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Role of polyphenols in the modulation of intestinal permeability and other related markers in the context of aging

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Abstract

The need for the identification of new, and possibly non-invasive, strategies directed towards improving health status in the older population is increasing due to the burden of chronic degenerative diseases and the worldwide challenge associated to the consequent impact on the health care system. In particular, the maintenance of a “homeostasis” balance between inflammatory and anti-inflammatory networks seems to be fundamental to improve the aging process. In this context, the exploitation of specific dietary patterns and food bioactives such as polyphenols (PPs) have been suggested for their possible effect in the prevention and management of age-associated functional and metabolic disorders. In the older subjects, these conditions may be related to intestinal permeability (IP), that is a barrier feature which contributes to the regulation of solute and fluid exchange between the lumen and tissues. In the present thesis 3 main tasks have been considered directed to: **A** – critically assess tools and methods to estimate polyphenol intake in different target groups of population; **B** – review the last updated literature regarding *in vivo* (animal model), *in vitro* and human studies focused on polyphenol and IP; **C**- provide data from a dietary intervention study developed within the MaPLE project to support the hypothesis that a polyphenol-rich diet (PR-diet) can improve IP in a group of older subject living in a well-controlled setting. Results from task A suggested that tea, coffee, red wine, fruit and vegetables are the main products providing PPs in different target populations. In addition, total flavonoids and specific subclasses have

been apparently associated with a low risk of diabetes, cardiovascular events and all-cause mortality even if the large variability in terms of methods for the evaluation and quantification of polyphenol intake makes still difficult to establish an evidence-based reference intake for these compounds. Task B results highlighted the lack of human intervention studies required to increase the understanding on the actual role of polyphenols not only from a mechanistic point of view.

Finally, in the task C we are able to show the effect of a polyphenol-rich dietary pattern, specifically developed for a target of older subjects, in the modulation of IP. In particular, a significant reduction of zonulin serum levels have been found. In addition, thanks to the accurate registration of food intake along the whole intervention study it has been possible to better demonstrate the high compliance to the dietary instruction and the significant increase of total polyphenol intake, thus, strengthening the trustworthiness of the observed effects.

In conclusion, the overall results of the present thesis seem to support the reliability of non-invasive dietary intervention (i.e. polyphenol-rich dietary pattern) for the management or prevention of IP and associated conditions in the older subjects and/or other sensitive target groups of population.

Riassunto

In considerazione dell'aumento della popolazione anziana e delle malattie croniche generalmente associate che hanno un impatto significativo sul sistema sanitario a livello mondiale, risulta fondamentale identificare nuove strategie non invasive volte a promuovere lo stato di salute di questo target di soggetti. In particolare, il mantenimento di una "omeostasi" tra i processi infiammatori e antinfiammatori sembra essere un punto critico per migliorare il processo di invecchiamento. In questo contesto, è stato suggerito l'utilizzo di specifici modelli dietetici e/o di composti bioattivi degli alimenti, come ad esempio i polifenoli (PPs) per il loro possibile effetto nella prevenzione e gestione dei disturbi funzionali e metabolici associati all'età. Nei soggetti più anziani, queste condizioni possono anche essere messe in relazione a una aumentata permeabilità intestinale (PI), che è una caratteristica della barriera intestinale che contribuisce alla regolazione dello scambio di soluti e fluidi tra lume e tessuti. Nella presente tesi sono stati considerati 3 ambiti di lavoro principali diretti a: A - valutare criticamente strumenti e metodi per stimare l'assunzione di PPs in diversi gruppi target di popolazione; B – effettuare una revisione aggiornata della letteratura riguardante gli studi sul ruolo dei PPs sulla PI effettuati *in vivo* (modello animale), *in vitro* e sull'uomo; C- ottenere risultati mediante uno studio di intervento dietetico, sviluppato nell'ambito del progetto MaPLE, per supportare l'ipotesi che una dieta ricca di PPs possa migliorare la PI in un gruppo di soggetti anziani che vivono in un ambiente controllato.

I risultati della parte A, suggeriscono che tè, caffè, vino rosso, frutta e verdura sono i principali prodotti che forniscono PPs in diversi gruppi di popolazione. Inoltre, emerge che i flavonoidi totali e alcune sottoclassi specifiche sono associate a un basso rischio di diabete, eventi cardiovascolari e mortalità per tutte le cause, anche se la grande variabilità in termini di metodi per la valutazione e la quantificazione dell'assunzione di PPs rende ancora difficile stabilire un quantitativo di riferimento per una assunzione adeguata di questi composti. I risultati della parte B hanno messo in luce la mancanza di studi di intervento effettuati sull'uomo che risultano necessari per aumentare la comprensione del ruolo effettivo dei PPs non solo dal punto di vista meccanicistico.

Infine, nella parte C, i risultati ottenuti attraverso uno studio di intervento, dimostrano l'effetto di un modello dietetico ricco di PPs e, sviluppato appositamente per un target di soggetti anziani, nella modulazione della PI. In particolare, è stata osservata una riduzione significativa dei livelli sierici di zonulina. Inoltre, grazie all'accurata registrazione dell'assunzione alimentare lungo l'intero studio di intervento è stato possibile dimostrare meglio l'elevata *compliance* dei soggetti e il significativo aumento dell'assunzione totale di PPs, rafforzando così l'affidabilità degli effetti osservati.

In conclusione, i risultati della presente tesi sembrano supportare l'efficacia e realizzabilità di un intervento dietetico non invasivo (ovvero ottenuto mediante inclusione di alimenti ricchi in PPs) per la gestione o la prevenzione della PI e delle condizioni associate presenti nei soggetti anziani e/o in altri gruppi sensibili della popolazione.

1. INTRODUCTION

Increased age is a recognized risk factor for numerous chronic diseases such as cardiovascular disease (CVD), type 2 diabetes, chronic obstructive pulmonary disease, cognitive decline and cancer (Nicoli et al., 2012).

It is also worth mentioned that the extension of the life expectancy registered in the last years is not generally paralleled by an increase of time of healthy life in the older subjects, thus, the increased higher incidence of age-related diseases poses a worldwide challenge to reduce the consequent impact on the health care system (i.e. high costs associated to the diagnosis, treatment, and care of older people) (Prasad et al.,2012).

The aging process involves numerous changes at different levels, able to affect the physiological, pathological and psychological condition (e.g. associated to cognitive decline). This can lead to the development of several diseases contributing to functional impairment and physical inability (Amarya et al., 2018). The latter is often associated with a generalized decline in functional status and, particularly, implicates impaired muscle function, decreased bone mass, immune dysfunction, anemia, reduced cognitive function and slow wound healing. All these modifications combined together may alter eating habits, reduce nutrient absorption and availability resulting in nutritional deficiencies. For example, Shlisky et al. (2017) reported that inadequate intakes of fruit, vegetables, legumes, whole grains, nuts or seeds and excess intakes of refined grain products, processed and fatty meats, fried

foods, and added sugars are present in older people with a poor or inadequate nutritional status.

Several studies seem to confirm that healthy dietary patterns are associated with reduced risk or improvement of many age-related chronic diseases (Guerriero et al., 2018). Consequently, an increased interest in research focused on nutritional strategies to improve health status in the older subject is growing. In addition *in vitro* and *in vivo* studies have shown that the beneficial properties attributed to dietary patterns rich in fruit and vegetables such as the Mediterranean diet are due, not only to the balanced content of essential nutrients, vitamins and minerals but also to the presence of other bioactive compounds able to modulate critical mechanisms involved in the promotion and progression of age-related diseases such as those at the base of low-grade inflammation (Guerreiro et al., 2018).

1.1 Polyphenols as protective dietary bioactives

Accumulating evidence underlines that bioactives such as polyphenols have potential health benefits, including anti-oxidant, anti-microbial, anti-inflammatory/immunomodulatory and anti-cancer properties thus increasing protection against chronic diseases (i.e. diabetes, obesity, CVD and neurodegenerative diseases, among others (Bonaccio et al., 2017; Guasch-Ferré et al., 2017; Ozdal et al., 2016).

Polyphenols are a large class of phytochemicals widely present in many fruits, herbs, vegetables, seed and cereals and even in beverages, such as coffee, tea, cocoa and wine.

These phytochemicals comprise a large variety of compounds with a main characteristic structure formed by one aromatic ring with one or more hydroxyl groups. The classification depends on the number of phenol rings that they contain and by the structural elements that bind these rings to one another. Briefly, polyphenols can be divided into flavonoids and non-flavonoids; among the flavonoids have been identified flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanins, while non-flavonoids phenolics are represented by phenolic acids, stilbenes, and lignans. A schematic overview of the classification is reported in Figure 1 of the published manuscript by Del Bo' et al., (2019) reported in Chapter 1.

It is recognized that the beneficial properties of polyphenols are conditioned by their bioavailability. Bioavailability depends on multiple factors including the type of bioactive compound, chemical structure, interaction with the food matrix and host-related factors (i.e. enzymes activity). Only very small amounts of the ingested compounds are absorbed. Many studies have shown that polyphenols can be absorbed by the intestinal mucosa as aglycones and in some cases as glucosides (Ozdal et al., 2016). Prior to pass into the blood stream, aglycones undergo different conjugation processes that occur in the small intestine, but above all in the liver. The most important modifications include methylation, sulfation and glucuronidation. Unabsorbed polyphenols reach the large intestine where they undergo an extensive metabolization by

gut microbiota to aglycones and/or simple phenolic acids. The released aglycones and phenolic acids/metabolites can be absorbed and further metabolized in the liver. Additionally, intestinal microbiota degrades aglycones and release simple aromatic compound, rendering them more available for absorption and conjugation. In this way, gut microbiota biotransforms polyphenols into metabolites that may be potentially more bioactive and bioavailable than their precursor structures (Ozdal et al., 2016). At the same time, several phenolic compounds and their metabolites have been suggested to improve gastrointestinal health by modulating gut microbiota composition and diversity, with the concomitant stimulation of beneficial bacteria and inhibition of pathogens, thereby acting as potential prebiotics. This two-way mutual interaction between polyphenols and intestinal microbiota may result in host health benefits (Ozdal et al., 2016).

1.2 Polyphenols-microbiota interaction (in the context of aging)

Gut microbiota composition has been demonstrated to change with age (Odamaki et al., 2016) and different factors related to the host or his environment can be involved, including the characteristics of the diet. Despite the increased research on the topic, the modifications of the microbial ecosystem occurring in the older subjects remain unclear.

It is widely recognized the significant contribution of the microbiota in the modulation of host immune system and this can become particularly critical in the older age. Claesson et al., (2011) have shown that the fecal microbiota of

older subjects is extremely variable and characterized by unusual phylum proportions. In addition, the same group demonstrated that older subjects who did not leave in the community but in residential care had a less diverse microbiota and increased parameters of frailty and inflammation (Claesson et al., 2012). Interestingly, the authors found a strong correlation of the microbiota composition with the diet and the residence location.

Several *in vitro*, animal and human studies suggest that a diet rich in fruits and vegetables can result in a greater abundance of beneficial bacteria in the gut, with a shift towards a presumably healthier microbiota. Increasing evidence supports that polyphenols can contribute to the modulation of more beneficial bacteria such as Lactobacilli and Bifidobacteria, while indicating a simultaneous reduction in pathogens, including “species of the” Clostridiales and Enterobacteriales. Furthermore, they have been shown to foster formation of short chain fatty acids (SCFAs) (Kaulmann and Bohn, 2016). Coherently, these data have been confirmed by other results recently reviewed by Cardona et al., (2013), which provided a detailed overview of the reciprocal interaction between polyphenols subclasses and gut microbiota. The authors highlighted the possible contribution of polyphenols to the improvement of gastrointestinal health pursued by the modulation of the intestinal ecosystem (i.e. the microbiota and the derived metabolic products).

1.3 Polyphenol intake and aging

The role of polyphenols in the prevention and counteraction of certain processes related to aging has been supported by several studies (Shen et al., 2012). More specifically, epidemiological studies showed that the intake of food products rich in polyphenols such as red wine could reduce the incidence of some neurological pathologies related to age, as in the case of Alzheimer's (Fischer et al., 2018). The regular intake of flavonoid-rich foods has been associated with up to 50% decrease in the risk of dementia, and also with a reduction in the incidence of degenerative diseases such as Parkinson's disease (Lefèvre-Arbogast et al., 2018). In addition, it has been demonstrated that some specific flavonoids such as flavones and flavanones, are associated with lower levels of oxidative and inflammatory markers, suggesting a potential ability to improve the systemic inflammatory state in humans (Fisher et al., 2012). For example, in the Italian MOLI-SANI study it has been reported a reduced inflammation when an increased polyphenols consumption was registered in the older population. The inverse association was explained by considering that polyphenols could (a) act as antioxidants or increase antioxidant gene expression, (b) lower endoplasmic reticulum stress signaling, (c) inhibit pro-inflammatory cytokines or endotoxin-mediated kinases and transcription factors implicated in metabolic disease, (d) suppress inflammatory gene expression or (e) activate transcription factors that antagonize chronic inflammation (Bonaccio et al., 2017; Pounis et al., 2015) . In the PREDIMED study (multicenter, randomized, nutritional intervention

trial) carried out in Spain, it has been demonstrated that older subjects with high intakes of polyphenols had a reduced risk (HR = 0.54; 95%CI: 0.33–0.91) of cardiovascular events (Tresserra-Rimbau et al., 2014). Moreover, in the InCHIANTI study it has been shown that polyphenol intake was associated with a lower risk of frailty or pre-frailty (Urpi-Sarda et al., 2015) and cognitive decline in older subjects. (Rabassa et al., 2016). Finally, it has been documented that a greater intake of these compounds was associated with increased well-being in later ages (Shlisky et al., 2017).

From several surveys it has been evidenced that in the older population, the main sources of polyphenols are drinks like tea, coffee, wine and finally citrus juices. As for the fruit and vegetables categories, potato along with apples seemed to be the most consumed (Manach et al., 2004). A complete and extensive review of the literature on the topic has been recently published by Del Bo' et al., (2019) and is reported in Chapter 1 of the present thesis.

It is noteworthy that a large part of the older population can have inadequate energy and nutritional intake due to different social and psychological factors but also problems related to reduced appetite and thirst or physical inability to follow an appropriate diet. This conditions in the older population can also reflect a reduced intake of polyphenols compared to other population groups, as evidenced by Zujko et al., (2015).

1.4 Inflammaging: definition and implications

As previously mentioned, advanced age is often accompanied by numerous alterations in the physiological system, including a decline in innate and acquired immune function, defined “immunosenescence”; all these conditions lead to a an increase in low-grade inflammation, termed “inflammaging” (Fulop et al., 2018).The term was first defined by Franceschi et al., (2000), as a chronic progressive increase in the concentrations of pro-inflammatory mediators in the blood- stream, including acute phase protein, cytokines such as tumor necrosis factor- α (TNF-α), interleukin-6 and 8 (IL-6 and IL-8), and adhesion molecules such as sICAM-1 and sVCAM-1. The levels of these inflammatory mediators have been shown to be higher in the older with respect to the younger adults (Calder et al., 2017). Inflammaging has been assumed to evolve in five phases: low-grade, controlled, asymptomatic, chronic, and systemic (Xia et al., 2016; Franceschi et al., 2018). It is a determinant in the trigger and development of the most common age-related diseases, including (a) atherosclerosis and other cardiovascular diseases, (b) metabolic syndrome, (c) type 2 diabetes and obesity, (d) sarcopenia and osteoporosis, (e) neurodegeneration, (f) major depression and mental health, and (g) cancer (Calder et al., 2017). According to literature, inflammaging is strongly associated with different kind of stressors, which induce a poor reaction in the weak organism of the older subjects, and with oxidative stress, that affects immune function. In addition, the overall balance of cytokines, such as IL-6 and TNF-α, plays a putative role in inflammaging as reported by

many researchers (Franceschi et al., 2018, Xia et al., 2016, Calder et al., 2017).

Results of various studies highlights that inflammaging reflects not a simple increase of pro-inflammatory markers, but an overall activation of the inflammatory systems, which is strongly related to morbidity and mortality. For instance, a recent longitudinal cohort study on almost one thousand Italian older adults demonstrated that higher circulating levels of inflammatory mediators such as IL-6, IL-1receptor, TNF-α receptor II were related to the onset of numerous chronic diseases, including hypertension, diabetes, ischemic heart disease, congestive heart failure, stroke, chronic obstructive pulmonary disease, cancer, Parkinson's disease, hip fracture, joint disease, anemia, chronic kidney disease, peripheral arterial disease and cognitive impairment (Fabbri et al., 2014).

It is noteworthy that the development of chronic inflammation has been associated to changes occurring in the gut microbiota during aging.

In fact, a reduced heterogeneity of the bacterial species in the gut has been observed in older person, as well as a decreased *Firmicutes*/*Bacteroidetes* ratio and a reduction of beneficial commensal microorganisms such as coprococcus and lactobacillus. These changes have been suggested to induce adverse consequences on host health since some Lactobacillus and Bifidobacterium can be involved in the regulation of genes encoding proteins involved in the integrity of the epithelial junctions (Yu et al., 2012). In addition, for instance, an increased abundance of Fusobacterium and Staphyloccocus species have been observed during aging and all these changes under

inflamed conditions can become pathogenic (Guglielmetti et al., 2013). Finally, it is well recognized that commensal bacteria are fundamental for the homeostasis and optimal function of the intestinal barrier.

