H] ⁺ : 271.1290 (100), 243.1340 (34), 86.0963 (31), 225.1234 (22), 132.1018 (21), 201.1233 (10), 247.1277 (2), 158.0439 (1)
M22	Glycine derivative	POS	MS ² 232.1299 [M+H] ⁺ : 215.1027 (100), 84.0806 (13), 214.1186 (6), 129.1020 (2), 197.0909 (1)
M23	Phenylalanine derivative	POS	MS ² 400.1860 [M+H] ⁺ : 235.1074 (100), 207.1126 (32), 382.1763 (21), 166.0862 (9)
M24	Proline derivative	POS	MS ² 229.1185 [M+H] ⁺ : 183.1127 (100), 116.0705 (35), 211.1078 (31), 155.1178 (11), 115.0501 (3), 70.0650 (2)
M25	Hydroxyarginine	POS	MS ² 191.1138 [M+H] ⁺ : 174.0871 (100), 157.0605 (89), 122.9243 (67), 129.0656 (22), 131.0813 (14), 111.0551 (9), 173.1032 (8), 68.0492 (7), 85.0757 (4), 114.0550 (2)
M26	Oxoarginine	POS	MS ² 174.0878 [M+H] ⁺ : 114.0549 (100), 70.0649 (11), 128.1069 (5), 112.0869 (4)
M27	(Epi)catechin sulfate	NEG	MS ² 369.0285 [M-H] ⁻ : 289.0717 (100), 230.9968 (84), 245.0819 (10), 205.0506 (1) pseudo-MS3 289.0700 [M-H-sulf] ⁻ : 245.0817 (100), 205.0507 (44), 191.0710 (19), 179.0351 (17), 231.0300 (7)
M28	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulfate	NEG	MS ² 305.0342 [M-H] ⁻ : 225.0771 (100), 101.0248 (13), 207.0665 (13), 163.0768 (10), 123.0455 (8), 179.0721 (0)
M29	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-O-glucuronide	NEG	MS ² 383.0984 [M-H] ⁻ : 207.0662 (100), 113.0246 (36), 175.0248 (23), 337.0279 (9), 163.0767 (8)
M30	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-3-O-sulfate	NEG	MS ² 287.0229 [M-H] ⁻ : 207.0663 (100), 163.0762 (1) pseudo-MS ³ 207.0665 [M-H-sulfate] ⁻ : 163.0768 (100), 122.0376 (8)
M31	5-(3',5'-Dihydroxy-phenyl)-γ-valerolactone-methyl-glucuronide	NEG	MS ² 397.1145 [M-H] ⁻ : 175.0251 (100), 221.0796 (26), 113.0243 (50)

Table S5 (continuation)

M	Metabolite	Ion mode	MS ² and pseudo-MS ⁿ spectra
M32	Phloroglucinol glucuronide	NEG	MS ² 301.0568 [M-H] ⁻ : 113.0247 (100), 175.0248 (35), 95.0142 (18), 85.0298 (12), 125.0244 (3) pseudo-MS ³ 125.0200 [M-H-sulfate] ⁻ : 97.0298 (100)
M33	Phloroglucinol sulfate	NEG	MS ² 204.9812 [M-H] ⁻ : 125.0247 (100) pseudo-MS ³ 125.0247 [M-H-sulfate] ⁻ : 97.0298 (100), 69.0349 (3), 81.0349 (3), 95.0142 (2)
M34	2-Hydroxyhippuric acid	POS	MS ² 196.0600 [M+H] ⁺ : 121.0280 (100), 178.0496 (9)
		NEG	MS ² 194.0452 [M-H] ⁻ : 150.0558 (100), 93.0344 (2) pseudo-MS ³ 150.0563 [M-H-CO ₂] ⁻ : 93.0349 (100), 121.0301 (5)
M35	Aminosalicyluric acid	POS	MS ² 211.0715 [M+H] ⁺ : 165.0654 (100), 136.0389 (17), 167.0811 (2), 193.0604 (1)
		NEG	MS ² 209.0567 [M-H] ⁻ : 165.0671 (100), 108.0458 (26), 191.0464 (20)
M36	Muconic acid	NEG	MS ² 141.0197 [M-H] ⁻ : 97.0298 (100), 113.0247 (24), 69.0348 (13)
M37	Protocatechuic acid glucoside	NEG	MS ² 315.0722 [M-H] ⁻ : 153.0195 (100), 152.0116 (38), 165.0194 (14), 109.0298 (12), 108.0219 (10) pseudo-MS ³ 153.0195 [M-H-glucoside] ⁻ : 109.0299 (100)
M38	Vanillic acid sulfate	NEG	MS ² 246.9923 [M-H] ⁻ : 203.0020 (100), 123.0455 (79), 167.0352 (17) pseudo-MS ³ 167.0300 [M-H-sulfate] ⁻ : 152.0115 (100), 123.0452 (76), 108.0218 (15)
M39	Hydroxyjasmonic acid	POS	MS ² 227.1282 [M+H] ⁺ : 209.1166 (100), 191.1060 (55), 85.0643 (11), 167.1061 (8), 163.1112 (4), 145.1007 (3), 149.0956 (3)
		NEG	MS ² 225.1100 [M-H] ⁻ : 181.1233 (100), 165.0921 (70), 207.1026 (45), 147.0815 (39), 163.1129 (35), 135.0817 (23)
M40	Hydroxydihydrojasmonic acid (I)	POS	MS ² 229.1400 [M+H] ⁺ : 211.1321 (100), 193.1216 (29), 133.1006 (3) pseudo-MS ³ 193.1200 [M+H-2(H ₂ O)] ⁺ : 175.1111 (100), 133.1006 (87), 147.1163 (27), 131.0850 (18), 149.0956 (11), 107.0850 (6), 91.0538 (6), 157.1007 (6), 165.1269 (4), 119.0851 (4), 105.0694 (4) pseudo-MS ⁴ 133.1000 [M+H-2(H ₂ O)-CH ₃ CO ₂ H] ⁺ : 91.05389 (100), 105.0695 (46)
		NEG	MS ² 227.1300 [M-H] ⁻ : 209.1182 (100), 165.1284 (96), 153.1285 (19), 191.1076 (18), 147.1180 (16), 197.1181 (11)
M41	Hydroxydihydrojasmonic acid (II)	POS	MS ² 229.1400 [M+H] ⁺ : 211.1322 (100), 193.1217 (68), 133.1003 (9) pseudo-MS ³ 193.1200 [M+H-2(H ₂ O)] ⁺ : 133.1005 (100), 175.1110 (79), 147.1163 (26), 131.0849 (14), 149.0956 (6) pseudo-MS ⁴ 133.1000 [M+H-2(H ₂ O)-CH ₃ CO ₂ H] ⁺ : 91.0539 (100), 105.0695 (63)
		NEG	MS ² 227.1280 [M-H] ⁻ : 165.1285 (100), 209.1183 (52)
M42	Ascorbic acid	NEG	MS ² 175.0243 [M-H] ⁻ : 115.0041 (100), 87.0096 (13), 59.0143 (11)
M43	Dopamine sulfate	POS	MS ² 234.0400 [M+H] ⁺ : 217.0159 (100), 137.0592 (60), 154.0858 (17), 188.1111 (9) pseudo-MS ³ 137.0600 [M+H-sulfate-NH ₃] ⁺ : 91.0538 (100), 119.0481 (88), 109.0642 (24)
		NEG	MS ² 232.0289 [M-H] ⁻ : 152.0719 (100) pseudo-MS ³ 152.0718 [M-H-sulfate] ⁻ : 122.0375 (100)
M44	Hypaphorine	POS	MS ² 247.1437 [M+H] ⁺ : 188.0702 (100) pseudo-MS ³ 188.0708 [M+H-(CH ₃) ₃ N] ⁺ : 146.0600 (100), 144.0807 (11), 170.0598 (3) pseudo-MS ⁴ 146.0599 [M+H-(CH ₃) ₃ N-COCH ₃] ⁺ : 118.0648 (100)
M45	Methylcysteine	POS	MS ² 136.0477 [M+H] ⁺ : 119.0158 (100) pseudo-MS ³ 119.0158 [M+H-NH ₃] ⁺ : 73.0280 (100)
M46	Pipecolic acid	POS	MS ² 130.0861 [M+H] ⁺ : 84.0804 (100), 130.0859 (6)
M47	Trigonelline	POS	MS ² 138.0546 [M+H] ⁺ : 94.0650 (100), 92.0492 (58), 138.0548 (51), 110.0598 (28)
M48	y-CEHC glucose	POS	MS ² 444.2215 [M+NH ₄] ⁺ : 265.1438 (100), 247.1334 (44), 151.0753 (13) pseudo-MS ³ 265.1441 [M+H-gluc] ⁺ : 247.1333 (100), 151.0754 (15), 130.0489 (2), 248.0919 (1)
M49	y-CEHC glucuronide	POS	MS ² 458.2027 [M+NH ₄] ⁺ : 265.1438 (100), 247.1333 (54), 151.0755 (16), 229.1227 (3), 405.1549 (2) pseudo-MS ³ 265.1441 [M+H-gluc] ⁺ : 247.1334 (100), 151.0755 (16), 149.0962 (1), 229.1228 (1) pseudo-MS ⁴ 247.1333 [M+H-gluc-H ₂ O] ⁺ : 229.1224 (100), 151.0754 (55), 203.1068 (19), 189.0911 (4), 109.0645 (3)

Table S5 (continuation)

M	Metabolite	Ion mode	MS2 and pseudo-MSn spectra
M50	Dihydroxydecanoic acid glucuronide	POS	MS ² 398.2000 [M+NH ₄] ⁺ : 363.1644 (100), 381.1747 (50), 335.1693 (47), 235.1172 (39), 345.1539 (38), 205.1432 (26), 187.1323 (20), pseudo-MS ³ 205.1437 [M+H-gluc] ⁺ : 169.1219 (100), 188.0702 (29), 187.132 (24), pseudo-MS ⁴ 187.1300 [M+H-gluc-H ₂ O] ⁺ : 169.1220 (100), 151.0750 (36), 133.1010 (11), 123.0800 (10), 141.1273 (9),
		NEG	MS ² 379.1613 [M-H] ⁻ : 203.1290 (100), 361.1504 (79), 185.1184 (72), 175.025 (19), 113.0248 (18), 193.0354 (18), 343.1399 (16), 243.1238 (5)
M51	Dodecanedioic acid glucuronide	POS	MS ² 407.1908 [M+H] ⁺ : 389.1800 (100), 371.1703 (31) MS ² 424.2182 [M+NH ₄] ⁺ : 389.1812 (100), 407.1915 (48), 231.1595 (15), 371.1705 (15) pseudo-MS ³ 231.1597 [M+H-gluc] ⁺ : 149.1326 (100), 195.1381 (74), 213.1489 (62), 177.1277 (21), 167.1442 (5), 159.1173 (3) pseudo-MS ⁴ 149.1321 [M+H-gluc-3(H ₂ O)-CO] ⁺ : 107.0852 (100), 93.0695 (83), 85.6035 (70), 118.0480 (68), 121.1010 (65)
		NEG	MS ² 405.1765 [M-H] ⁻ : 387.1659 (100), 361.1865 (82), 229.1445 (12), 167.1442 (10) pseudo-MS ³ 229.1444 [M-H-gluc] ⁻ : 167.1442 (100), 194.0607 (71), 93.0241 (68),
M52	Hydroxydodecanedioic acid glucuronide	POS	MS ² 389.1800 [M+H] ⁺ : 195.1377 (100), 213.1481 (36), 177.1273 (10), 371.1701 (7) pseudo-MS ³ 213.1487 [M+H-gluc] ⁺ : 195.1375 (100), 135.1164 (38), 177.1270 (13), 153.1270 (6), 133.1008 (5), 149.1320 (3), 139.0750 (2), 125.0594 (2), 167.1425 (2), 111.0438 (2), 159.1165 (2) pseudo-MS ⁴ 135.1200 [M+H-gluc-2(H ₂ O)-COCH ₃] ⁺ : 107.0852 (100), 93.0696 (87), 79.0539 (28)
		NEG	MS ² 387.1659 [M-H] ⁻ : 211.1332 (100), 193.0347 (95), 175.0241 (46), 113.0242 (34)
M53	Tetradecanedioic acid glucuronide	POS	MS ² 435.2200 [M+H] ⁺ : 417.2111 (100), 399.2008 (99), 223.1693 (14), 241.1794 (12), 259.1903 (10) pseudo-MS ³ 259.1900 [M+H-gluc] ⁺ : 223.1689 (100), 241.1794 (34), 205.1583 (10), 187.1478 (6) pseudo-MS ⁴ 241.1800 [M+H-gluc-H ₂ O] ⁺ : 223.1690 (100), 205.1584 (9), 187.1476 (2), 195.1741 (1), 177.1639 (1)
		NEG	MS ² 433.2072 [M-H] ⁻ : 257.1750 (100), 415.1959 (37), 371.2062 (22), 175.0244 (10), 239.1645 (10), 157.0137 (5), 389.2169 (5) pseudo-MS ³ 257.18 [M-H-gluc] ⁻ : 239.1649 (100), 129.0921 (95), 127.0766 (77), 211.1701 (66), 99.08169 (40), 177.1646 (22), 195.1753 (21)
M54	Dicarboxylic fatty acid C12:1, dihydroxy	POS	MS ² 261.1337 [M+H] ⁺ : 243.1228 (100), 225.1122 (21), 207.1011 (2) pseudo-MS ³ 207.100 [M+H-3(H ₂ O)] ⁺ : 189.0907 (100), 125.0593 (72), 147.0801 (54), 161.0957 (45), 109.0280 (19), 165.0906 (14), 163.1116 (9) pseudo-MS ⁴ 161.1000 [M+H-4(H ₂ O)-CO] ⁺ : 133.1007 (100), 143.0851 (85), 117.0695 (69), 119.0852 (48), 91.0539 (36), 105.0696 (32)
		NEG	MS ² 259.1187 [M-H] ⁻ : 215.1287 (100), 197.1179 (6), 241.1076 (5), 179.1082 (2) pseudo MS ³ 215.1300 [M-H-CO ₂] ⁻ : 197.1182 (100), 115.0766 (97), 141.0557 (38), 113.0610 (33), 97.0661 (28), 179.1076 (25), 129.0558 (23), 159.0662 (23), 153.1286 (18), 85.0661 (14)

Table S6. Replicated metabolites in the pooled samples experiment

M	Metabolite	RT	Experimental mass (m/z) [Annotation]	Pool 06 h	Pool 24 h
M01	Asp-Ala	0.91	205.0822 [M+H] ⁺	4.38x10 ⁻⁵	2.12x10 ⁻⁴
M03	Asp-Leu	2.86	247.1292 [M+H] ⁺ ; 248.1325 ¹³ C[M+H] ⁺ ; 245.1143 [M-H] ⁻ ; 227.1038 [M-H-H ₂ O] ⁻	5.91x10 ⁻⁵	4.90x10 ⁻⁵
M04	Asp-Met	1.43	265.0857 [M+H] ⁺	2.58x10 ⁻⁵	1.02x10 ⁻⁴
M05	Asp-Phe	3.75	281.1136 [M+H] ⁺ ; 282.1169 ¹³ C[M+H] ⁺ ; 279.0986 [M-H] ⁻	3.76x10 ⁻⁵	4.39x10 ⁻⁵
M07	Asp-Tyr	1.69	297.1086 [M+H] ⁺	2.69x10 ⁻⁵	4.39x10 ⁻⁵
M08	Asp-Val	1.33	233.1135 [M+H] ⁺	2.58x10 ⁻⁵	4.90x10 ⁻⁵
M12	Asp-Asn-Val / Asn-Asp-Val	1.30	347.1567 [M+H] ⁺	5.41x10 ⁻⁵	4.98x10 ⁻⁵
M13	Asp-Gly-(i)Leu	3.25	304.1509 [M+H] ⁺ ; 302.1357 [M-H] ⁻	2.32x10 ⁻²	3.30x10 ⁻⁴
M14	Asp-Gly-Tyr / Gly-Asp-Tyr	1.85	354.1302 [M+H] ⁺	1.94x10 ⁻⁴	1.57x10 ⁻³
M15	Asp-Gly-Val	1.48	290.1352 [M+H] ⁺ ; 288.1201 [M-H] ⁻	8.66x10 ⁻⁴	2.27x10 ⁻³
M19	Cyclo(His-Pro)	1.16	235.1194 [M+H] ⁺	1.97x10 ⁻⁴	1.89x10 ⁻²
M20	(i)Leucine derivative (I)	3.35	245.1143 [M-H] ⁻	4.38x10 ⁻⁵	4.39x10 ⁻⁵
M21	(i)Leucine derivative (II)	4.63	289.1399 [M+H] ⁺ ; 287.1248 [M-H] ⁻	2.46x10 ⁻³	1.88x10 ⁻⁴
M22	Glycine derivative	1.39	232.1295 [M+H] ⁺ ; 215.1029 [M+H-NH ₃] ⁺ ; 230.1148 [M-H] ⁻	1.30x10 ⁻²	1.76x10 ⁻¹
M24	Proline derivative	3.60	229.1186 [M+H] ⁺	1.61x10 ⁻³	5.26x10 ⁻⁴
M25	Hydroxyarginine	0.79	191.1141 [M+H] ⁺	1.81x10 ⁻⁴	3.30x10 ⁻⁴
M26	Oxoarginine	0.93	174.0875 [M+H] ⁺	1.89x10 ⁻²	7.13x10 ⁻⁴
M27	(Epi)catechin sulfate	4.25	369.0286 [M-H] ⁻	4.12x10 ⁻⁴	5.39x10 ⁻⁴
M28	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulfate	3.90	305.0336 [M-H] ⁻	4.63x10 ⁻⁴	5.56x10 ⁻⁴
M29	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-3-O-sulfate	4.63	287.023 [M-H] ⁻ ; 207.0664 [M-H-sulf] ⁻	8.95x10 ⁻⁵	3.55x10 ⁻⁴
M32	Phloroglucinol glucuronide	1.82	303.0716 [M+H] ⁺ ; 304.075 ¹³ C[M+H] ⁺ ; 325.0536 [M+Na] ⁺ ; 301.0565 [M-H] ⁻ ; 302.0598 ¹³ C[M-H] ⁻	2.26x10 ⁻⁴	3.35x10 ⁻⁴
M33	Phloroglucinol sulfate	1.32	224.0232 [M+NH ₄] ⁺ ; 204.9813 [M-H] ⁻ ; 205.9846 ¹³ C[M-H] ⁻ ; 206.9771 ²¹⁵ C[M-H] ⁻ ; 125.0247 [M-H-sulf] ⁻	3.71x10 ⁻⁵	4.90x10 ⁻⁵
M34	2-Hydroxyhippuric acid	5.27	196.0607 [M+H] ⁺ ; 121.0284 [M+H-glycine] ⁺ ; 194.0459 [M-H] ⁻ ; 150.0563 [M-H-CO ₂] ⁻ ; 239.0919 [M+C ₂ H ₅ N] ⁺	2.04x10 ⁻³	1.59x10 ⁻³
M35	Aminosalicyluric acid	1.74	211.0716 [M+H] ⁺	2.58x10 ⁻⁵	4.39x10 ⁻⁵
M37	Protocatechuic acid glucoside	2.18	315.0721 [M-H] ⁻	2.58x10 ⁻⁵	4.90x10 ⁻⁵
M38	Vanillic acid sulfate	2.80	246.9918 [M-H] ⁻	7.51x10 ⁻⁴	1.15x10 ⁻³
M39	Hydroxyjasmonic acid	5.13	227.1281 [M+H] ⁺ ; 225.1133 [M-H] ⁻	5.91x10 ⁻⁵	3.55x10 ⁻⁴
M40	Hydroxydihydrojasmonic acid (I)	4.80	229.1438 [M+H] ⁺ ; 211.1331 [M+H-H ₂ O] ⁺	2.04x10 ⁻³	4.50x10 ⁻³
M42	Ascorbic acid	0.90	175.025 [M-H] ⁻	1.39x10 ⁻⁴	4.79x10 ⁻⁴
M43	Dopamine sulfate	1.29	234.0434 [M+H] ⁺ ; 232.0287 [M-H] ⁻	3.54x10 ⁻⁵	6.69x10 ⁻⁵
M44	Hypaphorine	4.07	247.1445 [M+H] ⁺ ; 188.0708 [M+H-(CH ₃) ₃ N] ⁺ ; 144.0808 [M+H-(CH ₃) ₃ N-CO ₂] ⁺	7.00x10 ⁻³	9.19x10 ⁻⁴
M47	Trigonelline	0.88	138.055 [M+H] ⁺	2.58x10 ⁻⁵	4.39x10 ⁻⁵
M49	γ-CEHC glucuronide	6.39	458.2023 [M+NH ₄] ⁺ ; 459.2059 ¹³ C[M+NH ₄] ⁺ ; 501.2082 [M+H+C ₂ H ₅ O ₂] ⁺ ; 265.1438 [M+H-gluc] ⁺ ; 439.1609 [M-H] ⁻ ; 440.1642 ¹³ C[M-H] ⁻	7.26x10 ⁻³	2.88x10 ⁻⁴
M50	Dihydroxydecanoic acid glucuronide	5.51	363.1656 [M+H-H ₂ O] ⁺ ; 379.1609 [M-H] ⁻	4.72x10 ⁻⁴	9.27x10 ⁻²

Table S6 (continuation)

M	Metabolite	RT	Experimental mass (m/z) [Annotation]	Pool 06 h	Pool 24 h
M51	Dodecanedioic acid glucuronide	6.12	407.1919 [M+H] ⁺ ; 424.2182 [M+NH ₄] ⁺ ; 430.1712 ¹³ C[M+Na] ₂ ⁺ ; 231.1594 [M+H-gluc] ⁺ ; 405.1766 [M-H] ⁻ ; 406.18 ¹³ C[M-H] ⁻	2.26x10 ⁻²	8.67x10 ⁻¹
M52	Hydroxydodecadienoic acid glucuronide	7.00	389.1812 [M+H] ⁺ ; 406.2078 [M+NH ₄] ⁺ ; 213.1488 [M+H-gluc] ⁺ ; 387.166 [M-H] ⁻	2.60x10 ⁻²	1.52x10 ⁻¹
M53	Tetradecanedioic acid glucuronide	6.88	452.2494 [M+NH ₄] ⁺ ; 435.2229 [M+H] ⁺ ; 433.2078 [M-H] ⁻	1.56x10 ⁻⁴	5.48x10 ⁻³
M54	Dicarboxylic fatty acid C12:1, dihydroxy	5.05	261.1336 [M+H] ⁺	1.64x10 ⁻⁴	2.35x10 ⁻³

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Supporting Information

Food intake biomarkers of legume consumption
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4.3. Mediterranean diet, in-hospital exercise, and the incidence of hospitalization-associated disability in older patients

In this work, further insights on strategies to reduce the incidence of HAD in old patients have been provided. Particularly, the role of adherence to the MedDiet in the effect of a multicomponent training program on the functional status of acutely hospitalized older patients has been examined.

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Abstract

The aim of this study was to investigate the relationship between Mediterranean diet (MedDiet) adherence and response to an exercise and health education program to prevent hospitalization-associated disability (HAD) in acutely hospitalized older adults. In this secondary analysis, the functional status of a subset of 109 participants from AGECA-PLUS study with available data on MedDiet adherence (mean age 87, and range 75-98) was analyzed. Participants were randomized into the control group (n = 46, usual care) or the intervention group (n = 63, supervised exercise and health education) at admission. MedDiet adherence was measured with Mediterranean Diet Adherence Screener (MEDAS) and through urinary total polyphenols (UTP). Functional status was assessed with the Barthel Index. At discharge, patients in the intervention group who had low levels of MedDiet or UTP showed an increase in functional status [adjusted mean (95% CI) = 77.8 (70.8-84.8) points, $p = 0.005$, and adjusted mean (95% CI) = 78.0 (68.3-87.7) points, $p = 0.020$, respectively]. Older individuals over age 75 with low MedDiet adherence were likely to benefit more from a physical exercise and health education intervention.

Resum

L'objectiu d'aquest estudi va ser investigar la relació entre l'adherència a la dieta mediterrània (MedDiet) i la resposta a un programa d'exercici i educació sanitària per prevenir la discapacitat associada a l'hospitalització (HAD) en adults majors hospitalitzats. En aquesta anàlisi secundària, es va analitzar l'estat funcional d'un subconjunt de 109 participants de l'estudi AGE CAR-PLUS amb dades disponibles sobre l'adherència a la MedDiet (edat mitjana 87, i rang 75-98). Els participants van ser aleatoritzats en el grup de control ($n = 46$, atenció habitual) o en el grup d'intervenció ($n = 63$, exercici supervisat i educació sanitària) a l'admissió. L'adherència a la MedDiet es va mesurar amb el Mediterranean Diet Adherence Screener (MEDAS) i a través dels polifenols urinaris totals (UTP). L'estat funcional es va avaluar amb l'Índex de Barthel. A l'alta, els pacients del grup d'intervenció que tenien nivells baixos de MedDiet o d'UTP van mostrar una millora en l'estat funcional [mitjana ajustada (95% CI) = 77.8 (70.8-84.8) punts, $p = 0.005$, i mitjana ajustada (95% CI) = 78.0 (68.3-87.7) punts, $p = 0.020$, respectivament]. Els individus majors de 75 anys amb baixa adherència a la MedDiet probablement es beneficiarien més d'una intervenció d'exercici físic i educació sanitària.

Adherence to Mediterranean Diet and Response to an Exercise Program to Prevent Hospitalization-Associated Disability in Older Adults: A Secondary Analysis from a Randomized Controlled Trial

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Abstract

OBJECTIVES: To investigate the relationship between Mediterranean diet (MedDiet) adherence and response to an exercise and health education program to prevent hospitalization-associated disability (HAD) in acutely hospitalized older adults.

DESIGN: Randomized controlled trial.

SETTING AND PARTICIPANTS: Secondary analysis of a subset of 109 participants from AGE-CAR-PLUS study with available data on MedDiet adherence (mean age 87, and range 75–98).

INTERVENTION: Participants were randomized into the control group (n = 46, usual care) or the intervention group (n = 63, supervised exercise and health education) at admission.

MEASUREMENTS: MedDiet adherence was measured with MEDAS and through urinary total polyphenols (UTP). Functional status was assessed with the Barthel Index.

RESULTS: At discharge, patients in the intervention group who had low levels of MedDiet or UTP showed an increase in functional status [adjusted mean (95% CI) = 77.8 (70.8–84.8) points, $p = 0.005$, and adjusted mean (95% CI) = 78.0 (68.3–87.7) points, $p = 0.020$, respectively].

CONCLUSION: Older individuals over age 75 with low MedDiet adherence were likely to benefit more from a physical exercise and health education intervention.

Key words: Mediterranean dietary pattern, phenolic compounds, hospitalization-associated disability, functional ability, activities of daily living.

Introduction

In older patients, acute medical illness that necessitates hospitalization may often precipitate disability even when such illness is successfully treated. This leads to the subsequent inability to live independently and complete basic activities of daily living (ADLs) (1). Hospitalization-associated disability (HAD, i.e. loss of the ability to perform one or more ADLs independently at discharge) occurs in approximately one third of patients older than 70 and is associated with an increased length of stay, higher use of resources, institutionalization, hospital readmissions, long-term disability and mortality (2). Physical exercise interventions

have proven viable and safe in acutely hospitalized older patients and are effective for enhancing their functional status at discharge as well as for reducing the cost and length of hospitalization (3, 4). On the other hand, a healthy dietary pattern has been in the spotlight because of its effects on healthy aging (5). In fact, recent evidence suggests that healthy dietary patterns, such as the Mediterranean diet (MedDiet), the MIND diet, and the DASH diet, may reduce the risk of physical impairment, mobility disability, and frailty in community-dwelling and institutionalized older adults (6–8). The concentration of urinary total polyphenols (UTP) is a well-known objective biomarker of total dietary polyphenol exposure and a proxy measure of dietary fruit and vegetable intake (9). Moreover, UTP depends on the bioavailability of polyphenols, which tends to decrease during aging and may consequently impact their biological activity (10). Despite this interest, little research has been devoted to evaluating the relationship between UTP and age-related comorbidities, although this shows promising evidence. For instance, high UTP levels have been associated with a lower risk of physical performance decline (i.e. walking speed, rising from a chair, and standing balance), frailty, exhaustion, weakness, and cognitive decline in community-dwelling old adults (9, 11). However, to the best of our knowledge, no studies have explored the effect of the MedDiet and UTP on functional status in acute hospitalized older adults.

Hence, in this paper, we sought to investigate the relationship between MedDiet adherence and the response to an exercise and health education program aimed at preventing HAD in acutely hospitalized adults aged over 75 years during hospitalization.

Materials and methods

Study design

The Activity in GERiatric acute CARE Plus (AGE-CAR-PLUS) Health Education is a randomized control trial to assess the effectiveness of an intrahospital exercise and health education program during short hospital stays for improving

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functional capacity of patients aged 75 years or older. The details of the clinical trial (NCT03604640) are described elsewhere (<https://clinicaltrials.gov/ct2/show/NCT03604640>). Briefly, 260 patients (> 75 years) admitted to the acute care for older patients (ACE) unit of the Hospital General Universitario Gregorio Marañón (Madrid, Spain) were considered eligible to participate in the study and were thus randomized to a control group or intervention group. Participants allocated in the control group received standard hospital care. Participants allocated in the intervention group performed, during hospitalization, a multicomponent training program (30 minutes per session, twice a day on weekdays, lower limb strength training, balance training, walking, and inspiratory muscle training) and health education. Health education consisted of several informational activities. Each activity session taught the patient and caregiver how to perform the exercises to ensure continuity after discharge. Eligible participants were included in the control or intervention group in a time-dependent manner (i.e. using 4-week blocks) to avoid the co-presence of patients from both groups in the unit, so that participants were blinded to actual group assignment (Supplementary Figure 1).

The Institutional Review Board approved the protocol (Hospital Universitario Gregorio Marañón, Madrid, Spain), and all participants provided written informed consent. When it was not possible to obtain the informed consent directly from a patient for medical reasons (e.g. impaired cognitive function), proxy consent was obtained from their relatives. All procedures were performed in accordance with the ethical standards from the Declaration of Helsinki and its later amendments.

In this current work, we conducted a secondary analysis with 109 older participants from AGE-CAR-PLUS trial who had the Mediterranean Diet Adherence Screener (MEDAS) or urine sample available at admission (63 and 46 individuals in the intervention and in the control group, respectively) (Supplementary Figure 1).