1.5 The intestinal barrier

The intestinal barrier constitutes the physical and functional separation between the external environment and the gut lumen, exploiting a crucial role in our health by regulating various physiological processes: i) controlling selective passage of various essential substances and allowing the uptake/digestion of nutrients, ii) preventing the permeation of pathogens, toxins, antigens and pro-inflammatory agents in our body, and iii) mediating signal transduction and reacting with bioactive compounds (Lee et al., 2018; Nagpal et al., 2018). It is a complex structure mainly constituted by an external "physical wall" and an internal functional immunological barrier, whose interaction influences intestinal permeability (Bischoff et al., 2014). The physical barrier is made up of a single layer of polarized epithelial cells arranged in villi and crypts in the small intestine, and in crypts in the colon. In addition, overall gut barrier function is supported by multiple chemical and cellular processes; for instance, in the lumen, mucus, antimicrobial peptides and secretory immunoglobulins protect epithelium and impede bacterial adhesion and antigen uptake to the lamina propria, and likewise, immune cells and cytokines signaling are determinant (Galipeau and Verdu, 2016) . Bischoff et al, (2014), defined intestinal barrier as a "*functional entity separating the gut*

lumen from the inner host, and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensins, IgA), immunological elements (lymphocytes, innate immune cells), muscular and neurological elements". The most important key feature of the intestinal barrier is named intestinal permeability, which is a property of the barrier devoted to the regulation of solute and fluid exchange through mainly two pathways that modulate intestinal permeability: the transcellular and paracellular pathway. High molecular weight antigens, such as microbes or dietary antigens, cross the intestinal barrier via the transcellular pathway or via transport proteins; whereas, the paracellular pathway is involved in the transport of small molecules, ions, and solutes between epithelial cells (Lee et al., 2018).

1.5.1 TJs in the regulation of Intestinal permeability

TJs, are key regulators of the diffusive mechanisms from the gut lumen to the bloodstream and are located in interconnection spaces between endothelial cells with a complex structure. Over 50 different transmembrane proteins are involved in their composition (Bernardi et al., 2019), even if the mainly described and cited in literature are occludins, claudins and tricellulin (Tran and Greenwood-Van Meerveld, 2013), which are able to develop fibrils that cross the enterocytes' membranes giving shape to the structure. The claudins can be considered the structural pillars of TJ as showed by Furuse et al., (1998). However, other single-span transmembrane proteins mostly represented by junctional adhesion molecules (JAMs), are involved in the

maintenance of the epithelial structure, and in the immune regulation (Ulluwishewa et al., 2011). In addition, transmembrane proteins closely interact with intracellular proteins, such as the protein “family” of the zonula occludens (ZO-1, ZO-2 and ZO-3), which guarantee binding to the actin cytoskeleton (Ulluwishewa et al., 2011). A simple schematic overview of this complex structures is represented in Figure 2. Indeed, the disruption of these transcellular proteins structure leads to an increased paracellular permeability, that could cause the permeation of pro-inflammatory luminal molecules to the mucosal layer and to the bloodstream reflecting in the activation of the mucosal immune system, and causing prolonged inflammation and tissue damage, and, once in the bloodstream, systemic inflammatory responses. If these inflammatory processes become chronic, they could lead to several intestinal and systemic diseases, as stated above. Hence, the maintenance of the integrity of the intestinal barrier is essential for health (Man et al., 2015).

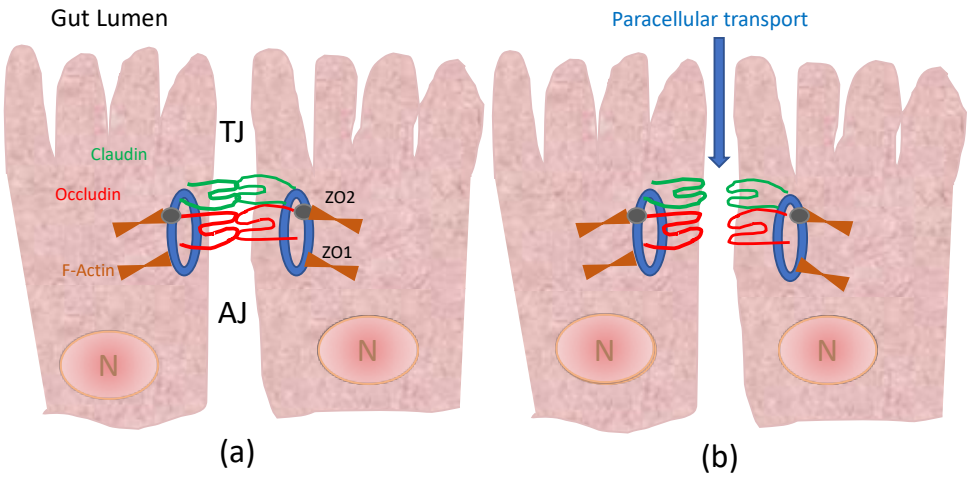

Figure 1 – schematic overview of intestinal epithelial TJs

a) assembly of TJs related proteins; b) paracellular transport across TJs opening – adapted from Lee et al., (2018).

1.6 Polyphenols and regulation of IP

Even if only limited data are available, polyphenols have been further hypothesized to influence intestinal permeability, via tight junctions' regulation. Several studies suggest that specific polyphenols might enhance gene expression and the production of proteins necessary for TJs integrity, such as claudin-5, occludin, and ZO-1 (Lee et al., 2018; Ulluwishewa et al., 2011). For instance, in a Caco-2 based models, quercetin, epigallocatechin gallate and resveratrol have been shown to protect TJ integrity through the inhibition of the redistribution of ZO-1, and through the prevention of lower expression of ZO-1 and occludin – induced by indomethacin action. Similar positive effects on cellular barriers *in vitro* and in animal studies have been documented by other trials by using other phenolic compounds (Bernardi et

al., 2019). Finally, based on the recent data collected by Yang et al., (2017), polyphenols could exert their positive effects in TJs regulation through various pathways and mediators, which interfere each other. In particular, possible regulatory pathways can be distinguished in: (a) inhibition of nuclear factor-κB signaling, (b) modulation of the activity of certain protein kinases, (c) regulation of the activity of key enzymes, and (d) reduction of reactive oxygen species.

1.7 Evaluation of IP

Intestinal permeability can be measured by many functional techniques or through the detection of biomarkers and these tests allow researchers to evaluate the status of mucosal barrier integrity and the pathophysiology of resultant diseases. As reported by Galipeau and Verdu, (2016), the gold standard for functional measurements of gut permeability in humans is the multi-sugar test. For example, the most used lactulose/mannitol test estimates urinary excretion of the two orally administered non-metabolized sugars after 6 hours from the intake. In animal studies the methods usually used are oral gavage of sugars, fluorescently labeled dextrans, polyethylene glycols (PEG), or 51Cr-ethylenediaminetetraacetic acid (EDTA) followed by detection in the urine or blood. Another tool is the measurement, after biopsy, of the electrical resistance across the membrane (i.e. Ussing chamber), which reflects active ion transport, and then the integrity of the tissue in relation to paracellular integrity. Additionally, the detection of bacteria or bacterial products have been

used as a sign of impaired intestinal barrier, including the presence of lipopolysaccharide (LPS) and of endotoxin core A antibodies. Finally, the assessment of the epithelial barrier morphology and of TJ expression are considered further useful tests.

1.8 Zonulin as marker of IP

An increased interest in the understanding the complex mechanisms that regulate intestinal epithelial paracellular pathway, have resulted by the identification of a ~47 kDa human protein analog to zonula occludens toxin (an enterotoxin which reversibly opens intracellular tight junctions), named zonulin (Fasano, 2011, 2012b; Sturgeon and Fasano, 2016). Although the physiological roles of zonulin is still not completely elucidated, its system appears to be involved in various functions, including TJ regulation responsible of the exchange of substances between the bloodstream and the intestinal lumen, and the defence against microorganism colonization of the intestine. Furthermore, *ex vivo* studies and various publications by Sturgeon and Fasano *et al., (2016),* confirmed that endogenous human zonulin was able to increase reversibly intestinal permeability by modulating intercellular TJs, and it was involved in innate immunity. Zonulin resulted overexpressed in people affected by autoimmune diseases, where TJ disassembly plays a crucial role, such as celiac disease and type 1 diabetes (Kim et al., 2018). The zonulin signaling pathway involves the opening of intestinal TJs. Zonulin binds to a specific surface receptor (EGFR) trough proteinase activated receptor 2

(PAR2), thereby activating a cascade signal: in further detail, the protein activates phospholipase C that hydrolyzes phosphatidyl inositol (PPI) to release inositol triphosphate (IP3) and diacylglycerol (DAG). Then PKC-α is activated either directly (via DAG) or through the release of intracellular Ca2+ (via IP3). Membrane-associated activated PKC-α catalyzes the phosphorylation of target proteins, including zonula occludens-1 (ZO-1) and myosin 1C, as well as polymerization of soluble G-actin in F-actin. This combination of TJ protein phosphorylation and actin polymerization triggers the rearrangement of the actin filaments and the consequent displacement of proteins (including ZO-1) from the junctional complex. Consequently, intestinal TJ becomes looser. Once the zonulin signaling is over, TJs return to their stationary state (Fasano, 2012a, 2012b).

The release of zonulin has shown to be triggered by several potential stimuli, such as small intestinal exposure to bacteria and gluten. Many enterotoxins, produced by enteric pathogens, provoke enteric infections which are associated with several pathological conditions, including allergic, autoimmune, and inflammatory diseases, by inducing impairment of the intestinal barrier. As a result, loss of barrier function generally leads to an uncontrolled translocation of antigens in the lamina propria, where they activate the immune system and the consequent release of cytokines, further intensifying the gut permeability (Fasano, 2012a; Nicoletti, 2015; Sturgeon and Fasano, 2016). With regard to the exposure to bacteria, an imbalanced microbiome or its inappropriate distribution along the gastrointestinal tract may be a cause of zonulin release, and of the subsequent barrier disassembly.

Furthermore, gut dysbiosis has been shown associated with aging and systemic inflammation, thus, with implication in the onset of inflammatory geriatric syndromes. These findings support the hypothesis that intestinal mucosal permeability and correlated inflammation may contribute, at least partly, to increases in chronic inflammation and accompanying disorders typical of older people. In accordance with this consideration, a recent publication by Qi *et al., (*2017), demonstrated that that serum concentration of zonulin and HMGB1 (high-mobility group box protein 1) – a nuclear protein that triggers inflammation and is commonly present in epithelial cells – were increased in older adults compared with younger ones; and that zonulin, but not HMGB1, was positively correlated to high levels of age-associated inflammatory cytokines (TNF-α and IL-6) and frailty measurements. Therefore, research on zonulin and zonulin signaling pathway seem to suggest the potential clinical relevance of zonulin as a biomarker of intestinal permeability and related conditions.

Since the identification of zonulin as marker of IP, the number of studies evaluating the prevalence of IP in different target groups of populations, as well as, the impact of some interventions has been increased. For example, Mokkala et al, (2016) showed an association between the richness and composition of gut microbiota and zonulin serum levels demonstrating that beneficial changes in commensal bacteria may beneficially affect intestinal permeability.

Nevertheless, the adequate evaluation of IP is a complex issue and it seems recommendable the development of protocols involving the combination of

different markers to better characterize possible dysfunctions at the barrier level able to modulate systemic responses.

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2. AIMS and OBJECTIVES

The overall aim of this PhD thesis is to provide scientific evidence on the role of diet in the modulation of intestinal permeability. Specifically, the main objectives of this Thesis have been divided in 3 chapters dedicated to:

CHAPTER 1. critically assess tools and methods to estimate polyphenols and their health effects in different target group of population obtained through a systematic review of the literature of the last 10 years;

CHAPTER 2. review all the results from *in vitro* and *in vivo* studies focused on the ability of polyphenols to modulate intestinal permeability in order to define actual knowledge and gaps;

CHAPTER 3. provide data supporting the hypothesis that a polyphenol-rich dietary pattern can improve intestinal permeability in a group of older subjects living in a well-controlled setting in order to minimize confounding factors.

3. RESULTS CHAPTERS

3.1 Chapter 1

Systematic review on polyphenol intake and health outcomes: is there sufficient evidence to define a health-promoting polyphenol-rich dietary pattern?

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Review

3.1.1 Systematic review on polyphenol intake and health outcomes: is there sufficient evidence to define a health-promoting polyphenol-rich dietary pattern?

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Abstract: Growing evidence support association between polyphenol intake and reduced risk for chronic diseases, even if there is a broad debate about the effective amount of polyphenols able to exert such protective effect. The present systematic review provides an overview of the last 10-year literature on the evaluation of polyphenol intake and its association with specific disease markers and/or endpoints. An estimation of the mean total polyphenol intake has been performed despite the large heterogeneity of data reviewed. In addition, the contribution of dietary sources was considered, suggesting tea, coffee, red wine, fruit and vegetables as the main products providing polyphenols. Total flavonoids and specific subclasses, but not total polyphenols, have been apparently associated with a low risk of diabetes, cardiovascular events and all-cause mortality. However, large variability in terms of methods for the evaluation and quantification of polyphenol intake, markers and endpoints considered, makes it still difficult to establish an evidence-based reference intake for the whole class and subclass of compounds. Nevertheless, the critical mass of data available seem to strongly suggest the protective effect of a polyphenol-rich dietary pattern even if further well targeted and methodologically sound research should be encouraged in order to define specific recommendations.

Keywords: polyphenol intake; polyphenol databases; dietary pattern; disease risk; cardiovascular and all-cause mortality

1. Introduction

The possibility to develop dietary guidelines for the intake of food bioactives with health promoting effects can be of utmost importance to try to evolve the concept of adequate nutrition to that of optimal nutrition. Clearly, this implies at least 2 levels of knowledge: 1) the availability of reliable data of food composition and food intake to estimate exposure to food bioactives and 2) the capacity to assess the amount needed to exert the protective activity.

Polyphenols have been suggested to exert a plethora of biological activities including antioxidant, anti-inflammatory, anti-microbial, anti-proliferative, pro-apoptotic activity and hormonal regulation capacity [1]. There is also increasing evidence that long-term intake can have favorable effects on the incidence of several cancers and other chronic diseases, including cardiovascular disease (CVD), type II diabetes, and neurodegenerative diseases [2]. More recently research has been focused on the impact of polyphenols on healthy aging and/or age-related diseases [3].

The emerging evidence, obtained through both animal models and human studies, on the direct and indirect role of polyphenols in the modulation of metabolic and functional features of the host, has enhanced the interest for an estimation of polyphenol intake in the general population or in at-risk target groups. In addition, the assessment of specificity in the protective properties of the single polyphenol classes/compounds (**Figure 1**) has been increased in the last years favored by the improvement of dedicated food databases (i.e.

Phenol-Explorer, USDA database) reporting more accurate and detailed polyphenols composition and considering factors affecting the intake such as the “retention factors” (i.e. the loss or gain of a compound during food processing). Despite the transformation of food intake data into polyphenol intake remains still a critical, even if improved, step of the process, the accuracy of self-reported methods to evaluate dietary patterns is often limited. In particular, it has been suggested that the notion that fruit and vegetables intake represents the main dietary sources of polyphenols could be over-reported [4]. Finally, as far as polyphenols are concerned, the low bioavailability and extensive metabolism demonstrated in numerous studies makes it difficult to clearly state recommendations on intake.

Nevertheless, the analysis of polyphenol intake data registered in several target population with different dietary patterns and lifestyle/exposure may help better understanding whether it is possible to identify a range of intake apparently associated to an overall reduced risk.

To this aim a comprehensive updated review on data and tools/methods used for the estimation of polyphenol intake was performed by considering differences in total and subclasses intake depending on factors related to dietary habits. In addition, main results on the association among polyphenol intake and specific endpoints of disease risk have been taken into account, when available, to suggest possible recommendation.

1.1. Search strategy and study selection

A literature search of all English language studies published was performed using PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and EMBASE (http://www.embase.com/) databases (updated December 2018) with the addition of other scientific papers of relevance fount in web sources or in previously published reviews. The search terms and strategy used for the study selection were: polyphenols *OR* flavonoids *OR* anthocyanins *OR* flavanols *OR* flavanones *OR* flavones *OR* flavonols *OR* isoflavones *OR* proanthocyanidins *OR* phenolic acids *OR* hydroxycinnamic acids *OR* hydroxybenzoic acids *OR* lignans *OR* stilbenes *AND* intake. Human studies were used as further criteria of literature search. The search was limited to the last 10 years of publication. Three independent reviewers (S.B., M.M. and M.T.) conducted the literature search in the scientific databases and assessed and verified the eligibility of the studies based on the title and abstract. Disagreement between reviewers was resolved through consultation with a third reviewer (P.R. or C.D.B.) to reach a consensus. Inclusion criteria: i) prospective, cohort and case-control studies analysing/estimating dietary total/classes/individual polyphenol intake; ii) studies reporting association between dietary total/classes/individual polyphenol intake and endpoints of disease risk and mortality; iii) studies published from January 2008 to December 2018. The exclusion criteria were: i)-dietary intervention studies; ii)-studies measuring polyphenols intake through urine excretion; iii)-studies performed in *in-vitro* or in animal models; iv)-studies reporting data on polyphenol intake from supplements (not food related); v)-studies evaluating the association between polyphenol intake and cancer risk/mortality

(numerous systematic reviews and meta-analysis have been recently performed); vi)-published articles in a language different from English and with no accessible translation.

1.2. Data extraction

For the papers meeting the inclusion criteria, the full text was retrieved, analysed and summarized in Tables. Data extraction was performed by three independent reviewers (S.B., M.M., M.T., P.R. and C.D.B.). The following information was collected: (i) first author name and year of publication; (ii) study design; (iii) number and subjects' characteristics; (iv) country; (v) tools used for estimating dietary polyphenols intake; (vi) polyphenol database source; (vii) overall results. For the studies evaluating the association with disease risk or mortality this information was included in the table. Additional revisions of contents have been performed by other reviewers (N.H.L., B.K. and B.C.).