Participants

The participant inclusion criterion was patients aged 75 and older admitted to the ACE unit. The exclusion criteria were: nonambulatory or dependent in all basic ADLs at baseline (i.e. two weeks before admission, as assessed by retrospective interview); having an unstable cardiovascular disease (or any other major medical condition contraindicating exercise), terminal illness, or severe dementia; expected length of hospitalization < three days; having a scheduled admission (which was usually associated with a length of hospitalization < three days) (4); or being transferred from another hospital unit.

Measurement of Mediterranean diet adherence

Determination of total polyphenols in urine samples

Spot urine samples were collected at admission (Supplementary Figure 1). Urine samples were aliquoted, processed, and stored at -80 °C until analysis. UTP were analyzed using Folin-Ciocalteu reagent after solid-phase

extraction utilizing Oasis HLB 96-well plates (Waters, Milford, MA), as described elsewhere (9). Total polyphenol results were further normalized with reference to the urinary refractive index to account for interindividual differences in the hydration status and micturition frequency (10). UTP concentrations were expressed as mg gallic acid equivalents (GAEs)/L (9). UTP has previously been positively correlated with adherence to the MedDiet (12). UTP levels were further divided into two median-based groups to categorize UTP levels as low (≤ 11.1 mg GAEs/L) and high (> 11.2 mg GAEs/L).

MEDAS questionnaire

At admission, the validated 14-item MEDAS questionnaire was used to briefly assess adherence to a traditional MedDiet (13). This questionnaire reports the habitual frequency of consumption or amount consumed of 12 main components of the MedDiet and two food intake habits considered characteristic of the Spanish MedDiet (13). Each of the 14 items was scored 1 or 0, depending on whether or not patients met each MedDiet condition. One point was assigned for using olive oil as the principal source of fat for cooking; for preferring white meat over red meat; for consuming ≥ 4 tablespoons of olive oil/day (including that used in frying, salads, meals eaten away from home, etc.); ≥ 2 servings of vegetables/day; ≥ 3 servings of fruit (including freshly squeezed juice)/day; < 1 serving of red meat, hamburger, or sausages/day; < 1 serving of animal fat (i.e. butter, margarine, or cream)/day; < 1 cup of sugar-sweetened and/or carbonated beverage/day; ≥ 7 cups of red wine/week; ≥ 3 servings of pulses/week; ≥ 3 servings of fish or seafood/week; < 2 servings of commercial pastry/week; ≥ 3 servings of nuts/week; ≥ 2 servings/week of a dish with a traditional sauce of tomatoes, garlic, onion, or leeks sautéed in olive oil (13). MEDAS scores were divided into two groups to categorize adherence as low (scores < 9) and high (scores ≥ 9) (14).

Endpoint assessment

The functional status with regard to the basic ADLs was determined at admission and at discharge. Functional status was evaluated with the Barthel Index (BI), which measures patients' ability to independently perform ten basic ADLs [feeding, transferring from bed to chair and back, walking on level surfaces, stair climbing, bowel and bladder control, using the toilet, bathing, grooming (e.g. tooth brushing, shaving), and dressing] (15). The sum score for this index ranges from 0 (total dependence) to 100 (complete independence) points.

Those medical and research staff who were in charge of the outcome assessment were not involved in supervising the intervention. However, assessors and care providers were not blinded to the assigned intervention.

Other variables

Upon admission, demographic (age, sex, place of living) and clinical characteristics [diagnosis at admission, presence of

Table 1. Main Characteristics of the Study Participants by Intervention Group and Mediterranean Diet Adherence (Intention-to-Treat Analysis)

Variables	Control		Intervention		p	Control		Intervention		p
	Low adherence ^c (n=31)	High adherence ^c (n=14)	Low adherence ^c (n=34)	High adherence ^c (n=26)		Low UTP levels ^d (n=16)	High UTP levels ^d (n=14)	Low UTP levels ^d (n=19)	High UTP levels ^d (n=21)	
Age [mean (SD)], y.	88.2 (4.8)	85.8 (4.2)	87.1 (4.3)	87.3 (4.7)	0.421	86.8 (4.7)	86.9 (5.6)	87.2 (4.6)	87.2 (4.5)	0.990
Female [n (%)]	16 (51.6)	7 (50.0)	17 (50.0)	9 (34.6)	0.565	9 (56.3)	7 (50.0)	7 (36.8)	8 (38.1)	0.601
BMI at admission ^b [mean (SD)], kg/m ²	20.5 (3.2) ^{ab}	21.2 (4.1) ^{ab}	22.9 (3.9) ^a	20.2 (2.4) ^a	0.014	19.8 (2.5)	23.3 (4.5)	21.6 (2.6)	20.9 (4.0)	0.088
Living at home [n (%)]	28 (90.3)	14 (100.0)	32 (94.1)	25 (96.2)	0.673	16 (100.0)	13 (92.9)	17 (89.5)	20 (95.2)	0.658
Charlson comorbidity index ^d [mean (SD)]	2.8 (1.8)	2.4 (1.7)	3.2 (1.9)	3.7 (2.7)	0.232	2.1 (2.2)	2.7 (1.5)	3 (2.0)	3.4 (2.2)	0.287
Geriatric syndromes										
Frailty phenotype ^a [n (%)]	15 (71.4)	8 (80.0)	14 (50.0)	16 (66.7)	0.255	8 (72.7)	9 (90.0)	10 (58.8)	14 (66.7)	0.407
Urinary incontinence ^e [n (%)]	18 (60.0)	6 (42.9)	17 (50.0)	12 (46.2)	0.659	10 (62.5)	6 (42.9)	12 (63.2)	11 (52.4)	0.624
Depression ^f [n (%)]	11 (36.7)	7 (50.0)	15 (44.1)	9 (34.6)	0.737	8 (50.0)	7 (50.0)	5 (26.3)	10 (47.6)	0.401
Falls ^g [n (%)]	13 (43.3)	3 (21.4)	10 (29.4)	8 (30.8)	0.464	6 (37.5)	4 (28.6)	7 (36.8)	5 (23.8)	0.765
Chronic pain ^h [n (%)]	10 (33.3)	2 (14.3)	9 (26.5)	10 (38.5)	0.407	6 (37.5)	3 (21.4)	9 (47.4)	6 (28.6)	0.416
Malnutrition ^h [n (%)]	6 (24.0)	5 (35.7)	2 (7.4)	8 (33.3)	0.074	5 (35.7)	2 (16.7)	4 (23.5)	6 (30.0)	0.301
Dementia ⁱ [n (%)]	5 (16.7)	1 (7.1)	2 (5.9)	4 (15.4)	0.507	1 (6.3)	1 (7.1)	3 (15.8)	3 (14.3)	0.756
Polypharmacy (≥7) ^j [n (%)]	20 (66.7)	12 (85.7)	28 (82.4)	21 (80.8)	0.360	12 (75)	10 (71.4)	16 (84.2)	18 (85.7)	0.693
Main admission diagnosis [n (%)]					0.834					0.338
Infectious	11 (35.5)	5 (35.7)	9 (26.5)	7 (26.9)		4 (25.0)	6 (42.9)	4 (21.1)	5 (23.8)	
Circulatory	8 (25.8)	6 (42.9)	7 (20.6)	7 (26.9)		6 (37.5)	4 (28.6)	7 (36.8)	3 (14.3)	
Digestive	4 (12.9)	1 (7.1)	4 (11.8)	3 (11.5)		2 (12.5)	1 (7.1)	1 (5.3)	4 (19.0)	
Respiratory	2 (6.5)	1 (7.1)	3 (8.8)	4 (15.4)		0 (0.0)	1 (7.1)	3 (15.8)	4 (19.0)	
Blood/myeloproliferative syn.	1 (3.2)	0 (0.0)	6 (17.6)	2 (7.7)		1 (6.3)	0 (0.0)	0 (0.0)	4 (19.0)	
Renal/urologic	3 (9.7)	0 (0.0)	2 (5.9)	2 (7.7)		2 (12.5)	1 (7.1)	2 (10.5)	1 (4.8)	
Others ^k	2 (6.4)	1 (7.1)	3 (8.7)	1 (3.8)		1 (6.3)	1 (7.1)	2 (10.5)	0 (0.0)	
Functional status score at admission ^l [median (IQR)]	50 (40.0)	72.5 (32.5)	57.5 (41.3)	72.5 (46.3)	0.250	67.5 (42.5)	55.0 (36.3)	60.0 (40.0)	70.0 (40.0)	0.922
FAC score at admission ^l [median (IQR)]	3 (2.0)	3.5 (3.0)	3.0 (2.0)	3.0 (2.0)	0.664	2.5 (3.0)	4.0 (1.0)	2.0 (2.0)	3.0 (1.0)	0.137
Independent ambulation at admission ^l [n (%)]	14 (46.7)	7 (50.0)	9 (27.3)	12 (46.2)	0.292	6 (37.5)	8 (57.1)	7 (36.8)	9 (42.9)	0.652
SPPB score at admission ^l [median (IQR)]	3.0 (5.0)	2.0 (4.0)	3.0 (3.8)	3.0 (5.5)	0.290	4.5 (7.0)	2.5 (3.3)	3.0 (5.5)	5.0 (5.5)	0.417
Length of hospitalization ^l [median (IQR)], d	5.0 (4.0)	4.0 (4.3)	5.0 (3.3)	5.5 (9)	0.108	4.5 (3.3)	5.0 (5.0)	5.0 (4.0)	5.0 (7.0)	0.786
MedDiet adherence score ^l [mean (SD)]	6.5 (1.4) ^a	9.8 (1.0) ^a	6.7 (1.4) ^a	9.9 (1.0) ^a	<0.001	7.9 (2.2)	7.5 (2.4)	8.7 (2.0)	8.2 (2.0)	0.420
UTP levels at admission ^l [mean (SD)], mg GAEs/L	10.8 (2.6)	10.7 (1.9)	10.7 (2.7)	10.7 (2.2)	0.999	8.6 (1.3) ^a	12.8 (1.3) ^a	8.5 (2.3) ^a	12.5 (0.8) ^a	<0.001

Note. BMI = body mass index; FAC = functional ambulatory classification; GAEs = gallic acid equivalents; IQR = interquartile range; MedDiet = Mediterranean diet; SD = standard deviation; SPPB = Short Physical Performance Battery; UTP = urinary total polyphenols. a. Frailty was defined as having ≥ 3 of 5 Fried criteria (17). b. Malnutrition was defined as having ≤ 7 of 14 Mini Nutritional Assessment–Short Form criteria (16). c. The “Others” category includes central nervous system, musculoskeletal, endocrine and neoplasia admission diagnosis. d. Functional status was assessed with the Barthel Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs: feeding, transferring, walking on level surfaces, stair climbing, bowel and bladder control, toileting, bathing, grooming and dressing (15). e. Independent ambulation was considered in the event of an FAC = 4 (19). f. A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence consisted in a MEDAS score ≥ 9 at admission. Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high levels of UTP consisted in > 11.2 mg GAEs/L at admission; § Variables containing missing values. The number of missing values in the different groups [low adherence + control, high adherence + control, low adherence + intervention, high adherence + intervention] [low UTP levels + control, high UTP levels + control, low UTP levels + intervention, high UTP levels + intervention] for the following covariables are: BMI at admission [6, 0, 3, 0] [2, 2, 0, 0]; Charlson comorbidity index [2, 0, 0, 0] [0, 0, 0, 0]; Frailty phenotype [10, 4, 6, 2] [5, 4, 2, 0]; Urinary incontinence [1, 0, 0, 0] [0, 0, 0, 0]; Depression [1, 0, 0, 0] [0, 0, 0, 0]; Falls [1, 0, 0, 0] [0, 0, 0, 0]; Chronic pain [1, 0, 0, 0] [0, 0, 0, 0]; Malnutrition [6, 0, 7, 2] [2, 2, 2, 1]; Dementia [1, 0, 0, 0] [0, 0, 0, 0]; Polypharmacy [1, 0, 0, 0] [0, 0, 0, 0]; FAC score at admission [1, 0, 1, 0] [0, 0, 0, 0]; Independent ambulation at admission [1, 0, 1, 0] [0, 0, 0, 0]; SPPB score at admission [3, 3, 2, 1] [2, 0, 1, 0]; Length of hospitalization [2, 0, 0, 0] [0, 0, 0, 0]; MedDiet adherence score [0, 0, 0, 0] [1, 0, 1, 2]; UTP levels at admission [12, 4, 17, 6] [0, 0, 0, 0]. Values without the same superscript differ (p < 0.05; Bonferroni post hoc test).

other geriatric syndromes (dementia, depression, falls, chronic pain, malnutrition (having ≤ 7 of the 14 Mini Nutritional Assessment–Short Form criteria (16)), urinary incontinence, frailty phenotype (having ≥ 3 of the five Fried’s criteria (17))), comorbidities (assessed by means of the Charlson Comorbidity Index (18)), independent ambulation (considered in the event of a functional ambulatory classification (FAC) = 4 (19)), lower extremity function and mobility (assessed by the Short Physical Performance Battery (SPPB) (20)), and polypharmacy (taking ≥ 7 drugs)] were also assessed.

Statistical analysis

Baseline characteristics of the participants were expressed as means and standard deviations (SD) for variables with normal distribution, medians and interquartile ranges (IQR) for variables with asymmetric distribution, and percentages for categorical variables. UTP and BI were not normally distributed and were thus normalized using a Box–Cox transformation ($\alpha = 0.00001$ and $\lambda = 0.25$) (9) and a log2 transformation, respectively.

Differences in characteristics between groups at admission were analyzed using the Student’s t-test, the chi-square test, or the Mann–Whitney U test, when appropriate.

One-factor analysis of variance (ANOVA) for repeated measures with the Bonferroni post hoc test was used to compare changes in BI in response to the intervention treatment and to the control group. In addition, it was also used to compare changes in functional status considering low and high levels of the following variables: a) adherence to MedDiet at admission and b) concentrations of UTP at admission. We studied the effect of the interaction of two factors: time as a within-participants factor (admission and discharge) and groups after adjustment for sex, age, body mass index (BMI) at admission, place of living, independent ambulation, Charlson Comorbidity Index, polypharmacy (≥ 7), cause of hospitalization (admission diagnosis), frailty phenotype, and additional creatinine clearance at admission, and chronic kidney disease. To assess the effects of individual factors, we finally calculated the differences between discharge and admission values for the BI and then we applied a one-factor analysis of covariance (ANCOVA) test adjusting for the above-mentioned variables and for functional status at admission.

Intention-to-treat analysis were performed using the methods of the last observation carried forward/backward (21, 22), and sensitivity analysis were performed using a complete-case analysis. We did not impute missing data of covariates, and thus only available data were used for analysis. Between- and within-group differences were expressed as estimated means and 95% CI. Finally, it should be noted that the AGE-CAR-PLUS trial was powered to assess the effectiveness of an intrahospital exercise and health education program for improving functional capacity of older patients, therefore, sample size calculations were based on the main outcomes of the trial. However, in this study, we performed an exploratory analysis of data acquired from the AGE-CAR-PLUS trial, thus, the statistical power has been calculated (23).

All statistical analyses were performed using the SPSS software package (SPSS 25.0, IBM, NY) and the significance level was a $p < 0.05$.

Results

The participants' characteristics according to intervention and MedDiet adherence groups are shown in Table 1 (intention-to-treat analysis) and in Supplementary Table 1 (complete-case analysis). The mean (SD) age of the participants was 87 (5) (range 75–98) and 46% were female. The median (IQR) length of hospitalization was 5.0 (4.0) days. No general between-group differences were found at the start of the study for sociodemographic and other clinical characteristics (all $p > 0.05$; Table 1 and Supplementary Table 1) although in the intervention group BMI at admission was slightly different between MedDiet adherence groups ($p < 0.05$). Participants in the intervention group performed a mean (SD) of 7.1 (5.8) training sessions. No adverse effects or falls were recorded during the intervention. BI [median (IQR)] at admission was similar among participants in both control and intervention groups (Table 1 and Supplementary Table 1). The mean (SD) adherence to the MedDiet was 7.9 (2.1) and it was similar between groups (Table 1). Individuals showed similar UTP

levels at admission between groups (Table 1 and Supplementary Table 1).

Participants in the intervention group presented a significantly better BI at discharge [adjusted mean (95% CI) = 75.7 (71.1–80.3) points] compared to admission [adjusted mean (95% CI) = 67.2 (62.8–71.5) points; $p = 0.002$] as well as to the control group [adjusted mean (95% CI) = 65.7 (59.4–71.9) points; $p = 0.014$] (Supplementary Table 2). No significant differences in functional status were noted in the control group during hospitalization ($p > 0.05$; Supplementary Table 2). The adjusted mean of change (95% CI) of functional status in the control group was 1.2 (-4.5–6.9) points and 9.3 (5.2–13.5) points in the intervention group ($p = 0.014$; Supplementary Table 2).

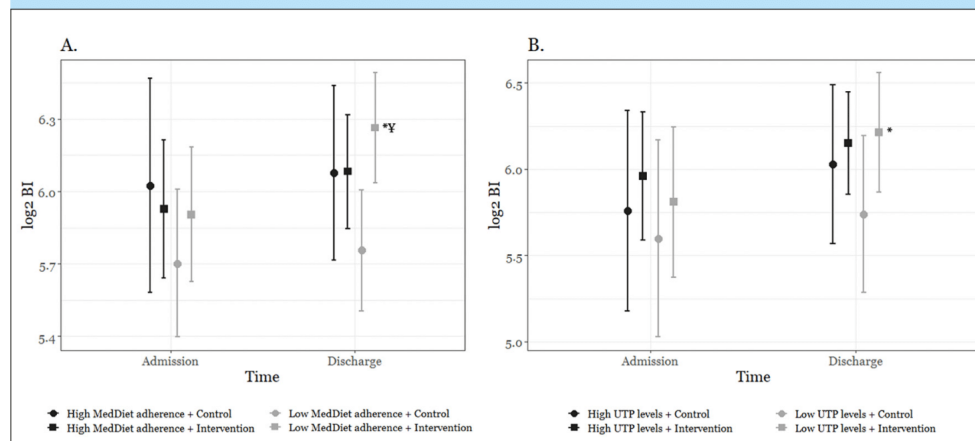
Differences in functional status during hospitalization (i.e. BI at discharge vs. BI at admission) between low and high levels of MedDiet adherence and UTP at admission are reported in Figure 1. Participants with low MedDiet adherence in the intervention group significantly increased their BI during hospitalization (Figure 1 and Supplementary Table 3). In addition, in the adjusted model, the functional status at discharge of these participants was significantly higher than that of the control group ($p < 0.05$). However, we did not observe significant increases in the control group or among those subjects with a high MedDiet adherence (all $p > 0.05$) (Figure 1 and Supplementary Table 3).

In the same vein, participants with low UTP levels at admission in the intervention group significantly increased their BI during hospitalization (Figure 1 and Supplementary Table 3). No differences in functional status during hospitalization were found among participants from either control group or with high levels of UTP at admission (all $p > 0.05$) (Figure 1 and Supplementary Table 3).

In addition, in the intervention group, we observed higher changes in BI in the low level of MedDiet adherence [mean (95% CI) of 12.3 (6.0–18.6)] and UTP groups [mean (95% CI) of 13.2 (4.6–21.8)] than in the high-level groups [MedDiet adherence (mean (95% CI) of 5.8 (-0.8–12.3); UTP (mean (95% CI) of 9.7 (2.3–17.0)], although they did not reach statistical significance (all $p > 0.05$) (Supplementary Table 4). It is remarkable that although not significant, changes in the control group were lower than in the intervention group (Supplementary Table 4). All these differences remained significant in sensitivity analyses (Supplementary Tables 2, 4 and 5).

In supplementary analyses, we explored the potential mediation effect of malnutrition on the relationship between MedDiet adherence and UTP levels and the response to the intervention through the Sobel test (24). We did not observe significant mediation pathways between MedDiet adherence or UTP levels and the response to the intervention through malnutrition [t (SE) = -0.174 (0.002), p -value = 0.861 for MedDiet adherence; and t (SE) = 0.522 (0.012), p -value = 0.601 for UTP levels] (25).

Figure 1. Functional Status at Admission and at Discharge of Participants in Both Control and Intervention Groups According to MedDiet Adherence (A.) and UTP Levels (mg GAEs/L) at Admission (B.) (Intention-to-Treat Analysis)



Note. BI, Barthel Index; CI = confidence interval; MedDiet = Mediterranean diet; UTP = urinary total polyphenols. Models are adjusted for sex, age, BMI at admission, place of living, independent ambulation, Charlson Comorbidity Index, polypharmacy (≥ 7), cause of hospitalization (admission diagnosis) and frailty phenotype. In the UTP levels model, we further adjusted for creatinine clearance at admission and chronic kidney disease. A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence consisted in a MEDAS score ≥ 9 at admission. Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high levels of UTP consisted in > 11.2 mg GAEs/L at admission. Functional status was assessed with the Barthel Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs: feeding, transferring, walking on level surfaces, stair climbing, bowel and bladder control, toileting, bathing, grooming and dressing (15). * $p < 0.05$; † Different from admission, † $p < 0.05$; ‡ Different from control group, ‡ $p < 0.05$ (ANOVA for repeated measures with Bonferroni post hoc test).

Discussion

To the best of our knowledge, this is the first work with hospitalized older patients aged over 75 to evaluate the relationship between a healthy dietary pattern and functional status following a physical exercise and health education intervention. This secondary analysis shows that, although the intervention with exercise has proven effective and safe overall (4), it has a greater effect in individuals who have low adherence to the MedDiet. In fact, participants who had a low MedDiet adherence significantly increased their functional status by an adjusted mean (95% CI) of 12.3 (6.0–18.6) points in the BI after intervention with exercise, which is considered clinically relevant (26). In contrast, no further differences between BI at admission and at discharge were noted in the control group or among those subjects with a high MedDiet adherence. Multi-domain interventions, where both exercise and nutritional interventions are optimally designed, tend to reveal a stronger effect on frailty, physical functioning, and muscle mass and strength than physical exercise alone (27). However, not all older people may benefit from the same type of exercise interventions in the same way. Valenzuela et al. (2020) reported interindividual variability in the responsiveness to physical exercise intervention in acutely hospitalized older adults. Interestingly, a worse nutritional status at admission was associated with a greater responsiveness to the intervention (28). Furthermore, in a multicentric prospective observational study, researchers observed that improvement in functional

status was higher in frail institutionalized older adults with lower vitamin D levels and poorer nutritional status after an oral nutritional supplementation and physical exercise than those with ≥ 20 ng/mL vitamin D plasmatic levels (29). Franzke et al. (2019) observed significant correlations between changes in micronutrient status and changes in performance parameters only among institutionalized older subjects who did not receive protein and vitamin supplementation (30). This is consistent with our results, although to the best of our knowledge, no results have been previously reported regarding baseline dietary patterns rather than nutritional status or nutritional supplementation. Actually, extensive literature supports the MedDiet as a dietary pattern for preventing chronic disease and malnutrition, as well as for maintaining health on aging (31). In this sense, taking into account the obtained results, we can see that those older individuals who were admitted with a low level of MedDiet adherence had a bigger improvement margin when following a simple in-hospital exercise and health education intervention than their peers with higher adherence. This led us to hypothesize that stronger interventions should be carried out among individuals with high MedDiet adherence as not all older patients may require the same exercise intensity to prevent HAD.

In the present study, statistically significant differences were also found in those subjects who were in the intervention group and who showed low levels of UTP at admission. In line with the results obtained for MedDiet adherence, we did not observe differences for the control group or high UTP levels. As far

as we are aware, no studies have evaluated levels of UTP in acutely hospitalized older patients, although previous results of the research group obtained in the InCHIANTI cohort have shown that the prevalence of frailty decreased with increasing UTP tertiles in community-dwelling older subjects of a mean age of 73 years, as did the prevalence of disability in more than one ADL and instrumental ADLs (9). Similarly, high UTP concentrations were also associated with a lower risk of physical performance decline and mortality over nine years of follow-up (11, 32). In fact, numerous studies have suggested protective associations between polyphenol intake and some age-related pathologies (33). These protective effects may be attributable to their hermetic functions and their stimulus towards adaptive cellular responses, which, in turn, delay cellular aging.

Some limitations should be considered. This study is a single-center study performed in a frail population (i.e. the proportion of participants who were fully independent in all of the 10 ADLs at baseline was only 17%) and whose mean age (SD) was 87 (5) years, which could restrict the extrapolation of our findings to younger or more functional patients. However, while limited by a small sample size, the main strength of our study is precisely the unusual inclusion of acutely hospitalized older participants aged 75 to 98 with a high proportion of the frailty phenotype (~50%). Another limitation of our study is that assessors, care providers, and participants were blinded to the intervention with exercise. In addition, the present study population was aged 75 and older and therefore may be less reliable in recalling food intake than younger subjects, although MEDAS is a country-specific questionnaire and has been previously validated for older subjects (13). To account for that, we used UTP as an objective biomarker of polyphenol-rich food intake and thus a proxy biomarker of MedDiet (9). Furthermore, participants with severe dementia at admission were excluded. The cross-sectional design nested in a randomized clinical trial did not allow us to assess causality between UTP or MedDiet adherence and functional status.

A shortcoming of this current investigation is the potential for type II statistical error. The sample size for the AGE CAR-PLUS (n = 252; final enrollment n = 260) was selected to permit the detection of a recovery rate up to 60% with a statistical power of 80% ($\alpha = 0.05$). For the present secondary analysis, the estimated effect size for MedDiet assessed through the MEDAS questionnaire (n = 105) revealed a statistical power of 60%. The estimated effect size for MedDiet assessed through the UTP levels (n = 70) revealed a statistical power of 55%. Given the exploratory nature of this analysis and moderated statistical power, outcomes need to be interpreted cautiously. The impact of MedDiet on the response to the exercise program was a secondary outcome and the AGE CAR-PLUS trial was not powered for this outcome. As a result, our findings offer a hypothesis-generator and provide a basis for further research rather than firm evidence. Hence, further studies that include an appropriate sample size should be designed focusing on MedDiet interventions in an ACE context.

Finally, some outcome-influential variables, such as nutritional supplementation, emotional status, lifestyle, and physical activity levels prior to hospitalization, were not

directly assessed and therefore our analyses were not controlled for these potential confounders which might have conceivably biased the study outcomes.

Conclusion

In conclusion, this study showed that hospitalized older patients over age 75 (mean age 87, and range 75–98) with low MedDiet adherence were likely to benefit more from a physical exercise and health education program than those with high values. These results suggest that adherence to the MedDiet could represent a potential indicator of those older patients aged over 75 with a putative better response to exercise interventions. Therefore, the inclusion of MedDiet adherence or UTP levels in the variables assessed at admission of older patients could allow more accurate and personalized strategies to be designed with a view to preventing HAD.

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Ethics declarations: Conflict of Interest: All authors have completed the ICMJE uniform disclosure form and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical standards: The Institutional Review Board approved the protocol (Hospital Universitario Gregorio Marañón, Madrid, Spain), and all participants provided written informed consent. When it was not possible to obtain the informed consent directly from a patient for medical reasons (e.g. impaired cognitive function), proxy consent was obtained from their relatives. All procedures were performed in accordance with the ethical standards from the Declaration of Helsinki and its later amendments.

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SUPPLEMENTARY MATERIAL

Supplementary Figure 1. CONSORT 2010 Flow Diagram of This Study. ACE = acute care for older patients; BI = Barthel Index; MEDAS = Mediterranean Diet Adherence Score.

Supplementary Table 1. Main Characteristics of the Study Participants by Intervention Group and MedDiet Adherence (Complete-Case Analysis).

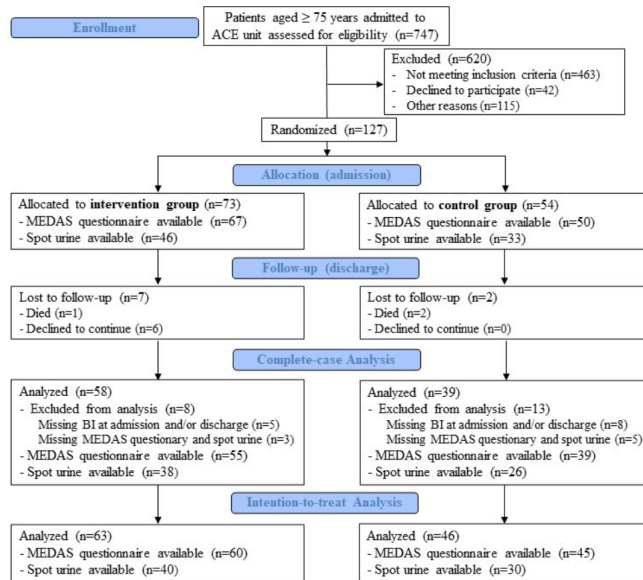
Supplementary Table 2. Functional Status at Admission and at Discharge of Participants According to Intervention Group.

Supplementary Table 3. Functional Status at Admission and at Discharge of Participants in Both Control and Intervention Groups According to MedDiet Adherence and UTP Levels (mg GAEs/L) at Admission (Intention-to-Treat Analysis).

Supplementary Table 4. Changes in Functional Status During Hospitalization of Participants in Both Control and Intervention Groups According to MedDiet Adherence and UTP Levels (mg GAEs/L) at Admission.

Supplementary Table 5. Functional Status at Admission and at Discharge of Participants in Both Control and Intervention Groups According to MedDiet Adherence and UTP Levels (mg GAEs/L) at Admission (Complete-Case Analysis).

- 1 **Supplementary Figure 1. CONSORT 2010 Flow Diagram of This Study.** ACE = acute care for older
- 2 patients; BI = Barthel Index; MEDAS = Mediterranean Diet Adherence Score.



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5 **Supplementary Table 1.** Main Characteristics of the Study Participants by Intervention Group and Mediterranean Diet Adherence

6 (Complete-Case Analysis).