2. Results

2.1. Study selection

The study selection process according to PRISMA guidelines is reported in **Figure 2**. A total of 3004 records were identified from the database search (PubMed and EMBASE) and other sources. After removing 48 duplicate articles, 2956 studies were screened and 2566 were excluded based on title and/or abstract. The full text of eligible studies (n = 390) was read; 299 studies were excluded because not meeting the inclusion criteria (n = 282) or not of

interest/pertinent (n = 17). At the end of the selection process, 91 papers were included.

2.2. Study characteristics

The main characteristics of the 91 included studies are reported in **Tables 1-4;** 45 studies focused only on polyphenol intake in specific target populations, 24 studies assessed the association between polyphenols and cardiovascular/diabetes risk (1 study included also data on CV mortality), 9 studies focused specifically on the association with mortality for cardiovascular and all other events, while 13 studies evaluated the association between polyphenol intake with others outcomes (e.g. frailty, bone fractures).

2.3. Dietary intake of polyphenols

Table 1 shows reported data from literature focused on polyphenols intake. A total of 45 studies were found and analyzed [3-47]. Most of the studies were performed in Europe, North America and Asia (**Figure 3A**). The researches (**Figure 3B**) were carried out in the adult + older population (63%) or only adults (20%), while few studied reported data specifically in older subjects (7%), in children and adolescents (7%); the dietary intake of polyphenols was assessed generally through 24-h dietary records (24-h DR; 56%) and food frequency questionnaire (FFQ; 31%) as reported in **Figure 3C**. The main scientific databases (**Figure 3D**) used for the estimation of polyphenol intake were USDA (22%) and Phenol-Explorer (PE; 20%). However, most of the studies combined USDA with PE and other databases and/or scientific sources (24%). Total polyphenol intake for the overall population was estimated to be about 900 mg/day; this value varied according to differences in target groups of subjects. The main food sources of polyphenols were represented by tea, coffee, red wine, fruit and vegetables.

Table 1. Polyphenol intake registered in adults.

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Song et al. [5]	8809 subjects (NHANES 1999–2000 and 2001–2002) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (1,2)	**Total flavonoids** Mean intake = 189.7 ± 11.6	**Flavan3-ols** Mean intake = 156.5 ± 11.3 **Flavanones** Mean intake = 14.4 ± 0.6 **Flavanols** Mean intake = 12.9 ± 0.4 **Anthocyanidins** Mean intake = 3.1 ± 0.5 **Flavones** Mean intake = 1.6 ± 0.2 **Isoflavones** Mean intake = 1.2 ± 0.2	Tea (82.8%) Citrus juices (4.3%) Wine (2.1%) Citrus fruits (1.8%)	Different total flavonoids intake was observed between tea consumers (21% of the population) and tea non-consumers (697.9 vs. 32.6 mg/day respectively) with flavonols and flavan-3-ols as main compounds
Ilow et al. [6]	203 subjects W = 121 M = 82 Age = 50 year	Poland	FFQs 48 food items	USDA database (1)	**Total flavonoids** (median) M+F = 610.8 M = 612.0 F = 609.2	n.a.	Tea Fruit Vegetable	The flavonoid intake in tea was the same in women as in men. Tea flavonoids constituted about 96% of all the consumed flavonoids in this population
Otaki et al. [7]	514 subjects W = all M = 0 Age = 58 ± 10 year	Japan	1 24-h WDR	FFF (functional food factor) database	**Total polyphenols-**	* **Total flavanols** Mean = 1277 ± 1403 * **Total isoflavones** Mean = 215.7 ± 147.3 * **Total flavonols** Mean = 58.4 ± 62.7 * **Total flavanones** Mean = 30.5 ± 145.8 * **Total flavones** Mean = 15 ± 51.6 * data expressed in µmol/day	Green tea Onion Soy processed food (tofu, natto and miso)	The study showed higher total flavonoid intake compared to previous studies performed in the Japanese population. The sources of flavonoids differed from those of Western countries. Green tea, soy foods and onion constituted the main sources of flavan-3-ols, isoflavones and flavonols, respectively. Grapefruits and citrus fruits were the main sources of flavanones, while Malabar spinach, green peppers and grapefruits the main sources of flavones
Chun et al. [8]	8809 subjects NHANES 1999–2000 (n = 4175) and 2001–2002 (n = 4634) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (1,2)	**Total flavonoids (1999–2000)** Mean intake = 209.8 ± 18.9 **Total flavonoids (2001–2002)** Mean intake = 204.5 ± 14.5	n.a.	Tea (76.8%) Citrus fruit juice (3.7%) Beers and ales (2.9%) Wine (2.4%) Citrus fruit (1.7%) Melon and berries (1.4%) Other vegetables (1.4%)	Daily intake of flavonoids was dependent on sociodemographic characteristics and lifestyle behaviors. Daily flavonoid intake was provided mainly by teas (i.e., catechins)

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Yang et al. [9]	128 subjects W = n.a. M = n.a. Age = 20–28 year	China	2 sFFQs 126 food items 2 7-day 24-h DRs (used to validate FFQs data)	Specifically developed database *	**Total flavonoids (FFQ1)** Mean intake = 45.39 ± 25.52 **Total flavonoids (FFQ2)** Mean intake = 46.94 ± 27.72 **Total flavonoids (24-h DRs)** Mean intake = 50.15 ± 35.83	FFQ 1 data: **Total flavonol** Mean intake = 34.74 ± 18.80 **Total flavone** Mean intake = 10.65 ± 7.02 FFQ 2 data: **Total flavonol** Mean intake = 35.75 ± 20.45 **Total flavone** Mean intake = 11.19 ± 7.57 24-h DRs data: **Total flavonol** Mean intake = 38.37 ± 28.59 **Total flavone** Mean intake = 11.78 ± 8.45	n.a.	The FFQ used had reasonable reproducibility (measured 1 year apart) and validity to estimate dietary intake of flavonols (quercetin, kaempferol, isorhamnetin) and flavones (apigenin, luteolin) in the Chinese population, as compared to the other type of assessment methods
Zhang et al. [10]	5046 subjects W = 2910 M = 2136 Age = 18–72 year	China	2 sFFQs 126 food items 2 7-day 24-h DRs (used to validate FFQs data)	Specifically developed database *	**Total flavonols-Flavones** Mean intake = 19.13 ± 8.28	**Flavonols** Mean intake Quercetin = 5.96 ± 3.09 Kaempferol = 4.14 ±1.95 Myricetin = 1.81 ± 1.24 Isorhamnetin = 2.34 ± 1.48 **Flavones** Mean intake Apigenin = 1.06 ± 0.56 Luteolin = 3.82 ± 1.88	Apple (12%) Potato (8%) Celery (7%) Eggplant (7%) Actinidia (5%)	The total intake of flavonols and flavones was higher in men than in women. Gender and above all age were independent predictors for total flavonols and flavones intake. Main food sources were vegetables (61%) and fruits (36%) while tea was only a minor source
Hanna et al. [11]	551 subjects W = 551 M = 0 Age = 40–79 year	Australia	Phytoestrogen frequency questionnaire 112-item	USDA and specific literature	**Total isoflavones-lignans** Mean = 8.44 ± 17.03 Median intake = 2.2 Min and max = 0.44–174	**Total isoflavones** Mean = 4.5 ± 10.07 Median = 0.03 Min and max = 0–98 Total Lignans Mean = 2.71 ± 3.04 Median intake = 1.83 Min and max = 0.16–33	Soy and soy product (tofu, miso, soy grits or cereal)	Isoflavone intake was significantly different depending on age, i.e., 40–49 years and 50–59 years age groups introduced higher isoflavone amount compared to 60–69 years and 70–79 years age groups. There was no significant difference in lignan intake among age groups
Péréz-Jimenéz et al. [12]	4942 subjects (SU.VI.MAX cohort 1994.1995) W = 2346 M = 2596 Age = 45–60 year	France	6 24-h DRs 736 food items	Phenol Explorer	**Total polyphenols** Mean intake = 1193 ± 510 Median intake = 1123	**Flavonoids** Mean intake = 506 ± 219 **Phenolic acids** Mean intake = 639 ± 273	Non-alcoholic beverages (55.2%) Fruit (17.3%) Alcoholic beverages (8.3%) Cocoa products (7.5%) Vegetables (6.8%) Cereals (3.9%)	Total polyphenol intake was higher in men than in women. Age had no significant influence on intake. Three beverages Coffee, tea, and red wine accounted for 44%, 9%, and 6% of the total polyphenol intake while fruit, cocoa products, vegetables, and cereals for 17%, 8%, 7%, and 4% of the total polyphenol intake confirming data from other Western populations

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Zamora-Ros et al. [13]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-SOFT)	USDA database expanded with Phenol Explorer 1877 food items	**Total flavonols-flavanones-flavones** Mean intake ± SEM = 66.76 ± 0.89 W = 70.32 ± 0.65 Min W = 37.2 mg/day (Sweden) Min M = 36.7 mg/day (Sweden) Max W = 97.0 mg/day (UK) Max M = 130.9 mg/day (UK)	**Flavonols** Min = 38.5% (South) Max = 47.4% (North) **Flavanones** Min = 46.6% (UK) Max = 52.9% (South) **Flavones** Min = 5.8% (North) Max = 8.6%. (South)	Citrus fruits Citrus-based juices Tea Wine Fruits Vegetables	A large variation in flavanols, flavanones and flavones intake across European regions was registered Overall, flavanones were the main compounds introduced and UK health-conscious group the highest consumers. The total intake was higher in women and dependent on sociodemographic and lifestyle factors. Main food sources differed being juices and tea intake higher in the north while citrus fruit, juices, vegetables and wine in the south
Wang et al. [14]	8809 subjects NHANES 1999–2000 (n = 4175) and 2001–2002 (n = 4634) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (3)	**Total proanthocyanidins (1999–2000)** Mean intake (PI) = 88.8 ± 6.3 **Total proanthocyanidins (2001–2002)** Mean intake (PII) = 100.0 ± 4.2	**Monomers** Mean intakePI = 20.9 ± 1.5 PII = 20.7 ± 1.4 **Dimers** Mean intake PI = 15.0 ± 1.0 PII = 15.9 ± 1.1 **Trimers** Mean intake PI = 4.7 ± 0.3 PII = 5.3 ± 0.2 **4–6mers** Mean intake PI = 13.5 ± 1.2 PII = 15.7 ± 0.5 **7–10mers** Mean intake PI = 9.4 ± 0.9 PII = 11.2 ± 0.5 **Polymers** Mean intake PI = 25.4 ± 2.8 PII = 31.4 ± 1.9	Tea Legumes Wines	A south to north gradient intake was observed. In general, a mean intake of 95 mg/day was found represented by polymers (30%), monomers (22%), dimers (16%), 4–6 mers (15%), 7–10 mers (11%), and trimers (5%). After adjustment for energy intake, the PA intake increased with age, in women and in alcohol consumer. Tea, legumes, and wines, contributed to about 48% of daily PA intake

Table 1. *Cont.*

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Knaze et al. [15]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-SOFT)	USDA database Phenol Explorer 1877 food items	**Total flavan-3-ols** Mean intake ± SE MED countries = 268.8 ± 2.6 Non-MED countries = 274.7 ± 1.9 UK = 406.6 ± 7.6 **Total monomers** Mean intake ± SE MED countries = 90.2 ± 0.7 UK = 182.4 ± 3.0 **Total proanthocyanidins** (Mean intake ± SE MED countries = 217.2 ± 2.2 Non-MED countries = 177.9 ± 1.5 UK = 198.4 ± 6.3 **Total theaflavins** (Mean intake ± SE) MED countries = 1.6 ± 0.1 Non-MED countries = 6.5 ± 0.1 UK = 25.9 ± 0.3	**Flavan-3-ols subclasses:** **Flavan-3-ol monomers** MED 18.6% non-MED 32.9% UK 44.9% **PA or condensed tannins** MED 80.8% non-MED 64.8% UK 48.8%; **Theaflavins** MED 0.6% non-MED 2.4% UK 6.4%	Tea Wine Fruits Pulses (UK)	Socio-demographic, anthropometric and lifestyle factors were associated with consumption of flavan-3-ols, PA and theaflavins. Differences among different countries were observed. Flavan-3-ol intake in the UK (health-conscious) was about 2-fold that of the MED countries and mainly due to tea providing theaflavins and epigallocatechins. Overall PA intake was higher in the MED countries, even if with large differences, and non-citrus fruit (i.e., apples and pears) and wine the main sources
Zamora-Ros et al. [16]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries of EPIC cohort	1 24-h DR (EPIC-SOFT)	USDA database expanded with Phenol Explorer 1877 food items	**Total anthocyanidin** W: Mean ± SE = 33.52 ± 0.39 Max intake = 44.08 (Turin, Italy) Min intake = 18.73 (Granada, Spain) M: Mean ± SE = 29.44 ± 0.53 Max intake = 64.88 (Turin, Italy) Min intake = 19.83 (Bilthoven, The Netherlands)	**Cyanidin** Mean intake W = 15.09 ± 0.23 M = 12.01 ± 0.31 **Delphinidin** Mean intake W = 2.71 ± 0.09 M = 2.26 ± 0.13 **Malvidin** Mean intake W = 9.94 ± 0.18 M = 10.27 ± 0.25 **Pelargonidin** Mean intake W = 3.02 ± 0.09 M = 2.19 ± 0.12 **Peonidin** Mean intake W = 1.64 ± 0.04 M = 1.49 ± 0.05 **Petunidin** Mean intake W = 1.13 ± 0.02 M = 1.23 ± 0.03	Fruits, nuts and seeds (38.1–61.2%) Wines (14.4–24.5%) Non-alcoholic beverages (15.8%) Vegetables (4.8–9.7%)	The highest total anthocyanidins (mainly cyanidins and malvidins). intake was recorded in the south European region. Women (central-southern regions) were the highest consumers. Main food sources were different depending on countries. Central and northern countries: non-citrus fruits (berries, apples and pears, and grapes), wine and non-alcoholic beverages (juices and soft drinks of anthocyanidin-rich fruits). Southern countries: wine, non-citrus fruits (grapes, stone fruits, apples and pears, and olives) and leafy vegetable. A possible underestimation of anthocyanidin intake have been hypothesized due to missing food composition data

Table 1. *Cont.*

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Beking et al. [17]	Subjects = n.a.	UK Ireland	FAO Food Balance Sheets	USDA Database (1) Lacking data from literature	**Total flavonoids** Mean intake Ireland = 176.8 UK = 182.2	Ireland (mean intake): **Anthocyanidins** = 60.3 **Flavanols** = 47.4 **Flavanones** = 29.0 **Flavones** = 5.8 **Flavonols** = 34.2 UK (mean intake): **Anthocyanidins** = 69.2 **Flavanols** = 52.4 **Flavanones** = 26.0 **Flavones** = 4.0 **Flavonols** = 30.3	Grapes and oranges (41.6% UK, 34.9% Ireland) Beer and wine (8.8% UK, 12.8% Ireland) Apples and onions (6.8% UK, 6.5% Ireland) Tea (4.0% UK, 5.3% Ireland).	Estimated dietary intake of anthocyanidins, flavanones, flavanols, flavonols, flavones, and all five combined is similar in the UK and Ireland. Anthocyanidins and flavanols were about 65% of total intake. Data on flavones and flavonols were in line with those obtained in food intake surveys in UK and US. In general, as more types of food flavonoids are analyzed and included in food composition databases, intake estimates are expected to rise and to be more accurate
Ilow et al. [18]	1520 subjects Cardiovascular Disease Prevention Program) W = 879 M = 641 Age = 49–50 year	Poland	FFQs 1 24-h DR	USDA Database (1)	**Total flavonoids** Mean intake W = 622.6 M = 616.9	**Flavan-3-ols** W = 93.6% of total flavonoid M = 94.2% **Flavonols** W = 4.0% M = 4.2% **Anthocyanidins** W = 0.9% M = 1.1% **Flavanones** W = 0.9% M = 0.9% **Flavones** W = 0.1% M = 0.1%	Tea (93.6%, 94.2%) Fruits (2.2%, 1.6%) Vegetables (1.4%, 1.1%) Fruit juices (0.7%, 0.8%) Chocolate (0.1%, 0.1%)	A higher flavonoid intake was reported in comparison with other studies. Tea was the main food source of total flavonoids and mainly of flavan-3-ols intake (from tea, fruits, fruit juices, chocolate)
Zujko et al. [19]	6661 subjects (Polish National Multicenter Health Survey, WOBASZ) W = 3529 M = 3132 Age = 20–74 year	Poland	1 24-h DR	Database of polyphenol contents in food products (developed by the authors) 118 items	**Total polyphenols** Mean intake W = 10,311,054 (20–40 years) 1089 (41–60 years) 947 (61–74 years) M = 1172 1251 (20–40 years) 1183 (41–60 years) 1076 (61–74 years)	n.a.	Beverages (tea, coffee) Vegetables (potato) Fruits (apples) Cereals (white bread)	Polyphenol intake was about 1 g independently from gender and age and apparently similar to that of other countries. However, patterns of consumption were different depending on gender and age groups