Variables	Control		Intervention		<i>p</i>	Control		Intervention		<i>p</i>
	Low	High	Low	High		Low UTP	High UTP	Low UTP	High UTP	
	adherence ^f (n=26)	adherence ^f (n=13)	adherence ^f (n=31)	adherence ^f (n=24)		levels ^f (n=13)	levels ^f (n=13)	levels ^f (n=19)	levels ^f (n=19)	
Age [mean (SD)], y.	88.4 (4.9)	86.1 (4.2)	87.3 (4.2)	87.4 (4.9)	0.514	87.4 (5.0)	86.3 (5.4)	87.2 (4.6)	87.5 (4.4)	0.908
Female [n (%)]	13 (50)	6 (46.2)	15 (48.4)	9 (37.5)	0.817	6 (46.2)	7 (53.8)	7 (36.8)	8 (42.1)	0.811
BMI at admission [§] [mean (SD)], kg/m ²	20.7 (3.5) ^{ab}	21.4 (4.2) ^{ab}	23.0 (3.8) ^a	20.4 (2.4) ^b	0.031	20.0 (2.7)	23.4 (4.6)	21.6 (2.6)	20.6 (4.0)	0.096
Living at home [n (%)]	23 (88.5)	13 (100)	29 (93.5)	23 (95.8)	0.635	13 (100)	12 (92.3)	17 (89.5)	18 (94.7)	0.904
Charlson comorbidity index [§] [mean (SD)]	2.7 (1.8)	2.3 (1.8)	3.2 (1.9)	3.5 (2.7)	0.290	2.2 (2.3)	2.5 (1.4)	3.0 (2.0)	3.5 (2.4)	0.368
<i>Geriatric syndromes</i>										
Frailty phenotype ^{as} [n (%)]	14 (73.7)	8 (80.0)	14 (53.8)	14 (63.6)	0.383	8 (80.0)	9 (90.0)	10 (58.8)	13 (68.4)	0.338
Urinary incontinence [§] [n (%)]	14 (56.0)	5 (38.5)	15 (48.4)	12 (50.0)	0.783	7 (53.8)	6 (46.2)	12 (63.2)	10 (52.6)	0.809
Depression [§] [n (%)]	10 (40.0)	6 (46.2)	12 (38.7)	8 (33.3)	0.893	6 (46.2)	7 (53.8)	5 (26.3)	9 (47.4)	0.395
Falls [§] [n (%)]	11 (44.0)	3 (23.1)	9 (29.0)	8 (33.3)	0.541	6 (46.2)	4 (30.8)	7 (36.8)	5 (26.3)	0.693
Chronic pain [§] [n (%)]	9 (36.0)	2 (15.4)	9 (29.0)	10 (41.7)	0.396	5 (38.5)	3 (23.1)	9 (47.4)	6 (31.6)	0.525

- RESULTS -

Malnutrition ^{b§} [n (%)]	4 (19.0)	4 (30.8)	2 (8.0)	7 (31.8)	0.074	3 (25.0)	2 (18.2)	4 (23.5)	5 (27.8)	0.418
Dementia [§] [n (%)]	4 (16.0)	1 (7.7)	2 (6.5)	4 (16.7)	0.642	1 (7.7)	1 (7.7)	3 (15.8)	3 (15.8)	0.808
Polypharmacy [§] (≥ 7) [n (%)]	16 (64.0)	11 (84.6)	26 (83.9)	19 (79.2)	0.291	10 (76.9)	9 (69.2)	16 (84.2)	16 (84.2)	0.720
Main admission diagnosis [n (%)]					0.907					0.443
<i>Infectious</i>	7 (26.9)	5 (38.5)	8 (25.8)	7 (29.2)		3 (23.1)	5 (38.5)	4 (21.1)	4 (21.1)	
<i>Circulatory</i>	7 (26.9)	5 (38.5)	6 (19.4)	7 (29.2)		5 (38.5)	4 (30.8)	7 (36.8)	3 (15.8)	
<i>Digestive</i>	4 (15.4)	1 (7.7)	4 (12.9)	3 (12.5)		2 (15.4)	1 (7.7)	1 (5.3)	4 (21.1)	
<i>Respiratory</i>	2 (7.7)	1 (7.7)	3 (9.7)	3 (12.5)		0 (0.0)	1 (7.7)	3 (15.8)	3 (15.8)	
<i>Blood/myeloproliferative syn.</i>	1 (3.8)	0 (0.0)	5 (16.1)	1 (4.2)		1 (7.7)	0 (0.0)	0 (0.0)	4 (21.1)	
<i>Renal/urologic</i>	3 (11.5)	0 (0.0)	2 (6.5)	2 (8.3)		1 (7.7)	1 (7.7)	2 (10.5)	1 (5.3)	
<i>Others^c</i>	2 (7.6)	1 (7.7)	3 (9.6)	1 (4.2)		1 (7.7)	1 (7.7)	2 (10.6)	0 (0.0)	
Functional status score at admission ^{d§} [median (IQR)]	47.5 (45.0)	75.0 (30.0)	55 (40.0)	80.0 (48.8)	0.191	75.0 (45.0)	55.0 (37.5)	60.0 (40.0)	70.0 (45.0)	0.943
FAC score at admission [§] [median (IQR)]	3.0 (2.0)	4.0 (2.5)	3.0 (1.3)	3.0 (2)	0.584	3.0 (3.0)	4.0 (1.0)	2.0 (2.0)	3.0 (1.0)	0.296
Independent ambulation at admission ^{e§} [n (%)]	11 (42.3)	7 (53.8)	7 (23.3)	11 (45.8)	0.179	5 (38.5)	7 (53.8)	7 (36.8)	7 (36.8)	0.755

- RESULTS -

SPPB score at admission [§] [median (IQR)]	2.0 (6.0)	2.0 (4.0)	3.0 (3.5)	3.5 (5.8)	0.250	3.0 (6.5)	2.0 (3.0)	3.0 (5.5)	5.0 (6.0)	0.415
Length of hospitalization [§] [median (IQR)], d.	5.0 (5.0)	5.0 (4.5)	5.0 (3.0)	5.5 (7.8)	0.282	5.0 (3.5)	5.0 (5.0)	5.0 (4.0)	5.0 (4.0)	0.921
MedDiet adherence score [§] [mean (SD)]	6.5 (1.5) ^a	9.7 (0.9) ^b	6.7 (1.5) ^a	10.0 (1.0) ^b	<0.001	7.7 (2.1)	7.7 (2.4)	8.7 (2.0)	8.2 (2.2)	0.490
UTP levels at admission [§] [mean (SD)], mg GAEs/L	10.9 (2.5)	10.8 (2.0)	10.7 (2.8)	10.6 (2.1)	0.989	8.9 (1.0) ^a	12.8 (1.4) ^b	8.5 (2.3) ^a	12.5 (0.8) ^b	<0.001

7 *Note.* BMI = body mass index; FAC = functional ambulatory classification; GAEs = gallic acid equivalents; IQR = interquartile range; MedDiet =
8 Mediterranean diet; SD = standard deviation; SPPB = Short Physical Performance Battery; UTP = urinary total polyphenols.

9 ^a Frailty was defined as having ≥ 3 of 5 Fried criteria ¹. ^b Malnutrition was defined as having ≤ 7 of 14 Mini Nutritional Assessment–Short Form criteria ².

10 ^c The “Others” category includes central nervous system, musculoskeletal, endocrine and neoplasia admission diagnosis. ^d Functional status was assessed
11 with the Barthel Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs: feeding, transferring, walking
12 on level surfaces, stair climbing, bowel and bladder control, toileting, bathing, grooming and dressing ³. ^e Independent ambulation was considered in the
13 event of an FAC = 4 ⁴. ^f A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence consisted in a MEDAS
14 score ≥ 9 at admission. Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high levels of UTP consisted in > 11.2 mg GAEs/L at admission.

15 [§] Variables containing missing values. The number of missing values in the different groups [low adherence + control, high adherence + control, low
16 adherence + intervention, high adherence + intervention] [low UTP levels + control, high UTP levels + control, low UTP levels + intervention, high UTP
17 levels + intervention] for the following covariables are: BMI at admission [5, 0, 3, 0] [1, 2, 0, 0]; Charlson comorbidity index [1, 0, 0, 0] [0, 0, 0, 0]; Frailty

- 18 phenotype [7, 3, 5, 2] [3, 3, 2, 0]; Urinary incontinence [1, 0, 0, 0] [0, 0, 0, 0]; Depression [1, 0, 0, 0] [0, 0, 0, 0]; Falls [1, 0, 0, 0] [0, 0, 0, 0]; Chronic pain
19 [1, 0, 0, 0] [0, 0, 0, 0]; Malnutrition [5, 0, 6, 2] [1, 2, 2, 1]; Dementia [1, 0, 0, 0] [0, 0, 0, 0]; Polypharmacy [1, 0, 0, 0] [0, 0, 0, 0]; FAC score at admission
20 [0, 0, 1, 0] [0, 0, 0, 0]; Independent ambulation at admission [0, 0, 1, 0] [0, 0, 0, 0]; SPPB score at admission [3, 2, 2, 0] [1, 0, 1, 0]; Length of hospitalization
21 [1, 0, 0, 0] [0, 0, 0, 0]; MedDiet adherence score [0, 0, 0, 0] [0, 0, 1, 2]; UTP levels at admission [9, 4, 15, 5] [0, 0, 0, 0].
22 Values without the same superscript differ ($p < 0.05$; Bonferroni post hoc test).

23 **Supplementary Table 2.** Functional Status at Admission and at Discharge of Participants According to Intervention Group.

Barthel Index (points) ^a									
intention-to-treat analysis					complete-case analysis				
	Control		Intervention			Control		Intervention	
	N	mean (95% CI)	N	mean (95% CI)	N	mean (95% CI)	N	mean (95% CI)	
<i>Unadjusted model</i>									
Admission	46	62.2 (55.0–69.3)	63	64.0 (58.0–70.1)	39	62.9 (55.0–70.9)	58	64.3 (57.8–70.8)	
Discharge	46	64.8 (58.3–71.3)	63	73.5 (67.9–79.1) ^{**‡}	39	66.0 (58.9–73.1)	58	74.6 (68.7–80.4) ^{**‡}	
Change	46	2.2 (-2.4–6.8)	63	9.7 (5.8–13.7) [‡]	39	2.8 (-2.4–8.0)	58	10.5 (6.2–14.7) [‡]	
<i>Adjusted model</i>									
Admission	31	63.1 (57.2–69.0)	55	67.2 (62.8–71.5)	29	62.1 (55.9–68.3)	51	67.4 (62.8–72.0)	
Discharge	31	65.7 (59.4–71.9)	55	75.7 (71.1–80.3) ^{*‡}	29	65.1 (58.6–71.7)	51	76.4 (71.6–81.2) [‡]	
Change	31	1.2 (-4.5–6.9)	55	9.3 (5.2–13.5) [‡]	29	1.2 (-4.9–7.2)	51	10.1 (5.7–14.6) [‡]	

24 *Note.* CI = confidence interval. Adjusted models are adjusted for sex, age, BMI at admission, place of living, independent ambulation, Charlson Comorbidity
 25 Index, polypharmacy (≥ 7), cause of hospitalization (admission diagnosis) and frailty phenotype. In all models of changes in functional status, we adjusted
 26 for functional status at admission.

27 ^a Functional status was assessed with the Barthel Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic
 28 ADLs: feeding, transferring, walking on level surfaces, stair climbing, bowel and bladder control, toileting, bathing, grooming and dressing ³.

29 * Different from admission, * $p < 0.05$, ** $p \leq 0.001$; ‡ Different from control group, ‡ $p < 0.05$ (ANOVA for repeated measures with Bonferroni post hoc
 30 test).

31

32 **Supplementary Table 3.** Functional Status at Admission and at Discharge of Participants in Both
33 Control and Intervention Groups According to MedDiet Adherence and UTP Levels (mg GAEs/L) at
34 Admission (Intention-to-Treat Analysis).

	Time	Barthel Index (points) ^b			
		Control		Intervention	
		N	mean (95% CI)	N	mean (95% CI)
MEDAS score at admission					
<i>Unadjusted model</i>					
Low MedDiet adherence ^a	Admission	31	59.2 (50.5–67.9)	34	59.4 (51.1–67.8)
	Discharge	31	63.5 (55.5–71.6)	34	72.2 (64.5–79.9)**
High MedDiet adherence ^a	Admission	14	67.9 (54.8–80.9)	26	69.2 (59.7–78.8)
	Discharge	14	66.8 (54.8–78.8)	26	74.2 (65.4–83.0)
<i>Adjusted model</i>					
Low MedDiet adherence ^a	Admission	21	60.5 (53.2–67.7)	28	65.5 (58.8–72.1)
	Discharge	21	62.9 (55.2–70.5)	28	77.8 (70.8–84.8)* [§]
High MedDiet adherence ^a	Admission	10	68.3 (57.7–78.8)	24	68.7 (61.9–75.6)
	Discharge	10	70.9 (59.8–82.0)	24	72.8 (65.6–80.0)
UTP levels at admission					
<i>Unadjusted model</i>					
Low UTP levels ^a	Admission	16	60.6 (48.5–72.8)	19	62.4 (51.2–73.5)
	Discharge	16	67.8 (57.0–78.6)	19	76.8 (67–86.7)*
High UTP levels ^a	Admission	14	60.7 (47.7–73.7)	21	65.7 (55.1–76.3)
	Discharge	14	62.1 (50.6–73.7)	21	73.8 (64.4–83.2)
<i>Adjusted model</i>					
Low UTP levels ^a	Admission	10	59.8 (46.5–73.1)	16	66.0 (55.9–76.2)
	Discharge	10	64.5 (51.8–77.3)	16	78.0 (68.3–87.7)*
High UTP levels ^a	Admission	10	58.2 (44.7–71.7)	21	66.3 (57.7–75.0)
	Discharge	10	66.5 (53.6–79.5)	21	74.6 (66.3–82.9)

35 *Note.* CI = confidence interval; MedDiet = Mediterranean diet; UTP = urinary total polyphenols.

36 Adjusted models are adjusted for sex, age, BMI at admission, place of living, independent ambulation, Charlson
37 Comorbidity Index, polypharmacy (≥ 7), cause of hospitalization (admission diagnosis) and frailty phenotype.
38 In the UTP levels model, we further adjusted for creatinine clearance at admission and chronic kidney disease.

39 ^a A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence
40 consisted in a MEDAS score ≥ 9 at admission. Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high
41 levels of UTP consisted in > 11.2 mg GAEs/L at admission. ^b Functional status was assessed with the Barthel
42 Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs:
43 feeding, transferring, walking on level surfaces, stair climbing, bowel and bladder control, toileting, bathing,
44 grooming and dressing ³.

45 * Different from admission, ^{*} $p < 0.05$, ^{**} $p \leq 0.001$; [¥] Different from control group, [¥] $p < 0.05$ (ANOVA for
46 repeated measures with Bonferroni post hoc test).

47

48 **Supplementary Table 4.** Changes in Functional Status During Hospitalization of Participants in Both Control and Intervention Groups
 49 According to MedDiet Adherence and UTP Levels (mg GAEs/L) at Admission.

	Changes in Barthel Index (points) ^b							
	Intention-to-treat analysis				Complete-case analysis			
	Control		Intervention		Control		Intervention	
	N	mean (95% CI)	N	mean (95% CI)	N	mean (95% CI)	N	mean (95% CI)
MEDAS score at admission								
<i>Unadjusted model</i>								
Low MedDiet adherence ^a	31	3.1 (-2.7–8.8)	34	11.6 (6.1–17.1)	26	3.7 (-2.8–10.1)	31	12.3 (6.3–18.2) [‡]
High MedDiet adherence ^a	14	0.6 (-7.9–9.2)	26	7.2 (0.9–13.5)	13	1.3 (-7.9–10.5)	24	8.0 (1.2–14.8)
<i>Adjusted model</i>								
Low MedDiet adherence ^a	21	-0.2 (-7.2–6.8)	28	12.3 (6.0–18.6)	19	-0.3 (-7.9–7.3)	26	13.4 (6.7–20.1)
High MedDiet adherence ^a	10	4.1 (-5.9–14.1)	24	5.8 (-0.8–12.3)	10	3.7 (-6.6–14.0)	22	6.4 (-0.6–13.3)
UTP levels at admission								
<i>Unadjusted model</i>								
Low UTP levels ^a	16	6.4 (-1.7–14.4)	19	14.4 (7.0–21.7)	13	8.3 (-1.0–17.5)	19	14.4 (6.7–22.0)
High UTP levels ^a	14	0.7 (-7.9–9.3)	21	9.3 (2.3–16.4)	13	0.5 (-8.8–9.7)	19	10.2 (2.5–17.8)
<i>Adjusted model</i>								
Low UTP levels ^a	10	2.7 (-8.6–14.0)	16	13.2 (4.6–21.8)	9	4.0 (-8.0–16.1)	16	12.9 (4.1–21.6)
High UTP levels ^a	10	5.4 (-6.1–16.9)	21	9.7 (2.3–17.0)	10	5.2 (-6.7–17.2)	19	10.6 (2.6–18.6)

50 *Note.* CI = confidence interval; MedDiet = Mediterranean diet; UTP = urinary total polyphenols. All models are adjusted for functional status at admission.
51 Adjusted models are further adjusted for sex, age, BMI at admission, place of living, independent ambulation, Charlson Comorbidity Index, polypharmacy
52 (≥ 7), cause of hospitalization (admission diagnosis) and frailty phenotype. In the UTP levels model, we further adjusted for creatinine clearance at
53 admission and chronic kidney disease.

54 ^a A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence consisted in a MEDAS score ≥ 9 at admission.
55 Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high levels of UTP consisted in > 11.2 mg GAEs/L at admission. ^b Functional status was assessed
56 with the Barthel Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs: feeding, transferring, walking
57 on level surfaces, stair climbing, bowel and bladder control, toileting, bathing, grooming and dressing ³.

58 [¥] Different from control group, [¥] $p < 0.05$ (ANOVA for repeated measures with Bonferroni post hoc test).

59 **Supplementary Table 5.** Functional Status at Admission and at Discharge of Participants in Both
60 Control and Intervention Groups According to MedDiet Adherence and UTP Levels (mg GAEs/L) at
61 Admission (Complete-Case Analysis).

		Barthel Index (points) ^b				
		Time	Control		Intervention	
			N	mean (95% CI)	N	mean (95% CI)
MEDAS score at admission						
<i>Unadjusted model</i>						
Low MedDiet adherence ^a	Admission	26	59.4 (49.7–69.1)	31	58.9 (50.0–67.8)	
	Discharge	26	64.6 (55.8–73.5)	31	72.9 (64.8–81.0)**	
High MedDiet adherence ^a	Admission	13	70.0 (56.3–83.7)	24	70.4 (60.3–80.5)	
	Discharge	13	68.8 (56.3–81.3)	24	75.8 (66.6–85.0)	
<i>Adjusted model</i>						
Low MedDiet adherence ^a	Admission	19	59.3 (51.5–67.0)	26	65.8 (58.9–72.8)	
	Discharge	19	62.3 (54.1–70.5)	26	78.9 (71.5–86.2)* ^y	
High MedDiet adherence ^a	Admission	10	66.8 (56.2–77.5)	22	68.9 (61.7–76.0)	
	Discharge	10	69.6 (58.4–80.9)	22	73.2 (65.7–80.7)	
UTP levels at admission						
<i>Unadjusted model</i>						
Low UTP levels ^a	Admission	13	61.2 (47.2–75.1)	19	62.4 (50.8–73.9)	
	Discharge	13	70.0 (57.7–82.3)	19	76.8 (66.7–87.0)*	
High UTP levels ^a	Admission	13	60.0 (46.1–73.9)	19	65.5 (54.0–77.1)	
	Discharge	13	61.5 (49.3–73.8)	19	74.5 (64.3–84.6)	
<i>Adjusted model</i>						
Low UTP levels ^a	Admission	9	58.5 (44.6–72.5)	16	65.7 (55.5–75.9)	
	Discharge	9	65.4 (51.9–78.8)	16	77.4 (67.6–87.2)*	
High UTP levels ^a	Admission	10	56.3 (42.6–70.0)	19	68.1 (58.9–77.3)	
	Discharge	10	65.5 (52.4–78.7)	19	76.2 (67.4–85.1)	

62 *Note.* CI = confidence interval; MedDiet = Mediterranean diet; UTP = urinary total polyphenols. Adjusted
63 models are adjusted for sex, age, BMI at admission, place of living, independent ambulation, Charlson
64 Comorbidity Index, polypharmacy (≥ 7), cause of hospitalization (admission diagnosis) and frailty phenotype.
65 In the UTP levels model, we further adjusted for creatinine clearance at admission and chronic kidney disease.

66 ^a A low level of MedDiet adherence consisted in a MEDAS score < 9 and a high level of MedDiet adherence
67 consisted in a MEDAS score ≥ 9 at admission. Low levels of UTP consisted in ≤ 11.1 mg GAEs/L and high
68 levels of UTP consisted in > 11.2 mg GAEs/L at admission. ^b Functional status was assessed with the Barthel
69 Index of independence in ADLs, which uses a total 0 to 100 score and includes the following 10 basic ADLs:
70 feeding, transferring, walking on level surfaces, stair climbing, bowel and bladder control, toileting, bathing,
71 grooming and dressing ³.

72 * Different from admission, * $p < 0.05$, ** $p \leq 0.001$; ‡ Different from control group, § $p < 0.05$ (ANOVA for
73 repeated measures with Bonferroni post hoc test).

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86

4.4. The role of fish intake in the prevention of cognitive decline in older adults

In this study, the consumption of fish was evaluated as a key and sustainable element of the Mediterranean diet, serving as a protective factor against cognitive decline in free-living older persons.

Publication 4: Alba Tor-Roca, Tomás Meroño, Cristina Andres-Lacueva, Antonio Miñarro, Andrea Unión-Caballero, Nicole Hidalgo-Liberona, Montserrat Rabassa, Alex Sánchez-Pla, Luigi Ferrucci, Stefania Bandinelli, Toshiko Tanaka, Mireia Urpi-Sarda, Antonio Cherubini. Fish intake is inversely associated with cognitive decline in community-dwelling older adults: the InCHIANTI study. Prepared to be submitted to J Gerontol A Biol Sci Med Sci.

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Abstract

Dietary intake of fish has been associated with a lower risk of Alzheimer's disease and dementia. However, inconsistent findings have been reported regarding cognitive decline (CD), especially in older adults. A prospective cohort study including 883 community-dwelling older adults (mean age 74 years, 56% women) living in Tuscany (Italy) followed for 15 years was analyzed (InCHIANTI study). Dietary intake based on validated food frequency questionnaires and clinical information were assessed four times during follow-up. Total fish intake was expressed as servings per week. Plasma concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were quantified at baseline. CD was defined as a decline of ≥ 3 points in MMSE between two consecutive follow-up evaluations. Time-dependent Cox regression models adjusted for confounders were used to assess the association between total fish intake and CD. At baseline, the mean $\pm$ SD total fish intake was 1.7 ± 1.2 servings/week. Participants with a higher total fish intake were younger, more educated and physically active, and had lower prevalence of impaired renal function and depression. Total fish intake was positively correlated with circulating EPA and DHA at baseline ($r = 0.214$, $p < 0.001$). Total fish intake was also

significantly correlated with PUFA intake over time (repeated measures correlation coefficient = 0.308, $p < 0.001$). During the 15 years of follow-up, 521 participants developed CD. A higher fish intake was significantly associated with a lower risk of CD in adjusted models [HR (95% CI) per 1 serving/week increase = 0.90 (0.83–0.98)]. In secondary analyses, no mediation was observed for plasma levels of EPA and DHA, dietary PUFA intake, or incidence of cardiovascular disease. In conclusion, total fish intake was inversely associated with CD in Mediterranean older adults in a follow-up over 15 years. Further studies are required to better understand the molecular mechanisms involved in the neuroprotective effects of fish.

Resum

El consum de peix s'ha associat amb un menor risc d'Alzheimer i demència. No obstant això, s'han observat resultats inconsistents pel que fa al declivi cognitiu (CD), especialment en adults grans. Es va analitzar un estudi de cohort prospectiu que inclou 883 adults majors que viuen a la comunitat (edat mitjana 74 anys, 56% dones) que habiten a la Toscana (Itàlia) seguits durant 15 anys (estudi INCHIANTI). La ingesta dietètica basada en qüestionaris de freqüència alimentària validats i la informació clínica es van avaluar quatre vegades durant el seguiment. La ingesta total de peix es va expressar com a ració per setmana. Les concentracions de plasma d'àcid eicosapentaenoic (EPA) i àcid docosahexaenoic (DHA) es van quantificar a l'inici del seguiment. El CD es va definir com una disminució de ≥ 3 punts en el MMSE entre dues avaluacions de seguiment consecutives. Es van utilitzar models de regressió de Cox dependents del temps ajustats per confusors per avaluar l'associació entre la ingesta total de peix i el CD. A l'inici del seguiment, la ingesta total mitjana $\pm$ SD de peix era de 1.7 ± 1.2 racions/setmana. Els participants amb una ingesta més alta de peix total eren més joves, més educats i físicament actius i tenien una prevalença més baixa d'IRF i depressió. La ingesta de peix total es va correlacionar positivament amb l'EPA i el DHA circulants a l'inici del seguiment ($r = 0.214$, $p < 0.001$). La ingesta total de peix també es va correlacionar significativament amb la ingesta de PUFAs durant el seguiment (coeficient correlació mesures repetides = 0.38, $p < 0.001$). Durant els 15 anys de seguiment, 521 participants van desenvolupar CD. Una major ingesta de peix total es va associar significativament amb un menor risc de CD en models ajustats [HR (95% CI) per cada increment de ració/setmana = 0.90 (0.83–0.98)]. En anàlisis secundàries, no es va observar cap mediació per als nivells de plasma d'EPA i DHA, la ingesta dietètica de PUFAs, o la incidència de malalties cardiovasculars. En conclusió, la ingesta de peix total es va associar inversament amb el CD en

adults majors mediterranis en un seguiment de 15 anys. Es requereixen més estudis per comprendre millor els mecanismes moleculars implicats en els efectes neuroprotectors del peix.

Fish intake is inversely associated with cognitive decline in community-dwelling older adults: the InCHIANTI study

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Abstract

Background: Fish consumption has been associated with a lower risk of Alzheimer's disease and dementia. However, inconsistent findings have been reported regarding cognitive decline (CD), especially in older adults.

Methods: A prospective cohort study including 883 community-dwelling older adults (mean age 74 years, 56% women) living in Tuscany (Italy) followed for 15 years was analyzed (InCHIANTI study). Dietary intake based on validated food frequency questionnaires and clinical information were assessed four times during follow-up. Total fish intake was expressed as servings per week. Plasma concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were quantified at baseline. CD was defined as a decline of ≥ 3 points in MMSE between two consecutive follow-up evaluations. Time-dependent Cox regression models adjusted for confounders were used to assess the association between total fish intake and CD.

Results: At baseline, the mean $\pm$ SD total fish intake was 1.7 ± 1.2 servings/week. Participants with a higher total fish intake were younger, more educated, and physically active and had lower prevalence of impaired renal function and depression. Total fish intake was positively correlated with the sum of circulating EPA and DHA at baseline ($r = 0.214$, $p < 0.001$). Total fish intake was also significantly correlated with PUFA intake over time (repeated measures correlation coefficient = 0.308, $p < 0.001$). During the 15 years of follow-up, 521 participants developed CD. A higher total fish intake was significantly associated with a lower risk of CD in adjusted models [HR (95% CI) per 1 serving/week increase = 0.90 (0.83–0.98)]. In secondary analyses, no mediation was observed for plasma levels of EPA and DHA, dietary PUFA intake, or incidence of cardiovascular disease.

Conclusions: In a 15-year follow-up, total fish intake was inversely associated with CD in Mediterranean older adults. Further studies are required to better understand the molecular mechanisms involved in the neuroprotective effects of fish.

Keywords: seafood, cognition, omega-3 fatty acids, prospective study

Abbreviations

AD, Alzheimer's disease

ADLs, activities of daily living

ApoE, Apolipoprotein E

BMI, body mass index

CES-D, Center for Epidemiologic Studies Depression Scale

CD, cognitive decline

COPD, chronic obstructive pulmonary disease

CVD, cardiovascular disease

DHA, docosahexaenoic acid

EPA, eicosapentaenoic acid

EPIC, European Prospective Investigation into Cancer and Nutrition

FA, fatty acid

FDR, false discovery rate

FFQ, food frequency questionnaire

IRF, impaired renal function

LOCF, Last Observation Carried Forward

MCI, mild cognitive impairment

MMSE, Mini-Mental State Examination

PUFA, polyunsaturated fatty acid

RDA, Recommended Dietary Allowance

STROBE-nut, Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology

Introduction

The global aging of the population is a demographic trend that will increase in the coming decades. Nearly 12% of the worldwide population will be 65 years old or over by 2030 (1). Age is a major risk factor for cognitive decline (CD), which might be the early stage of cognitive impairment and dementia. Ultimately, CD and dementia are major causes of disability and dependency among older people, and they have been associated with an increased risk of poor quality of life and mortality (2–4).

Several modifiable risk factors have been identified that could delay or prevent the progression of CD and dementia. Dietary factors may be involved in the onset of CD, both directly and through their influence on other independent risk factors (e.g., diabetes, heart disease, and stroke) (5–7). Indeed, the Nutrition for Dementia Prevention Working Group acknowledged that a healthy diet may have preventive potential for CD (8). In recent years, there has been a consensus among numerous epidemiological studies conducted in both Mediterranean and non-Mediterranean countries on fish consumption and its benefits for brain health. Fish and seafood, a high-protein food group of the Mediterranean diet (MedDiet) and a source of long-chain omega-3 fatty acids (FAs) [i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], is one of the food groups most consistently associated with a decreased risk of dementia and AD (9,10). In fact, a recent UK Biobank study showed that among all the individual food groups of the MedDiet, only fish consumption was associated with a decreased risk of dementia (11). However, inconsistent findings have been reported regarding CD, perhaps due to differences in age at baseline of the study population, length of follow-up, the domains included in the global cognitive outcome, and the methods of assessment. For instance, Qin et al. reported that the benefits of fish consumption in delaying CD were limited to adults aged ≥ 65 years (12). On the other hand, in a meta-analysis pooling five cohorts of older adults ($n = 23,688$) with a median follow-up range of 3.9–9.1 years, no significant inverse association was found between fish intake and global cognition - assessed by diverse composite scores - within any of the individual cohorts (13). This finding was in line with van de Rest et al., who reported protective effects of seafood on decline in some cognitive domains but not globally among older participants from the Rush Memory and Aging Project (follow-up average of 4.9 years) (14). However, other observational studies with similar follow-up durations evaluating global cognitive function in older adults through different batteries of tests suggested that fish intake had a protective effect (12,15,16).

To the best of our knowledge, there are no studies evaluating the role of fish intake in CD in older adults from a Mediterranean region with a follow-up longer than 10 years. The current study aims to investigate the association between fish intake and CD over 15 years of follow-up in the InCHIANTI study, a large cohort of older participants.

Methods

The current observational study was conducted and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology guidelines (STROBE-nut) guidelines (17) (Supplementary Table S1).

Study design

The InCHIANTI study is a prospective, population-based cohort study of older adults living in Bagno a Ripoli and Greve in Chianti, two Italian towns adjacent to the city of Florence (Tuscany, Italy). From 1998 to 2000, baseline data were collected during a home interview prior to a full medical and functional evaluation conducted by trained personnel. Follow-up measurements were performed at 3, 6, 9, and 15 years after baseline. A detailed description of the rationale and the study design is provided elsewhere (18). All participants signed an informed consent form. The study protocol was approved by the Italian National Institute of Research and Care of Aging Institutional Review Board and review board at the National Institutes of Health (Baltimore, MD, USA).

Study population

The study randomly sampled 1,260 community-dwelling adults aged ≥ 65 years from the population registries, of whom 1,155 agreed to participate. Of these, we excluded participants with missing data in dietary questionnaires ($n = 16$), those who died within the first 3 years of follow-up ($n = 123$), individuals with dementia at baseline ($n = 42$), and participants without two consecutive Mini-Mental State Examination (MMSE) evaluations during the follow-up ($n = 91$) (Supplementary Figure S1). In total, 883 participants and 2,038 observations were included for the analyses.

Dietary intake assessment

Habitual dietary intake was assessed by trained interviewers at baseline and 3, 6, and 9 years after enrollment, using the Italian version of the food frequency questionnaire (FFQ) developed and validated in the European Prospective Investigation into Cancer and Nutrition-Italy (EPIC-

Italy) study (19). Participants reported the frequency of consumption and usual portion size of 198 food and beverage items. Energy and nutrient intake data were calculated using the Food Composition Database for Epidemiological Studies in Italy (19).

Total fish intake (g/d) was estimated as the sum of two food items: fish (i.e., lean and fatty fish) and seafood (i.e., crustaceans and mollusks). The standard serving size of fish was considered to be 100 g and consumption in servings of fish/week was further calculated. Total fish intake (servings/week) was secondarily categorized using cutoff values based on sex- and follow-up-specific quartiles (Supplementary Table S2). Tests for trend were calculated employing quartile-based scores 1–4 used as continuous variables. Adherence to the MedDiet was assessed according to the method developed by Trichopoulou et al. (20). The consumption of EPA and DHA was estimated by excluding linoleic and linolenic acids from total polyunsaturated fatty acid (PUFA) intake. PUFA intake was energy-adjusted using the residual method (21).

Covariates assessment

Data on sociodemographic (age, sex) and lifestyle variables were collected using standardized questionnaires. Education level was assessed as years of education, and self-reported smoking habits were categorized as never, former, and current smokers. Income was self-reported indicating whether participants' current income was adequate for their needs. Physical activity during the year prior to the visit was self-reported and classified as sedentary (hardly any physical activity), light (light-intensity physical activity 2–4 h/week), or moderate to intense (light-intensity physical activity > 4 h/week or moderate-intensity physical activity 1–2 h/week) (22). Height and weight were measured, and body mass index (BMI) was calculated in kg/m². Depression was evaluated using the Center for Epidemiologic Studies Depression Scale (CES-D), and subjects with a CES-D score ≥ 16 were defined as being in a depressed mood (22). Apolipoprotein E (ApoE) variant genotypes (e2, e3, e4) were defined by two single-nucleotide polymorphisms (SNPs), namely rs429358 and rs7412, as described elsewhere (19). Impaired renal function (IRF) was defined as a glomerular filtration rate < 60 ml/min. Mild cognitive impairment (MCI) was defined as MMSE < 18 (19). Other comorbidities and their definitions are detailed elsewhere (19).

Quantification of plasma polyunsaturated fatty acids

Plasma PUFAs measurement method has been described previously (23). Briefly, blood samples were collected in the morning after a 12-hour overnight fast. Aliquots of serum and plasma were

immediately obtained and stored at -80°C until analyzed. Plasma EPA and DHA were quantified using heptadecanoic acid as an internal standard, followed by the preparation of fatty acid methyl esters and their identification through gas chromatography (23). For the present analysis we considered the sum of EPA and DHA ($n = 747$), expressed as percentage of total plasma FAs (mg/L), given that their primary dietary source is fish (24). In the InCHIANTI study, plasma concentrations of EPA and DHA were only available at baseline.

Outcome assessment

Cognitive function was measured using the MMSE and CD was defined as a decline of ≥ 3 points in MMSE between two consecutive follow-up evaluations (19,22). Number of participants, time periods, and CD cases during the follow-up are shown in Supplementary Figure S2.

Statistical analysis

Missing information on dietary data and income during the follow-up (3.7% and 1.9%, respectively) were imputed by the Last Observation Carried Forward (LOCF). For other covariates, the baseline median was assigned to missing values (5.4%).

Data on total fish consumption were analyzed both as a categorical (sex- and follow-up-specific quartiles) and as a continuous (servings/week) variable. Baseline characteristics of the participants were expressed as means $\pm$ SDs for continuous variables and percentages for categorical variables. Descriptive analyses of baseline characteristics across quartiles of total fish intake (servings/week) were tested using the Pearson chi-square test (χ^2) for categorical variables or one-way ANOVA for continuous variables. The Bonferroni post-hoc test was used for multiple comparisons. Intraclass correlation coefficients were calculated to assess changes in total fish intake during follow-up using linear mixed models with participants as a random effect. Pearson correlation analysis was performed to examine the relation between total fish intake and circulating EPA and DHA at baseline, while the association between total fish intake and consumption of PUFAs during the follow-up was assessed using repeated measures correlation.

Time-dependent Cox models were used to estimate HRs and 95% CIs for the association between total fish intake (servings/week) and CD risk. Time-to event was defined as the time elapsed from baseline to that at which CD was first detected (Supplementary Figure S2). Model 1 was adjusted for age at baseline, sex, and total energy intake (kcal/d). Model 2 was additionally adjusted for BMI, years of education, income, smoking status, alcohol intake (g/d), and physical

activity. Model 3 was further adjusted for ApoE isoforms and comorbidities: cardiovascular disease (CVD), hypertension, diabetes, cancer, depression, chronic obstructive pulmonary disease (COPD), and IRF.

Effect modification by age at baseline, sex, BMI, physical activity, depression, MCI at baseline, hypertension, IRF, ApoE haplotypes, CVD, and compliance with Recommended Dietary Allowance (RDA) for protein in older adults (> 1 g/kg/d) (25) was assessed by adding product terms in the fully adjusted time-dependent Cox models (26). The Benjamini-Hochberg false discovery rate (FDR) was used for multiple testing correction.

Sensitivity analyses were carried out by excluding subjects who developed CD during the first visit ($n = 170$). Additional models were created censoring participants aged over 80 ($n = 195$), reporting an excessive intake of alcohol (40 g/d for women and 60 g/d for men (27)) ($n = 59$), with prevalent diabetes ($n = 267$) or disability [> 1 activities of daily living (ADLs)] ($n = 35$) at recruitment, or with imputations in dietary data or in other covariables ($n = 178$). Finally, we conducted further analysis adjusting for the MedDiet adherence score.

In order to clarify the role of plasma EPA and DHA, and PUFA intake in the association between total fish intake and CD, we followed the procedure for establishing potential mediating effects proposed by Baron and Kenny (28). In a similar way, we evaluated the potential mediation pathway of higher total fish intake and lower CD risk, through a lower CVD risk.

All statistical analyses were performed using SPSS (SPSS 27.0, IBM, NY) and R 4.2.3 (R foundation, Vienna) software and its related packages. A significance level of $p < 0.05$ was considered statistically significant.

Results

Baseline characteristics

The study included a total of 883 participants, 56% of whom were women, with a mean $\pm$ SD age of 74 ± 7 years at baseline. At baseline, the mean MMSE was 26 ± 3 and total fish intake was 1.7 ± 1.2 servings/week. A total of 70% of the individuals consumed at least one serving of fish per week, which was slightly higher than the global Italian population aged 65 or older (58% – data from 2005) (29). Participants in the highest total fish intake group (3.2 ± 1.3 servings/week) were younger, had a higher level of education, and undertook more intense physical activity than those in the lowest total fish intake group (0.5 ± 0.3 servings/week) (Table 1). Furthermore, they

were also less likely to have IRF or depression and were more likely to meet the RDA for protein intake than participants with the lowest total fish intake. Daily energy intake (kcal/d), animal:plant protein ratio (g/d), and adherence to MedDiet were significantly greater in the higher quartiles of total fish consumption. PUFA intake (g/d) and circulating EPA and DHA (% FAs, mg/L) were also higher in those who consumed more fish. Total fish intake was positively correlated with the sum of circulating EPA and DHA at baseline ($r = 0.214$, $p < 0.001$). Total fish intake was also significantly correlated with PUFA intake over time (repeated measures correlation coefficient = 0.308, $p < 0.001$). On the other hand, circulating EPA and DHA were not correlated with consumption of PUFAs at baseline ($p > 0.05$).

Association between fish intake and cognitive decline

Based on data available from FFQ, total fish intake was stable during the follow-up and there were no statistically significant changes between the different evaluations (Supplementary Table S2). In the study, 521 participants developed CD after a median $\pm$ IQR follow-up of 14.4 ± 9.5 years (Supplementary Figure S2). Supplementary Table S3 shows the baseline characteristics of the studied population by CD status at follow up. The associations between total fish consumption and CD are shown in Figure 1 and Supplementary Table S4. In the fully adjusted time-dependent Cox model (Model 3), subjects who reported a higher total fish intake were at lower risk of CD [HR (95% CI) per one serving/week increase = 0.90 (0.83–0.98)] (Figure 1 and Supplementary Table S4). Similar results were obtained in categorical analysis where those in the highest quartile of total fish intake had significantly lower risk of developing CD compared to the lowest quartile of total fish intake [HR_{Q4vs.Q1} (95% CI) = 0.72 (0.55–0.93); p -trend = 0.011] (Supplementary Table S4). We observed analogous results between sexes, although the results were only statistically significant in women in the fully adjusted model (Supplementary Table S5).

After multiple testing correction, we did not find any significant interaction between total fish intake and age, sex, BMI, physical activity, depression, MCI at baseline, hypertension, IRF, ApoE haplotype, CVD, or protein RDA (all p -values > 0.05) (Supplementary Table S6).

In a series of sensitivity analyses, the results were similar after: (1) excluding subjects who developed CD in the first visit [HR (95% CI) = 0.91 (0.83–1.00)]; (2) censoring participants aged over 80 [HR (95% CI) = 0.90 (0.83–0.99)]; (3) excluding individuals reporting an excessive intake of alcohol [HR (95% CI) = 0.90 (0.83–0.98)]; (4) excluding participants with prevalent diabetes at baseline [HR (95% CI) = 0.91 (0.83–0.99)]; (5) censoring subjects with a disability at recruitment

[HR (95% CI) = 0.89 (0.82–0.97)]; (6) excluding participants with missing data on food intake or in covariables [HR (95% CI) = 0.89 (0.82–0.97)]; (7) further adjusting for MedDiet [HR (95% CI) = 0.91 (0.84–0.99)]. Neither plasma levels of EPA and DHA, nor dietary PUFAs, nor CVD were shown to mediate the relationship between total fish intake and CD in this cohort (data not shown).

Discussion

In this population-based prospective cohort study with participants aged ≥ 65 years from the InCHIANTI study, we found that a higher consumption of fish was associated with a lower CD risk over 15 years of follow-up. When using total fish intake as a continuous variable, the risk of CD was reduced by 10% per single serving of fish/week increase after adjusting for major potential cofounders. Moreover, those who consumed two or more fish meals per week had an $\approx 30\%$ lower risk of CD than subjects with less than a weekly consumption in models considering total fish intake as a categorical variable.

Previous studies have generally reported significant inverse associations between fish intake and the risk of adverse cognitive outcomes, albeit most of them have focused on MCI, dementia, and AD (30). Prospective studies on fish intake and CD have been scarce and have shown mixed results. For instance, the total consumption levels of seafood were not associated with change in global cognition after 4 years of follow-up in 5,988 women (mean age, 72 years) from the Women's Health Study Cognitive Cohort (31). Similarly, in a community-based epidemiologic study, older adults who consumed more than one seafood meal per week did not demonstrate a slower decline in global cognition scores (14). In contrast, prospective data from the Zutphen Elderly Study showed that moderate fish consumption was associated with a decreased risk of CD (32). These findings were further supported by Morris et al., who suggested that the rate of CD was slower among older adults who consumed more than one fish meal per week (15). Some of these incongruities may be due to differences in length of follow-up or sample size, as pointed out in a recent meta-analysis (13). According to the authors, no significant relationships between fish intake and CD within any of the individual French and US cohorts (i.e., the Three-City Study, the Nurses' Health Study, the Women's Health Study, the Chicago Health and Aging Project, and the Rush Memory and Aging Project) were found. However, when the authors combined the cohorts, they found a significant trend whereby an increased intake of fish was associated with slower global CD (13).

CVD may have connections with CD and dementia as they share some risk factors, and vascular mechanisms are believed to partly trigger the etiology of CD (33,34). Because CVD accelerates CD and anticipates the onset of dementia (35), fish intake might positively affect cognition, even without directly affecting the brain, through the beneficial effect on CVD. Fish intake has been associated with a decreased risk of acute coronary syndrome and other cardiovascular-related outcomes (36). However, our results do not show significant mediating effects of CVD in the association between fish and CD. This apparent lack of agreement can be attributed to the low percentage of participants with CVD or the relatively small sample size. To the best of our knowledge, this mediation approach has not been explored in previous research on fish consumption and CD, thus the comparison with other works is difficult. Future studies on the current topic are therefore required to elucidate the role of cardiovascular burden in the association between fish intake and CD.

The health effects of regular fish intake have been generally attributed to EPA and DHA (24), which have a leading role in both cerebrovascular and cardiovascular health (37). Indeed, EPA and DHA are precursors of lipid mediators with neuroprotective and anti-inflammatory capacities, as well as a potential function in neural plasticity (38). When we evaluated whether the relationship between fish and CD could in some way be mediated by PUFA intake or circulating EPA and DHA, we did not find any evidence of a mediation effect. The lack of mediation by PUFA intake could be attributed to the fact that, in this cohort, PUFA intake has not been associated with circulating EPA and DHA concentrations at baseline, which are an objective measure of these long-chain omega-3 FA exposure. This might be related to i) interindividual variability in the absorption and metabolism of PUFAs (39) and ii) because the variable we have used to indirectly assess EPA and DHA intake may include other FAs (e.g., arachidonic acid). Unfortunately, it was not possible to investigate the mediation effect of circulating PUFAs (i.e., EPA and DHA) within a multiple-time points approach as they were only measured at baseline. Another possible explanation for the absence of PUFA intake mediation is the varied content of EPA and DHA in fish and seafood and that, despite the significant correlation between the consumption of fish and PUFAs, the latter may include other FAs (e.g., arachidonic acid) whose food sources differ from fish and seafood.

On the other hand, fish is a significant source of additional nutritional components that have been associated with cognitive function, such as vitamin B6, vitamin B12, vitamin D, and vitamin E (40). Therefore, the observed neuroprotective effect of fish might not solely be related to the

content of long-chain omega-3 FAs but instead to the combination of these components, as also hypothesized by others (15).

ApoE-ε4 is the main susceptibility gene variant for AD (41) and it has recently been suggested that it predicts CD (42). Although evidence indicates that the ApoE genotype might modulate the effects of EPA and DHA on the risk of CD and AD (43), we did not observe any interactions between the consumption of fish and the ApoE haplotype. This is in line with previous findings that led to the hypothesis that the relationship between fish and cognition is probably similar independent of the inherited susceptibility to AD (10,13,44). However, further work is required since many observational studies have reported significant associations only in ApoE-ε4 carriers, while others have found a more pronounced protective effect of fish among noncarriers of ApoE-ε4 (14,45,46).

The main limitations of this study are the relatively small sample size and the use of a three-point difference in consecutive MMSE evaluations to define CD, although there is no consensus as to which tool is more suitable for detecting CD, nor cutoff values of MMSE scores (47). In addition, we cannot rule out survival bias given the mean age of the population included in this study (~74 years old) or bias in the dietary recall data. However, the FFQ was country specific and had been previously validated (48), and participants with dementia at baseline were excluded. Another shortcoming of this current investigation is that the food database used in the InCHIANTI study did not allow us to differentiate between lean and fatty fish intake, which significantly differ in PUFA content and could therefore have a distinct impact on cognitive and cardiovascular outcomes (10,49,50). Finally, information on PUFA supplementation and circulating EPA and DHA during follow-up evaluations was not available. Despite these limitations, the strengths of the present study are the population-based design, the long follow-up, the repeated measures of diet and cognitive tests, and controlling for several potential confounders, including depressive symptomatology and ApoE isoforms (51).

In conclusion, this study shows that a higher intake of fish is inversely associated with CD over 15 years among community-dwelling older adults from a Mediterranean region. We found no evidence of any mediation effect from either PUFAs or CVD, and further studies are required to enable a better comprehension of the mechanisms involved in the neuroprotective role of fish and seafood.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the responsible for the InCHIANTI study (Dr. Luigi Ferrucci) on reasonable request. Data of the InCHIANTI study is available to all researchers upon justified request using the proposal form available on the InCHIANTI website (<http://inchiantistudy.net/wp/how-to-submit-a-proposal/>).

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Tables and Figures

Table 1. Baseline characteristics of the studied population by quartiles of total fish consumption (servings/week) in the InCHIANTI study (n = 883).

Data are expressed as mean $\pm$ SD or number (%) as appropriate. Comparisons among quartiles of total fish intake (servings/week) with Pearson's chi-square test or one-way ANOVA as appropriate. Values with distinct superscript differ.

Figure 1. Multivariate association between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.

Time-dependent Cox regression model, including total fish intake (servings/week) adjusted for age at baseline, sex, total energy intake (kcal/d), BMI, years of education, income, smoking status, alcohol intake (g/d), physical activity, ApoE isoforms, and comorbidities (CVD, hypertension, diabetes, cancer, depression, COPD, IRF). N (CD) = 1,925 (494). Shaded areas are 95% CI.

Table 1. Baseline characteristics of the studied population by quartiles of total fish consumption (servings/week) in the InCHIANTI study (n = 883).

	Q1 (n = 225) (< 1.00 in men; < 0.71 in women)	Q2 (n = 227) (1.00–1.66 in men; 0.73–1.33 in women)	Q3 (n = 209) (1.67–2.40 in men; 1.34–1.99 in women)	Q4 (n = 222) (> 2.40 in men; > 2.03 in women)	p
Age at baseline, y	74.4 ± 6.8 ^{ab}	75.1 ± 7.1 ^a	73.3 ± 6.5 ^{bc}	72.0 ± 5.6 ^c	< 0.001
Female	123 (56.4)	125 (56.1)	126 (56.8)	124 (56.4)	0.999
BMI, kg/m ²	26.8 ± 3.9 ^a	27.1 ± 4 ^{ab}	27.8 ± 4.2 ^b	27.6 ± 3.8 ^{ab}	0.025
Education, y.	5.3 ± 3.1	5.3 ± 3.4	5.4 ± 3.1	6.1 ± 3.7	0.039
Income, inadequate	101 (46.3)	109 (48.9)	117 (52.7)	119 (54.1)	0.122
Physical activity, sedentary	50 (22.9) ^a	44 (19.7) ^{ab}	31 (14.0) ^{ab}	26 (11.8) ^b	0.012
Current smoking	28 (12.8)	37 (16.6)	32 (14.4)	33 (15.0)	0.855
Hypertension	131 (60.1)	136 (61.0)	131 (59.0)	124 (56.4)	0.777
MMSE, score	25.4 ± 2.7	25.5 ± 2.9	25.6 ± 3.0	25.9 ± 3.0	0.403
ApoE, ε4	34 (16.5) ^{ab}	44 (21.2) ^b	29 (13.6) ^{ab}	20 (9.5) ^a	0.009
IRF	90 (41.3) ^a	75 (33.6) ^{ab}	68 (30.6) ^{ab}	61 (27.7) ^b	0.018
CVD	42 (19.3)	48 (21.5)	50 (22.5)	40 (18.2)	0.653
Depression	87 (39.9) ^a	80 (35.9) ^a	71 (32.0) ^{ab}	53 (24.1) ^b	0.004
Diabetes	29 (13.3)	20 (9.0)	30 (13.5)	29 (13.2)	0.396
Cancer	15 (6.9)	13 (5.8)	11 (5.0)	12 (5.5)	0.848
COPD	16 (7.3)	20 (9.0)	13 (5.9)	17 (7.7)	0.662
MCI	2 (0.9)	2 (0.9)	5 (2.3)	3 (1.4)	0.626

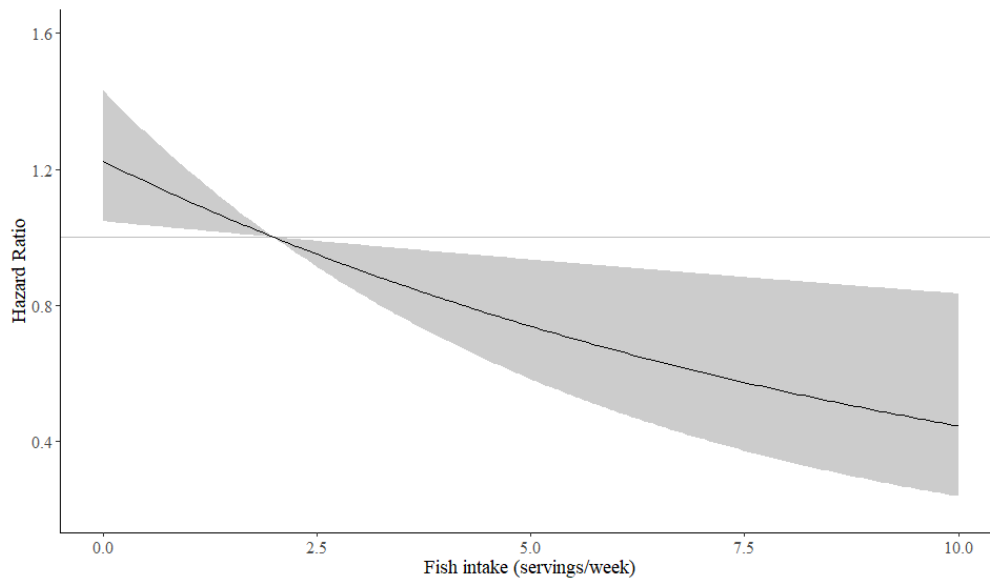
- RESULTS -

Energy intake, kcal/d	1,842.5 ± 583.2 ^a	1,907.6 ± 564.1 ^{ab}	1,916.4 ± 516.5 ^{ab}	2,043.2 ± 553.4 ^b	0.002
Alcohol intake, g/d	13.9 ± 19.2	13.8 ± 17.1	14.8 ± 22.2	16.8 ± 23.6	0.405
Total fish intake, servings/week	0.5 ± 0.3 ^a	1.2 ± 0.2 ^b	1.8 ± 0.3 ^c	3.2 ± 1.3 ^d	< 0.001
Fish intake, servings/week	0.4 ± 0.3 ^a	1.1 ± 0.2 ^b	1.6 ± 0.3 ^c	2.9 ± 1.2 ^d	< 0.001
Seafood intake, servings/week	0.0 ± 0.1 ^a	0.1 ± 0.2 ^{ab}	0.2 ± 0.2 ^b	0.4 ± 0.6 ^c	< 0.001
MDS, points	3.7 ± 1.5 ^a	3.7 ± 1.5 ^a	4.5 ± 1.6 ^b	4.9 ± 1.5 ^c	< 0.001
Ratio animal:plant protein intake, g/d	1.6 ± 0.6 ^a	1.8 ± 0.7 ^{ab}	1.9 ± 0.6 ^b	2.1 ± 0.7 ^c	< 0.001
Protein RDA, < 1 g/kg/d	98 (45.0) ^a	100 (44.8) ^a	82 (36.9) ^{ab}	69 (31.4) ^b	0.007
PUFA intake, g/d	0.2 ± 0.1 ^a	0.2 ± 0.1 ^a	0.2 ± 0.1 ^b	0.3 ± 0.1 ^c	< 0.001
EPA and DHA, % cFAs (mg/L)	2.6 ± 0.8 ^a	2.8 ± 0.8 ^{ab}	3.0 ± 0.8 ^{bc}	3.1 ± 1.0 ^c	< 0.001

Note: ApoE, Apolipoprotein E. BMI, body mass index. cFAs, circulating fatty acids. COPD, chronic obstructive pulmonary disease. CVD, cardiovascular disease. DHA, docosahexaenoic acid. EPA, eicosapentaenoic acid. IRF, impaired renal function. MCI, mild cognitive impairment. MDS, Mediterranean diet score. MMSE, Mini-Mental State Examination. PUFA, polyunsaturated fatty acid. RDA, Recommended Dietary Allowance.

Data are expressed as mean ± SD or number (%) as appropriate. Comparisons among quartiles of total fish intake (servings/week) with Pearson's chi-square test or one-way ANOVA as appropriate. Values with distinct superscript differ ($p < 0.05$; Bonferroni post hoc test).

Figure 1. Multivariate association between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.



Note: Time-dependent Cox regression model, including total fish intake (servings/week) adjusted for age at baseline, sex, total energy intake (kcal/d), BMI, years of education, income, smoking status, alcohol intake (g/d), physical activity, ApoE isoforms, and comorbidities (CVD, hypertension, diabetes, cancer, depression, COPD, IRF). N (CD) = 1,925 (494). Shaded areas are 95% CI.

Supplementary Material

Supplementary Table S1. STROBE-nut checklist.

Supplementary Table S2. Total fish intake (servings/week) according to sex- and follow-up-specific quartiles in the InCHIANTI study.

Supplementary Table S3. Baseline characteristics of the studied population by cognitive decline trajectory in the InCHIANTI study (n = 883).

Supplementary Table S4. Multivariate associations between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.

Supplementary Table S5. Multivariate associations between total fish intake (servings/week) and cognitive decline by sex during 15 years of follow-up in the InCHIANTI study.

Supplementary Table S6. Effect modification of multivariate associations between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.

Supplementary Figure S1. Flowchart of participants from the InCHIANTI study included in the analysis.

Supplementary Figure S2. Flowchart of participants across follow-up assessments in the InCHIANTI study.

Supplementary References

Supplementary Table S1. STROBE-nut checklist.

		Reporting Item	Page Number
Title and abstract			
Title	<u>#1a</u>	Indicate the study's design with a commonly used term in the title or the abstract	
None	<u>#nut-1</u>	State the dietary/nutritional assessment method(s) used in the title or in the abstract.	Title p.1;
Abstract	<u>#1b</u>	Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract p.3
Introduction			
Background / rationale	<u>#2</u>	Explain the scientific background and rationale for the investigation being reported	Introduction p.6-7
Objectives	<u>#3</u>	State specific objectives, including any prespecified hypotheses	Introduction p.7
Methods			
Study design	<u>#4</u>	Present key elements of study design early in the paper	Methods p. 7
Setting	<u>#5</u>	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	
None	<u>#nut-5</u>	Describe any characteristics of the study settings that might affect the dietary intake or nutritional status of the participants, if applicable.	Methods p. 7-8
Eligibility	<u>#6a</u>	Cohort study: Give the eligibility criteria and the sources and methods of selection of participants. Describe methods of follow-up. Case-control study: Give the eligibility	

		criteria and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. Cross-sectional study: Give the eligibility criteria, and the sources and methods of selection of participants.	
None	<u>#nut-6</u>	Report any particular dietary, physiologic, or nutritional characteristics that were considered when selecting the target population.	Methods p. 8
None	<u>#6b</u>	Cohort study: For matched studies, give matching criteria and number of exposed and unexposed. Case-control study: For matched studies, give matching criteria and the number of controls per case.	n/a
Variables	<u>#7</u>	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
None	<u>#nut-7.1</u>	Clearly define foods, food groups, nutrients, or other food components (e.g., preparation method, taxonomical descriptors, classification, chemical form).	Methods p. 8-9
None	<u>#nut-7.2</u>	When calculating dietary patterns, describe the methods to obtain them and their nutritional properties.	Methods p. 9
Data sources and measurement	<u>#8</u>	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for exposed and unexposed groups if applicable.	
None	<u>#nut-8.1</u>	Describe the dietary assessment method(s) (e.g., portion size estimation, number of days and items recorded, how it was developed and administered, and how quality was ensured); report if and how supplement intake was assessed.	Methods p. 8-9

None	<u>#nut-8.2</u>	Describe and justify food-composition data used; explain the procedure to match food composition with consumption data; describe the use of conversion factors, if applicable	Methods p. 8
None	<u>#nut-8.3</u>	Describe the nutrient requirements, recommendations, or dietary guidelines and the evaluation approach used to compare intake with the dietary reference values, if applicable	n/a
None	<u>#nut-8.4</u>	When using nutritional biomarkers, additionally use the STROBE-ME; report the type of biomarkers used and usefulness as dietary exposure markers	
None	<u>#nut-8.5</u>	Describe the assessment of nondietary data (e.g., nutritional status and influencing factors) and timing of the assessment of these variables in relation to dietary assessment	Methods p. 9-10
None	<u>#nut-8.6</u>	Report on the validity of the dietary or nutritional assessment methods and any internal or external validation used in the study, if applicable	Methods p. 8
Bias	<u>#9</u>	Describe any efforts to address potential sources of bias	
None	<u>#nut-9</u>	Report how bias in dietary or nutritional assessment was addressed (e.g., misreporting, changes in habits as a result of being measured, data imputation from other sources).	Methods p. 9
Study size	<u>#10</u>	Explain how the study size was arrived at	Methods p. 8
Quantitative variables	<u>#11</u>	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	
None	<u>#nut-11</u>	Explain categorization of dietary/nutritional data (e.g., use of N-tiles and handling of nonconsumers) and the choice of reference category, if applicable.	Methods p. 10
Statistical methods	<u>#12a</u>	Describe all statistical methods, including those used to control for confounding	Methods p. 10

Subgroups and interactions	<u>#12b</u>	Describe any methods used to examine subgroups and interactions	Methods p. 11
Missing data	<u>#12c</u>	Explain how missing data were addressed	Methods p. 10
Loss to follow up	<u>#12d</u>	Cohort study: if applicable, explain how loss to follow-up was addressed. Case-control study: if applicable, explain how matching of cases and controls was addressed. Cross-sectional study: if applicable, describe analytical methods taking account of sampling strategy.	n/a
Sensitivity analysis	<u>#12e</u>	Describe any sensitivity analyses	Methods p. 10-11
None	<u>#nut-12.1</u>	Describe any statistical method used to combine dietary or nutritional data, if applicable.	n/a
None	<u>#nut-12.2</u>	Describe and justify the method for energy adjustments, intake modeling, and use of weighting factors, if applicable	Methods p. 9
None	<u>#nut-12.3</u>	Report any adjustments for measurement error (i.e., from a validity or calibration study).	n/a
Results			
Participants	<u>#13a</u>	Report numbers of individuals at each stage of study—e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed. Give information separately for exposed and unexposed groups if applicable.	Supplementary Figure S2
Non-participation	<u>#13b</u>	Give reasons for non-participation at each stage	n/a

Participant journey	<u>#13c</u>	Consider the use of a flow diagram	
None	<u>#nut-13</u>	Report the number of individuals excluded on the basis of missing, incomplete, or implausible dietary and nutritional data.	Supplementary Figure S1
Descriptive data	<u>#14a</u>	Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	Results p. 12-13; Table 1
Missing data	<u>#14b</u>	Indicate number of participants with missing data for each variable of interest	Methods p. 10
Follow-up time	<u>#14c</u>	Cohort study: Summarize follow-up time (e.g., average and total amount)	Results p.13
None	<u>#nut-14</u>	Give the distribution of participant characteristics across the exposure variables, if applicable; specify if food consumption for the total population or consumers only was used to obtain results	Results p. 13; Table 1
Outcome data	<u>#15</u>	Cohort study: report numbers of outcome events or summary measures over time. Case-control study: report numbers in each exposure category, or summary measures of exposure. Cross-sectional study: report numbers of outcome events or summary measures.	Results p. 13
Main results	<u>#16a</u>	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results p. 13; Figure 1; Supplementary Table S4
Category boundaries	<u>#16b</u>	Report category boundaries when continuous variables were categorized	Methods p. 9-10

Relative and absolute risks	<u>#16c</u>	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
None	<u>#nut-16</u>	Specify if nutrient intakes are reported with or without the inclusion of dietary supplement intake, if applicable.	Discussion p.18
Other analyses	<u>#17</u>	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	Results p. 13-14; Supplementary Table S4; Supplementary Table S6
None	<u>#nut-17</u>	Report any sensitivity analysis (e.g., exclusion of misreporters or outliers) and data imputation, if applicable	Methods p.10; Results p. 14
Discussion			
Key results	<u>#18</u>	Summarize key results with reference to study objectives	Discussion p. 14
Limitations	<u>#19</u>	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	
None	<u>#nut-19</u>	Describe the main limitations of the data sources and assessment methods used and implications for the interpretation of the findings	Discussion p. 17
Interpretation	<u>#20</u>	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	
None	<u>#nut-20</u>	Report the nutritional relevance of the findings, given the complexity of diet or nutrition as an exposure.	Discussion p. 15
Generalizability	<u>#21</u>	Discuss the generalizability (external validity) of the study results	Discussion p. 15-16

Other Information

Funding	<u>#22</u>	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Funding p.18-19
Ethics	<u>#nut-22.1</u>	Describe the procedure for consent and study approval from ethics committee(s).	Methods p. 8
Data statement	<u>#nut-22.2</u>	Provide data collection tools and data as online material or explain how they can be accessed	Availability of data and materials p. 19

Supplementary Table S2. Total fish intake (servings/week) according to sex- and follow-up-specific quartiles in the InCHIANTI study.