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Lee et al. [20]	8502 subjects W = n.a. M = n.a. Age = >2 year	Korea	1 24-h DR	Phytonutrient database (Korea National Academy of Agricultural Science)	**Total polyphenols-**	Subjects meeting the recommendations **Anthocyanidins** = 73 ± 4.8 **Hesperitin** = 25.4 ± 3.2 **Catechin** = 24.8 ± 1.4 **Quercetin** = 9.1 ± 0.3 **Isoflavones** = 25.8 ± 2.8 **Gallic Acid** = 18.9 ± 2.6 Subjects not meeting the recommendations: **Anthocyanidins** = 8.7 ± 0.3 **Hesperitin** = 3.5 ± 0.5 **Catechin** = 2.2 ± 0.2 **Quercetin** = 2.9 ± 0.1 **Isoflavones** = 5.4 ± 0.5 **Gallic acid** = 4.3 ± 0.7	Fruits Onions Soybeans Nuts	Flavonoids (anthocyanidins, hesperitin, quercetin, catechin, and isoflavones), and one phenolic compound (gallic acid) were significantly higher among subjects who met the recommendations for fruit and vegetable consumption compared with those who did not
Zamora-Ros et al. [21]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-Soft)	USDA database (1)	**Total polyphenols-**	**Total thearubigins** M: Min = 0.9 Max = 532.5 W: Min = 1.2 Max = 455.6	Tea	Large differences in dietary thearubigins (TR) estimations intake across European countries; TR intake is low in Spanish men and high in men from UK; TR contributed < 5% to the total flavonoid intake in Greece, Spain and Italy while contributed 48% to the total flavonoids intake in UK
Tresserra-Rimbau et al. [22]	7200 subjects (PREDIMED) W = n.a. M = n.a. Age = 55–80 year	Spain	FFQs	Phenol Explorer 137 foods item	**Total polyphenols** Mean intake = 820 ± 323	**Flavonoids** = 443 ± 218 **Phenolic acids** = 304 ± 156 **Other polyphenols** = 71.2 ± 46.7	Fruits (44%) non-alcoholic beverages i.e., coffee (55%), vegetables (12%) alcoholic beverages (10%) Olive oil (11%)	Coffee and fruits resulted the main sources of polyphenols even if olives and olive oil represented significant and peculiar Mediterranean dietary sources of polyphenols (i.e., hydroxycinnamic acids, other phenolic acids, lignans and other polyphenols) with respect to other countries

Table 1. *Cont.*

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Vogiatzoglou et al. [23]	15,371 subjects W = 8278 M = 7093 Age = 14–80 year	Germany	1 24-h DR (EPIC-SOFT)	FLAVIOLA Database	**Total polyphenols-**	**Total flavanols** Mean intake = 385.9 Min = 195.8 Max = 840.7 **Proanthocyanidins** Mean intake = 196.4 Min = 138.7 Max = 300.3 **Flavan-3-ol monomers** Mean intake = 119.8 Min = 18.3 Max = 414.3 **Theaflavins** Mean intake = 69.7 Min = 38.8 Max = 126.1	Data are referred to total flavanols: Pome fruits (27%) Black tea (25%) Non-alcoholic beverages (46%) Green/fruit herbal tea (10–16%) Berries (6%)	Women had slightly higher intakes of total flavanols than men in all age groups, except for the elderly. There was a steep age gradient with an increase in total flavanols, flavan-3-ol monomers, and theaflavins across the age groups. Proanthocyanidins were the main contributor of total flavanols in both men and women
Grosso et al. [24]	10,477 subjects (HAPIEE study) W = 5340 M = 5137 Age = 45–69 year	Poland	FFQs 148 items	Phenol Explorer	**Total polyphenols** Mean intake = 1740.7 ± 630.2 Median intake = 1662.5	**Total flavonoids** Mean intake = 897.6 ± 423.4 **Total phenolic acids** Mean intake = 800.2 ± 345.8	Coffee (40%) Tea (27%) Chocolate (8%)	Intakes were slightly higher in men than in women, but when adjusted for energy intake, women had a higher intake of polyphenols than men. Age had significant influence on total and energy-adjusted polyphenol intake, being higher among younger participants
Witkowska et al. [25]	6661 subjects W = 3529 M = 3132 Age = 20–74 year	Poland	24-h DR	Phenol Explorer USDA database (1–3)	**Total polyphenols** Mean Intake = 989.3 ± 360	**Total flavonoids** Mean Intake USDA = 524.6 ± 155 PE = 403.5 ± 150 **Total phenolic acids** Mean Intake USDA = n.a. PE = 556.3 ± 204	Total polyphenols (PE): Non-alcoholic beverages (75%) Total flavonoids: Non-alcoholic beverages: (PE 78.5%) (USDA 90%)	Flavonoids estimated through various databases might substantially differ. The use of several databases can truly reflect the real intake but it will be difficult to comparison for which only one method has been used for calculations

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Kim et al. [26]	11,474 Subjects W = n.a. M = n.a. Age = ≥ 19 year	Korea	1 24-h DR	USDA database (1) Korean-targeted flavonoid database	**Total flavonoids** Mean Intake ± SE = 96.6 ± 1.34 Median = 70.4 P10 – P90 = 22.8 – 192	**Total anthocyanidins** Mean Intake ± SE = 26.4 ± 0.9 Median = 6.36 P10 – P90 = 0 – 68.1 **Total flavanols** Mean Intake ± SE = 25.5 ± 1.8 Median = 1.08 P10 – P90 = 0 – 43.2 **Total flavanones** Mean Intake ± SE = 8.15 ± 0.39 Median = 0P10 – P90 = 0 – 25.1 **Total flavones** Mean Intake ± SE = 0.87 ± 0.03 Median = 0.45 P10 – P90 = 0.13 – 1.86 **Total flavonols** Mean Intake ± SE = 24.6 ± 0.42 Median = 16.8 P10 – P90 = 4.88 – 50.2 **Total isoflavones** Mean Intake ± SE = 21.9 ± 0.39 Median = 12.1 P10 – P90 = 0.27 – 53.9	Kimchi (traditional fermented vegetable product) (12%) Green tea (9%) Persimmon (7%) Soybean (7%) Onion (7%) Tofu (6%) Radish (5%) Tangerine (5%) Apple (4%) Pear (3%)	Total Flavonoid intake was lower in Korea than in western countries. A major difference came from tea intake and also by the lower flavonoid density of major sources (kimchi, persimmon, tangerine, onion, radish etc.) in Korea than those (tea, citrus fruit, apples, pears, wine, etc.) in western countries. Contrast the isoflavone intake was much higher than the estimates for western countries due to high intakes of soybeans, tofu, and fermented soy pastes
Zamora-Ros et al. [27]	36,037 Subjects W = 23,009 M = 13,028 Age = 35–74 year	10 European countries of EPIC cohort	1 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake ± SEW = 1192 ± 6 M = 1177 ± 8 highest in Denmark M = 1786 W = 1626 lowest in Greece M = 744 W = 584	**Total flavonoids:** Mean intake ± SEW = 546 ± 4 M = 492 ± 5 **Total phenolic acids** Mean intake ± SEW = 625 ± 6 M = 593 ± 5 **Total lignans** Mean intake ± SEW = 3.6 ± 0.1 M = 2.5 ± 0.2 **Total stilbenes** Mean intake ± SEW = 2.4 ± 0.0 M = 3.0 ± 0.1	MED countries: Coffee (36%) Fruits (25%) Wine (10%) Non-MED countries: Coffee (41%) Tea (17%) Fruits (13%)	Mean intake of polyphenols was three times higher in men from Denmark than in women from Greece. Stratifying by region, mean of total polyphenols intake was in non-MED countries due to the higher intake of phenolic acids. The study showed a large heterogeneity in both the nature of polyphenols and levels of intake across the countries due to different habits and socio-demographics status

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Vogiatzoglou et al. [28]	30,000 subjects W = n.a M = n.a Age = 18–64 year	14 Countries	2–7 24-h DR	FLAVIOLA Database	**Total flavonoids:** Mean Intake = 428 ± 49 central region = 506 ± 75 northern region = 348 ± 20 southern region = 301 ± 27 Median Intake = 164 ± 55 central region = 249 ± 87 northern region = 56 ± 22 southern region = 47 ± 7	**Theaflavins and thearubigins** Mean intake = 168 ± 39 Median intake = 89 ± 38 **Proanthocyanidins:** Mean intake = 124 ± 7 Median intake = 27 ± 5 **(Epi)catechin** Mean intake = 24 ± 2Median intake = 7 ± 2 **Gallated compounds** Mean intake = 53 ± 12 Median intake = 28 ± 12 **Anthocyanidins** Mean intake = 19 ± 2 Median intake = 3 ± 1 **Flavonols** Mean intake = 23 ± 2 Median intake = 8 ± 2 **Flavanones** Mean intake = 14 ± 2 Median intake = 1 ± 0 **Flavones** Mean intake = 4 ± 1 Median intake = 1 ± 0 **Flavonoids (monomeric)** Mean intake = 136 ± 14 Median intake = 49 ± 15	Non-alcoholic beverages Fruits	Large regional differences, both in the type of flavonoids consumed and the distribution of intake. Intakes of anthocyanidins (in particular cyanidin) and flavanones (in particular hesperetin) were highest in the Northern Region, in particular in Finland. Within the Central Region, there was also a large variability of intake between countries. While overall flavonoid intake in Ireland was the highest in Europe, the intake of anthocyanidins was the lowest overall, and intake of flavanones was also very low. France was included in the Southern Region as dietary intake was more comparable with intake in Italy and Spain. However, there are some important differences, and the intake of flavan-3-ols and anthocyanidins in France is considerably higher than in the other countries of the Southern Region
Sebastian et al. [29]	5420 subjects W = 2758 M = 2662 Age = >20 year	USA	1 24-h DR	USDA database (1)	**Total flavonoids** Mean intake = 251 ± 16.8 IQR = 18.8–272 W Mean intake = 241 ± 15.2 IQR = 16.3–272 M Mean intake = 263 ± 20.4 IQR = 20.4–271	Mean intake: **Total flavonols** = 19.4 ± 0.91 IQR = 6.05–25.4 **Total flavones** = 0.9 ± 0.1 IQR = 0.1–1.1 **Total flavanones** = 13.1 ± 0.88 IQR = 0.00–5.15 **Total isoflavones** = 1.7 ± 0.3 IQR = 0–0 **Total flavanols** = 204 ± 15.6 IQR = 3.07–189 **Total anthocyanidins** = 11.6 ± 1.07 IQR = 0–9.92	Tea (80%) Fruit Vegetables	A positive association between flavonoid intake and dietary quality suggest that a diet high in flavonoids is synonymous with greater compliance with national guidance. Individuals with higher flavonoids intake not only consume more fruit and vegetables but also eat more healthfully

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Kozłowska et al. [30]	151 subjects Polish = 91 Spanish = 60 Polish W = 74 M = 17 Spanish W = 36 M = 24 Total: W = 110 M = 41 Age = n.a.	PolandSpain	FFQs	USDA Database (1)	**Total flavonoids** Mean intake Polish students = 801 Spanish students = 297	n.a.	Polish Students: Black and green tea Oranges Orange juice Spanish Students: Oranges Green tea Orange Juice	Flavonoid consumption in Polish students was more than two times higher than in the Spanish students. The main sources of flavonoids in Spanish and Polish diets were different as black tea in the Spanish group provided weekly about 236 mg of flavonoids, over 12 times less than in the Polish group. On the other hand, the Spanish diet was richer than the polish diet in sources of flavonoids such as oranges, chickpeas, dried parsley, onions, strawberries, almonds or pomelo
Zujko et al. [31]	6661 subjects M = 3132 W = 3529 Age = 20–74 year	Poland	1 24-h DR	Database developed by the authors	**Total flavonoids** Mean intake = 276 **W (20–40 year)** = 278 CI95% = 266–290 **M (20–40 year)** = 304CI95% = 291–317 **W (41–60 year)** = 275 CI95% = 264–286 **M (41–60 year)** = 291 CI95% = 279–311 **W (61–74 year)** = 238 CI95% = 227–249 **M (61–74 year)** = 268 CI95% = 256–280	n.a.	Beverages (47%) Fruit and fruit jams (27%) Tea (22%) Vegetables (18%) Apples (12%) Coffee (8%)	The consumption of tea, coffee, and apples was associated with the largest contributions to the flavonoid content. In comparison to the young and middle age participants, the elderly consumed less beverages and vegetables with a lower level of flavonoids
Taguchi et al. [32]	610 subjects M = 569 W = 41 Age = 52–89 year	Japan	FFQs	Database developed by the author	**Total polyphenols** Mean intake = 1492 ± 665	n.a.	Coffee (43.2%) Green tea (26.6%)	The present study showed that a population of elderly Japanese (mostly men) consumed higher amounts of polyphenols than previous data in Japanese adults, and coffee and green tea were the largest sources of polyphenols in their daily life

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Sun et al. [33]	887 subjects W = 887 Age = 12–18 year	China	FFQs 4 24-h DR	Flavonoids database developed by the authors	**Total flavonoids** Mean intake = 20.60 ± 14.12	**Total flavonol** = 16.29 ± 11.91 **Quercetin** = 5.51 ± 4.00 **Kaempferol** = 5.49 ± 3.68 **Myrucetin** = 2.29 ± 1.84 **Isorhamntin** = 3.00 ± 2.37 **Total flavones** = 4.31 ± 2.21 **Luteolin** = 3.27 ± 1.63 Apigenin = 1.03 ± 0.58	Apple (11.7%) Potatoes (9.9%) Lettuce (7.3%) Oranges (7.0%) Chinese Cabbage (4.7%) Tomatoes (4.2%) Celery (4.2%) Soyabean Sprouts (4.2%) Leeks (3.9%) Aubergine (3.9%)	The dietary flavonoid intakes among female adolescents in the Suihua area were similar to those reported in previous studies. In the present study, apples, potatoes, lettuce, oranges, soyabean sprouts and leeks were the main food sources of flavonols, whereas tomatoes, aubergine, white radishes, celery and sweet potatoes were the main sources of flavones
Kim et al. [34]	9801 subjects W = 5032 M = 4769 Age = >19 year	US	2 24-h DR	USDA databases (1,2)	**Total flavonoids** Mean intake = 200.1 ± 8.9	**Total flavonols** Mean intake = 15.9 ± 0.4 **Total flavones** Mean intake = 1.2 ± 0.1 **Total flavanones** Mean intake = 12.2 ± 0.5 **Total flavanols** Mean intake = 158.4 ± 8.5 **Total anthocyanidins** Mean intake = 11.5 ± 0.7 **Total isoflavones** Mean intake = 0.9 ± 0.1	Tea Citrus fruit juices Berries Citrus fruit Wine Apples	Flavonoid intake increased with age from 19 to 30 years until 50–70 years in both men and women. After adjusting for energy intake, flavonoid density of women was greater than those of men ($p < 0.0001$). The difference of flavonoid density among ethnicity was reduced after adjusting for energy intake. Flavonoid density of alcohol non-consumer was greater than that of alcohol consumer ($p < 0.05$)
Burkholder-Cooley et al. [35]	77,441 subjects W = 50,336 M = 27,105 Age = 57 year	USA Canada	FFQs	Phenol Explorer USDA database (1-2)	**Total polyphenols** Mean intake coffee consumers = 1370 ± 1069 non-coffee consumers = 541 ± 368	**Total flavonoids** Mean intake non-coffee consumer = 305 ± 238 coffee consumer = 273 ± 213 **Total phenolic acids** Mean intake non-coffee consumers = 125 ± 106 coffee consumers = 986 ± 1030	Coffee Fruit Vegetables Fruit juice Legumes (including soya)	Significant differences in mean adjusted total polyphenol intakes were observed between dietary patterns. 34% of the participants reported coffee consumption in the FFQ. In the group of non-coffee consumers vegans reported the highest intake of total polyphenols followed by pesco-vegetarians, lacto-ovo vegetarians, semi-vegetarians and non-vegetarians. In the group of coffee consumers non- vegetarians reporting the highest intakes, followed by vegans, semi-vegetarians, pesco-vegetarians and lacto-ovo-vegetarians

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Pounis et al. [36]	14,029 subjects W = 7048 M = 6981 Age = n.a.	Italy	EPIC-FFQs specifically adapted for the Italian population 164 food items	Eurofir-eBASIS USDA database	**Total polyphenols** -	Median intake: **Total flavonols** = 17.0 **Total flavones** = 0.7 **Total flavanones** = 32.4 **Total flavanols** = 51.2 **Total anthocyanidins** = 144 **Total isoflavones** = 23.5 **Total lignans** = 80	Seasonal fruits Citrus fruits Leafy vegetable Grain Root vegetables Onions Garlic	Total energy intake was positively associated with the consumption of all polyphenol classes and sub-classes in both genders. Men or older participants seemed to have higher intakes of most of the polyphenols compared with women or younger participants. No significant sex difference was observed for lignans. Educational level did not account for differences in most of flavonoid and lignan intake among participants. No/former smokers presented higher intake of polyphenols. Participants with higher physical activity level consumed greater quantities of all classes of polyphenols
Ivey et al. [37]	1063 subjects W = 1063 M = 0 Age = >75 year Mean age = 80 ± 3 year	Australia	sFFQs	Phenol Explorer USDA database [(1–3)]	**Total flavonoids** USDA database [(1–3)] Mean intake = 834 ± 394 PE Mean intake = 487 ± 243	**Total flavonols** USDA = 30 ± 17 PE = 104 ± 61 **Total flavanols** USDA = 666 ± 345 PE = 327 ± 179 **Total flavones** USDA = 4 ± 3 PE = 13 ± 7 **Total flavanones** USDA = 40 ± 36 PE = 33 ± 31 **Total anthocyanidins** USDA = 88 ± 77 PE = 11 ± 11	n.a.	The mean flavonol PE intake of the cohort was nearly 350% greater than the flavonol USDA estimate. This difference may be, in part, due to the fact that the PE database provides data for five additional groups of flavonol compounds which were not expressed in USDA. Furthermore, the USDA database does not include the flavonol content data of chocolate

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Godos et al. [38]	1937 subjects W = n.a. M = n.a. Age = >18 year	Italy	FFQs 110 food items	Phenol Explorer	**Total polyphenols** Mean intake = 663.7 ± 608.1	**Total flavonoids** Mean intake = 258.7 ± 199.1 **Total flavonols** Mean intake = 57 ± 45.6 **Total flavanols** Mean intake = 93.9 ± 118.2 **Total flavanones** Mean intake = 37.9 ± 42.0 **Total flavones** Mean intake = 8.4 ± 10.2 **Total anthocyanins** Mean intake = 55.4 ± 55.3 **Total isoflavones** Mean intake = 4.0 ± 14.4 **Total phenolic acids** Mean intake = 362.7 ± 516.0 **Total stilbenes** Mean intake = 1.9 ± 3.5 **Total lignans** Mean intake = 2.8 ± 2.6	Nuts (29%) Non-alcoholic beverages (23%) Fruits (20%) Vegetables (15%) Alcoholic beverages (7%)	Compared to other Mediterranean cohorts the main differences with all the other cohorts was the contribution of nuts. In this population nuts were among the main contributors of hydroxybenzoic acids, which in other cohorts were generally provided by tea and red wine.
Miranda et al. [39]	1103 subjects W = 678 M = 425 Age = >20 year	Brazil	1 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake ± SE = 377.5 ± 15.3 Median intake = 300.3 IQR = 154.1–486.9	**Mean ± SE** **Phenolic acids** = 284 ± 15.9 **Hydroxycinnamic acids** = 281.2 ± 15.9 **Hydroxybenzoic acids** = 3.4 ± 0.4 **Flavonoids** = 54.6 ± 3.5 **Flavanones** = 16.1 ± 1.9 **Flavonols** = 14.6 ± 0.9 **Flavanols** = 11.4 ± 0.8 **Anthocyanins** = 6.8 ± 1.1 **Flavones** = 3.6 ± 0.3	Coffee (70.5%) Citrus fruit (4.6%) Tropical fruit (3.4%)	The polyphenol intake was three times lower than the estimated value compared with other countries probably due to sociodemographic differences and food choices. Older subjects (>60 y) consumed more flavonoids and tyrosol than adults (20–59 y) and also more fruits.
Burkholder-Cooley et al. [40]	899 subjects W = 602 M = 297 Age = 58 ± 13.2 year	USA Canada	24-h DR FFQs	Phenol Explorer USDA database (1,2)	**Total polyphenols** FFQs Mean intake = 717 ± 646 24-h DR Mean intake = 402 ± 345	n.a.	Coffee Fruit juice	Beverages and fruit were key contributors to total daily polyphenol intake. Subjects could over-report the frequency of intake of fruit and fruit juice in the FFQ even if a positive correlation with 24-h DR is observed.

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Bawaked et al. [41]	3534 subjects W = 2015 M = 1509 Age = 2–24 year	Spain	1 24-h DR	USDA database [(1)] Phenol Explorer	**Total Flavonoids** Mean intake = 70.7 ± 84.1 Median intake = 48.1 25th–75th percentile = 19.3–93.1	**Total flavonols** Mean intake = 15.6 ± 30.6 Median intake = 5.9 25th–75th percentile = 1.8–17.2 **Total flavones** Mean intake = 2.2 ± 9.1 Median intake = 0.3 25th–75th percentile = 0.0–1.1 **Total flavanones** Mean intake = 19.7 ± 34.1 Median intake = 0.1 25th–75th percentile = 0.0–28.1 **Total flavan-3-ols** Mean intake = 25.2 ± 47.1 Median intake = 14.1 25th–75th percentile = 4.7–28.1 **Total anthocyanins** Mean intake = 7.7 ± 27.1 Median intake = 0.3 25th–75th percentile = 0.0–4.2 **Total isoflavones** Mean intake = 0.1 ± 1.4 Median intake = 0.0 25th–75th percentile = 0.0–0.0	Fruit (42.8%) Cocoa powder and chocolate (23.5%) Vegetables (spinach, onions, artichokes and lettuce) (22%)	Higher adherence to the Mediterranean diet was correlated with higher flavonoids intake. Fruits were the main source of dietary flavonoids
Zamora-Ros et al. [42]	115,315 subjects W = 115,315 M = 0 Age = >25 year	Mexico	sFFQs 140 food items	Phenol Explorer	**Total polyphenols** Median intake = 694 Min and max = 536 and 750 25th–75th percentile = 413–1103	**Total flavonoids** Median intake = 235 Min and max = 188–270 25th–75th percentile = 141–367 **Total phenolic acid** Median intake = 361 Min and max = 243 and 439 25th–75th percentile = 166–690	Total polyphenol: Coffee (29%) Decaffeinated coffee (19%) Total flavonoids: Apple (19%) Orange and mandarins (13%) Orange juice (12%)	Large heterogeneity in intakes of individual polyphenols among Mexican women, but a moderate heterogeneity across Mexican states. Main food sources were also similar in the different states

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Ziauddeen et al. [43]	9374 subjects W = 5075 M = 4299 Children (age < 18 year) = 4636 Adults or older (age > 18 year) = 4738 Age > 1.5 year	UK	4D-FR	Phenol Explorer	**Total polyphenols** Mean intake by age ranges = (1.5–3 year) = 266.6 ± 166.1 (4–10 year) = 388.8 ± 188.8 (11–18 year) = 455.0 ± 263.2 (19–34 year) = 635.9 ± 448.9 (35–49 year) = 846.1 ± 514.1 (50–64 year) = 1053.2 ± 545.3 (65+ year) = 1035.1 ± 544.3	**Total flavonoids** (1.5–3 year) = 212.2 ± 151.7 (4–10 year) = 312.1 ± 170.3 (11–18 year) = 355.4 ± 230.9 (19–34 year) = 433.8 ± 335.1 (35–49 year) = 568.3 ± 398.2 (50–64 year) = 714.5 ± 415.2 (65+ year) = 716.2 ± 404.9 **Phenolic acids** (1.5–3 year) = 54.3 ± 24.8 (4–10 year) = 76.5 ± 43.2 (11–18 year) = 99.6 ± 63.4 (19–34 year) = 201.3 ± 228.5 (35–49 year) = 276.2 ± 232.6 (50–64 year) = 336.7 ± 292.0 (65+ year) = 317.6 ± 297.0 **Stilbenes** (1.5–3 year) = 0.1 ± 0.2 (4–10 year) = 0.1 ± 0.1 (11–18 year) = 0.1 ± 0.4 (19–34 year) = 0.8 ± 2.4 (35–49 year) = 1.6 ± 3.8 (50–64 year) = 1.9 ± 4.1 (65+ year) = 1.3 ± 3	Non-alcoholic beverages Fruits	Polyphenol intake increased with age ($p < 0.001$) and was higher in males with exception of adults aged between 19–34 and 50–64 that showed higher levels in females
Karam et al. [44]	211 subjects W = 112 M = 99 Age = 55–80 year	Spain	2 24-h DR	Phenol Explorer USDA databases pecific literature. (449 food items; 245 polyphenol containing products considered)	**Total polyphenols** Mean intake = 332.7 ± 197.4 Median intake = 299.5 IQR = 250.4 **Energy adjusted** Mean intake = 187.5 ± 100.5 Median intake = 172.9 IQR = 140.3	**Flavonoids** = 170.3 ± 144.4 **Flavanols** = 46.0 ± 57.7 **Flavonols** = 22.7 ± 29.9 **Flavanones** = 30.7 ± 50.6 **Flavones** = 10.7 ± 20.3 **Anthocyanin** = 36.7 ± 61.9 **Dihydrochalcones** = 0.3 ± 1.8 **Isoflavonoids** = 19.3 ± 71.1 **Phenolic acids** = 100.0 ± 130.0 **Lignans** = 7.2 ± 15.6 **Stilbenes** = 2.6 ± 4.4	Total polyphenol: Red wine 17.7% Artichoke 6.2% Soy milk 5.4% Total flavonoids: Red wine 26.8% Soy milk 10.8% Orange 9.5%	Flavonoids were the highest ingested polyphenols in the older population under analysis. Polyphenol intake was generally higher in female (adjusted for energy intake), in subjects aged 64–67 y, in physically active and alcoholic product drinkers
Rossi et al. [45]	241 subjects W = n.a M = n.a Age = 6–12 year	Argentine	sFFQs	Phenol Explorer Lacking data from literature	**Total polyphenols** Mean intake = 412	**Phenolic acid** Mean intake = 310 **Flavonoids** Mean intake = 94.1	Mate (60%) Tea (19%) Coffee (5%) Onion (3%)	Low intake of polyphenols was found in this scholar population of high region of the northwest Argentine due to the very low consumption of fruits and vegetables

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Wisnuwardani et al. [46]	2428 subjects (HELENA study) W = 1289 M = 1139 Age = 12.5–17.5 year	Different European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria, Spain)	2 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake = 329 Median intake = 326 Q1 = 167 Q4 = 564 Mean intake by age ranges (12.5–13.99 year) = 346 ± 0.1 (14–14.99 year) = 345 ± 0.2 (15–15.99 year) = 356 ± 0.2 (16–17.49 year) = 396 ± 0.2	**Total flavonoids** (12.5–13.99 year) = 267 ± 0.1 (14–14.99 year) = 256 ± 0.1 (15–15.99 year) = 253 ± 0.1 (16–17.49 year) = 271 ± 0.1 **Phenolic acids** (12.5–13.99 year) = 75 ± 0.1 (14–14.99 year) = 75 ± 0.1 (15–15.99 year) = 85 ± 0.1 (16–17.49 year) = 104 ± 0.1 **Stilbenes** (12.5–13.99 year) = 0.038 ± 0.0 (14–14.99 year) = 0.048 ± 0.0 (15–15.99 year) = 0.046 ± 0.0 (16–17.49 year) = 0.060 ± 0.0 **Lignans** (12.5–13.99 year) = 1.0 ± 0.0 (14–14.99 year) = 1.0 ± 0.0 (15–15.99 year) = 1.1 ± 0.0 (16–17.49 year) = 1.1 ± 0.0	Fruit (apple and pear 16%) (23%) Chocolate products (19.2%) Fruit and vegetable juices (16%)	Total polyphenol intake was lower compared to intake of adults reported in previous studies. Polyphenol intake differed largely among countries. Overall, intake for flavonoids was = 75–76% of total polyphenol, for phenolic acids was = 17–19% of total polyphenol and for stilbenes and lignans was = <1% of total polyphenol.
Kent et al. [47]	79 subjects (The Blue Mountains Eye Study) W = 45 M = 34 Age mean = 70.1 year Age = 60–80 year	Australia	12 24-h DR (weighed)	USDA database (1)	**Total flavonoids** Mean intake = 678.69 ± 498.53 Median intake = 581.84 IQR = 619.58	**Anthocyanins** Mean intake = 6.73 ± 12.7 Median intake = 1.05 IQR = 7.88 **Flavonols** Mean intake = 28.04 ± 33.29 Median intake = 24.06 IQR = 21.21 **Flavones** Mean intake = 1.87 ± 4.78 Median intake = 0.55 IQR = 2.11 **Flavan 3-ols** Mean intake = 596.17 ± 494.95 Median intake = 499.72 IQR = 622.95 **Flavanones** Mean intake = 21.43 ± 61.46 Median intake = 2.15 IQR = 12.14	n.a.	Substantial within-individual variation and between individual variation was documented for both total flavonoid intake and intake of flavonoid subclasses. The within-individual variation was in the range 80–140% while the between individual variation was in the range 60–117%. It is speculated that a minimum of 6-day weighed food records is necessary to obtain a reliable estimate of flavonoid intake.

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Vitale et al. [48]	2573 subjects (TOSCA.IT Study) W = n. a. M = n. a. Age = 50–75 year Mean = 62.2 ± 0.1 year	Italy	FFQs (Epic)	USDA [(1)] Phenol Explorer Lacking data from literature	**Total polyphenols** Mean intake = 683.3 ± 5.8 Mean intake (mg/1000 Kcal/day) Mean = 376.6 ± 3.2 W = 374.0 ± 4.9 M = 378.7 ± 4.1 Mean intake by geographical area North = 387.4 ± 6.0 Center = 355.2 ± 6.1 South = 381.9 ± 4.5 Mean intake by age <60 year = 367.9 ± 4.7 60–65 year = 376.1 ± 5.8 >65 year = 388.4 ± 6.1	**Total flavonoids** Mean intake = 324.7 ± 4.1 **Phenolic acids** Mean intake = 324.2 ± 3.0 **Lignans** Mean intake = 4.1 ± 0.06 **Stilbenes** Mean intake = 3.5 ± 0.11 **Other polyphenols** Mean intake = 27.0 ± 0.27	Non-alcoholic beverages (coffee 54%, tea 27%), fruits (apple 37%, orange 13%), alcoholic beverages (red wine 93%) and vegetables (artichokes 40%, spinach 20%, onions 18%)	A lower intake of polyphenols has been registered in diabetic subjects compared with other groups, showing a different dietary pattern in this type of Italian population.
Nascimento-Souza et al. [49]	620 subjects W = 330 M = 290 Age = 60–98 years	Brazil	Multiple 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake = 1198.6 ± 693.8 Median = 1052.7 IQR = 740.5–1477.9 Mean intake by sex W Mean intake = 1097.6 ± 616 Median = 949.4 IQR = 692.4–1407.9 M Mean intake = 1313.5 ± 757.3 Median = 1169.2 IQR = 844.7–1610.3 Mean intake by age 60–74 years Mean intake = 1197.8 ± 619.3 Median = 1092.4 IQR = 806.9–1502.9 >75 years Mean intake = 1310.2 ± 699.4 Median = 1186.9 IQR = 818.3–1582.2	**Total flavonoids** Mean intake = 444.7 ± 345.1 **Phenolic acids** Mean intake = 729.5 ± 545.4 **Lignans** Mean intake = 13.6 ± 25.5	Non-alcoholic beverages (coffee 45.8%), beans (32.8%), polenta (1.3%)	The intake of polyphenols was in a range similar to that reported for other populations, in particular European countries, but it differs for the main food contributors (high in beans and polenta, low in fruits and vegetables)

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Nascimento-Souza et al. [49]	620 subjects W = 330 M = 290 Age = 60–98 years	Brazil	Multiple 24-h DR	Phenol Explorer	**Mean intake energy-adjusted** Mean = 1198.6 ± 591.1 Median = 1102.8 IQR = 817.3–1504.8 Mean intake by sex W Mean intake = 1183.8 ± 545.4 Median = 1097.6 IQR = 816.7–1494.8 M Mean intake = 1215.4 ± 639.8 Median = 1116.0 IQR = 829.5–1537.2 Mean intake by age 60–74 years Mean intake = 1197.8 ± 619.1 Median = 1092.4 IQR = 806.9–1502.9 >75 years Mean intake = 1200.7 ± 522.1 Median = 1143.9 IQR = 858.5–1508.6	**Total flavonoids** Mean intake = 444.7 ± 345.1 **Phenolic acids** Mean intake = 729.5 ± 545.4 **Lignans** Mean intake = 13.6 ± 25.5	Non-alcoholic beverages (coffee 45.8%), beans (32.8%), polenta (1.3%)	The intake of polyphenols was in a range similar to that reported for other populations, in particular European countries, but it differs for the main food contributors (high in beans and polenta, low in fruits and vegetables)

Legend: * Cao J, Zhao XJ, Wu K, Zhang Y, and Zhang YQ: Simultaneous determination of five flavonoid compounds in vegetables and fruits by high performance liquid chromatography. Chinese J Prev Med Inf 7, 525–527,2008. n.a. = not available; 24-h DR = 24 h dietary recall; M = men. W = women; FR = food record; FFQ = food frequency questionnaire. (1) USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation.

2.4. Polyphenol intake and cardiovascular diseases/diabetes risk

In **Table 2** the results of studies that examined the association between polyphenol intake and cardiovascular diseases risk are reported [50–73]. Seven out of 24 studies were conducted in United States (US), 2 in South America, 12 in Europe, 3 in Asia (**Figure 4A**). Most of the studies were carried out in the adult population – including older subjects (63%) while the remaining studies were performed in adult population (37%) i.e. aged < 65 years (**Figure 4B**).

Food intake was mainly assessed through FFQs (63%) or with 24-h DR (29%); 1 study adopted the FFQ in combination with other tools, while 1 study used other assessment methods (**Figure 4C**).

The main databases used were USDA (42%) and PE (25%). Three studied combined USDA and PE, while the rest of the studies evaluated polyphenol intake with different databases alone or in combination such as Epic Nutrient database, EuroFIR, U.K. Food Standard Agency, Flavonoid Korean Database (**Figure 4D**).

The association between polyphenol intake and cardiovascular disease risk and diabetes was evaluated by considering several outcomes such as: HDL-cholesterol, triacylglycerols (TAGs), TAG: HDL-cholesterol ratio, HOMA-IR (Homeostatic Model Assessment of Insuline Resistence), Body Mass Index (BMI), cardiovascular events (CV events), stroke events, hypertension and type 2 diabetes (T2D).

On the whole, 12 studies reported an inverse association between polyphenol intake and CV events. In some studies a significant decreased CV risk was observed at the highest quartile of total polyphenol intake (1170 mg/day for Spain and 2632 mg/day for Poland) [61,71] while no effect was demonstrated in other studies performed in Spain and Iran (1248 mg/day and 2459 mg/day

respectively) [65,66]. Ten studies evaluated the association with polyphenol subclasses, mainly total flavonoids but only 3 found a significant inverse association with CV events [59,62,65] with intake ranging from 115 to 944 mg/day.

As regard T2D, 1 study performed in Poland showed an increased protection for total polyphenol intake higher than 2632 mg/day while mixed results were found in the other studies focused on total flavonoids and/or subclasses only in some cases able to demonstrate significant T2D risk reduction [53,54,67,69]. Finally, 1 study [59] reported an inverse association for both CV and T2D with the highest quartile of total flavonoids (585 mg/day).