Total fish intake (servings/week)	0–3 years		3–6 years		6–9 years		9–15 years	
	n	median (IQR)	n	median (IQR)	n	median (IQR)	n	median (IQR)
Total								
Q1	210	0.5 (0.5)	141	0.5 (0.6)	99	0.5 (0.5)	48	0.4 (0.4)
Q2	215	1.2 (0.3)	145	1.3 (0.4)	96	1.1 (0.3)	49	1.1 (0.3)
Q3	211	1.8 (0.4)	144	2.1 (0.3)	99	1.8 (0.4)	50	1.7 (0.4)
Q4	212	2.8 (1.1)	143	3.2 (1.1)	97	2.9 (1.1)	49	3.2 (1.2)
Males								
Q1	93	0.7 (0.4)	63	0.6 (0.5)	48	0.6 (0.4)	24	0.4 (0.3)
Q2	93	1.3 (0.3)	67	1.4 (0.5)	46	1.2 (0.3)	24	1.2 (0.3)
Q3	94	2.0 (0.3)	66	2.2 (0.4)	47	1.8 (0.3)	25	2.0 (0.5)
Q4	93	3.0 (1.2)	65	3.5 (1.2)	47	3.1 (1.4)	24	3.2 (0.8)
Females								
Q1	117	0.4 (0.4)	78	0.4 (0.7)	51	0.3 (0.2)	24	0.5 (0.4)
Q2	122	1.1 (0.3)	78	1.2 (0.4)	50	1.0 (0.1)	25	1.1 (0.4)
Q3	117	1.7 (0.3)	78	2.0 (0.3)	52	1.7 (0.3)	25	1.7 (0.3)
Q4	119	2.6 (1.0)	78	3.0 (1.2)	50	2.9 (0.6)	25	2.9 (1.7)

Note: IQR, interquartile range.

Supplementary Table S3. Baseline characteristics of the studied population by cognitive decline trajectory in the InCHIANTI study (n = 883).

	Without CD (n = 362)	With CD (n = 521)	p
Age at baseline, y.	72.9 ± 6.5	74.3 ± 6.7	0.002
Female	177 (48.9)	321 (61.6)	< 0.001
BMI, kg/m ²	27.2 ± 3.6	27.3 ± 4.2	0.649
Education, y.	6.2 ± 3.7	5.1 ± 2.9	< 0.001
Income, inadequate	173 (47.8)	273 (52.4)	0.092
Physical activity, sedentary	47 (13.0)	104 (20.0)	0.013
Current smoking	47 (13.0)	83 (15.9)	0.018
Hypertension	215 (59.4)	307 (58.9)	0.890
MMSE, score	25.9 ± 2.8	25.4 ± 2.9	0.010
ApoE, ε4	44 (12.8)	83 (16.8)	0.273
IRF	112 (30.9)	182 (34.9)	0.216
CVD	66 (18.2)	114 (21.9)	0.186
Depression	102 (28.2)	189 (36.3)	0.012
Diabetes	37 (10.2)	71 (13.6)	0.129
Cancer	21 (5.8)	30 (5.8)	0.979
COPD	26 (7.2)	40 (7.7)	0.783
MCI	5 (1.4)	7 (1.3)	0.962
Energy intake, kcal/d	1,997.2 ± 545.1	1,879.2 ± 563.1	0.002
Alcohol intake, g/d	15.5 ± 20.8	14.3 ± 20.6	0.389
Total fish intake, servings/week	1.8 ± 1.2	1.6 ± 1.2	0.088
Fish intake, servings/week	1.6 ± 1.1	1.5 ± 1.1	0.230

Seafood intake, servings/week	0.2 ± 0.4	0.1 ± 0.3	0.031
MDS, points	4.4 ± 1.5	4.0 ± 1.6	< 0.001
Ratio animal:plant protein intake, g/d	1.8 ± 0.7	1.8 ± 0.7	0.666
Protein RDA, < 1 g/kg/d	141 (39.0)	208 (39.9)	0.771
PUFA intake, g/d	0.2 ± 0.1	0.2 ± 0.1	0.811
EPA and DHA, % cFAs (mg/L)	2.9 ± 1.0	2.9 ± 0.8	0.748

Note: ApoE, Apolipoprotein E. BMI, body mass index. cFAs, circulating fatty acids. COPD, chronic obstructive pulmonary disease. CVD, cardiovascular disease. DHA, docosahexaenoic acid. EPA, eicosapentaenoic acid. IRF, impaired renal function. MCI, mild cognitive impairment. MDS, Mediterranean diet score. MMSE, Mini-Mental State Examination. PUFA, polyunsaturated fatty acid. RDA, Recommended Dietary Allowance.

Data are expressed as mean ± SD or number (%) as appropriate. Comparisons between groups with Pearson's chi-square test or Student's t-test as appropriate.

Supplementary Table S4. Multivariate associations between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.

	n (CD)	Q1 (n = 210)	Q2 (n = 215)	Q3 (n = 211)	Q4 (n = 212)	p trend	continuous (per 1 serving/week increase)
Model 1	2,008 (521)	1 (ref.)	1.02 (0.82–1.28)	0.95 (0.75–1.19)	0.71 (0.55–0.91)	0.008	0.89 (0.83–0.97)
Model 2	2,008 (521)	1 (ref.)	1.04 (0.83–1.29)	0.98 (0.78–1.23)	0.75 (0.58–0.96)	0.025	0.91 (0.84–0.99)
Model 3	1,925 (494)	1 (ref.)	0.99 (0.79–1.25)	0.95 (0.75–1.20)	0.72 (0.55–0.93)	0.014	0.90 (0.83–0.98)

Note: Time-dependent Cox models were used to assess the risk of CD by quartiles of total fish intake (servings/week) and as a continuous variable (per 1 serving/week). Results are presented as hazard ratios (95% CI). Model 1 was adjusted for age at baseline, sex, and total energy intake (kcal/d). Model 2 was additionally adjusted for BMI, years of education, income, smoking status, alcohol intake (g/d), and physical activity. Model 3 was further adjusted for ApoE isoforms and comorbidities: CVD, hypertension, diabetes, cancer, depression, COPD, and IRF. CD was defined as a ≥ 3 points decline in MMSE between two consecutive follow-up evaluations (1,2).

Supplementary Table S5. Multivariate associations between total fish intake (servings/week) and cognitive decline by sex during 15 years of follow-up in the InCHIANTI study.

	n (CD)	Q1	Q2	Q3	Q4	p trend	continuous (per 1 serving/week increase)
Males							
n		95	98	96	96		
Model 1	919 (200)	1 (ref.)	0.88 (0.61–1.28)	0.90 (0.63–1.30)	0.69 (0.46–1.03)	0.092	0.89 (0.79–1.00)
Model 2	919 (200)	1 (ref.)	0.89 (0.62–1.29)	0.96 (0.66–1.38)	0.72 (0.48–1.08)	0.176	0.91 (0.80–1.02)
Model 3	889 (190)	1 (ref.)	0.87 (0.59–1.28)	0.96 (0.66–1.39)	0.71 (0.47–1.06)	0.165	0.90 (0.80–1.01)
Females							
n		123	125	126	124		
Model 1	1,089 (321)	1 (ref.)	1.12 (0.85–1.48)	0.97 (0.72–1.30)	0.71 (0.51–0.99)	0.034	0.90 (0.81–1.00)
Model 2	1,089 (321)	1 (ref.)	1.13 (0.86–1.49)	0.98 (0.73–1.31)	0.74 (0.53–1.04)	0.070	0.91 (0.82–1.02)
Model 3	1,036 (304)	1 (ref.)	1.05 (0.79–1.40)	0.92 (0.68–1.23)	0.69 (0.49–0.98)	0.028	0.89 (0.79–0.99)

Note: Time-dependent Cox models were used to assess the risk of CD by quartiles of total fish intake (servings/week) and as a continuous variable (per 1 serving/week). Results are presented as hazard ratios (95% CI). Model 1 was adjusted for age at baseline, sex, and total energy intake (kcal/d). Model 2 was additionally adjusted for BMI, years of education, income, smoking status, alcohol intake (g/d), and physical activity. Model 3 was further adjusted for ApoE isoforms and comorbidities: CVD, hypertension, diabetes, cancer, depression, COPD, and IRF. CD was defined as a ≥ 3 points decline in MMSE between two consecutive follow-up evaluations (1,2).

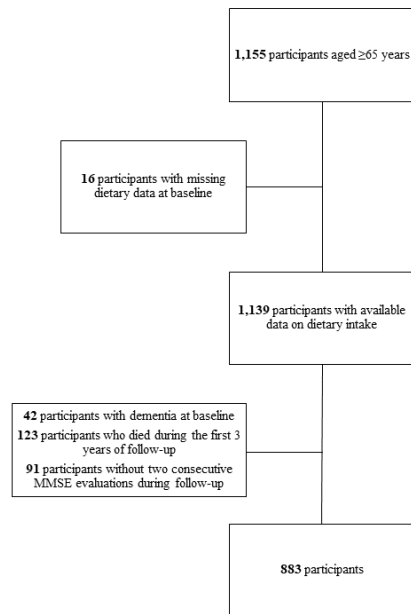
Supplementary Table S6. Effect modification of multivariate associations between total fish intake (servings/week) and cognitive decline during 15 years of follow-up in the InCHIANTI study.

	p for interaction	FDR corrected p for interaction
Age at baseline	0.879	0.942
Sex	0.942	0.942
BMI, kg/m ²	0.835	0.942
Physical activity	0.383	0.702
Hypertension	0.810	0.942
IRF	0.165	0.363
Depression	0.028	0.309
MCI	0.065	0.357
ApoE	0.818	0.942
CVD	0.154	0.363
Protein RDA	0.158	0.363

Note: ApoE, Apolipoprotein E. BMI, body mass index. CVD, cardiovascular disease. FDR, false discovery rate. IRF, impaired renal function. MCI, mild cognitive impairment. RDA, Recommended Dietary Allowance.

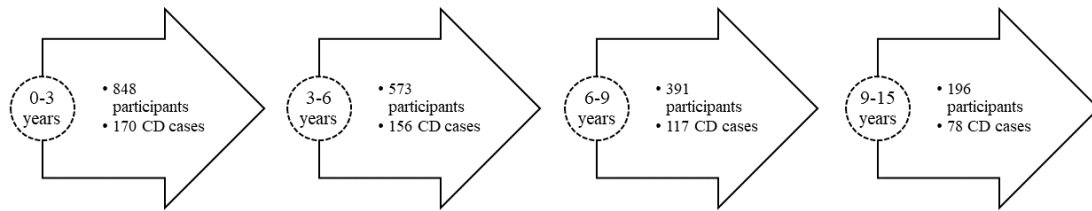
Effect modification was assessed by adding product terms in the fully adjusted time-dependent Cox models (3). Benjamini-Hochberg FDR was utilized for multiple testing correction.

Supplementary Figure S1. Flowchart of participants from the InCHIANTI study included in the analysis.



Note: MMSE, Mini-Mental State Examination.

Supplementary Figure S2. Flowchart of participants across follow-up assessments in the InCHIANTI study.



Note: CD, cognitive decline.

Supplementary References

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4.5. Adherence to the Mediterranean diet assessed by a novel dietary metabolomic score and cognitive decline in older adults

In this research, targeted metabolomics analysis has been performed to develop an *a priori* MedDiet adherence score based on serum biomarkers. The association between the metabolomic score and cognitive decline in community-dwelling older adults has been evaluated.

Publication 5: Alba Tor-Roca, Alex Sánchez-Pla, Aniko Korosi, Mercè Pallàs, Paul J Lucassen, Pol Castellano-Escuder, Ludwig Aigner, Raúl González-Domínguez, Claudine Manach, Francisco Carmona, Esteban Vegas, Catherine Helmer, Catherine Feart-Couret, Sophie Lefèvre-Arbogast, Jeanne Neuffer, Hyunah Lee, Sandrine Thuret, Cristina Andres-Lacueva, Cécilia Samieri, Mireia Urpi-Sarda. A Mediterranean diet-based metabolomic score and cognitive decline in older adults: A case-control analysis nested within the Three-City Cohort Study. Accepted at Mol Nutr Food Res.

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Abstract

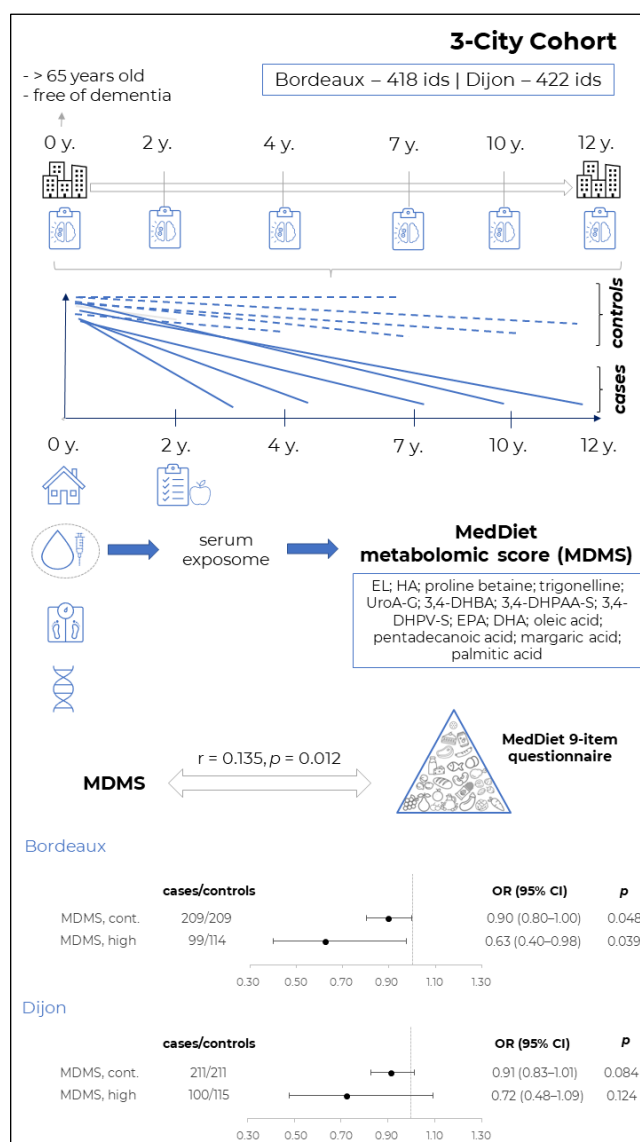
Evidence on the Mediterranean diet (MedDiet) and age-related cognitive decline (CD) is still inconclusive partly due to self-reported dietary assessment. The aim of the current study was to develop a Mediterranean diet metabolomic score (MDMS) and investigate its association with CD in community-dwelling older adults. Participants from the Three-City Study from the Bordeaux (n = 418) and Dijon (n = 422) cohorts who were free of dementia at baseline were included. Repeated measures of cognition over 12 years were collected. A MDMS was designed based on serum biomarkers related to MedDiet key food groups and using a targeted metabolomics platform. Associations with CD were investigated through conditional logistic regression (matched on age, sex, and education level) in both sample sets. The MDMS was found to be inversely associated with CD (OR (95% CI) = 0.90 (0.80–1.00); $p = 0.048$) in the Bordeaux (discovery) cohort. Results were comparable in the Dijon (validation) cohort, with a trend towards significance (OR (95% CI) = 0.91

(0.83–1.01); $p = 0.084$). A greater adherence to the MedDiet, here assessed by a serum MDMS, was associated with lower odds of CD in older adults.

Resum

L'evidència sobre la dieta mediterrània (MedDiet) i el declivi cognitiu (CD) relacionat amb l'edat encara no és conclouent en part a causa de l'avaluació dietètica autorreportada. L'objectiu del present estudi va ser desenvolupar un *score* metabolòmic de MedDiet (MDMS) i investigar la seva associació amb CD en adults majors que viuen a la comunitat. Es van incloure participants de l'Estudi de les Tres Ciutats de Bordeus ($n = 418$) i Dijon ($n = 422$) que no presentaven demència a l'inici del seguiment. Es van recollir mesures repetides de cognició durant 12 anys. Es va dissenyar un MDMS basat en biomarcadors de sèrum relacionats amb grups d'aliments clau de la MedDiet i utilitzant una plataforma metabolòmica. Les associacions amb el CD van ser investigades a través de la regressió logística condicional (aparellats per edat, sexe i nivell d'educació) en ambdós conjunts de mostres. Es va observar que el MDMS estava inversament associat amb el CD (OR (95% CI) = 0.90 (0.80–1.00); $p = 0.048$) a la cohort de Bordeus (descobriment). Els resultats van ser comparables amb els de la cohort de Dijon (validació), amb una tendència cap a la significació (OR (95% CI) = 0.91 (0.83–1.01); $p = 0.084$). Una major adherència a la MedDiet, aquí avaluada a través d'un MDMS sèric, es va associar amb menors probabilitats de CD en adults grans.

Figure 8. Graphical abstract of the manuscript entitled "A Mediterranean diet-based metabolomic score and cognitive decline in older adults: A case-control analysis nested within the Three-City Cohort Study" (Publication 5).



A Mediterranean diet-based metabolomic score and cognitive decline in older adults: A case-control analysis nested within the Three-City Cohort Study

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Abbreviations: AD, Alzheimer's disease. ApoE-ε4, Apolipoprotein E-ε4 genotype. CD, cognitive decline. CI, confidence interval. CVD, cardiovascular disease. DASH, dietary approaches to stop hypertension. DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. FFQ,

food frequency questionnaire. HA, hippuric acid. IQRs, interquartile ranges. MD, Mediterranean diet. MDS, Mediterranean diet score. MIND, Mediterranean-DASH Intervention for Neurodegenerative Delay. MDMS, Mediterranean diet metabolomic score. MMSE, Mini-Mental State Examination. OR, odds ratio. SFAs, saturated fatty acids. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 24-HDR, 24 h dietary recall. 3C Study, Three-City Cohort. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate.

Keywords: Alzheimer's disease, cognitive dysfunction, cognitive impairment, aging, metabolite

Abstract

Scope

Evidence on the Mediterranean diet (MD) and age-related cognitive decline (CD) is still inconclusive partly due to self-reported dietary assessment. The aim of the current study was to develop a MD metabolomic score (MDMS) and investigate its association with CD in community-dwelling older adults.

Methods and Results

We included participants from the Three-City Study from the Bordeaux (n = 418) and Dijon (n = 422) cohorts who were free of dementia at baseline. Repeated measures of cognition over 12 years were collected. A MDMS was designed based on serum biomarkers related to MD key food groups and using a targeted metabolomics platform. Associations with CD were investigated through conditional logistic regression (matched on age, sex, and education level) in both sample sets. The MDMS was found to be inversely associated with CD (OR (95% CI) = 0.90 (0.80–1.00); $p = 0.048$) in the Bordeaux (discovery) cohort. Results were comparable in the Dijon (validation) cohort, with a trend towards significance (OR (95% CI) = 0.91 (0.83–1.01); $p = 0.084$).

Conclusions

A greater adherence to the MD, here assessed by a serum MDMS, was associated with lower odds of CD in older adults.

1 Introduction

Dementia is a rapidly growing public health problem affecting around 50 million people worldwide. Every year, there are nearly 10 million new cases, and this figure is projected to triple by 2050 due to the global aging population and its anticipated impact and costs for society are huge.^[1] Dementia is characterized by progressive cognitive decline (CD), a major cause of disability and dependency among older people.

While there is currently no treatment for dementia, certain lifestyle factors have been associated with a delay in the age-at-onset or with a slowing down of disease progression. In fact, a healthy diet is thought to have great preventive potential for CD, both directly and through its role in reducing other risk factors (such as hypertension and type 2 diabetes).^[2] Healthy dietary patterns have indeed been associated with a lower risk of dementia^[3-5] and better cognitive performance.^[6] Also, several observational studies have concluded that a high adherence to in particular the Mediterranean diet (MD) is associated with a decreased risk of mild cognitive impairment and Alzheimer's disease (AD),^[7] and with better episodic memory and global cognition.^[8] Two other related dietary patterns also associated with better cognitive performance are the Dietary Approaches to Stop Hypertension (DASH)^[9] and the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diets.^[10,11] However, evidence of the associations between dietary patterns and cognitive function is still inconclusive.^[12] For instance, a meta-analysis of randomized controlled trials investigating MD effects on cognition, or on brain morphology and function, concluded that the associations were mostly non-significant, with small effect sizes.^[13] Gauci et al. also found conflicting results in studies investigating the effects of the DASH and MIND diets on neurocognitive function.^[14]

Some of these inconsistencies may be attributable to the fact that current epidemiological data on dietary patterns mostly rely on self-reported questionnaires, which may misreport the actual food consumption and thereby underestimate the inter- and intraindividual variability in bioavailability of bioactive food components that could arise from numerous

factors (e.g., sex, age, genetic background, chronic diseases, liver function and gut microbiota).^[15]

To overcome these limitations, the use of a panel of dietary biomarkers would constitute an alternative (or complement) to provide a more specific assessment of dietary exposure and to better evaluate the association between dietary exposure and health outcomes.^[16] Prior studies have indeed identified protective associations between urinary total polyphenols, individual plant-derived metabolites - including phenolic acids such as 3-hydroxyhippuric acid and 3-hydroxyphenylacetic sulfate, urinary urolithin derivatives, lutein, ergothioneine, and equol - and CD, as well as negative associations with metabolites related to generally considered unhealthy dietary components, such as saccharin and propionic acid.^[17–23] Yet, studies using combinations of diverse biomarkers to monitor compliance with specific dietary patterns are scarce.

Here, we developed a MD metabolomic score and investigated its association with CD in two sample sets from the centers of Bordeaux and Dijon, as of the Three-City (3C) Cohort.

2 Experimental Section

2.1 Study Design and Population

We conducted a nested case-control study using data from the 3C Study, a population-based cohort on dementia that includes older persons (≥ 65 years) from three French cities.^[24] A detailed description is provided elsewhere.^[17–19,24–29] Briefly, sociodemographic and lifestyle characteristics, medical information, neuropsychological testing, blood pressure, anthropometric measurements, and fasting blood samples were collected at baseline from 1999 to 2000, and follow-up visits were then scheduled every 2–3 years for repeated health and neuropsychological assessment.^[24] The study was performed in accordance with the principles contained in the Declaration of Helsinki. The Consultative Committee for the Protection of Persons participating in Biomedical Research at Kremlin-Bicêtre University Hospital (Paris, France) approved the 3C Study protocol, and all participants provided written consent.

In this nested case-control study, two sample sets were built among participants from the centers of Bordeaux and Dijon of the 3C Study, with Bordeaux as the discovery set and Dijon as the set used for external validation.^[17,18] From the entire Bordeaux and Dijon cohorts, we selected eligible participants for the present study if they were not diagnosed with dementia at baseline, had available serum samples, and had at least one repeated cognitive evaluation over the subsequent 12 years.

To build the case-control samples on CD, we defined a composite score of global cognition at each follow-up visit as the average Z-scores of five neuropsychological tests (Mini-Mental State Examination (MMSE), Benton Visual Retention Test, Isaac's Set Test, Trail-Making Test part A, and Trail-Making Test part B).^[17,18] Individual slopes of cognitive change were then estimated using linear mixed models, as previously detailed.^[18] Cases were defined as the participants with the worst CD slopes. Then, we matched each case to a control (i.e., a participant with a CD slope better than the population median) based on age at baseline, sex, and education level. Overall, 209 cases were individually matched to a control in Bordeaux, leading to a discovery sample of 418 subjects. In Dijon, 211 cases were successfully matched to 211 controls, leading to a validation sample of 422 subjects.

2.2 Targeted Metabolomics Analysis of Serum Samples

We conducted a targeted metabolomics analysis of serum samples using a multi-metabolite platform for the simultaneous detection and quantification of food-related metabolites, microbiota derivatives, and endogenous metabolites following the methodology detailed in previous publications.^[17,30] The samples were randomly distributed for metabolomics analysis utilizing an Infinity UHPLC system (Agilent, Santa Clara, CA, USA) coupled to a QTRAP 6500 mass spectrometer equipped with an Ion Drive Turbo V ion source (Sciex, Framingham, MA, USA) using the operating conditions described elsewhere.^[17]

2.3 Mediterranean Diet Metabolomic Score

In line with the Mediterranean diet score (MDS) defined by Trichopoulou et al.^[31] the MD metabolomic score (MDMS) was developed de novo using a 14-point linear scale that incorporated two putative dietary metabolomic biomarkers of seven key components of the MD (vegetables, legumes, fruits, cereals, dairy, fats, and fish) (Table 1 and Table S1, Supporting Information).^[31,32]

The metabolites for the MDMS were selected based on three criteria: a) metabolites reported as putative biomarkers of key MD food groups^[33–47], b) metabolites available in the serum metabolomic panel described previously^[17,30,48] and c) metabolites with described health-related properties (Table S1, Supporting Information). Detailed information about the eligibility criteria of the metabolites for the MDMS is shown in Table S1 (Supporting Information). In this MDMS score, we excluded the meat item from the MDS as it is not possible to establish a set of metabolites that can reliably discriminate between meat intake, products of human muscle catabolism, and other protein-rich foods (e.g., fish and seafood), as well as between different meat sources (e.g., red meat vs. poultry).^[44] We did not include an alcohol-related item either since low levels of alcohol have shown slight reductions in health-related harm (i.e., ischemic heart disease and diabetes in very specific populations) but an increased risk of other health-related conditions, including cirrhosis, infectious diseases, cardiovascular disease, early dementia and cancer.^[49,50]

We thus worked with n=14 serum biomarker components. We decided to group four categories of foods (vegetables, fruits, legumes, and cereals) as a plant-based food group since some of the biomarkers described could be ubiquitously present among all these food groups. Enterolactone (EL), hippuric acid (HA), proline betaine; trigonelline; 3,8-dihydroxy-urolithin glucuronide (UroA-G), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3',4'-dihydroxyphenylacetic acid sulfate (3,4-DHPAA-S), and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate (3,4-DHPV-S) represented the plant-based food group. Docosahexaenoic acid (DHA) and eicosapentaenoic

acid (EPA) depicted the fish group, while pentadecanoic acid and margaric acid represented the dairy products group. Finally, the dietary intake of MUFAs and saturated fatty acids (SFAs) were depicted by oleic acid and palmitic acid, respectively (Table S1 and Table S2, Supporting Information).

Following the same design used by Trichopoulou et al.^[31] we assigned a value of 0 or 1 to each dietary biomarker with the use of the sex-specific median as the cutoff (Table S3, Supporting Information). For dietary biomarkers of beneficial components (vegetables, legumes, fruits, cereals, and fish), subjects whose circulating level was below the median were assigned a value of 0, and individuals whose level was at or above the median were assigned a value of 1. According to the MDS defined by Trichopoulou et al.^[31,32] dairy products are presumed to be detrimental. For this reason, the scoring for dietary biomarkers of dairy products was inverted.^[31,32] Finally, for the relation between MUFAs and SFAs, we assigned a value of 1 to those individuals whose oleic acid concentration was at or above the median and a value of 0 to those whose concentrations were below it. The scoring was inverted for palmitic acid concentrations. Oleic acid and palmitic acid are the most abundant dietary and plasma FAs.^[47,51] Moreover, oleic acid is a biomarker of olive oil intake, the main source of MUFAs in the MD context.^[31,52]

Thus, the total MDMS ranged from 0 (minimal adherence) to 14 (maximal adherence). The MDMS was secondarily categorized, using cutoff values based on the sex- and cohort-specific medians, as low (< 7 points) and high ($\geq$ 7 points) adherence for both sexes and cohorts.

2.4 Dietary Data

Details of dietary habits assessment are described elsewhere.^[53,54] Briefly, we used a comprehensive food frequency questionnaire (FFQ)^[53] and a 24 h dietary recall (24-HDR)^[54] administered in 3C Bordeaux at the first follow-up visit in the period 2001–2002 (2–3 years after baseline when blood was drawn). For foods from the FFQ, data were available for 351

Bordeaux sample subjects, and for MUFAs and SFAs intake from the 24-HDR, data were available for 359 subjects. A MDS based on the FFQ and the 24-HDR was available for 349 subjects from Bordeaux (Table S4, Supporting Information).

2.5 Statistical Analysis

Baseline characteristics of the participants were expressed as means and standard deviations (SDs) for variables with normal distribution, medians and interquartile ranges (IQRs) for variables with asymmetric distribution, and percentages for categorical variables. Baseline characteristic comparisons were tested using conditional logistic regression models. Correlation analyses were performed to examine the relation between MDMS and MDS, and between food groups and dietary biomarkers of MD key food groups in the Bordeaux cohort.