Table 2. Polyphenol intake and CVD/Diabetes risk.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Huffman et al. [50]	Cohort study	507 subjects W = 263 M = 244 Age = 43–65 year	USA	FFQs	USDA database (1)	**Total flavonoids** Median intake: without diabetes = 280 (387 IQR) with diabetes = 222 (260 IQR)	↓ LDL associated with higher flavanones intake in the group with diabetes ↓ LDL associated with higher flavan-3-ols, and flavanones intake in the group without diabetes ↓ LDL associated with lower polyflavonoids intake in the group without diabetes ↑ HDL associated with higher anthocyanidins and flavan-3-ols intake in the group without diabetes ↓ HDL associated with lower polyflavonoids intake in the group without diabetes There was no relationship between HDL and flavonoids for the group with diabetes.
Pellegrini et al. [51]	Cross-sectional study	242 subjects W = 91 M = 151 Age = 60 year	Italy	3D-WR	Information provided by specific literature [a]	**Total lignans** Mean (95%CI) Q1 = 382 (332–433) Q2 = 586 (537–636) Q3 = 788 (739–837) Q4 = 1101 (1051–1152)	Total lignans intake are not associated with vascular inflammation and endothelial dysfunction
Cassidy et al. [52]	Cohort study (from NHS I, NHS II, and from HPFS)	156,957 subjects W = 133,914 M = 23,043 Age = 25–75 year	USA	FFQs	USDA database (1–3) EuroFIR	**Total flavonoids** NHS I Mean = 358 Q1 = 93 Q5 = 944 NHS II Mean = 413 Q1 = 103 Q5 = 1122 HPFS Mean = 376 Q1 = 115 Q5 = 933	↓ 6% hypertension incidence risk associated with higher total flavonoids' intake (Q5 vs. Q1; RR = 0.94; 95% CI: 0.90–0.99) in NHS I Total flavonoids' intake was not significantly associated with the risk of hypertension incidence in NHS II (RR = 1.01; 95% CI: 0.95–1.07) e HPFS (RR = 1.06; 95% CI: 0.97–1.16)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Wedick et al. [53]	Cohort study (from NHS I, NHS II, and from HPFS)	200,894 subjects W = 159,560 M = 41,334 Age = 25–75 year	USA	FFQs 118–131-item	USDA database (1)	**Total flavonoids** NHS I Q1 = 105.2 Q2 = 174.8 Q3 = 249.2 Q4 = 369.1 Q5 = 718.1 NHS II Q1 = 112.1 Q2 = 182.5 Q3 = 256.1 Q4 = 378.4 Q5 = 770.3 HPFS Q1 = 112.5 Q2 = 182.2 Q3 = 251.7 Q4 = 352.9 Q5 = 624.3	↓ 15% type 2 diabetes risk associated with higher total flavonoids' intake (Q5 vs. Q1; HR = 0.85; 95% CI: 0.79–0.92) in NHS I Total flavonoids' intake was not significantly associated with the risk of hypertension incidence in NHS II (HR = 0.99; 95% CI: 0.89–1.11) e HPFS (HR = 0.92; 95% CI: 0.81–1.04)
Zamora-Ros et al. [54]	Center stratified subcohort from Cohort study (EPIC-InterAct sub-cohort)	12,403 subjects W = 11,067 M = 5768 Age = 52.4 ± 9.1 year	8 European countries	24-h DR	Phenol Explorer USDA database (1–3)	**Flavanols** Mean = 334 ± 286 Median = 246 5th–95th percentile = 60.9–938 **Flavonols** Mean = 24.8 ± 16.0 Median = 20.4 5th–95th percentile = 7.8–57.4 Proanthocyanidins Mean = 183 ± 140 Median = 15 15th–95th percentile = 41.7–423	↓8% type 2 diabetes risk associated with higher consumption of myricetin (Q5 = >5.38 vs. Q1 = <0.37; cut off for each quintile) (P-trend = 0.001; HR = 0.92; 95% CI: 0.88, 0.96). ↓14% type 2 diabetes risk associated with higher consumption of proanthocyanidin dimers (Q5 = >49.5 vs. Q1 = <14.1; cut off for each quintile) (P-trend = <0.003; HR = 0.94; 95% CI: 0.90, 0.99). ↓7% type 2 diabetes risk associated with higher consumption of (-)-Epicatechin (Q5 = >28.75 vs. Q1 = <6.76; cut off for each quintile) (P-trend = <0.040; HR = 0.93; 95% CI: 0.89, 0.98). ↓6% type 2 diabetes risk associated with higher consumption of (+)-Catechin (Q5 = >20.08 vs. Q1 = <5.50; cut off for each quintile) (P-trend = <0.005; HR = 0.94; 95% CI: 0.91, 0.98). ↓2% type 2 diabetes risk associated with higher consumption of (+)-Gallocatechin (Q5 = >3.45 vs. Q1 = <0.04; cut off for each quintile) (P-trend = <0.027; HR = 0.98; 95% CI: 0.97, 0.99).

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Zamora-Ros et al. [55]	Cohort study (EPIC cohort)	15,258 subjects W = 9484 M = 5774 Age = 52.4 ± 9.1 year	Denmark, France, Germany, Greece, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom	FFQs (98–266-item) Diet histories Food record	EPIC Nutrient Database based on: Phenol Explorer USDA database [(1)]	**Total flavonoids** Mean intake = 414.9 ± 311.7 median intake = 326.7 5th percentile = 93.2 95th percentile = 1050.4 Median intake Q1 = 126.8 Q2 = 223.7 Q3 = 326.7 Q4 = 478.4 Q5 = 817.5	↓10% type 2 diabetes risk associated with higher consumption of total flavonoids (HR 0.90 [95% CI 0.72–1.07; P value trend = 0.040) ↓18% type 2 diabetes risk associated with higher consumption of flavanols (HR 0.82 [95% CI 0.68–0.99; P value trend = 0.012) ↓27% type 2 diabetes risk associated with higher consumption of flavan-3-ol monomers (HR 0.73 [95% CI 0.57–0.93; P value trend = 0.029) ↓19% type 2 diabetes risk associated with higher consumption of flavonols (HR 0.81 [95% CI 0.69–0.95; P value trend = 0.020) Conversely lignans did not show any association (HR 0.88 [95% CI 0.72–1.07] P value trend = 0.119)
Jacques et al. [56]	Cohort study (Framingham Heart Study Offspring cohort)	2915 subjects W = 1341 M = 1574 Age = 54 y CL = 53.8–54.5 year	USA	FFQs	USDA database [(1–3)]	**Total flavonoids** Median = 210 Min = 2 Max = 1963 Median intake Q1 = 85 Q2 = 165 Q3 = 272 Q4 = 537	Total flavonoids' intake was not significantly associated with the risk of diabetes incidence (HR = 0.89; 95% CI: 0.75–1.05) ↓ risk of diabetes incidence associated with flavanols (HR = 0.68; 95% CI: 0.54–0.86) P-trend = 0.001
Tresserra-Rimbau et al. [57]	Cohort study (PREDIMED cohort)	7172 subjects W = 3923 M = 3249 Age = 67 ± 6 year	Spain	FFQs	Phenol Explorer	**Total polyphenols** Median intake Q1 = 562 Q2 = 701 Q3 = 800 Q4 = 917 Q5 = 1170	↓ 46% CV events risk associated with higher total polyphenol intake (Q5 vs. Q1; HR = 0.54; 95%CI: 0.33–0.91) ↓ CV events risk associated with several polyphenols' subclasses:Lignans (HR = 0.51; 95% CI: 0.30–0.86) Flavanols (HR = 0.40; 95% CI: 0.23–0.72) Hydroxybenzoic acids (HR = 0.47; 95% CI: 0.26–0.86)
Jennings et al. [58]	Cross-sectional-study	1997 subjects W = 1997 M = 0 Age = 18–76 year	UK	FFQs (131-item)	USDA database [(1–3)]	**Total flavonoids** Mean intake = 1170 ± 639 IQR = 617–1700	Total flavonoids were not significant associated with cardiovascular outcomesTotal flavonoids inversely associated with biomarkers of insulin resistance and inflammation: ↓ HOMA-IR, insulin, hs-CRP associated with anthocyanins intake (Q5 vs. Q1) ↓ HOMA-IR, insulin, adiponectin associated with flavones intake (Q5 vs. Q1)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Ponzo et al. [59]	Cohort study	1658 subjects W = 878 M = 780 Age = 45–64 year	Italy	FFQs	USDA Database (1-2-3) extended with information from a European database	**Total flavonoids** Median intake T1 = 89 T2 = 251.4 T3 = 532.3	↓ 54% non-fatal CV events risk associated with higher flavonoid intake (T3 vs. T1; HR = 0.46; 95% CI: 0.28–0.75) ↓ non-fatal CV events risk associated with several flavonoids' subclasses: Proanthocyanids (HR = 0.43; 95% CI: 0.27–0.70) Flavan-3-ols (HR = 0.42; 95% CI: 0.26–0.68) Anthocyanidins (HR = 0.56; 95% CI: 0.36–0.89) Flavanones (HR = 0.48; 95% CI: 0.29–0.77) Flavonols (HR = 0.53; 95% CI: 0.34–0.83) Total and subclasses of flavonoids were not significantly associated with the risk of CV mortality ↓ all-cause mortality associated with the T3 of several flavonoid subclasses: Flavan-3-ols (HR = 0.68; 95% CI 0.48–0.96) Anthocyanidins (HR = 0.66; 95% CI 0.46–0.95) Flavanones (HR = 0.59; 95% CI 0.40–0.85)
Jacques et al. [60]	Cohort study (Framingham Heart Study Offspring cohort)	2880 subjects W = 1302 M = 1578 Age = 54 year CL = 53.8–54.5	USA	FFQs	USDA database (1–3)	**Total flavonoids** **Exam 5 (1991–1995)** Median = 212 25th = 124 75th = 372 **Exam 8 (2005–2008)** Median = 259 25th = 157 75th = 436	Total flavonoids' intake was not significantly associated with the risk of incidence of CVD events (RR = 0.93; 95% CI: 0.82–1.06)
Yeon et al. [61]	Cohort study	4186 subjects W = 2575 M = 1611 Age = 40–59 year	Korea	24-h DR	USDA Database (1)	**Flavanones** W = 29.24 ± 4.17 M = 21.26 ± 4.37 **Flavones** W = 0.48 ± 0.04 M = 0.36 ± 0.02 **Flavonols** W = 17.06 ± 0.55 M = 15.72 ± 0.59	↓ insulin (β-coefficient = −0.0067; p for trend = 0.0092) and HOMA (β-coefficient = −0.0016; p for trend = 0.0239) associated with flavonols intake in men ↓ insulin (β-coefficient = −0.0008; p for trend = 0.0063) and HOMA (β-coefficient = −0.0002; p for trend = 0.0119) associated with flavanones intake in women
Oh et al. [62]	Cohort study	7963 subjects W = 7963 M = 0 Age = >30 years	Korea	24-h DR	Flavonoid Korean Database	**Total flavonoids** Mean Intake: Normal fasting glucose group = 107.40 ± 1.69 Type 2 diabetes mellitus group = 97.81 ± 8.11	↓prevalence of type 2 diabetes associated with intake of flavones above the 25th percentile (≥0.25 mg/day) compared with intake below the 25th percentile (OR = 0.593, 95% CI: 0.414–0.847)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Goetz et al. [63]	cohort study	20,024 subjects W = 11,253 M = 8771 Age = >45 years	US	FFQs (107-item)	USDA database (1–3)	**Total flavonoids** Median intake (range) Q1 = 34.3 (<50.8) Q2 = 66.6 (50.9–83.4) Q3 = 102.9 (83.5–127.0) Q4 = 156.9 (127.1–208.3) Q5 = 296.8 (≥ 208.4)	↓risk of incident acute ischemic stroke (HR = 0.72; 95% CI: 0.55, 0.95; P-trend = 0.03) was associated with flavanone intake, but not total or other flavonoid subclasses. Associations did not differ by sex race, or region for any flavonoid measure.
Goetz et al. [64]	Cohort study	16,678 subjects W = 9798 M = 6880 Age = >45 years	US	FFQs (107-item)	USDA database (1–3)	**Total flavonoids** W: Mean intake = 234 Median intake = 131 M: Mean intake = 227 Median intake = 131	↓incident CHD associated with consumption of anthocyanidin and proanthocyanidin. Anthocyanidins Q1 vs. Q5; HR = 0.71; 95% CI: 0.52–0.98; P-trend = 0.04; proanthocyanidins Q1 vs. Q5; HR = 0.63; 95% CI: 0.47–0.84; P-trend = 0.02). There was no significant effect modification by age, sex, race, or region of residence
Miranda et al. [65]	Cohort study	550 subjects W = 346 M = 204 Age = 20–59 years Age older adults = >60 years	Brazil	2 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake = 392.6 Median intake = 360.6	↓hypertension associated with highest tertiles of some classes of polyphenols: tyrosols (OR = 0.33; 95% CI 0.18–0.64), alkylphenols (OR = 0.45; 95% CI 0.23–0.87), lignans (OR = 0.49; 95% CI 0.25–0.98), as well as stilbenes (OR = 0.60; 95% CI 0.36–0.98), and other polyphenols (OR = 0.33; 95% CI 0.14–0.74). ↓hypertension associated with middle tertiles of total polyphenols and phenolic acids.There was no significant association for total flavonoids
Cassidy et al. [66]	Cohort study (HPFS cohort)	43,880 subjects M = 43,880 W = 0 Age = 32–81 years	UK	FFQs	USDA database (1)	**Anthocyanins** Q1 = 1.9 Q2 = 4.5 Q3 = 7.8 Q4 = 13.7 Q5 = 26.3 intake range = 0–613 IQR = 3.9–15.7 **Flavanones** Q1 = 7.5 Q2 = 23.6 Q3 = 43.5 Q4 = 64.5 Q5 = 103.9 intake range = 0–728 IQR = 18.8–70.9	↓total or fatal MI risk associated with higher anthocyanin intake (HR = 0.87; 95% CI: 0.75–1.00; P = 0.04; P-trend = 0.098); this association was stronger in normotensive participants (HR = 0.81; 95% CI: 0.69–0.96; P-interaction = 0.03). Anthocyanin intake was not associated with stroke risk. ↓ischemic stroke associated with higher flavanone intake (HR = 0.78; 95% CI: 0.62–0.97; P = 0.03, P-trend = 0.059); with the greatest magnitude in participants aged > 65 years (P-interaction = 0.04). Flavanone intake was not associated with MI or total stroke risk

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Kim et al. [67]	Cohort study	4042 subjects W = 1970 M = 2072 Age = >19 years	US	2 24-h DR	USDA Database (1-2-3)	**Total flavonoids** Mean intake Q1 = 12.5 Q2 = 59 Q3 = 197.6 Q4 = 585.5	Changes in percentages of cardiovascular risk factors with a 100% increase in flavonoid intake: ↑ 0.54% HDL-cholesterol associated with higher total flavonoid intake ↓ 1.25% TAG and ↓ 1.60% TAG:HDL-cholesterol ratio associated with anthocyanidin intake ↓ 1.31% TAG and ↓ 1.83% TAG:HDL-cholesterol ratio associated with total flavonoid intake ↓ 3.18% insulin and ↓ 3.10% HOMA-IR were associated with flavone intake ↓ 3.11% insulin and ↓ 4.01% HOMA-IR were associated with isoflavone intake ↓ 0.60% BMI associated with anthocyanidin intake
Rizzi et al. [68]	Cohort study	443 subjects W = 175 M = 268 Age = 20–85 years	Italy	24-h DR	USDA Database (1) Phenol Explorer EIO Database	**Total polyphenols** range intake T1 = 99.4–804.5 T3 = 1288.0–4342.2	High polyphenols intake was not associated with significant differences in the lipid profile compared with low polyphenols intake
Grosso et al. [69]	Cohort study (HAPIEE study)	5806 subjects W = 3075 M = 2731 Age = 45–69 years	Poland	FFQs (148-item)	Phenol Explorer	**Total polyphenols** Mean intake Q1 = 1026.7 ± 212 Q2 = 1469.6 ± 102.2 Q3 = 1872.6 ± 136.7 Q4 = 2632.1 ± 608	↓ 32% of risk of type 2 diabetes in the whole population associated with highest intake of total polyphenol (Q4 vs. Q1)
Witkowska et al. [70]	Cohort study	2599 subjects W = 2599 M = 0 Age = 20−74 years	Poland	24-h DR (367-item)	Phenol Explorer	**Total polyphenols** Mean intake Q1 = 948.2 ± 236 Q2 = 1523.2 ± 142 Q3 = 2016.3 ± 154 Q4 = 2975.8 ± 724	↓ 1.1% odds ratio of CVD in postmenopausal women with higher dietary polyphenol intake (per 100 mg/day)
Grosso et al. [71]	Cohort study (HAPIEE study)	8821 subjects W = 4530 M = 4291 Age = 50–65 years	Poland	FFQs (148-item)	Phenol Explorer	**Total polyphenols** n.a.	↓ metabolic syndrome associated with the highest quartile of polyphenol intake (OR = 0.80; 95% CI: 0.64–0.98 and OR = 0.70; 95% CI: 0.56–0.86 for both men and women, respectively). ↓ blood pressure, waist circumference, high lipoprotein cholesterol, and triglycerides associated with high total polyphenol intake in women. ↓ fasting plasma glucose associated with high total polyphenol intake in both genders.