To study the association between MDMS and CD, we estimated odds ratios (ORs) and their 95% confidence interval (CI) using logistic regression models conditioned on matching variables (i.e., age at baseline, sex, and education level) (Model 1). For multivariable-adjusted analyses, we considered potentially modifiable risk factors for dementia (i.e., BMI, diabetes, alcohol intake, smoking status, hypertension, depression, hypercholesterolemia)^[55,56] (Model 2) as well as genetic factors (i.e., Apolipoprotein E- ϵ 4 genotype (ApoE- ϵ 4))^[56] and the total number of medications regularly consumed^[17] (Model 3). The goodness of fit of the proposed models was evaluated using the likelihood-ratio chi-square test of the full model versus the null model, where p-value below 0.05 was considered to be a good fit.

Effect modification by age at baseline, sex, education level, BMI, diabetes, alcohol intake, smoking status, hypertension, depression, hypercholesterolemia, the total number of medications regularly consumed, and ApoE- ϵ 4 was assessed by adding product terms in the fully adjusted regression models. Bonferroni correction was utilized for multiple testing correction ($p < 0.05/12$, statistical threshold at $\alpha = 0.004$).

For covariates, missing values comprised < 2% of the samples and the reference category was assigned to missing data (for categorical variables) or the median value (for continuous variables). All statistical analyses were performed using the SPSS software package (SPSS 25.0, IBM, NY) and the significance level was a 2-tailed $p < 0.05$.

3 Results

3.1 Characteristics of the Study Populations

Clinical and demographic characteristics of the study populations at baseline are shown in Table 1. The participants were matched for age, sex, and education level within the two samples, and these characteristics were similar between the discovery (i.e., Bordeaux) and validation (i.e., Dijon) sample sets. As expected, we observed lower scores for MMSE tests assessed in this study among subjects with CD. Medication use was significantly higher in cases, as well as the prevalence of diabetes. In the Bordeaux cohort, the presence of ApoE-ε4 was higher among cases than among controls, although the percentage was similar in the Dijon set. Cases from the Dijon cohort also showed a significantly higher percentage of participants with depression compared to controls (Table 1). However, in the Bordeaux set, cases and controls had a similar percentage of subjects with depression (Table 1).

3.2 Mediterranean Diet Metabolomic Score

The correlations among FFQ dietary components of MD key food groups and concentrations of serum biomarkers in the Bordeaux set are presented in Table S5 (Supporting Information). Nearly all MD key food groups had a positive and significant correlation with their serum metabolites. The strongest correlation was between the intake of fish and seafood and the sum of EPA and DHA (Table S5, Supporting Information). The plant-based food group (i.e., total intake of vegetables, fruits, legumes, and cereals) and the sum of their selected biomarkers were also positively correlated (Table S5, Supporting Information). Dairy intake and

serum pentadecanoic acid and margaric acid were not correlated. MDS was also positively correlated with MDMS ($r = 0.135$, $p = 0.012$) in the Bordeaux cohort ($n = 349$).

3.3 MDMS and CD

The associations between MDMS and CD are shown in Figure 1. In the discovery set, CD was not associated with MDMS in the basic model (Figure 1A, Model 1). This association was unchanged after further adjustment for CD risk factors (i.e., BMI, diabetes, alcohol intake, smoking status, hypertension, depression, and hypercholesterolemia) (Figure 1A, Model 2). However, after additionally adjusting for the total number of medications regularly consumed and for ApoE- ϵ 4 genotype (Model 3), subjects with a higher MDMS were less likely to experience CD (OR (95% CI) = 0.90 (0.80–1.00); $p = 0.048$, MDMS as continuous variable) (Figure 1A, Model 3). This was consistent with the results observed in Dijon, with a trend toward significance in this validation sample (OR (95% CI) = 0.91 (0.83–1.01); $p = 0.084$ for Model 3 and MDMS as a continuous variable) (Figure 1B, Model 3). The omnibus test suggested a significant improvement in fit of the models relative to the null ($\chi^2(10) = 34.292$; $p \leq 0.001$ and $\chi^2(10) = 37.670$; $p \leq 0.001$ in Bordeaux and Dijon, respectively, for Model 3, MDMS as continuous variable).

Stratified analyses were performed to assess the association between MDMS and the risk of CD in various subgroups (Table S6, Supporting Information). We noticed a close to significant interaction between MDMS and hypertension in the Bordeaux cohort (no hypertension, adjusted OR (95% CI) = 1.19 (0.95–1.50), compared with hypertension, adjusted OR (95% CI) = 0.83 (0.66–1.05); p for interaction = 0.006). None of the other variables, including age at baseline, sex, education level, BMI, diabetes, alcohol intake, smoking status, hypercholesterolemia, depression, the total number of medications regularly consumed, and ApoE- ϵ 4, significantly modified the association between MDMS and the risk of CD in the Bordeaux cohort. No interaction between MDMS and the abovementioned variables was found

in the Dijon cohort (all p for interactions > 0.004 for multiple testing correction) (Table S6, Supporting Information).

4 Discussion

Leveraging targeted large-scale metabolomics data from the 3C Study, we here developed a Mediterranean diet metabolomic score (MDMS) based on serum biomarkers of MD key food groups. Our findings show that a higher MDMS is prospectively associated with a lower risk of CD in older adults free of dementia at baseline (median follow-up 12 years).

These results are in line with some prior prospective studies, where a high adherence to the MD, as assessed using dietary questionnaires, has been associated with a slower decline in global cognition in older adults.^[57,58] However, despite the efforts made to reduce the heterogeneity of the scoring systems used for assessing adherence to the MD, it is still challenging to elucidate why current literature still reports mixed results for cognitive function and the MD in older adults.^[57,59] For instance, in the Three-City Cohort, Féart et al. suggested that a higher adherence to the MD, using the MDS, was associated with a slower cognitive decline, as based on MMSE score, but not with the risk of incident dementia or AD.^[32] In another large-scale prospective study including 16,058 women from the Nurses' Health Study aged ≥ 70 years, authors found that an alternate MDS was related to moderately better cognition overall, but not to change in cognition over 4 repeated measures after 6 years of follow-up.^[60] By contrast, in the EPIC-Spain Dementia Cohort Study, recent results showed that MD appeared to be protective and lowered the incidence of dementia.^[61] Also, Scarmeas et al. reported that a higher MD adherence was associated with a reduced risk of AD.^[62]

One source of these biases could be linked to the modality of assessing dietary intake, as most studies used dietary records, which lack accuracy and are prone to random bias and systematic errors. As a matter of fact and as potentially expected, the MDMS was weakly correlated with the MDS, as were most of the MD key food groups with their metabolites. The

MDMS was created *a priori* based on the current state of the art on food intake biomarkers^[63,64] and aiming at taking a step forward by surrogating traditional indicators of dietary patterns intake (i.e., FFQ and 24-HDR derived scores) and thus their inherent prone-to-subjectivity and lack of accuracy.^[65–67] For instance, dairy intake and serum pentadecanoic and margaric acids were not correlated in this cohort. This may be attributable to the heterogeneity of fat content through different dairy products in the marketplace or within a specific product resulting from differences in processing (e.g., skimming, among others), as well as in the quality of the milk used as raw material (e.g., cattle breed and feeding, among others).^[45] This particular case might serve as a revealing example of the need of better dietary definition, since in the MDS defined by Trichopoulou et al. the dairy products are presumed to be detrimental on account of their fat content.^[31]

The weakness of correlation between the other food groups and their dietary biomarkers could be attributed to i) the variability of phytochemical or fatty acid content through a food group or a specific product resulting from differences in harvesting or between species (e.g., fatty fish have higher levels of long chain n-3 PUFA than lean fish and seafood),^[68] among others; ii) the presence of the selected metabolites in other food sources not included in the MD key food groups (e.g., coffee, tea and cocoa products);^[69] and iii) the presence of other metabolites that could contribute to the real content of a MD key food group (i.e., MUFA and SFA).

In a move to obtain more revealing information from dietary intake data and to overcome the above-mentioned problems, food biomarkers could be valuable in this respect as they can capture not only food composition but also their bioavailability. In this sense, several works have highlighted promising results when evaluating single food biomarkers in relation to CD. Rabassa et al. showed in their 3-year follow-up study that higher levels of urinary total polyphenols, a biomarker of fruit and vegetable intake, were associated with a lower risk of CD in older adults.^[21] Interestingly, in the Three-City Cohort, distinct associations were found

between several serum food-derived metabolites and CD.^[17–19] Yet, in another move aimed at obtaining more meaningful evidence, nutritional researchers have begun examining biomarkers as measures of dietary patterns rather than of single nutrients or dietary components. Unlike individual compounds, dietary patterns better reflect a person's habitual diet and provide a more integrated measure of the synergistic and accumulative effects of a combination of dietary factors.

To date, few studies have captured metabolomic signatures related to the MD through predictive and discriminant analysis, and some have studied their association with health outcomes, such as cardiovascular disease (CVD) and its risk factors.^[70–75] To the best of our knowledge, our current work is the first study to have developed an *a priori* dietary metabolomic score based on serum biomarkers of MD food groups and to evaluate it in association with CD. Specifically, the MDMS comprises a set of serum phytochemical and fatty acid metabolites that reflect the individual bioavailability. Moreover, some of them have been recognized as exposure markers of the key MD food groups to which, in turn, the protective effects on health of the MD have been attributed to (Supporting Information).^[76]

Validation is important but has rarely been carried out in prior food metabolomics studies, likely because it is challenging in research on nutrimental metabolomics and cognitive impairment as there is an intrinsic and large heterogeneity across studies, and profound intervariability arising from factors like gut microbiota and/or health status,^[15,77] and the complexity of outcomes based on long-term trajectories such as CD and different types of dementia. For example, despite belonging to the same cohort study, the features of our validation sample differed somewhat from the discovery sample (e.g., participants were older, showed a higher percentage of depression and ApoE-ε4 carriers, and had more cardiovascular risk factors at baseline). This could explain the weaker association we found in the Dijon (validation) sample compared to the discovery one, even though both sample sets were positively related to a greater MDMS, indicating a protective effect.

The major strengths of our study are the population-based and prospective design with repeated cognitive assessment over up to 12 years, a rigorous case-control sampling, and the application of a powerful targeted metabolomics approach to serum samples collected at baseline, i.e., at an age prior to the appearance of any dementia symptoms, which reduces the possibility of reverse causation. In addition, the use of two separate nested case-control sample sets noticeably enhances the reliability of our findings.

However, also several limitations warrant further discussion. First, serum samples for metabolomics analysis were only available at baseline, and thus we could not examine prior exposures or changes during the follow-up. An approach based on multiple sampling would have been of great interest to investigate the trajectory of CD. Moreover, although we adjusted the model by several potential confounders including ApoE- ϵ 4, residual confounding factors cannot be dismissed. Another limitation is that the MDMS scoring system is based on sex-specific median values across metabolite concentrations of the studied populations. Therefore, the score may not be related to a healthy level of concentrations per se. Although we evaluated the reproducibility of the results in a separate nested case-control sample set, this metabolomic score deserves to be validated also in other cohorts and geographic areas, and its association with other chronic diseases should be examined.

In summary, a greater adherence to the MD assessed by a novel Mediterranean diet metabolomic score was associated with a lower risk of CD in older adults during a 12-year follow-up. The development of dietary metabolic scores based on dietary patterns may help further refine dietary assessment measures and will hopefully contribute to a better understanding of the biological mechanisms via which diet impacts cognitive health in the aging population.

Author Contributions

Conceptualization: A.T.-R., C.M., A.K., P.J.L., L.A., S.T., C.H., C.S., C.A.-L. M.U.-S.; data curation: R.G.-D., P.C.-E., F.C., E.V., A.S.-P.; formal analysis: A.T.-R., M.U.-S.; funding acquisition: C.M., A.K., P.J.L., L.A., M.P., S.T., C.H., C.S., C.A.-L.; investigation: A.T.-R., A.S.-P., A.K., M.P., P.J.L., P.C.-E., L.A., R.G.-D., C.M., F.C., E.V., C.H., C.F.-C., S.L.-A., J.N., H.L., S.T., C.A.-L., C.S., M.U.-S.; methodology: R.G.-D., M.P., A.S.-P., S.L.-A., C.A.-L., M.U.-S.; supervision, C.S., C.A.-L., M.U.-S.; writing – original draft: A.T.-R., M.U.-S.; writing – review & editing: A.T.-R., A.S.-P., A.K., M.P., P.J.L., P.C.-E., L.A., R.G.-D., C.M., F.C., E.V., C.H., C.F.-C., S.L.-A., J.N., H.L., S.T., C.A.-L., C.S., M.U.-S.. All authors have read and approved the final manuscript.

Institutional Review Board Statement

The 3C protocol was approved by the Consultative Committee for the protection of persons participating in biomedical research at Kremlin-Bicêtre University hospital in France (IRB approval number 9928, Kremlin Bicêtre, 10 June 1999).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data used in the manuscript will be made available by the corresponding author upon request pending application and approval.

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Tables

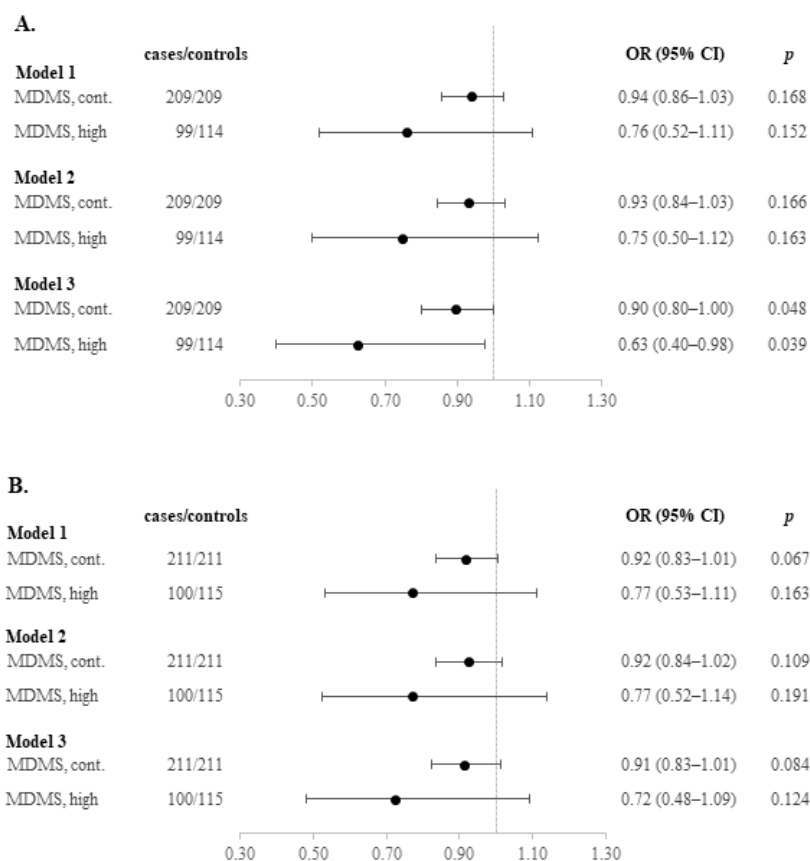
Table 1. Clinical and demographic characteristics of the discovery (Bordeaux, n = 418) and validation (Dijon, n = 422) case-control samples

	Bordeaux cohort			Dijon cohort		
	Controls (n = 209)	Cases (n = 209)	p^a	Controls (n = 211)	Cases (n = 211)	p^a
Age (years)	75.7 (4.2)	75.9 (4.5)	-	76.2 (4.7)	76.5 (5.2)	-
Sex, female (n (%))	138 (66)	138 (66)	-	133 (63)	133 (63)	-
Educational level, $\geq$ secondary school (n (%))	60 (28.7)	61 (29.2)	-	60 (28.4)	60 (28.4)	-
BMI (kg/m ²)	25.8 [4.3]	26.4 [5.6]	0.12	24.9 [4.4]	25.4 [6]	0.099
Number of medications regularly consumed	4.1 (2.4)	4.9 (2.7)	0.003	4.0 [3.0]	5.0 [4.0]	< 0.001
MMSE (points)	28.0 [2.0]	28.0 [3.0]	< 0.001	29.0 [1.0]	26.0 [4.0]	< 0.001
Diabetes, yes (n (%))	12 (5.7)	27 (12.9)	0.017	12 (5.7)	27 (12.8)	0.017
ApoE- ϵ 4, carrier (n (%))	25 (12.0)	54 (25.8)	0.001	44 (20.9)	56 (26.5)	0.187
Alcohol intake (g/d)	9.6 [19.2]	9.6 [17.8]	0.263	9.6 [16.5]	9.6 [17.8]	0.822
Current smoking, yes (n (%))	9 (4.3)	10 (4.8)	0.695	4 (1.9)	13 (6.2)	0.150
Depression, yes (n (%))	15 (7.2)	20 (9.6)	0.386	25 (11.8)	47 (22.3)	0.005
Hypercholesterolemia, yes (n (%))	128 (61.2)	127 (60.8)	0.922	119 (56.4)	126 (59.7)	0.482
Hypertension, yes (n (%))	159 (76.1)	164 (78.5)	0.564	175 (82.9)	177 (83.9)	0.789
MDS (points) ^b	4.3 (1.6)	4.3 (1.7)	0.404	-	-	-
MDMS (points)	6.7 (2.1)	6.4 (2.2)	0.168	6.8 (2.0)	6.5 (2.0)	0.067

ApoE-ε4, Apolipoprotein E-ε4 genotype. MDS, Mediterranean diet score. MDMS, Mediterranean diet metabolomic score. MMSE, Mini-Mental State Examination. Values are mean (SD), median [IQR], or number of subjects with the characteristic (%). ^a Estimated using conditional logistic regression models. ^b Data available from 349 subjects from the Bordeaux cohort.

Figures

Figure 1. Multivariate conditional logistic regression models for association between MDMS (continuous and categorical) and CD in the 3C discovery (Bordeaux, n = 418) (A.) and validation (Dijon, n = 422) (B.) cohorts. OR (95% CI)



CD, cognitive decline. CI, confidence interval. MDMS, Mediterranean diet metabolomic score. OR, odds ratio. 3C, Three-City.

Model 1 is unadjusted. Model 2 is adjusted for BMI, diabetes, alcohol intake, hypertension, smoking status, depression, and hypercholesterolemia. Model 3 is further adjusted for ApoE-ε4 and number of medications.

The CD variable was built defining the composite score of global cognition at each follow-up visit.^[17,18] Individual slopes of cognitive change were then evaluated using linear mixed models, as detailed in the previous publication.^[18] Cases were defined as the participants with the CD worst slopes.

Supporting Information

Table S1. Metabolites included in the MDMS as biomarkers of key MD food groups*

MDMS metabolite	metabolite class	food
EL*	enterolignans (microbial metabolites of lignans)	fiber-rich fruits and vegetables, grains, nuts and legumes ^[1,2]
HA*	hydroxybenzoic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, coffee, cocoa, grains, nuts, legumes, olive oil, and wine ^[1,3–10]
proline betaine*	proline derivatives	citrus fruits (mainly), Chinese artichoke, and rye and wheat bran ^[1,11–13]
Trigonelline*	pyridinium derivatives	coffee, legumes, and rye and wheat bran ^[1,14]
UroA-G*	uroolithins (microbial metabolites of ellagitannins)	berries, wine, pomegranate, and nuts ^[1,15]
3,4-DHBA*	hydroxybenzoic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, coffee, cocoa, grains, nuts, legumes, olive oil, and wine ^[1,3–10]
3,4-DHPAA-S*	hydroxyphenylacetic acids (microbial metabolites of polyphenols)	fruits and vegetables, tea, cocoa, olives and olive oil, and wine ^[1,3–5,9,10]
3,4-DHPV-S*	hydroxyphenylvalerolactones (microbial metabolites of flavan-3-ols)	procyanidin-rich foods (berries, apple, tea, cocoa, wine, nuts, and legumes) ^[1,4,5,7,8,10,16]
DHA	n-3 PUFA	total fish and shellfish ^[17]
EPA	n-3 PUFA	total fish and shellfish ^[17]
margaric acid	LCFA	dairy products ^[18]
pentadecanoic acid	LCFA	dairy products ^[18,19]
oleic acid	LCFA	dietary MUFAs ^[20,21]
palmitic acid	LCFA	dietary SFAs ^[20]

DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. HA, hippuric acid. LCFA, long-chain fatty acids. MDMS, Mediterranean diet metabolomic score. MD, Mediterranean diet. n-3 PUFA, n-3 polyunsaturated fatty acids. UroA-G, 3,8-dihydroxy-uroolithin glucuronide. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate

***METABOLITES SELECTION CRITERIA FOR THE DEVELOPMENT OF MDMS**

The metabolites for the MDMS were selected based on three criteria: a) metabolites reported as putative biomarkers of key MD food groups^[17–20,22–32] and b) metabolites available in the serum metabolomic panel described previously.^[1,33,34] c) metabolites with described health-related proprieties. Specifically, the metabolomic panel from D-CogPlast project consisted of 206 metabolites,^[33] 35% of which were food-related metabolites and 15% were gut microbiota-derived metabolites.^[33,34]

Six in eight selected putative biomarkers of the plant-based MD food group were microbiome-generated metabolites (enterolactone (EL), hippuric acid (HA), 3,8-dihydroxy-urolithin glucuronide (UroA-G), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3',4'-dihydroxyphenylacetic acid sulfate (3,4-DHPAA-S), 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate (3,4-DHPV-S)), and the remaining two were betaine derivatives.

Concretely, distinct diversity of polyphenols classes (i.e., hydroxybenzoic and hydroxyphenylacetic acids, hydroxycoumarins, hydroxyphenylvalerolactones and lignans) representative of some different metabolism phases (e.g., 3,4-DHPV-S is the degradation product of (epi)catechin, arising from the pyrogallol C-ring opening; 3,4-DHBA comes from the decarboxylation of caffeic acid or the demethylation of vanillic acid) were considered, although some of these compounds have also been described in certain foods.^[35] Circulating levels of microbiome-generated metabolites last longer than those derived from the host itself and thus reflect long-term effect on health.^[36] In fact, we also chose those metabolites which have showed a potential role as well on health. For instance, the microbiota-derived flavonoid metabolites (e.g., 3,4-DHBA and γ -valerolactones) not only preserve the antioxidant and anti-inflammatory activity of the native flavonoid but also have bioactive properties that promote mitochondrial health and cerebrovascular microcapillary function.^[37] EL has been reported to exert antiproliferative and anti-inflammatory activities in vitro,^[38] exhibits blood-brain barrier permeability, protects murine microglia against inflammation^[39] and has been associated with more favorable cardiometabolic risk factors.^[40] On the other hand, a study showed that UroA

was the most common urolithin specie produced in nature.^[41] Moreover, the production of UroA is variable and takes place in only approximately 40% of the human elderly population,^[42] which restricts its positive impacts on health conditions (e.g., mitochondrial, cardiometabolic and muscle health) as well as those from its food sources (i.e., berries, wine, pomegranate, and nuts) indeed.^[43–46] HA, a terminal metabolite of multiple microbial catabolites, has been suggested to exhibit in vitro myoprotective properties and may contribute to a better glycemic control and β -cell function, as well as to lessen the risk of developing age-related cognitive disorders.^[47,48] 3,4-DHPAA has showed a decrease in the formation of amyloid fibrils, a protection from cytotoxicity and oxidative stress, a regulation of inflammatory pathways in vitro,^[49–52] and significant reductions in high sensitivity C-reactive protein in patients with type 2 diabetes.^[53] In addition, we gave priority to dihydroxylated metabolites rather than monohydroxylated metabolites given their higher antioxidant capacity^[54] and marked anti-inflammatory properties, which indicate their potential value as beneficial agents.^[54]

Among all the DHBA available in our multi-metabolite platform, we selected the 3,4- isomer position, as 3,4-DHBA has showed potent inhibitory effects, as well as antioxidant, anti-inflammatory, antihyperglycemic and neuroprotective activities.^[55,56] Moreover, at physiological pH, anthocyanins easily convert to 3,4-DBHA, which is also abundantly formed and absorbed in the large intestine due to microbial metabolization.^[57]

With regard to non-polyphenols metabolites, trigonelline appears to have neuroprotective and antidiabetic effects^[58,59] and has been suggested as a therapeutic agent for cardiovascular disorders^[60] given its anti-inflammatory and antioxidant proprieties.^[61] In fact, it has been associated with a lower risk of type 2 diabetes among a generally healthy population^[62] and it has been associated to a urine molecular signature in the metabolic syndrome.^[63] Finally, proline betaine is a well-known biomarker of citrus intake^[11,64] which has been selected in this study as citrus is a fruit group highly consumed in the Southern Europe and is one of the top dietary sources of polyphenol intake in the 3C Study.^[65,66] Moreover, proline betaine has been negatively correlated with glucose and insulin concentrations, blood lipid profiles, BMI and

inflammation marker hsCRP^[13,67] as well as inversely associated with blood pressure.^[68] In fact, citrus intake has been suggested to be associated with global cognition benefits,^[69] as well as with lower risk of incident dementia,^[70,71] and cardiovascular risk factors.^[72–75]

Table S2. Concentrations of MDMS dietary biomarkers by levels of MDMS in the 3C discovery (Bordeaux, n = 418) and validation (Dijon, n = 422) cohorts

MDMS metabolites (µg/L)	Bordeaux cohort (n = 418)				Dijon cohort (n = 422)			
	Total	Low MDMS (n = 205)	High MDMS (n = 213)	p	Total	Low MDMS (n = 207)	High MDMS (n = 215)	p
EL	6.1 (1.5)	5.7 (1.6)	6.5 (1.2)	0.009	10.0 (5.7)	8.1 (5.2)	11.8 (5.6)	0.006
HA	1985.2 (2267.5)	1477.2 (1878.4)	2474.0 (2495.7)	< 0.001	1434.0 (1492.5)	1098.2 (1030.8)	1757.3 (1773.4)	< 0.001
proline betaine	249.2 (241.3)	208.6 (224.4)	288.3 (250.9)	< 0.001	296.5 (284.4)	282.0 (297.3)	310.5 (271.5)	0.140
trigonelline	13.9 (15.4)	12.0 (16.2)	15.7 (14.4)	0.001	10.5 (11.3)	9.9 (12.6)	11.2 (9.8)	0.047
UroA-G	0.7 (2.8)	0.3 (1.5)	1.0 (3.6)	0.004	0.8 (2.6)	0.3 (1.6)	1.3 (3.2)	< 0.001
3,4-DHBA	43.0 (80.8)	27.2 (43.0)	58.3 (102.9)	< 0.001	28.1 (75.9)	23.4 (101.1)	32.5 (38.0)	< 0.001
3,4-DHPAA-S	9112.4 (24221.3)	5221.9 (15125.7)	12856.8 (30084.8)	< 0.001	8889.1 (22819.1)	8704.4 (23539.6)	9067.0 (22156.8)	< 0.001
3,4-DHPV-S	6.6 (11.2)	3.9 (5.6)	9.1 (14.2)	< 0.001	8.4 (16.3)	6.4 (14.1)	10.4 (17.9)	0.001
DHA	6490.5 (2381.6)	6167.8 (1928.9)	6801.1 (2716.3)	0.006	5379.9 (895.5)	5167.8 (794.3)	5584.1 (940.6)	< 0.001
EPA	3000.1 (795.2)	2855.6 (463.9)	3139.3 (998.5)	< 0.001	3223.9 (486.3)	3114.8 (387.4)	3329.0 (546.1)	< 0.001
margaric acid	5558.5 (2280.7)	6141.5 (2070.6)	4997.4 (2336.8)	< 0.001	4608.6 (448.7)	4684.9 (461.1)	4535.2 (424.7)	< 0.001
pentadecanoic acid	5247.3 (2129.7)	5834.0 (2207.8)	4682.6 (1891.5)	< 0.001	4212.1 (680.3)	4408.1 (664.8)	4023.4 (642.0)	< 0.001
oleic acid	68038.3 (27325.2)	66700.6 (26934.5)	69325.8 (27698.2)	0.473	76072.0 (26349.5)	75030.2 (26006.4)	77075.1 (26697.8)	0.401
palmitic acid	35210.2 (13848.5)	35458.1 (13947.0)	34971.6 (13781.7)	0.627	18559.4 (5099.8)	18492.4 (4925.1)	18624.0 (5273.2)	0.859

Values are mean (SD). DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. HA, hippuric acid. MDMS, Mediterranean diet metabolomic score. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 3C,

Three-City. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate

Table S3. Concentrations of MDMS dietary biomarkers (µg/L) by sex-specific medians in the 3C discovery (Bordeaux, n = 418) and validation (Dijon, n = 422) cohorts

MDMS metabolite	median (µg/L)	Bordeaux cohort (n = 418)		Dijon cohort (n = 422)	
		males (n = 142)	females (n = 276)	males (n = 156)	females (n = 266)
EL	< median	5.2 (1.2)	5.0 (1.2)	6.1 (3.0)	4.9 (2.8)
	≥ median	7.1 (0.7)	7.3 (0.8)	15.3 (3.6)	14.3 (3.7)
HA	< median	866.0 (395.6)	800.7 (381.3)	546.7 (192.6)	589.3 (249.7)
	≥ median	3229.6 (2617.6)	3105.1 (2792.0)	1912.3 (1330.5)	2518.7 (1866.2)
proline betaine	< median	66.0 (29.1)	85.8 (38.7)	94.5 (40.0)	102.0 (47.4)
	≥ median	357.1 (239.6)	451.4 (233.5)	415.9 (262.5)	539.5 (290.7)
trigonelline	< median	4.7 (2.5)	4.8 (3.0)	3.4 (2.1)	3.9 (2.2)
	≥ median	18.3 (9.3)	25.3 (19.8)	14.4 (9.8)	19.1 (13.7)
UroA-G	< median	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	≥ median	5.1 (3.0)	8.8 (7.6)	6.0 (3.3)	5.7 (4.7)
3,4-DHBA	< median	19.9 (17.5)	1.2 (4.1)	4.1 (6.9)	1.7 (4.0)
	≥ median	97.2 (81.8)	68.9 (109.8)	47.5 (28.8)	57.1 (125.3)
3,4-DHPAA-S	< median	0.0 (0.0)	845.3 (1116.0)	423.3 (955.3)	1408.8 (1480.3)
	≥ median	13366.9 (20301.1)	19975.7 (36555.3)	11009.7 (22301.8)	20090.8 (33765)
3,4-DHPV-S	< median	1.9 (1.3)	1.0 (0.9)	0.9 (0.8)	0.9 (0.7)
	≥ median	13.6 (14.0)	10.9 (13.8)	15.7 (15.6)	16.2 (22.7)
DHA	< median	5058.3 (802.3)	4927.0 (643.5)	4817.4 (334.8)	4668.9 (323.0)
	≥ median	7999.1 (1890.9)	8014.8 (2765.6)	6124.8 (874.9)	5983.9 (741.2)
EPA	< median	2601.1 (158.2)	2612.4 (116.2)	2949.4 (122.0)	2896.4 (128.1)
	≥ median	3449.9 (832.7)	3361.8 (1036.1)	3597.2 (665.6)	3493.6 (403.3)
margaric acid	< median	3874.4 (1014.5)	3842.2 (781.5)	4272.8 (169.3)	4248.1 (160.0)
	≥ median	6989.3 (1441.5)	7405.0 (2168.3)	4967.1 (384.1)	4955.9 (344.6)
pentadecanoic acid	< median	3243.3 (967.4)	3852.2 (845.9)	3672.6 (314.6)	3679.4 (296.5)
	≥ median	6573.1 (2101.6)	6991.3 (1529.4)	4572.2 (422.2)	4850.0 (530.9)
oleic acid	< median	45939.9 (11711.6)	47191.4 (12749.5)	56327.0 (12694.6)	54107.8 (12206.9)
	≥ median	83203.0 (16105.8)	92450.0 (22296.8)	98210.2 (15334.8)	96632.8 (20054.6)
palmitic acid	< median	25222.8 (5963.9)	24828.0 (5043.9)	14774.5 (2448.1)	14392.4 (2316.8)
	≥ median	43709.5 (8755.8)	46357.9 (13344.3)	23282.6 (2917.5)	22176.2 (4098.4)

Values are mean (SD). DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. HA, hippuric acid. MDMS, Mediterranean diet metabolomic score. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 3C, Three-City. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate

Table S4. Dietary intake of MD key food groups (FFQ) and nutrients (24-HDR) in subjects from the Bordeaux cohort (n = 351)

MD key food groups	median (p25, p75)
Plant-based foods (serv/wk)	54.0 (46.0, 63.0)
Vegetables (serv/wk)	18.0 (14.0, 24.0)
Fruits (serv/wk)	14.0 (9.8, 17.0)
Legumes (serv/wk)	0.5 (0.3, 1.0)
Cereals (serv/wk) ^a	23.0 (18.0, 25.0)
Fish and seafood (serv/wk)	2.5 (1.5, 3.5)
Dairy products (serv/wk)	14.0 (13.0, 20.0)
Ratio of MUFAs/SFAs (g/d) ^b	0.8 (0.6, 1.0)
MUFAs (g/d)	19.4 (13.1, 27.0)
SFAs (g/d)	24.6 (16.7, 34.1)

MD, Mediterranean diet. SFAs, saturated fatty acids. g/d, grams/day. serv/wk, servings/week. 24-HDR, 24 h dietary recall. ^a Cereals included cereals (including bread), pasta, and rice. ^b Data available from 359 subjects from the Bordeaux set.