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Sohrab et al. [72]	Cohort study	1265 Subjects W = 711 M = 554 Age = 19–74 years	Iran	FFQs	Phenol Explorer	**Total polyphenols** Median Intake (range) T1 = 827 (≤1128) T2 = 1425 (1129–1819) T3 = 2459 (≥1820) **Total flavonoids** Median intake (range) T1 = 38.4 (≤52.8) T2 = 69.5 (52.9–88.4) T3 = 115.1 (≥88.5)	Total polyphenols were not significant associated with metabolic syndrome ↓ 31% metabolic syndrome risk (OR = 0.69; 95% CI: 0.48–0.98, P-trend: 0.04) associated with total flavonoid intake (T3 vs. T1)
Mendonça et al. [73]	Cohort study (SUN cohort)	17,065 Subjects W = 10,358 M = 6707 Age = 20–89 years	Spain	FFQs 136-item	Phenol Explorer USDA database	**Total polyphenols** Mean Intake Q1 = 396 (±134) Q2 = 526 (±149) Q3 = 653 (±149) Q4 = 812 (±156) Q5 = 1248 (±405) **Total flavonoids** Mean Intake Q1 = 186 (±72) Q2 = 234 (±86) Q3 = 302 (±97) Q4 = 424 (±105) Q5 = 772 (±330)	Total polyphenols were not significant associated with cardiovascular events (HR = 0.61; 95% CI: 0.33–1.13 P for trend 0.28) Total flavonoids were not significant associated with cardiovascular events (HR = 0.53; 95% CI: 0.29–0.98 P for trend 0.09)

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire. (1) = USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) = USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) = USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation. a = Milder et al. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matair- esinol. Br J Nutr 2005.; Valsta et al. Phyto-estrogen database of foods and average intake in Finland. Br J Nutr 2003.; Mazur et al.Adlercreutz H. Lignan and isoflavonoid concentrations in tea and coffee. Br J Nutr 1998.; Mazur et al. Natural and anthropogenic environ- mental oestrogens: the scientific basis for the risk assessment. Naturally occurring oestrogen in food. Pure Appl Chem 1998.

2.5. Polyphenols intake and all-cause/cardiovascular mortality

In **Table 3** the association between polyphenol intake and all-cause mortality is reported with a specific focus on cardiovascular mortality. A total of 10 studies [73–82] (**Figure 5A**) were found; most of the them (50%; 5 out of 10) were performed in Europe (Spain, Italy and The Netherland), 2 in USA, 2 in Australia and 1 was performed including USA, Canada and Australia. Five out of 10 trials (50%) involved older subjects (> 65 years), 3 studies were performed in adults while 2 trails included both adult and older subjects (**Figure 5B**). The food intake was assessed mainly by FFQ (60%; 6 out of 10 studies); however, some studies (30%) associated FFQs with other tools for the evaluation of food intake (i.e. computerized dietary history questionnaire). One study combined FFQ with EPIC questionnaire (**Figure 5C**). The evaluation of polyphenol intake was estimated by USDA database (30%; 3 out of 10 studies), or a combination of USDA with others database (40%), or USDA with PE (20%; 2 out of 10 studies). When polyphenol content of specific food-products was missing in available databases, data were obtained from the literature. One study estimated polyphenol intake, in particular monomeric flavan-3-ol, by considering their content in 120 commonly consumed plant foods and beverages obtained by combining results from reverse-phase HPLC and data from literature (**Figure 5D**).

Overall, one study that investigated the association with total polyphenol intake and all-cause mortality failed to demonstrate a significant effect [75]. Similar findings were also reported by considering the association between total flavonoids and CV mortality [73]. On the contrary, a reduction of mortality risk for cardiovascular events and all–cause mortality was associated with total flavonoid intake in the highest quintiles ranging from 360 mg/day [78] to about 800 mg/day [80]. The impact of the single subclasses has been evaluated in some of the studies, but the effects were conflicting depending on the subject's characteristics (i.e. age, sex) and cause of mortality.

Generally, the models adjusted for the age, as confounding factor, reported a protection also for specific flavonoid subclasses such as isoflavones, flavan-3-ols, flavones. The effects in some cases were found both in women and men. However, generally adjustments for the different confounding factors (i.e. BMI, smoking and alcohol habits, energy intake, physical activity, medications, etc…) affected the significance of the associations.

Table 3. Association between polyphenol intake and all-cause/cardiovascular mortality.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
McCullough et al. [74]	Cohort study (American Cancer Society's CPS-II Nutrition Cohort Study)	98,469 subjects W = 60,289 M = 38,180 Mean Age W = 70 years Mean Age M = 69 years	USA	FFQs (152 food items)	USDA database [1-2-3] other research publications	**Total flavonoids** Mean intake (energy-adjusted) **Men** Mean intake = 268 Median intake = 203 10th–90th percentile = 99–498 **Women** Mean intake = 268 Median intake = 201 10th–90th percentile = 92–522	**Cardiovascular mortality** **Age-adjusted model:** Inverse association were observed for high total flavonoid, anthocyanidins (median 22.2 (≥16.7) mg/day), flavan-3-ols (median 63.7 (≥37.2) mg/day), flavones (median 3.0 (≥2.1) mg/day), flavonols (median 27.2 (≥20.6) mg/day), proanthocyanidins (median 379.4 (≥253.6) mg/day) and isoflavones (median 0.713 (≥0.142) mg/day) in both the sex. Inverse association for flavanones (median 49.9 (≥35.4) mg/day) in women. **Multivariable-adjusted model** [3]: No association in men. Inverse association for high total flavonoid, anthocyanidin, flavan-3-ol intake in women. Subjects with high total flavonoid consumption (median 512.5 (≥359.7) mg/day) showed a low risk of death (−18%) in both the sex.Inverse association for high anthocyanidin, flavan-3-ol, flavones, flavanol and proanthocyanidin intake by considering women + men. **Ischemic heart disease mortality** **Age-adjusted model**: Inverse association for high anthocyanidin and flavone intake in both the sex. Inverse association for high total flavonoid intake in men and women + men; high flavanone intake in women + men; high flavanol intake in women and women + men; high proanthocyanidin intake in women + men; high isoflavone intake in men and women + men. **Multivariable-adjusted model** [3]: Inverse association for high flavone intake in women and women + men **Stroke mortality** **Age-adjusted model**: Inverse association for high total flavonoid intake in men, and high flavones intake in men and women + men. **Multivariable-adjusted model** [3]: Inverse association for high total flavonoid intake in men

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Zamora-Ros et al. [75]	Cohort study(Invecchiare in Chianti study)	807 subjects W = 447 M = 360 Age = 74.3 ± 6.9 years Survived W = 313 M = 240 Age = 71.8 ± 5.3 years Died W = 134 M = 140 Age = 79.2 ± 7.2 years	Italy	FFQs (Italian version) Urinary polyphenol assessment	Phenol Explorer USDA database	**Total polyphenols** Mean intake = 594 ± 196 **Survived** Mean intake = 600 ± 201 **Died** Mean intake = 584 ± 185	No association between total dietary polyphenols and all-cause mortality
Zamora-Ros et al. [76]	Cohort study (EPIC Spain Study)	40,622 subjects W = 25,298 M = 15,324 Age = 29–69 years	Spain	FFQs computerized diet history questionnaire developed and validated specifically for the EPIC study in Spain	USDA database Phenol-explorer UK Food Standards Agency (10% missing values in 1877 food items)	**Total flavonoids** Mean intake = 387.3 ± 280.2 Median intake = 329.8 25th percentile = 218.4 75th percentile = 489.6 **Total lignans** Mean intake = 1.0 ± 0.5 Median intake = 0.9 25th percentile = 0.7 75th percentile = 1.2	**Multivariable-adjusted model** [2]: subjects with high flavanones (>51.3 mg/day), flavanols (>28.0 mg/day) and total flavonoids intake (>447.8 mg/day) showed a low risk of all-cause mortality (flavanones: 0.60 (95% CI = 0.38–0.94) and flavonols: 0.59 (95% CI = 0.40–0.88). This reduction was due entirely to a decrease in mortality from CVD. Proanthocyanidins were the most important contributor (66%) to total flavonoid intake, followed by flavanones (11%), flavan-3-ol monomers (9%), anthocyanidins (7%), and flavonols (6%), flavones (1%), isoflavones (0.1%), and theaflavins (<0.1%). No evidence of an association between dietary flavonoid or lignan intake and mortality from cancer or other causes.
Ivey et al. [77]	Cohort study	1063 Subjects W = 1063 Age > 75 years	Australia	FFQs developed by the AntiCancer Council of Victoria	USDA	**Total flavonoids** **Flavonol**: 31 ± 14 **Flavan-3-ol**: 431 ± 279 **Proanthocyanidin**: 215 ± 147 Flavone: 3 ± 2 **Flavanone**: 53 ± 38 **Anthocyanidin**: 37 ± 26 **Isoflavone**: 5 ± 6	**Unadjusted model**: Subjects with high intake of total flflavonol (>35 mg/day), flflavan3-ol (>563 mg/day), flavone (>3 mg/day) and flavanone (>61 mg/day) showed a reduced risk of atherosclerotic vascular disease mortality. **Age- and energy-adjusted model and multivariate-adjusted model** [4]: Subjects with high intake of total flflavonol (>35 mg/day), flflavan3-ol (>563 mg/day) showed a reduced risk of atherosclerotic vascular disease mortality No association was observed for the other flavonoid subclasses Tea contributed 59% of total flavonoid intake; the major contributors were flavonols (65%) and flavan-3-ols (93%). **Multivariate-adjusted model** [4]: Subjects with high intake of flavonols derived from tea and non-tea sources (≥12 mg/day and ≥27 mg/d, respectively) showed a low risk of atherosclerotic vascular disease mortality.

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Ivey et al. [78]	Cohort study	1063 subjects W = 1063 M = 0 Age > 75 years	Australia	FFQs (93 items) Beverage questionnaire (to assess tea and coffee consumption)	Phenol Explorer (47 foods recorded as not containing flavonoids or not present in the database) USDA database [1-2-3] (19 foods recorded as not containing flavonoids or not present in the database)	**Total flavonoids (PE)** Mean intake = 674 ± 326 Median intake = 648 IQR = 449–872 **Total flavonoids (USDA)** Mean intake = 696 ± 322 Median intake = 668 IQR = 468–889	Subjects with high total flavonoid intake (≥813 mg/day USDA; ≥788 mg/day PE) showed a low risk of all-cause mortality and cardiovascular mortality
Ponzo et al. [59]	Cohort study	1658 subjects W = 878 M = 780 Age = 45–64 year	Italy	FFQs	USDA Database [1-2-3] extended with information from an European database	**Total flavonoids** Median intake T1 = 89 T2 = 251.4 T3 = 532.3	Total and subclasses of flavonoids were not significantlyassociated with the risk of CV mortality The third tertile of flavan-3-ols (HR = 0.68; 95% CI 0.48–0.96), anthocyanidins (HR = 0.66; 95% CI 0.46–0.95) and flavanones (HR = 0.59; 95% CI 0.40–0.85) was inversely associated with all-cause mortality
Dower et al. [79]	Cohort study (Zutphen Elderly Study)	774 subjects W = 0 M = 774 Age = 65–84 years	The Netherlands	Cross-check dietary history (adapted for the Dutch setting) [5]	Monomeric flavan-3-ol contents of 120 commonly consumed plant foods and beverages were determined with the use of reverse-phase HPLC with ultraviolet and fluorescence detection. (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg), and (+)-gallocatechin concentrations were determined as reported in previous published papers [6]	**Total epicatechins** Mean intake = 15.2 ± 7.7 Range intake = 0.01–60.6	**Coronary heart disease mortality** Subjects with high epicatechin intake (>18 mg/day) showed a low (−38%) risk of CHD mortality **Cardiovascular disease mortality** Subjects with high epicatechin intake (>18 mg/day) showed a low (−46%) risk of CVD mortality in men with prevalent CVD but not in men who were free of CVD The major dietary sources of epicatechin intake were tea (7.8 mg/day; 51% of total epicatechin intake), apples (4.3 mg/day; 28% of total epicatechin intake), cocoa (1.1 mg/day; 7% of total epicatechin intake), and other sources (2.0 mg/day; 13% of total epicatechin intake)

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Ivey et al. [80]	Cohort study (Nurses' Health Study II)	93,145 subjects W = 93.145 M = 0 Age = 25–42 years	USA	FFQs	USDA database	**Total flavonoids** Mean intake = 379 ± 374	**Age-adjusted model**: subjects with high total flavonoids intake (≥518 mg/day) showed a 19% reduction of overall mortality in the 18-year follow-up period. Subjects with high flavan-3-ols (≥86 mg/day), proanthocyanidins (≥356 mg/day) and anthocyanin intake (≥17 mg/day) showed a low risk of mortality for CVD and other causes. **Multivariable adjusted model** [1]: no association High consumption (more than once per week) of red wine, tea, peppers, blueberries and strawberries was associated with reduced risk of total and cause-specific mortality.
Zhang et al. [81]	Cohort study	6235 subjects with breast cancer W = 6235 M = 0 Age = 51.8 ± 10.6 years	USA, Canada, Australia	FFQs	USDA database	**Total isoflavones** Mean intake: 1.8 ± 3.9 Median intake: 0.7 IQR intake: 1.2	Quartile 4 (≥1494 mg/day) associated with a 21% decrease in all-cause mortality. This result was limited to women with negative tumor hormone receptors and those not treated with hormonal therapy for breast cancer

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Pounis et al. [82]	Cohort study (Moli-sani cohort Study)	21,302 subjects W = 10,980 M = 10,322 Age = 54–56 years	Italy	FFQs	Eurofir-eBASIS FCTs USDA database (1-2-3)	Data reported as polyphenol antioxidant content (PAC)-score [6] (−28 to 28)	**Risk for all-cause mortality** Women: low risk for high intake of flavones (>1.12 mg/day), flavanones (>46.5 mg/day), isoflavones (>32.7 mg/day), and lignans (>116.1 mg/day) had a low risk. **After adjustments for potential confounders (model 4)** [7]: the effects remained significant for Q4 (4–13) and Q5 (>13) of PAC-score Men: low risk for some quintile of intake; flavonols (Q2: 11.2–15.1 mg/day and Q5: >25.8 mg/day), flavones (Q3: 0.61–0.81 mg/day), flavanones (Q2: 22–29 mg/day), isoflavones (Q2: 16.5–21 mg/day, Q3: 21–25.7 mg/day and Q5: >32.7 mg/day), lignans (Q3: 72.8–90.3 mg/day). **After adjustments for potential confounders (model 4)** [8]: the effects remained significant for Q2 (−13 to −4), Q3 (−4 to 4) and Q4 (4–13) of PAC-score **Vascular causes** Women: no association Men: low risk of mortality for Q2 (−13 to −4) and Q3 (−4 to 4) **Other causes** Women and men at Q2 (−13 to −4) and Q4 (4–13) of PAC-score showed a low mortality risk from other causes

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire. [1] BMI, smoking status, menopausal status, family history of diabetes/cancer/myocardial infarction, multivitamin supplement use, aspirin use, race, type 2 diabetes, hypercholesterolaemia, hypertension, physical activity, alcohol consumption and energy intake and the Alternative Health Eating Index (minus alcohol) score. [2] Cox proportional hazards regression models were stratified by center, age (1 year) and sex and adjusted for BMI, education level, physical activity, tobacco smoking, alcohol lifetime, total energy, vitamin C and fiber intake. [3] Age, smoking, beer and liquor intake, history of hypertension, history of cholesterol, family history of myocardial infarction, BMI, physical activity, energy intake, aspirin use, hormone replacement therapy (in women only), and sex (in combined model only) by using Cox proportional hazards regression. [4] age, previous CVD, previous diabetes, energy expended in physical activity and history of smoking. [5] Keys A et al., Acta Med Scand Suppl 1966;460:1–392. [6] Arts ICW et al., J Agric Food Chem 2000;48:1752–7; Arts ICW et al., J Agric Food Chem 2000;48:1746–51. [7] Pounis et al., European Journal of Clinical Nutrition 2016; 70;338–345. [8] Age, energy intake, smoking habits, social status, physical activity level and INFLA-score. Eurofir-eBASIS: European Food Information Resource—Bioactive Substances in Food Information Systems; FCTs: Italian Food Composition Tables; (1) USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation.

2.6. Polyphenols intake and other outcomes

Table 4 shows the associations between polyphenol intake and other outcomes in a total of 13 studies [83–95]. The associations were evaluated for endothelial function (1 study), kidney function (1 study), bone health (i.e. bone mineral density, frailty and fractures; 3 studies), eyes health (i.e. cataract and macular degeneration; 2 studies), physical performance decline (1 study), dementia (1 study), cognitive decline (1 study) and pubertal development (1 study).

Six out of 13 studies (46%) were performed in Europe, 3 in Australia, 2 in the USA and in Asia **Figure 6A**). Over than a half of the studies (58%) were carried out in the older population while 33% included adult and older subjects. 1 study was performed only in adults and 1 in adolescents (**Figure 6B**). The most frequent tools used for the evaluation of the diet were the FFQs (77%; 10 studies), 1 study used a 24-h DR while 2 studies combined FFQs with other tools (**Figure 6C**). Half of the studies (50%) used USDA database, or a combination of USDA with PE (3 studies) or USDA with other databases (2 studies). Only one study performed the estimation using PE, while one study used a different specific database for the calculation of polyphenol intake (**Figure 6D**). An overall association between high intake of polyphenols and subclasses, and different outcomes was observed. Conversely, in the InCHIANTI study urinary total polyphenols, but not total dietary polyphenols, were associated with a lower probability of frailty or pre-frailty [89] and cognitive decline [87]. Flavonoids have been associated with a higher endothelial function (>640 mg/day) [83], a lower risk of reduced forced vital capacity and spirometric restriction of the lung (≈ 290 mg/day) [93], a higher bone mineral density (≈ 490 mg/day). In addition, flavonoids have been inversely associated with bone fractures (≈ 1500 mg/day) [88,90] and macular degeneration (≈ 875 mg/day) [94]. Proanthocyanidins (≥ 229 mg/day) were inversely associated with risk of renal failure events and kidney insufficiency, while isoflavones (> 3 mg/day) with a better pubertal development [84,86].