Table S5. Correlations between intakes of MD food groups (FFQ) and nutrients (24-HDR) and serum dietary biomarkers in subjects from the Bordeaux cohort (n = 351)

MD key food group	serum metabolite	ρ coefficient ^c	<i>p</i>
Plant-based food group: vegetables, fruits, legumes, and cereals ^a	EL; HA; proline betaine; trigonelline; UroA-G; 3,4-DHBA; 3,4-DHPAA-S and 3,4-DHPV-S ^[33-43]	0.155	0.004
Fish and seafood	DHA and EPA ^[44]	0.240	< 0.001
Dairy products	margaric acid and pentadecanoic acid ^[45,46]	0.070	0.189
MUFAs & SFAs ^b			
MUFA intake	oleic acid ^[47,52]	0.110	0.037
SFA intake	palmitic acid ^[47]	0.168	0.001

DHA, docosahexaenoic acid. EL, enterolactone. EPA, eicosapentaenoic acid. FFQ, food frequency questionnaire. HA, hippuric acid. MD, Mediterranean diet. SFAs, saturated fatty acids. UroA-G, 3,8-dihydroxy-urolithin glucuronide. 24-HDR, 24 h dietary recall. 3,4-DHBA, 3,4-dihydroxybenzoic acid. 3,4-DHPAA-S, 3',4'-dihydroxyphenylacetic acid sulfate. 3,4-DHPV-S, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone sulfate. ^a Cereals included cereals (including bread), pasta, and rice. ^b Data available from 359 subjects from the Bordeaux set. ^c Correlations between total intake of each MD key food group (serv/wk) and nutrients (g/d), and the sum of their selected dietary biomarkers (μ g/L).

Table S6. Multivariate conditional logistic regression models for association between MDMS and CD in the 3C discovery (Bordeaux, n = 418) and validation (Dijon, n = 422) cohorts in various subgroups. OR (95% CI)

Discovery set				Validation set			
variables	cases/controls	OR (95% CI)	<i>p</i> for interaction	variables	cases/controls	OR (95% CI)	<i>p</i> for interaction
Sex			0.763	Sex			0.954
Male	71/71	0.85 (0.59–1.23)		Male	78/78	0.91 (0.64–1.28)	
Female	138/138	0.88 (0.61–1.27)		Female	133/133	0.91 (0.64–1.29)	
Age, y			0.720	Age, y			0.719
< 75.9 (median)	105/104	0.88 (0.65–1.21)		< 76.5 (median)	106/105	0.94 (0.67–1.31)	
≥ 75.9	104/105	0.89 (0.65–1.22)		≥ 76.5	105/106	0.92 (0.66–1.29)	
Educational level			0.256	Educational level			0.799
< secondary school	148/149	0.86 (0.76–0.98)		< secondary school	151/151	0.91 (0.80–1.02)	
≥ secondary school	61/60	0.99 (0.87–1.12)		≥ secondary school	60/60	0.93 (0.83–1.05)	
BMI, kg/m ²			0.214	BMI, kg/m ²			0.891
< 26.1 (median)	99/112	0.82 (0.59–1.14)		< 25.1 (median)	98/113	1.00 (0.71–1.40)	
≥ 26.1	110/97	0.87 (0.63–1.21)		≥ 25.1	113/98	0.94 (0.67–1.33)	
Polypharmacy (≥ 5)			0.097	Polypharmacy (≥ 5)			0.168
No	98/126	0.98 (0.73–1.33)		No	77/136	1.19 (0.85–1.65)	
Yes	111/83	0.93 (0.68–1.25)		Yes	134/75	0.99 (0.71–1.38)	
Diabetes			0.627	Diabetes			0.223
No	182/197	0.90 (0.81–1.01)		No	184/199	0.93 (0.84–1.04)	
Yes	27/12	0.82 (0.73–0.92)		Yes	27/12	0.76 (0.68–0.84)	
ApoE-ε4 carrier			0.138	ApoE-ε4 carrier			0.415

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No	155/184	0.92 (0.82–1.03)		No	155/167	0.89 (0.79–1.00)	
Yes	54/25	0.73 (0.65–0.81)		Yes	56/44	0.99 (0.88–1.11)	
Hypertension			0.006	Hypertension			0.540
No	45/50	1.19 (0.95–1.50)		No	34/36	0.98 (0.76–1.27)	
Yes	164/159	0.83 (0.66–1.05)		Yes	177/175	0.90 (0.70–1.16)	
Smoking status			0.827	Smoking status			0.675
Never	141/136	0.90 (0.80–1.02)		Never	129/136	0.91 (0.80–1.04)	
Former or current	68/73	0.88 (0.78–0.99)		Former or current	82/75	0.90 (0.79–1.03)	
Depression			0.034	Depression			0.272
No	189/194	0.86 (0.76–0.97)		No	164/186	0.89 (0.80–1.00)	
Yes	20/15	1.31 (1.16–1.47)		Yes	47/25	1.06 (0.94–1.18)	
Alcohol intake, g/d			0.526	Alcohol intake, g/d			0.182
< 9.9 (median)	113/121	0.95 (0.69–1.30)		< 9.6 (median)	90/87	1.13 (0.80–1.60)	
≥ 9.9	96/88	0.91 (0.66–1.25)		≥ 9.6	121/124	0.99 (0.70–1.40)	
Hypercholesterolemia			0.780	Hypercholesterolemia			0.414
No	82/81	0.91 (0.78–1.06)		No	85/92	0.96 (0.82–1.12)	
Yes	127/128	0.88 (0.76–1.03)		Yes	126/119	0.88 (0.75–1.03)	

ApoE-ε4, Apolipoprotein E-ε4 genotype. CI, confidence interval. OR, odds ratio. 3C, Three-City.

The cognitive decline (CD) variable was built defining the composite score of global cognition at each follow-up visit.^[33,77] Individual slopes of cognitive change were then evaluated using linear mixed models, as detailed in the previous publication.^[77] Cases were defined as the participants with the worst CD slopes.

P-values for interaction were calculated by adding product terms in the fully adjusted conditional logistic regression models. Each subgroup analysis was adjusted, if not stratified, for BMI, diabetes, alcohol intake, hypertension, smoking status, depression, hypercholesterolemia, ApoE-ε4, and number of medications.

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5. **General discussion**

It is well known that people across the globe are living longer. By 2030, for the first time in recorded history, the old will begin to outnumber the young (United Nations - Department of Economic and Social Affairs, 2019). However, in many cases, these latter years are accompanied by a greater burden of disease and declines in intrinsic capacity (World Health Organization, 2015).

In recent decades, researchers have identified factors that, alone or combined, have the potential to improve health and functioning, and avert age-related conditions. In this sense, evidence suggest that, even among old people, a healthy diet promotes lower risk of major chronic disease and mortality, and overall wellbeing (Afshin et al., 2019; Shan et al., 2023).

The primary advantage of studying dietary patterns appears to lie in the synergy among combinations of different nutrients, food components and foods, instead of the isolated factors themselves (F. B. Hu, 2002; Jacobs & Steffen, 2003). Moreover, the physical and chemical interactions between and within the food matrices are essential to comprehend their meaningful health effects (Aguilera, 2019).

Within this context, the MedDiet is of particular interest since it has been associated with reduced risk of all-cause mortality, CVD, overall cancer, neurodegenerative diseases, and diabetes incidence in several observational cohort studies and clinical trials (Dinu et al., 2018). This dietary pattern has traditionally been characterized by a high intake of vegetables, legumes, fruits, nuts, cereals, and olive oil but a moderately high intake of fish, a low-to-moderate intake of dairy products, a low intake of saturated fat, meat, and poultry, and a regular but moderate intake of ethanol, primarily in the form of wine and generally during meals (Willett et al., 1995).

Various types of self-report instruments (e.g., 24HR and FFQ) have been developed for the assessment of food intake to obtain more reliable evidence for food-health relationship. While these methods are error-prone due to recall bias and measurement error, the use of BFI, measured in biological samples, may provide a more objective estimate of actual intake. In this context, the application of nutritional metabolomics holds promise for the discovery of BFIs and, consequently, for the development of a robust and unbiased strategy for measuring adherence to dietary patterns, such as the MedDiet (Ulaszewska et al., 2019).

The understanding of the discovery of bioactive compounds and BFIs using metabolomics and its related tools was conducted in this thesis (**Objective #1**) with a special focus on legumes, a sustainable and inexpensive protein food source from the MedDiet with age-related health protective effects (Martini et al., 2021; Willett et al., 2019; Zargarzadeh et al., 2023). The research presented a comprehensive reviewed analysis of phytochemical content in pulses (i.e., lentils, beans, chickpeas, soybean, and peas), wherein phenolic compounds depicted as the most extensively described compounds. In parallel, phytic acid, saponins, phytosterols, tocopherols, lignans, phenolic acids, isoflavones, flavonols, and flavanols were reported to have been identified within all types of legumes, and 14 compounds (mostly phytosterols and isoflavones) were found to be distributed among all groups. By employing metabolomic tools, qualitative differences among legumes were visualized for the first time. Notably, beans exhibited the most differentiated profile primarily due to their content in flavonols, while lentils, chickpeas, and peas revealed a closer portrayal (on account of phenolic acids, isoflavones, and flavonols). Moreover, niches for further research were identified since some types of legumes - i.e., peas and chickpeas - showed a smaller number of identified phytochemicals. Future endeavors should also focus on comprehensively elucidating the characterization of less-identified compounds, such as tocopherols and non-phenolic compounds.

This qualitative analysis was further complemented by a randomized crossover postprandial intervention study aimed at identifying reliable BFIs of pulses. Numerous metabolites were associated with the intake of some of the most consumed pulse varieties (i.e., lentils, chickpeas, and white beans). For instance, urinary levels of trigonelline were increased after intake of the three types of pulses. Of note, beans and peas were reported to contain alkaloids in the preceding qualitative review. Other discriminant metabolites increased in urine after pulse intake were tentatively identified as phloroglucinol glucuronide and phloroglucinol sulfate. Interestingly, phloroglucinol was detected only in lentils food test while their derivatives were described in the previous work in beans, lentils, peas, and peanuts. In addition, some phytosterols (i.e., stigmasterol, campesterol, and β -sitosterol) were depicted in the test foods from the intervention as well as in all pulses in the review. γ -tocopherol was also described in legumes from both works, which was in good agreement with the identification of urine γ -carboxyethyl hydroxychroman conjugates (tocopherol-derived molecules) in the intervention (Lodge et al., 2000).

Nonetheless, it was difficult to identify discriminant biomarkers for individual pulse types as most biomarkers were shared among two or three varieties. The study showed that catechins were particular to lentils and that their derived metabolites (i.e., γ -valerolactones) were observed only in urine as a result of host and gut metabolism, which slightly differs from what was reported in the review. According to the previous work, catechins were described in all legumes, and condensed tannins were reported in all legumes except chickpeas and peas. Urinary vanillic acid also achieved higher levels after the intake of lentils, while in the review it was depicted in beans, chickpeas, lentils, and peanuts. Notably, vanillic acid was not detectable in the test foods in the GCxGC-MS analyses. Furthermore, while the intervention study found no potential BFIs for chickpeas intake but an elevated urine level of protocatechuic acid glucoside, the review described up to 6 putatively discriminant bioactive compounds, mainly isoflavones. Of note, protocatechuic acid and its derivatives were described as potential discriminant phytochemicals of beans, peanuts, lentils, and peas in the previous review, although it was not identified in any test food from the intervention. Regarding dry beans, nonprotein nitrogen components and jasmonic derivatives appeared to be particular to white bean intake although jasmonic acid was not detectable in the test foods. Since these classes of compounds were dismissed from consideration as phytochemicals in the qualitative analysis, it is not possible to make comparisons between both studies.

Adherence to the MedDiet has usually been evaluated through dietary surveys and derived *a priori* scores, which reflect the complexity of diet through the quantification and weighing of its individual components. A simple tool developed and validated within the PREDIMED study group is the MEDAS questionnaire, which conveniently determines adherence to the traditional MedDiet in older Spanish adults (Schröder et al., 2021). This tool was employed in this thesis to account for the relationship between a healthy diet and functional status in hospitalized adults aged over 75 (**Objective #2**). Among older subjects, acute medical conditions necessitating hospitalization often lead to subsequent impairments in cognitive and physical function which span multiple domains including independent living and the ability to perform basic ADLs (e.g., bathing, dressing, walking, feeding, etc.). The research focused on an exercise and health education intervention to prevent HAD (i.e., loss of the ability to complete one or more ADLs without assistance at hospital discharge), showing that it was more effective for those with low adherence to the MedDiet at baseline. This was in line with previous works which reported interindividual variability in the responsiveness to exercise intervention in acutely hospitalized older adults (Abizanda et al., 2015; Franzke et al., 2019; Valenzuela, Ortiz-Alonso, et al., 2020).

Specifically, older patients with poorer nutritional status or no nutritional supplementation were those with larger functional status improvement. We hypothesized that patients reporting low adherence to the MedDiet could present, in turn, other unmeasured habits that would have jointly induced to deficits in different health areas. By having a worse general condition, the margin of improvement could be greater than in those reporting high adherence when performing the intervention of exercise and health education.

In this study, the MedDiet adherence was assessed also through UTP levels, and comparable results were obtained. Although no studies have evaluated levels of UTP in acutely hospitalized older patients, findings from the InCHIANTI cohort indicated that increasing UTP levels were linked to reduced frailty and disability prevalence (Urpi-Sarda et al., 2015). Furthermore, elevated UTP concentrations were associated with a decreased risk of physical performance decline and mortality over a nine-year follow-up period (Rabassa et al., 2016; Zamora-Ros et al., 2013). The similarities between the results obtained with both MedDiet assessment methods provide an impetus for the adoption of more objective measurements, a step that not only enhances the precision of exposure assessment but also encompasses considerations of compound bioavailability and their potential interactions (Xu et al., 2021). The UTP is a measurement of total antioxidant capacity and might be a better estimation of polyphenol exposure than dietary assessment (Urpi-Sarda et al., 2015). Moreover, total antioxidant capacity in urine has been positively correlated with daily intake of fruits, vegetables, nuts, and olive oil, and consequently, with adherence to the MedDiet (Ruiz et al., 2019).

On the other hand, the number of older people living with dementia is rising worldwide. The trajectory of CD is established by a complex interplay of genetic and environmental factors (World Health Organization, 2021). In spite of controversies still raging around, a growing body of evidence supports nutrition overall as a potentially modifiable risk due to its potential ability to reduce the risk of age-related cognitive pathologies (Dalile et al., 2022).

A key element of healthy dietary patterns, such as the MedDiet, is the inclusion of protein sources primarily from legumes, nuts, and fish (Willett et al., 2019). The consumption of fish has been linked to a reduced risk of AD and dementia. Nevertheless, mixed results have been reported concerning CD, particularly among older adults. Therefore, the third objective of this thesis focused on evaluating the relationship between fish intake and CD in community-dwelling older adults (**Objective #3**). A total of 70% of the participants in the InCHIANTI Study consumed at least one serving of fish per week, which was slightly higher than the Italian global reference

population aged 65 or older (Italian National Institute of Statistics, 2005). The findings suggested a potential protective role of fish consumption against CD (defined as $\geq$ a 3-point decline in MMSE between two consecutive follow-up evaluations), particularly in the context of a Mediterranean population. Furthermore, while literature continues to accumulate around the idea that ApoE genotype influences cognitive and neurological responses to glucose and lipid substrates (Farmer et al., 2019), results from this study aligned with some prior research, supporting the hypothesis that the relationship between fish intake and cognition likely remains consistent despite inherited susceptibility to AD (Keenan et al., 2020; Samieri et al., 2018).

The study also delved into potential mechanisms underlying this relationship and explored the role of CVD and omega-3 fatty acids. Although these factors are known to influence cognitive health (Qiu & Fratiglioni, 2015; Shahidi & Ambigaipalan, 2018), no conclusive evidence of a mediation effect was found. This fact could be accounted for in part by several factors, including the lack of correlation between reported PUFAs and circulating EPA and DHA at baseline, the heterogenous profile of FAs reported as PUFAs, and the varied content of EPA and DHA in fish. Moreover, the putative neuroprotective effect of fish may derive not only from long-chain omega-3 FAs but from a combination with other nutritional components from fish and seafood such as vitamin B₆, vitamin B₁₂, vitamin D, and vitamin E (Scarmeas et al., 2018).

Regarding the advantages of research on dietary patterns, current evidence suggests a stronger link between cognitive outcomes and healthy dietary patterns, such as the MedDiet, as compared to individual nutrients or food groups (Scarmeas et al., 2018). This could be attributed to the synergistic benefits derived from the diverse array of ingredients within these diets. Moreover, evidence pertaining to the effects of fish and other food groups on cognitive function are mostly restricted to specific subcomponents (ingredients within that food group) (Dalile et al., 2022).

For this reason, and in pursuit of more robust evidence, nutritional researchers have taken a novel approach by investigating biomarkers as indicators of dietary patterns, rather than isolated nutrients or dietary components. While limited research has explored metabolomic signatures associated with the MedDiet using predictive and discriminant analysis, some investigations have also explored their associations with health outcomes, including CVD and its risk factors (Almanza-Aguilera et al., 2017; Galié et al., 2021; J. Li et al., 2020; McCullough et al., 2019; Sobiecki et al., 2023; Tong et al., 2020; Vázquez-Fresno et al., 2015). However, there is no *a priori* MedDiet score based on serum biomarkers of its key food groups published to date.

With this in mind, an *a priori* dietary metabolomic score was developed selecting a set of serum phytochemical and fatty acid metabolites that reflect not only the individual bioavailability but also the protective effects on health attributed to the MedDiet (Schwingshackl et al., 2020) (**Objective #4**). Moreover, certain metabolites were chosen based on the knowledge derived from prior works incorporated in this thesis, such as trigonelline and 3,4-DHPV-S, which have been identified as putative biomarkers of legume intake. In line with expectations, the MDMS showed a weak correlation with the MDS, as did most of the MedDiet key food groups with their representative metabolites. This may be attributable to the variability of phytochemical or fatty acid content through a food group or a specific product resulting from differences in processing, harvesting or between species (Münger et al., 2018; U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020), the presence of the selected metabolites in other food sources not included in the MedDiet key food groups (e.g., coffee, tea, and cocoa products) (Wishart et al., 2022), as well as to the existence of other metabolites that could contribute to the real content of a MedDiet key component (i.e., MUFAs and SFAs). The weakness of correlation reveals the need for better dietary definition and accuracy. For instance, in the MDS defined by Trichopoulou et al., the dairy products are presumed to be detrimental on account of their fat content, but no distinction based on this is made during the dietary assessment (Trichopoulou et al., 2003).

The MDMS was found to be inversely associated with CD in a discovery set of community-dwelling older people and results were comparable in the validation cohort, with a trend towards significance. Validation is important but has rarely been carried out in prior nutritional metabolomics studies, especially in nutrimental metabolomics and cognitive impairment research due to inherent heterogeneity across populations (Naska et al., 2017; Newgard, 2017) and the complexity of outcomes based on long-term trajectories (e.g., CD and dementia). Our validation sample differed from the discovery sample, which may have explained the weaker association found in the validation sample compared to the discovery one, though both showed a positive relationship with a higher MDMS, indicating a protective effect.

The findings derived from this doctoral thesis offer substantiating evidence that adherence to a healthy diet pattern rich in phytochemicals and unsaturated fatty acids, such as the MedDiet, may exert a contributory effect in preventing functional and cognitive decline in older people. In addition, these results underscore the need to refine nutritional metabolomic methodologies for their full application in forthcoming research.

6. Conclusions

This thesis has studied the effect of the Mediterranean diet and its components on cognitive and functional ageing through dietary assessment instruments and nutrimentalomics. The primary conclusions of this doctoral thesis are presented below for each of the stated objectives:

- i. A phytochemical footprint from different varieties of legumes was described from literature. Phenolic compounds comprised the main group of phytochemicals reported. Metabolomics data analysis tools were used for the first time for visualizing qualitative differences between legumes. Beans showed the most differentiated profile, while lentils, chickpeas, and peas revealed a high number of common compounds. Future research should focus on the characterization of underrepresented legumes and compounds.
- ii. A broad list of metabolites, identified through untargeted metabolomics, were candidates to be used as biomarkers of legume intake. Some metabolites and their families were found to be specific for certain pulses, while others appeared to be more generic indicators of legume consumption. On the other hand, some metabolites derived from pulse intake were consistent with the analysis of food tests. The next step would be the development and application of a quantitative method for the compounds identified in this work. Additionally, it is essential to validate these candidate biomarkers using larger subject samples and under different experimental conditions, including free-living conditions.
- iii. Hospitalized older individuals over age 75 with low Mediterranean diet adherence were likely to benefit more from a physical exercise and health education intervention than those with high values. Adherence to the Mediterranean diet could represent a potential indicator of those older patients aged over 75 with a putative better response to exercise interventions. Therefore, the inclusion of Mediterranean diet in the variables assessed at hospital admission of older patients could allow more accurate and personalized strategies to be designed with a view to preventing hospital-associated disability.
- iv. In a population-based prospective cohort study including participants aged ≥ 65 years from a Mediterranean region, higher consumption of fish was associated with lower

cognitive decline risk over 15 years of follow-up. However, no evidence of any mediation effect from either polyunsaturated fatty acids or incidence of cardiovascular disease was found. Further studies are required to enable a better comprehension of the mechanisms involved in the neuroprotective role of fish and seafood.

- v. A greater adherence to the Mediterranean diet assessed by a novel Mediterranean diet metabolomic score was associated with a lower risk of cognitive decline in older adults free of dementia at baseline during a 12-year follow-up. The development of dietary metabolic scores based on dietary patterns may help further refine dietary assessment measures and will hopefully contribute to a better understanding of the biological mechanisms via which diet impacts cognitive health in the aging population.

The results obtained in this doctoral thesis provide evidence that adherence to a healthy diet pattern rich in phytochemicals and unsaturated fatty acids, such as the Mediterranean diet, may contribute to the prevention of cognitive and functional decline in older people. In addition, these findings reinforce the need to refine nutritional metabolomic techniques for their full and promising application in future research on dietary patterns.

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8. Appendices

8.1. Supplementary tables

The tables summarizing the characteristics and results of the papers included in the *Biomarkers of polyphenol intake and cognitive function in older adults* section (1.3.3.1) are shown below:

Table A.1. Summary of the characteristics, designs and results of the experimental-based studies reviewed.

Reference (year)	Study design / duration / N (% women)	Subjects' characteristics / Age (SD)	Intervention	Cognitive measurements	Polyphenol measurements	Main results
Ringman et al. (2012)	Randomized, double-blind, placebo-controlled trial 6 m. (with open-label extension to 12 m.) 30 subjects Int 1: 9 (67%) Int 2: 10 (70%) Ctrl: 11 (55%)	Elderly subjects with mild-to-moderate AD 73.9 y. Int 1: 76.7 (5.6) y. Int 2: 75.3 (6.9) y. Ctrl: 70.2 (12.4) y.	Int 1: 2 g/day Curcumin C3 Complex® capsules Int 2: 4 g/day Curcumin C3 Complex® capsules Ctrl: placebo capsules	<i>At baseline and at the 6- and 12- m. visits</i> <i>Primary outcome:</i> - ADAS-Cog <i>Secondary outcomes:</i> - NPI - ADCS-ADL scale - MMSE	Plasma levels of Cu, THCu, Gluc-Cu, Gluc-THCu Use of LC/MS/MS 6-m. mean concentration: Cu: 2.67 ± 1.69 ng/mL THCu: 6.87 ± 4.91 ng/mL 3-h after administration: Cu: 7.76 ± 3.23 mg/mL THCu: 3.73 ± 2.0 ng/mL Gluc-Cu: 96.05 ± 26 ng/mL Gluc-THCu: 298.2 ± 140.04 ng/mL	- No differences in ADAS-Cog, NPI, ADCS-ADL, or MMSE scores between groups.
Gleason et al. (2008)	Randomized, double-blind, placebo-controlled, pilot trial 6 m. 30 subjects Int: 15 (46.7%) Ctrl: 15 (53.3%)	Elderly nondemented subjects 73.7 y. Int: 73.0 (7.9) y. Ctrl: 74.3 (6.3) y.	Int: 100 mg/day purified isoflavone glycosides (85% daidzin and genistin; Novasoy® brand isoflavones) capsules Ctrl: placebo tablets	<i>At baseline and at the 1-, 3-, 6- and 8- m. visits</i> - BSRT - Paragraph Recall Test - RCFT - VSLT - BNT - FAS word fluency test (letter fluency) - Animal fluency test (category fluency)	Plasma levels of total isoflavones, genistein, daidzein and equol (<i>baseline and at the 1- and 6- m. visits</i>) Use of GC-MS (enzymatic hydrolysis of plasma) Total isoflavones Int: 1-6m: $.224.6 (188.6) - 345.8 (183.5)$ nmol/L Ctrl: 1-6m.: $8.4 (2.7) - 8.6 (3.8)$ nmol/L Genistein	- Significant increase of plasma isoflavone levels between placebo and intervention group at 1- and 6-m. visits - Better performance in the following tests among isoflavone-treated group compared to placebo group: ▪ RCFT ▪ Category fluency-test (m. 3 and 6 only)

Gleason et al. (2015)	Randomized, double-blind, parallel-group trial	Elderly subjects with AD	Int: 100 mg/day purified isoflavone glycosides (85% daidzin and genistin; Novasoy® brand isoflavones) capsules Ctrl: placebo capsules	<i>At baseline and at the 3- and 6-m. visits</i>	- RCFT copy - GPT - SCWT - Mazes - TMT-B	Int: 1-6m.: 173.5 (115.0) – 186.5 (105.3) nmol/L Ctrl: 1-6m.: 2.8 (1.6) – 3.1 (2.5) nmol/L Daidzein Int: 1-6m.: 157.6 (88.8) – 154.9 (84.4) nmol/L Ctrl: 1-6m.: 2.8 (1.3) – 2.9 (1.5) nmol/L Equol Int: 1-6m.: 4.7 (5.6) – 3.4 (3.7) nmol/L Ctrl: 1-6m.: 2.8 (3.7) – 2.7 (0.2) nmol/L Plasma levels of genistein, daidzein and equol (at baseline, and at the 3- and 6- m. visits) Use of GC-MS (enzymatic hydrolysis of plasma)	▪ GPT - Better performance in the following tests among placebo group compared to isoflavone-treated group: ▪ TMT-B ▪ SCWT
					- MMSE - List Learning - Paragraph Recall Test - BVRT - Complex Figure Recall - Phonemic fluency test - Animal fluency test - Digit Symbol - Digit Span - SCWT - Mazes - TMT-A; TMT-B	Int: 3-6m.: 277.4 (188.4) – 338.6 (308.8) ng/mL Ctrl: 3-6m.: 2.1 (3.7) – 15.6 (35.4) ng/mL Daidzein Int: 3-6m.: 197.3 (150.5) – 206.6 (156.8) ng/mL Ctrl: 3-6m.: 1.1 (2.3) – 10.6 (37.8) ng/mL Equol Int: 3-6m.: 20.3 (50.7) – 19.6 (40.5) ng/mL	- Significant increase in plasma isoflavone concentrations in intervention group. - No significant differences in MMSE between groups. MMSE decline in both groups. - Significant correlation between total plasma equol, GPT and Phonemic Fluency.

John et al. (2014)	Single-centre, randomized, double- blind, placebo- controlled trial 2.5 y. 300 subjects Int: 150 (100%) Ctrl: 150 (100%)	Elderly healthy postmenopausal women 61.0 y. Int: 61.0 (7.0) y. Ctrl: 61.0 (7.0) y.	Int: 25 g/day isoflavone-rich soy protein (91 mg of aglycone isoflavones: 52 mg genistein, 36 mg daidzein, 3 mg glycitein) Ctrl: milk protein- matched placebo	- Complex Figure Copy - GPT <i>At baseline and at the 2.5-year visit</i> - WTAR - TMT-B - BNT - CVLT, immediate recall - CVLT, delayed recall - Faces I, immediate recall - Faces II, delayed recall - SDMT - Shipley Abstraction Test - Letter-Number Sequencing, from WAIS test - Block Design, from WAIS test - Judgment of Line Orientation - Category fluency - EBMT, immediate recall - EBMT, delayed recall	Ctrl: 3-6m.: 0.2 (0.5) – 0.1 (0.6) ng/mL Urine excretion and plasma levels of isoflavones (at baseline and at the 6- m. visit) Use of LC-MS Genistein, daidzein, glycitein, equol, dihydrogenistein, dihydrodaidzein, o- desmethylangolensin	- No association between baseline overnight urine individual isoflavones, total isoflavones and their metabolites and baseline measures of global cognition or cognitive factor score. - No significant association between change in overnight urine individual isoflavones, total isoflavones, and their metabolites and change in the global cognition score or changes in the verbal and visual episodic memory factors. - Inverse association between change in overnight urine individual isoflavones, total isoflavones, and their metabolites and change in the general intelligence factor. Greater magnitude of association in women
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						who had undergone surgical menopause than in those experiencing natural menopause. - Similar but attenuated results in plasma. - In <i>post-hoc</i> analyses, SDMT, TMT-B, Shipley Abstraction Test, Letter-Number Sequencing, Block Design, Category fluency and BNT showed non-significant negative associations with change in isoflavones and their metabolites. Larger effects were seen for Letter-Number Sequencing, Category fluency, Shipley Abstraction Test and TMT- B. - No significant difference in cognitive tasks between dose-timing groups. - No significant acute effect on cognition. - No significant differences in the excretion rates of
Igwe et al. (2017)	randomized, crossover, bioequivalence/non-inferiority, pilot trial 3 hours on 2 different occasions with a 2-wk washout period	Elderly healthy subjects 77.0 (6.0) y.	Int 1: 1 x 300 mL QGP juice at baseline, 1 and 3 hours (369 mg anthocyanins) Int 2: 3 x 100 mL QGP juice at baseline, 1 and 3 hours (123 mg anthocyanins/serving)	<i>At baseline and at the 6-hour on each study day (2-wk washout period)</i> - TMT - RAVLT	Urine excretion of anthocyanins (at baseline and at the 0-2, 2-6, 6-12 and 12-24 hours after QGP juice intake on each study day) Use of HPLC-DAD/MS	

	12 subjects (75%)			- Pattern and Letter Comparison task - Reaction task - Stroop task	Sum of cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-glucoside monoglucuronide, cyanidin monoglucuronide, peonidin-3-glucoside, peonidin monoglucuronide and pelargonidin monoglucuronide 1 x 300 mL (369mg): 871 (602) µg/24h 3 x 100 mL (123mg/dose): 693 (458) µg/24h	native QGP anthocyanins and their metabolites profile between dose-timing groups.
McNamara et al. (2018)	randomized, double-blind, placebo-controlled trial 76 subjects Int 1: 19 (57.9%) Int 2: 17 (41.2%) Int 3: 20 (65%) Ctrl: 20 (50%)	Elderly subjects with mild, self-perceived CD with aging 68.0 y. Int 1: 68 (3.9) y. Int 2: 69 (5.2) y. Int 3: 68 (4.7) y. Ctrl: 67 (4.9) y.	Int 1: 2 x 12.5 mg blueberry powder/day (417 GAEs, 269-mg C3G eq.) Int 2: 4 x fish oil capsule (400-mg EPA + 200-mg DHA/dose) Int 3: Int 1 + Int 2 Ctrl: placebo packets and corn oil capsules	<i>At baseline and at the 6- and 12-m. visits</i> - DEX - TMT-A; TMT-B - Controlled Oral Word Production - HVLT	Urine excretion of anthocyanins (at baseline and at the 3- m. visit) Use of LC-MS/MS deglycosylated glucuronidated anthocyanins (approximately 60% of total), glycosidic forms of anthocyanins (2% of total), and the native (food) form of anthocyanins (0.2% of total). total anthocyanins (100%) Int 1: 535 (wk 0) 468 (wk 12) ng/mg Cr	- No differences in urinary anthocyanin and anthocyanin aglycone glucuronide conjugates excretion among the 4 groups over intervention. - Decrease in glycoside forms of anthocyanins over 12 wk in the placebo groups but no change in the 2 BB-supplemented groups. - Fewer cognitive symptoms in the BB-treated group reported

Int 2: 411 (wk 0) 469 (wk 12) ng/mg Cr	(DEX test) at 24-wk and 48-wk.
Int 3: 403 (wk 0) 396 (wk 12) ng/mg Cr	- Improved recognition memory (HVLT-discrimination test) in the BB-treated group at 24-wk but not at 48-wk.
Ctrl: 570 (wk 0) 342 (wk 12) ng/mg Cr	- No effect in any cognitive domain for the combined fish oil and BB-treated group at 24-wk.
aglycone conjugates (65%)	
Int 1: 353 (wk 0) 310 (wk 12) ng/mg Cr	
Int 2: 267 (wk 0) 291 (wk 12) ng/mg Cr	
Int 3: 265 (wk 0) 290 (wk 12) ng/mg Cr	
Ctrl: 372 (wk 0) 207 (wk 12) ng/mg Cr	
glycoside anthocyanins (2%)	
Int 1: 9.67 (wk 0) 9.40 (wk 12) ng/mg Cr	
Int 2: 11.43 (wk 0) 6.58 (wk 12) ng/mg Cr	
Int 3: 7.45 (wk 0) 9.40 (wk 12) ng/mg Cr	
Ctrl: 16.90 (wk 0) 5.12 (wk 12) ng/mg Cr	
native form of anthocyanins (0.2%)	
Int 1: 0.920 (wk 0) 1.37 (wk 12) ng/mg Cr	
Int 2: 0.390 (wk 0) 0.70 (wk 12) ng/mg Cr	
Int 3: 0.980 (wk 0) 1.86 (wk 12) ng/mg Cr	

Bookheimer et al. (2013)	randomized, double-blind, placebo-controlled trial	Non-demented, elderly right-handed subjects with self-reported age-related memory complaints	Int: 236.59 mL PJ/day Ctrl: placebo drink	<i>At baseline and at the 28- day visit</i>	Ctrl: 0.230 (wk 0) 0.460 (wk 12) ng/mg Cr Plasma levels of Uro-A-glucuronide (at baseline and at the 1- m. visit) Use of LC-MS	- Significantly better memory performance after intervention in PJ group, but not in placebo group.
	1 m. 28 subjects Int: 15 (73.3%) Ctrl: 13 (76.9%)	62.7 y. Int: 63.1 (8.0) y. Ctrl: 62.0 (7.8) y.		- Buschke-Fuld selective reminding test - Experimental unrelated word pair associates learning task, based on the Wechsler memory scale paired associates learning - Functional MRI with: - Verbal memory task - Visual memory task	Int: unpaired t (baseline vs day 28) = 3.75; df = 24, $p < 0.001$ Ctrl: $p \geq 0.05$	- Greater t2 vs t1 improvement in total recall measure in the PJ group compared to the placebo group. - Greater consistent long-term retrieval score in the PJ group compared to the placebo group.
Bensalem et al. (2019)	bicentric, randomized, double-blind, placebo-controlled trial	Elderly subjects with lower level of memory performance	Int: 2 x 300 mg/day PEGB capsules (258 mg flavonoids) Ctrl: placebo capsules	<i>At baseline and at the 6- m. visit</i>	Urine excretion of phenolic metabolites (at baseline and at the 6- m. visit) Use of UHPLC-MS/MS	- Almost significant association between changes in urinary concentration of biomarkers of PEGB consumption (flavan-3-ols) and changes in the performances at the VRMFR test.
	6 m. 190 subjects (71.1%) Int: 92 Ctrl: 98	64.7 (2.9) y.		- CANTAB-PALTEA test - VRMFR - VRMR - SSP test - Reverse SSP test	(+)-catechin Ctrl: 0-6m: 9.2 (0.6) – 9.8 (0.7)	

Int: 0-6m: 9.4 (0.7) – 1,14.7 (7.2) $p < 0.001$ (-)-epicatechin Ctrl: 0-6m: 3.0 (0.5) – 3.3 (0.6) Int: 0-6m: 3.6 (0.6) – 38.6 (3.2) $p < 0.001$ (epi)catechin glucuronide Ctrl: 0-6m: 205.8 (16.2) – 211.9 (17.2) Int: 0-6m: 233.1(23.7) – 3,069.8 (206.39) (epi)catechin sulfate Ctrl: 0-6m: 726.6 (85.7) – 777.0 (88.1) Int: 0-6m: 749.6 (105.0) – 14,444.7 (935.7) $p < 0.001$ (epi)catechin glucuronide sulfate Ctrl: 0-6m: 112.2 (12.9) – 121.0 (11.8) Int: 0-6m: 124.6 (15.5) – 2,089.4 (131.8) $p < 0.001$ (epi)catechin methyl- glucuronide Ctrl: 0-6m: 84.4 (6.5) – 104.8 (11.9) Int: 0-6m: 91.0 (8.7) – 1,368.0 (83.29) $p < 0.001$	- Association between variation of urinary concentration of native and conjugated derivatives of (epi)catechins and increase in the immediate VRMFR total correct responses. - Association between variation of (epi)catechins and their conjugates and immediate VRMFR in decliners*. - Association of change in urinary concentration of PEGB biomarkers (flavan-3- ols), (epi)catechins, and their conjugated metabolites with the VRMFR-delayed recognition total correct responses.
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(epi)catechin methyl-sulfate

Ctrl: 0-6m: 1.345.4 (152.8)

– 1,558.2 (157.1)

Int: 0-6m: 1,463.2 (176.0)

– 14,412.1 (853.5)

$p < 0.001$

β -type procyanidins dimers

Ctrl: 0-6m: 2.5 (0.6) – 3.4 (0.7)

Int: 0-6m: 4.4 (1.0) – 30.9 (3.7)

$p < 0.001$

hydroxyphenyl- γ -valerolactone

Ctrl: 0-6m: 2.9 (0.9) – 7.3 (2.9)

Int: 0-6m: 4.1 (0.9) – 44.5 (5.3)

$p < 0.001$

dihydroxyphenyl- γ -valerolactone

Ctrl: 0-6m: 7,692.9 (669.4)

– 8,331.9 (753.4)

Int: 0-6m: 8,309.0 (937.7)

– 28,470.6 (1864.4)

$p < 0.001$

4-hydroxyphenylacetic acid

Ctrl: 0-6m: 585.5 (38.7) – 613.0 (52.1)

Int: 0-6m: 514.9 (42.9) – 810.1 (61.2)

Ochiai et al. (2019)	randomized, double-blind, placebo-controlled crossover trial	Elderly MCI patients 73.7 (6.0) y.	Int: 2 x 553.6 mg/day chlorogenic acids/100mL water Ctrl: placebo beverage	<i>At baseline and at the 12- and 28-wk visits (4-wk washout period)</i> - MMSE - ADAS-Cog - TMT-A - TMT-B	<i>p</i> < 0.001 3-hydroxybenzoic acid Ctrl: 0-6m: 8.3 (1.6) – 13.0 (2.6) Int: 0-6m: 6.5 (0.7) – 20.5 (5.4) <i>p</i> < 0.001 gallic acid Ctrl: 0-6m: 5.5 (1.1) – 4.2 (0.7) Int: 0-6m: 3.7 (0.7) – 13.7 (1.1) <i>p</i> < 0.001 Plasma levels of chlorogenic acids (at baseline and at the 12- and 28-wk visits) Use of LC-ESI-MS/MS 3-caffeoylquinic acid 0.5 ± 1.4 (Ctrl.) 0.6 ± 1.4 (Int.) ng/mL; <i>p</i> = 0.691 4-caffeoylquinic acid 0.7 ± 1.9 (Ctrl.) 0.9 ± 2.1 (Int.) ng/mL; <i>p</i> = 0.386 5-caffeoylquinic acid 1.0 ± 2.6 (Ctrl.) 1.6 ± 3.1 (Int.) ng/mL; <i>p</i> = 0.150 3-feruloylquinic acid 2.6 ± 4.7 (Ctrl.) 3.0 ± 6.5 (Int.) ng/mL; <i>p</i> = 0.705 4-feruloylquinic acid 2.1 ± 3.9 (Ctrl.) 1.9 ± 3.6 (Int.) ng/mL; <i>p</i> = 0.827 5-feruloylquinic acid	- Reduction in the number of TMT-B errors in the active beverage condition as compared with the placebo group after a 12-wk period. - No difference between groups in test completion time. - No significant change was between groups in the times taken to complete the MMSE, ADAS-Cog and TMT-A. - No significant correlation between change in blood chlorogenic acids levels and number of TMT-B

Rutledge et al. (2021)	randomized, double-blind, placebo-controlled, parallel study	Elderly healthy subjects 67.6 y. Int: 67.8 (4.6) y. Ctrl: 67.3 (4.8) y.	Int: 24 g/day lyophilized, cultivated blueberries (36 mg total phenolics, 19.2 mg anthocyanins) Ctrl: placebo powder	<i>At baseline and at the 45- and 90-day visits</i> - TST <i>At baseline and at the 90-day visit</i> - CVLT-II	1.0 ± 1.6 (Ctrl.) 1.5 ± 2.3 (Int.) ng/mL; $p = 0.264$ Dicafeoylquinic acids not detected Plasma levels of anthocyanins and phenolic acid metabolites (after overnight fast and after breakfast + dose at baseline and at the 45- and 90-day visits) Use of UHPLC-TQMS	errors before and after intake of the active.
					Intervention group by day interactions at fasting: Intervention group by day interactions at postprandial: Intervention group effects at fasting (baseline; day 45; day 90); $p \leq 0.05$: Hippuric acid Ctrl: 25.4 ± 7.2; 12.6 ± 3; 14.9 ± 3.7 Int: 20.2 ± 5.4; 80.8 ± 8.4; 71.1 ± 11.3 Intervention group effects at postprandial (baseline; day 45; day 90): Hippuric acid	- Negative association between change in ferulic acid-glucuronide and change in task switching errors. - Association between change in malvidin-3-O-galactoside and change in switching errors. - No significant association between syringic acid and change in switching errors. - Association between change in concentration of syringic acid and change in repetition errors. - Association between change in concentration of ferulic acid-glucuronide and change in repetition errors. - Negative association between change in hippuric acid and

Ctrl: 18.8 ± 3.3 ; 12.2 ± 2.5 ; 16.3 ± 3.8 change in repetition errors.
Int: 16.7 ± 4.3 ; 63.8 ± 5.7 ;

57.4 ± 8.5

Isovanillic acid

Ctrl: 114 ± 8.6 ; $113.9 \pm$

11.7 ; 106.9 ± 8.4

Int: 136.2 ± 11.9 ; $151.7 \pm$

16.1 ; 162.7 ± 17.5

Phloroglucinaldehyde

Ctrl: 3.6 ± 0.7 ; 2.8 ± 0.6 ;

3.3 ± 0.5

Int: 3.5 ± 1 ; 12.7 ± 2 ; 11.1

± 1.4

Syringic acid

Ctrl: 3.6 ± 1.9 ; 3.5 ± 1.4 ;

2.7 ± 0.9

Int: 3.9 ± 2.6 ; 91.3 ± 18.1 ;

71.3 ± 12.6

Trans-cinnamic acid

Ctrl: 24.9 ± 2.8 ; 28.3 ± 3.4 ;

24.5 ± 2.9

Int: 33.4 ± 2.6 ; 36 ± 6 ; 40.6

± 5.1

Ferulic acid-glucuronide

Ctrl: 58.4 ± 13.9 ; 40 ± 13.1 ;

38.9 ± 12.4

Int: 43.6 ± 10.4 ; 70 ± 15.3 ;

58.7 ± 13.8

Cyanidin-3-O-arabinoside

Ctrl: 0.5 ± 0.5 ; 0 ± 0 ; 0 ± 0

Int: 0.9 ± 0.9 ; 3.5 ± 1.9 ; 2.5

± 0.7

Cyanidin-3-O-galactoside

Ctrl: 0.8 ± 0.8 ; 0 ± 0 ; 0.7 ± 0.7

Int: 1.8 ± 1.8 ; 18.5 ± 6.2 ;
 11.2 ± 2.3

Cyanidin-3-O-glucoside

Ctrl: 0.4 ± 0.4 ; 0 ± 0 ; 0 ± 0

Int: 0 ± 0 ; 4.2 ± 2.2 ; 2.5 ± 0.9

Malvidin-3-O-arabinoside

Ctrl: 2 ± 2 ; 0 ± 0 ; 0 ± 0

Int: 1.9 ± 1.9 ; 9.9 ± 7.6 ; 7.8 ± 2.7

Malvidin-3-O-galactoside

Ctrl: 2.7 ± 2.7 ; 0 ± 0 ; 0 ± 0

Int: 2.8 ± 2.8 ; 29.5 ± 8.6 ;
 18 ± 4.5

Malvidin-3-O-glucoside

Ctrl: 1.7 ± 1.7 ; 0 ± 0 ; 0 ± 0

Int: 2 ± 2 ; 20.8 ± 8.3 ; 12.4 ± 3.5

Peonidin-3-O-galactoside

Ctrl: 0.4 ± 0.4 ; 0 ± 0 ; 0 ± 0

Int: 1.3 ± 1.3 ; 3.6 ± 3.1 ; 2.9 ± 2

Peonidin-3-O-glucoside

Ctrl: 0.5 ± 0.5 ; 0 ± 0 ; 0 ± 0

Int: 1.3 ± 1.3 ; 3.7 ± 2.4 ; 3.3 ± 1

Peonidin-3-O-xyloside

Ctrl: 0.5 ± 0.5 ; 0 ± 0 ; 0 ± 0

Int: 0 ± 0 ; 1.1 ± 0.5 ; 1.9 ± 0.5

Peonidin glucuronide

					Ctrl: 15.4 ± 15.4; 0 ± 0; 0 ± 0 Int: 0 ± 0; 150.7 ± 67.2; 41 ± 18.1 Petunidin-3-O-galactoside Ctrl: 1 ± 1; 0 ± 0; 0 ± 0 Int: 1.3 ± 1.3; 3.4 ± 1.9; 4.4 ± 1.2 Petunidin-3-O-glucoside Ctrl: 0.7 ± 0.7; 0 ± 0; 0 ± 0 Int: 0 ± 0; 2.6 ± 1.2; 1.4 ± 0.8	
Igwe et al. (2020)	randomized, crossover trial 4 m. with a 4-wk washout period 31 subjects Int→Ctrl sequence: 16 (63%) Ctrl→Int sequence: 15 (47%)	Elderly healthy subjects 70.0 y. Int→Ctrl sequence: 69.0 (10.0) y. Ctrl→Int sequence: 70.0 (4.8) y.	Int: 200 mL/day QGP nectar (3.7-5.3mg C3G eq./100mL) Ctrl: raspberry cordial beverage	<i>At baseline and at the 4-, 8-, 16- and 20-wk visits (4-wk washout period)</i> - RAVLT - Verbal fluency task - Digit-span backwards task - Stroop task - Counting span	Urine identification of anthocyanins and their common conjugated and methylated metabolites (at baseline and at the 4-, 8-, 16- and 20-wk visits) Use of UHPLC-PDA-ESI-MS/MS Cyanidin-3-glucoside Cyanidin monoglucuronide Cyanidin-3-rutinoside Peonidin monoglucuronide Peonidin-3-rutinoside	No significant effect of low anthocyanin on battery of cognitive tests.
Bergland et al. (2019)	open-label, pilot trial 4 m.	Elderly subjects with MCI or mild dementia and/or stable non-	Int: 4x 80 mg/day anthocyanin capsules (320 mg anthocyanins)	<i>At baseline and at the 16-wk visit</i>	Below the limits of quantification Plasma levels of anthocyanin metabolites (at baseline and at the 16-wk visit)	- Larger decrease of o-coumaric acid and dihydroferulic acid-4-O-sulfate in the

	38 subjects Int: 27 (33%) Ctrl: 20 (55%)	obstructive coronary artery disease 59.7 y. Int: 61.0 (11.1) y. [§] Ctrl: 58.0 (5.2) y. [§]	Ctrl: -	- CERAD battery (learning, recall and recognition) - TMT-A - TMT-B - Stroop Golden (word and color)	Use of LC-MS Dihydroferulic acid-4-O- sulfate -1.5 ± 0.7 [§] (Int.) 0.8 ± 0.95 [§] (Ctrl.) nmol/L; <i>p</i> = 0.01 o-Coumaric acid -116 ± 48.75 (Int.) -36 ± 27.5 (Ctrl.) nmol/L; <i>p</i> = 0.019	intervention group than in the control group. - Improvements in the intervention group for CERAD learning, recall and recognition and for STROOP test word and color.
Krikorian et al. (2020)	randomized, double- blind, placebo- controlled trial 4 m. 37 subjects Int: 16 (57%) Ctrl: 21 (53%)	Elderly subjects with MCI 77.1 y. Int: 77.3 (6.3) y. Ctrl: 77.0 (5.4) y.	Int: 2x 12 g/day whole freeze-dried blueberry fruit powder (401 GAEs, 258 mg C3G eq.) Ctrl: placebo powder	<i>At baseline and at the 16-wk visit</i> - TMT-A - TMT-B - Controlled Oral Word Association task - HVLT - SPAL - DEX	Urine levels of anthocyanins (after overnight fast and prior to the morning dose at baseline and at the 8-wk visit) Use of LC-MS/MS total anthocyanins Int: 1,841 (wk 0) 1,660 (wk 8) ng/mg Cr Ctrl: 2,290 (wk 0) 1,629 (wk 8) ng/mg Cr <i>p</i> ≥ 0.05 simple deglycosylated anthocyanins Int: 431 (wk 0) 359 (wk 8) ng/mg Cr Ctrl: 649 (wk 0) 360 (wk 8) ng/mg Cr <i>p</i> ≥ 0.05 major deglycosylated conjugates	- No between- or within-group difference in total urinary anthocyanins, including parent and metabolite forms. - Better SPAL and Controlled Oral Word Association task scores for the intervention group - Greater TMT-A trend for the intervention group. - No significant differences in TMT-B, HVLT and DEX. - Correlation between effects on cognition (semantic access and visual-spatial memory) and urinary content of parent anthocyanins,

Int: 1,213 (wk 0) 1,047 (wk 8) ng/mg Cr
Ctrl: 1,447 (wk 0) 1,093 (wk 8) ng/mg Cr
 $p \geq 0.05$

**minor deglycosylated
conjugates**

Int: 78.5 (wk 0) 58.6 (wk 8)
ng/mg Cr
Ctrl: 84.9 (wk 0) 66.1 (wk 8) ng/mg Cr
 $p \geq 0.05$

**total anthocyanidin
glycosides**

Int: 38.4 (wk 0) 109 (wk 8)
ng/mg Cr
Ctrl: 37.2 (wk 0) 26.9 (wk 8) ng/mg Cr
 $p: 0.002$

glycoside conjugates

Int: 32.1 (wk 0) 41.0 (wk 8)
ng/mg Cr
Ctrl: 30.6 (wk 0) 26.2 (wk 8) ng/mg Cr
 $p \geq 0.05$

parent (food) form

Int: 2.07 (wk 0) 47.2 (wk 8)
ng/mg Cr
Ctrl: 3.74 (wk 0) 0.450 (wk 8) ng/mg Cr
 $p < 0.001$

glycoside anthocyanin
conjugates and total
glycosidic anthocyanin
forms.

Note: AD, Alzheimer disease. ADAS-Cog, Alzheimer's disease Assessment Scale - Cognitive Subscale. ADCS-ADL, Alzheimer's disease Cooperative Study - Activities of Daily Living. A β ₁₋₄₂, Amyloid β . BNT, Boston Naming Test. BSRT, Buschke Selective Reminding Test. BVRT, Benton Visual Retention Test. CANTAB, Cambridge Neuropsychological Test Automated Battery. CD, Cognitive Decline. CES-D, Center for Epidemiologic Studies Depression Scale. Cr, creatinine. Ctrl, control. CSF, cerebrospinal fluid. Cu, curcumin. CV, coefficient of variation. CVLT, California Verbal Learning Test. C3G eq., cyanidin-3-glucoside equivalents. DEX, Dysexecutive Questionnaire. DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition.

EBMT, East Boston Memory Test. GAEs, Gallic Acid Equivalents. GC-MS, gas chromatography- mass spectrometry. Gluc, glucuronidated. GPT, Grooved Pegboard Test. HPLC-DAD/MS, High-performance liquid chromatography with photodiode array-mass spectrometry. HVLT, Hopkins Verbal Learning Test. Int, intervention group. LC-MS-MS, liquid chromatography-tandem mass spectrometry. m., month(s). MMSE, Mini Mental Status Examination. MRI, magnetic resonance imaging. NPI, Neuropsychiatric Inventory. PALTEA, Paired Associate Learning total errors adjusted. PEGB, Polyphenol-rich Extract from Grape and Blueberry. PJ, pomegranate juice. QGP, Queen Garnet plum. RCFT, Rey Complex Figure Test. SCWT, Stroop Color and Word Test. SD, standard deviation. SDMT, Symbol Digit Modalities Test. SPAL, Spatial Paired Associate Learning Test. SSP, Spatial Span or Speech Sounds Pick. THCu, tetrahydrocurcumin. TMT-A, Trail Making Test part A. TMT-B, Trail Making Test part B. Uro, urolithin. VRMFR, Verbal recognition memory – free recall. VRMR, Verbal recognition memory – recognition. VSLT, Visual Spatial Learning Test. WAIS, Wechsler Adult Intelligence Scale. wk, week. WTAR, Wechsler Test of Adult Reading. y., years.

*Decliners: subgroup with advanced CD derived from the stratification of the cohort in quartiles based on PAL score at baseline.

⁵Calculated as: SD = IQR/1.35.

Table A.2. Summary of the characteristics, designs and results of the observational studies reviewed.

Reference (year)	Design / Duration / N (% women)	Subjects' characteristics / Age (SD)	Cognitive measurements	Polyphenols measurements	Main results
Rabassa et al. (2015)	Prospective cohort 3 years 652 (55.4%)	Elderly healthy subjects (CD diagnostic criteria: Decrease in MMSE score of ≥ 3 points from baseline to 3-years follow-up and the worst 10% of the TMT-A and TMT-B distribution after subtracting baseline from 3-year follow-up scores (in seconds) or discontinuation of the test at follow-up)	<i>At baseline and after 3 years of follow-up</i> - MMSE - TMT-A; TMT-B	<i>Method:</i> Folin-Ciocalteu assay <i>At baseline:</i> Urine excretion of total polyphenols (expressed as mg GAEs/day) Median (IQR) = TUP _{T1} : 103 (82–113); TUP _{T2} : 148 (138–163); TUP _{T3} : 218 (196–254)	- Association between TUP levels and lower risk of substantial CD on the MMSE and on the TMT-A, comparing extreme tertiles and in lineal regression models - No association between TUP levels and risk of substantial CD on the TMT-B.
Yuan et al. (2016)	Case-control 276 subjects Cases: 138 (64.5%) Controls: 138 (64.5%)	73.4 (6.4) y. Elderly subjects with MCI (cases diagnostic criteria: > 13/14 points for individuals with no formal education, 19/20 for individuals with 1-6 years of education and 24/25 for individuals with ≥ 7 years of education) 64.5 y. Cases: 65 (0.5) y. Controls: 64 (0.6) y.	<i>At baseline</i> MoCA (MCI diagnosis)	<i>Method:</i> LC-MS <i>At baseline:</i> Plasma flavonoids (luteolin, quercetin, naringenin, apigenin, kaempferol, hesperetin) levels	No difference of plasma flavonoids levels between the MCI and control subjects.
Valls-Pedret et al. (2012)	Cross-sectional in a longitudinal cohort 447 (52%)	Elderly subjects at high cardiovascular risk 66.9 (6.38*) y.	<i>At baseline</i> - MMSE - RAVLT (immediate recall and delayed recall) - Verbal paired associates test - SVF test	<i>Method:</i> Folin-Ciocalteu assay <i>At baseline:</i> Urine excretion of total polyphenols (expressed as mg GAEs/g of creatinine)	- Association between TUP excretion and immediate memory recall score (RAVLT immediate recall) – dose-relationship. - Almost significant association between TUP excretion and delayed

				- Digit span test, from WAIS test - CTT, part I and II	memory recall score (RAVLT delayed recall) – dose-relationship. - No association between TUP excretion and other cognitive tests.
Igase et al. (2017)	Cross-sectional, observational 152 subjects Cases[§] : 60 (74.0%) Controls[§] : 92 (66.0%)	Elderly healthy subjects [Case or equol-producer diagnostic criteria: urine equol level > 1000 nmol/L after intake of 2 x 50 mg isoflavones (soy food)] 69.2 y. Cases[§] : 69.1 (9.4) y. Controls[§] : 69.1 (9.0) y.	<i>At baseline</i> - TDAS - MCI screening test	<i>Method</i> : immunochromatographic strip test <i>After isoflavones intake</i> : Urine excretion of equol	- Higher score in TDAS cognitive test among equol producers compared to non-equol producers. - Lower prevalence of MCI among equol producers compared to non-equol producers.
Rabassa et al. (2020)	Prospective cohort 3 years 119 (47.9%)	Elderly healthy subjects (CD diagnostic criteria: decrease in MMSE score of ≥ 3 points from baseline to 3-years follow-up) 73.0 (5.9) y.	<i>At baseline and after 3 years of follow-up</i> MMSE	<i>Method</i> : HPLC-Q-ToF-MS <i>At baseline</i> : Urine metabolome	- Association between change in cognitive function (continuous variable) and urine excretion of Uro-A, Uro-A glucuronide, Uro-A sulphate, Uro-A sulpho-glucuronide, Uro-B and Uro-B glucuronide. -Inverse association between cognitive decline (dichotomous variable) and urine excretion of Uro-A, Uro-A sulphate, Uro-B, Uro-B glucuronide and hydroxyphenylacetic acid.

Note: CD, Cognitive Decline. CTT, Color Trail Test. GAEs, Gallic Acid Equivalents. IQR, interquartile range. MCI, Mild Cognitive Impairment. MMSE, Mini-Mental State Examination. MoCA, Montreal Cognitive Assessment. RAVLT, Rey Auditory Verbal Learning Test. SD, standard deviation. SVF test, Semantic verbal fluency test. TMT-A, Trail Making Test, part A. TMT-B, Trail Making Test, part B. TDAS, Touch Panel-type Dementia Assessment Scale. TUP, Total Urinary Polyphenols. Uro, urolithin. WAIS, Wechsler Adult Intelligence Scale.

*SD not reported and estimated as one quarter of the typical range of data values (Higgins et al., 2022).

[§]Cases were defined as equol producers and controls were defined as equol non- producers.

8.2. Supplementary references

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