Table 4. Polyphenol intake and other outcomes.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Fisher et al. [83]	Analytical	19 subjects W = 11 M = 8 Age = 72 ± 7 years	US	FFQs	USDA database (1–3) (22 food item)	**Total flavonoids** Median intake = 2428 mg/week Median = 347 Q1–Q4 = 1242–4789 mg/week	Habitual dietary intake of flavonoids was associated with higher endothelial function evaluated as reactive hyperemia (RH)-PAT response. Subjects with habitual flavonoid intake (>4500 mg/week) had significantly higher (RH)-PAT response
Ivey et al. [84]	Prospective	948 subjects W = 948 M = 0 Age = ≥75 years	Australia	FFQs	USDA database (1-2-3)	**Total Proanthocyanidins** Mean intake = 215 ± 147 Min-max = 18–1728	Over 50% of total proanthocyanidin intake were from fruit (89 ± 63 mg/day), chocolate (43 ± 75 mg/day), and alcoholic beverages (32 ± 86 mg/day). Subjects with habitual proanthocyanidin intake (≥229 mg/day) had lower risk of moderate chronic kidney insufficiency and renal failure events
Zhang et al. [85]	Cross-sectional	3317 subjects W = 2239 M = 1078 Age = 60.2 years	China	FFQs (79-item)	USDA database (1–3) Hong Kong database of isoflavones [1]	**Total flavonoids** Median intake **W(Q1)** = 53.3 IQR = 40.5–66.3 W(Q2) = 110.0 IQR = 92.2–132.1 W(Q3) = 232.4 IQR = 194.8–274.4 **W(Q4)** = 486.9 IQR = 402.2–584.4 **M(Q1)** = 63.1 IQR = 44.9–94.6 M(Q2) = 207.9 IQR = 174.4–237.2 M(Q3) = 351.8 IQR = 297.6–392.2 **M(Q4)** = 555.3 IQR = 479.6–618.2	High total flavonoid intake (Q4 vs. Q1) was associated with higher bone mineral density (BMD) in women, but not in men. A dose dependent positive relationship was found for all BMD measured sites. In addition, a significant association was found also for flavonoid subclasses (flavonols, flavan-3-ols, flavones, and proanthocyanidins)
Urpi- Sarda et al. [86]	Cross-sectional (Invecchiare CHIANTI Study)	811 subjects W = 446 M = 364 Age = >65 years	Italy	FFQs	Phenol explorer USDA database	**Total polyphenols** Mean intakeAll (N = 811) = 595.2 ± 195.6 Non-frail (n = 418) = 608.5 ± 199.8 Prefrail (n = 321) = 587.3 ± 195.9 Frail (n = 72) = 550.5 ± 158.7 1T < 509.2 2T = 509.2–645.2 3T < 645.2	No association between total dietary polyphenols and frailty and pre-frailty in older subjects

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Rabassa et al. [95]	Cross-sectional (Invecchiare CHIANTI Study)	652 subjects W = 361 M = 291 Mean Age = 73	Italy	FFQs	Phenol explorer USDA database	**Total polyphenols** Median intake All (n = 652) = 574 IQR = 472–701 1T = 430 IQR = 354–470 2T = 574 IQR = 543–610 3T = 766 IQR = 701–855	No association between total dietary polyphenols and any cognitive test in older subjects
Myers et al. [87]	Prospective	1188 subjects W = 1188 Age = >70 years	Australia	FFQs Beverage questionnaire	USDA database [1-2-3]	**Total flavonoids** Median intake **Tea Low consumer** = 266 IQR = 191–361 **Tea Moderate consumer** = 845 IQR = 672–959 **Tea High consumer** = 1570 IQR = 1325–1915	Higher intake of black tea and flavonoids was associated with lower hospitalization (30–40% reduction) for fractures in older women at high risk
Ma et al. [88]	Case-control	249 subjects (cases) 66 subjects (controls) W = 182 M = 133 Age = 50–70 years	China	FFQs 3 24-h DR	USDA database	**Total flavonoids** **Cases** Median intake = 51.13 IQR = 38.06–64.21 **Controls** Median intake = 64.92 IQR = 53.66–75.61	Total dietary anthocyanidin, flavan-3-ol, flavanone, flavone, and flavonol intake was not associated with age related cataract risk. Only quercetin and isorhamnetin intake appeared to be associated with the risk in this population
Rabassa et al. [89]	Cross-sectional (Invecchiare CHIANTI Study)	368 subjects W = 199 M = 169 Age = >65 years	Italy	FFQs	USDA database [1–3] Phenol explorer 236 food items	**Total polyphenols** **Baseline** Median intake = 556 IQR = 462–682 **3-year follow-up** Median = 539 IQR = 429–656 **6-years** Median = 513 IQR = 415–619 **9-years** Median = 500 IQR = 407–595	Total dietary polyphenol (TDP) intake was higher in older subjects and women with higher physical activity level. No association between TDP and physical performance decline was found

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Garcia-Larsen et al. [90]	Cross-sectional (GA^2LEN study)	2599 subjects W = 1516 M = 1083 Age = 47.2 ± 14.5 years	Denmark Finland Sweden UK Portugal Belgium Germany Netherlands Poland	FFQs (250-item)	USDA database [1–3]	**Total flavonoids** Median intake = 291.2 IQR = 126.8–569.4	Total flavonoid intake and pro-anthocyanidins was positively associated with a good ventilatory function (forced vital capacity), while a negative association with spirometric restriction was found in the cohort. In particular, subjects with total flavonoid intake at the highest quintile had a 42% lower risk of reduced forced vital capacity
Gopinath et al. [91]	Cohort study (Blue Mountains Eye Study)	2856 subjects W = 1597 M = 1259 Age = ≥ 49 years	Australia	FFQs (145-item)	USDA database [1-2-3]	**Total flavonoids** Median intake = 875 Q1 ≤ 410.6 Q2 = 412.4–881.5 Q3 = 881.6–1232.3 Q4 ≥ 1232.4	Total flavonoids and subclasses (e.g., flavonols and flavanones), were associated with age-related macular degeneration (AMD) among older adults. The consumption of oranges and orange juice, contributing to total flavanone intake, was found to significantly affect AMD risk
Pounis et al. [92]	Cross-sectional (Moli-sani study)	9659 subjects W = 4551 M = 5108 Age = ≥35 years	Italy	FFQs (164-item)	Eurofir–e BASIS USDA database [1-2-3]	**Flavonols** Median intake (Q1–Q3) W = 15.4 (11.1–21.2) M = 19.1 (14.1–26.0) **Isoflavones** Median intake (Q1–Q3) W = 23.3 (17.9–31.0) M = 23.7 (18.1–31.1) **Flavones** Median intake (Q1–Q3) W = 0.77 (0.53–1.10) M = 0.65 (0.44–0.95) **Flavanones** Median intake (Q1–Q3) W = 31.1 (22.9–42.1) M = 35.0 (26.1–45.9)	Higher polyphenol intake was associated with better pulmonary function (forced vital capacity, and forced expiratory volume in the first second) in the population under study. A potential anti-inflammatory activity of polyphenols was hypothesized in men where a reduction in C-reactive protein and white blood cells was observed

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Pounis et al. [92]	Cross-sectional (Moli-sani study)	9659 subjects W = 4551 M = 5108 Age = ≥35 years	Italy	FFQs (164-item)	Eurofir–e BASIS USDA database (1-2-3)	**Flavanols** Median intake (Q1–Q3) W = 41.6 (24.4–73.0) M = 66.1 (36.3–108.8) **Anthocyanidins** Median intake (Q1–Q3) W = 145.3 (99.8–209.3) M = 148.0 (101.9–216.3) **Lignans** Median intake (Q1–Q3) W = 82.7 (61.1–109.8) M = 81.2 (61.1–107.2)	Higher polyphenol intake was associated with better pulmonary function (forced vital capacity, and forced expiratory volume in the first second) in the population under study. A potential anti-inflammatory activity of polyphenols was hypothesized in men where a reduction in C-reactive protein and white blood cells was observed
Lefevre-Arbogast et al. [93]	Cohort study (The 3C Bordeaux cohort)	1329 subjects W = 824 M = 505 Mean Age = 75.8 years	France	24-h DR	Phenol Explorer	**Total polyphenols** Mean intake **All subjects** = 1071 ± 570 **Incident dementia** = 1029 ± 542 (n = 256) **No dementia** = 1081 ± 576 (n = 1073)	Polyphenol intake was associated with a decreased risk of all-cause dementia and of Alzheimer disease (AD) over 12 years. Subjects in the higher quintile of intake had a ≈ 50% lower risk of both dementia and AD. The pattern of polyphenol intake associated with the reduced risk was characterized by flavonoids (e.g., dihydroflavonols, anthocyanins, isoflavonoids, and flavanones), stilbenes (including resveratrol), lignans, and additional isolated polyphenols (hydroxybenzaldehydes, naphthoquinones, and furanocoumarins)
Segovia-Siapco et al. [94]	Cross-sectional (The Teen Food and Development Study)	248 subjects W = 0 M = 248 Age = 12–18 years	USA	Web-FFQs (151-item)	Nutrition Data Systems for Research (NDS-R) Specific database [2]	**Total Isoflavones** Mean intake = 22.1 Min and max = 18.3–26.0	Moderate (3–20 mg/day) and high (>20 mg/day) consumers of soy isoflavones nearly follow the same pattern for pubertal development. Whether soy isoflavones play a role in the rate of maturation and sequencing of pubertal development in boys cannot be determined based on our study findings

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire; sFFQs = semi-quantitative FFQ. (1) USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation. [1] Chan SG, Murphy PA, Ho SC, Kreiger N, Darlington G, So EK, Chong PY (2009) Isoflavonoid content of Hong Kong soy foods. J Agric Food Chem 57:5386–5390. [2] Jaceldo-Siegl K, Fraser GE, Chan J, Franke A, Sabaté J (2008).

3. Discussion

The great interest for the protective role of polyphenols is demonstrated by the rapid increase of publications evaluating the mechanisms of action of these heterogeneous/complex and multi-target compounds, and also by the studies focused on association between polyphenol intake and different diseases or mortality. In particular, the association of both total or polyphenol subclasses with different types of cancer has been largely addressed in recent reviews and meta-analyses even if the effects are often nulls [96–102].

The present study analyzed the literature on polyphenol intake assessment *per se* or in relation to CVD, diabetes, other health outcomes or mortality.

As expected, the review of data obtained from different studies underlines a consistent difference in the estimated polyphenol intake which may be attributed to different methodological issues such as the type of tool administered to assess the intake, the database used for the calculation of polyphenol intake and the type of polyphenols under evaluation.

It is well known that dietary intake is difficult to measure, and single methods (i.e. questionnaires) cannot perfectly estimate dietary exposure. This is particularly critical especially for micronutrients and bioactive compounds. FFQs, and sometimes 24-h DR, represent the main tools used within the epidemiological studies to assess dietary intake. They have different characteristics; for example, FFQs consist in a pre-finite list of foods and beverages (the number of items queried typically ranges from 80 to 120) with response categories to indicate usual frequency of consumption over the time period queried. Conversely, the 24-h DR consists of an open-ended questionnaire administered by a trained interviewer able to collect detailed information about all foods and beverages consumed by the subjects in the previous 24 hours. Both questionnaires present several limitations; for example, FFQs lack of detailed information about food preparation, specific food and beverages consumed, as well as different brands. Moreover, the pre-specified food list does not necessarily reflect the eating behavior of the population under study and the presence of systematic errors must be partially

mitigated through appropriate statistical modeling that take into consideration the adjustments for cofounding factors such, as an example, age and energy intake. Regarding 24-h DR it requires multiple days to assess usual intake. In addition, multiple administrations are also recommended when 24-h DRs are used to examine diet impact on health outcomes or other parameters. On the other hand, it has been reported that the assessment of total flavonoid intake requires at least 6 days of weighed food records, and between 6 and 10 days to determine intake of specific flavonoid subclasses with an acceptable degree of accuracy [47]. Most of the studies analyzed in the present review did not perform a multiple evaluation of food intake as highly recommended thus, an under or overestimation of total polyphenols and their classes/subclasses intake cannot be excluded.

Another important critical point for the estimation of polyphenol intake is the choice of the databases. The most commonly used are USDA and Phenol-Explorer. USDA database focuses predominantly on flavonoids as aglycones (anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones), while Phenol-Explorer, in addition to the above mentioned flavonoids (mainly as glycosides), provides data also of the precursors (chalcones, dihydrochalcones and dihydroflavonols) and information on total polyphenols measured by Folin-Ciocalteu [25]. Despite both data sources are systematically extended to reflect most accurately phenolic contents in food, it is clear that they show several limitations. First of all, since they provide information on different classes of polyphenols, the comparison of the results obtained on the basis of the various data sources may differ. For example, some studies reported that the intake of flavonoids are generally higher when calculated using the USDA databases in relation to the Phenol-Explorer database [103]. In addition, despite they provide information on a wide range of foods, the list does not include all food and polyphenol sources; this represents a critical aspect since missing data have to be found by using different databases and/or by consulting the scientific literature with an increase of risk of bias. Moreover, the effect of seasonality, storage and

cooking process is not always considered but certainly, it could represent a critical point. Finally, in view of these issues, it should be remarked that all databases allow only an estimation of dietary polyphenols intake. In this regard, it is noteworthy that databases do not consider non-extractable polyphenols thus contributing to an overall under estimation of intake [104]. This is relevant since these compounds seem to have potential protective properties exerted through gut microbiota metabolites production [105].

In the present review, we found that most of the studies used USDA and Phenol-Explorer databases alone, in combination, or together with other databases and/or data sources (i.e. specific scientific publications). An estimation of polyphenol intake data obtained from reviewed studies using FFQs and from those using 24-h DR, seem to provide comparable results in terms of total polyphenol intake (FFQs 910 mg; 24-h DR 890 mg), total flavonoids (FFQs 360 mg; 24-h DR 380 mg) and total phenolic acids (FFQs 410 mg; 24-h DR 450 mg). In addition, it is noticeable that generally data come from single evaluations instead of multiple evaluations of food intake as recommendable, thus an under or overestimation of polyphenols and/or specific subclasses cannot be excluded.

Polyphenol intake is also affected by intrinsic factors such as the geographical area, the population characteristics in term of age, gender and socio-cultural factors and above all the dietary habits. In this regard, we have found that the intake of total polyphenols is higher in Japan (about 1500 mg/day) compared to European countries and North and South America (about 900 mg/day and 800 mg/day respectively). Within Europe, we found a large variability of intake between countries; Poland and France had the highest intake of total polyphenols (above 1000 mg/day), followed by Italy (about 650 mg/day) and Spain (about 300 mg/day). Conversely, within the EPIC study, Denmark showed the highest intake of total polyphenols (1786 mg/day) while Greece the lowest (584 mg/day)[27].

Regarding total flavonoids, Poland and Australia had the highest intake (about 600 mg/day) while USA and South America the lowest (about 200 and 400

mg/day, respectively) followed by Asia (China and Korea, at about 60 mg/day). Finally, regarding total phenolic acids, France, Poland and Brazil had the highest intake (above 600 mg/day), while USA, Italy and Spain the lowest (about 300 mg/day). These data were also in accordance with the results obtained within EPIC study, which showed a high flavonoid and phenolic acid intake in non-Mediterranean countries [15] associated to different dietary habits. For example, in the North and Central Europe, non-alcoholic beverages, in particular tea and coffee, are the main polyphenol contributors, while in South Europe the main contributors are fruits alcoholic beverages (e.g. red wine). In Asia, such as China and Korea, apples and vegetables seem to be the main polyphenol sources, while green tea in the Japanese population. Finally, tea, citrus and legumes seem to be the main polyphenol contributors in the USA.

As far as gender differences in polyphenol intake are concerned, data in literature are not univocal even if more studies suggest a higher intake in females compared to males, above all when standardization for energy intake is taken into account. In addition, differences in polyphenol sources selected seem to be dependent on gender (e.g. higher contribution of fruit and vegetables in females compared to males who are higher consumers of alcoholic beverages and coffee).

Notwithstanding, most of the data available have been assessing polyphenol intake in adults, a large number of studies considered also the intake in older subjects. Nine studies specifically reported results on total polyphenol and/or subclasses in target of older populations (2 Australia, 2 Spain, 1 Brazil, 1 Italy, 1 Poland, 1 UK and 1 Japan). Total polyphenol intake ranged from about 333 mg/day in Spain [44] to 1492 mg/day in Japan [32]. In addition, those considering total flavonoid intake registered values from about 170 mg/day in Spain [44] to about 834 mg/day in Australia [103]. When available the contribution of phenolic acids was approximately 30-40% of the total polyphenol intake. Studies considering different age classes found controversial results, even if generally, all studies reported differences in food

habits affecting polyphenol intake. For example, Vitale et al. [48] showed that flavonoid and stilbene increased with age in the TOSCA.IT study, being higher in over 65 years subjects compared to those with age lower than 65 years. Accordingly, Miranda et al [39] reported that older subjects (> 60 years) from a Brazil cohort consumed more flavonoids and tyrosol than adults (20-59 years) and also more fruits. Moreover, Zamora-Ros et al [106], showed an increased intake of flavonoids, stilbenes, lignans and other polyphenols with age, while no effect on total polyphenol intake in the EPIC cohort. Other studies reported no differences in polyphenol intake depending on age, or a slight increase after energy adjustment [43,49]. Others (Zujko et al [19]) showed lower levels of flavonoid intake in older Brazilian subjects who generally consumed less beverages and vegetables. Finally, Karam et al [44] found an increased energy adjusted polyphenol intake by age classes in older adults from Mallorca island showing also the impact of factors such as gender, educational level and lifestyle significantly affecting eating habits. Large differences in food selection depending on region/country have been underlined reflecting a different pattern of polyphenol intake.

Only 3 studies reported data on children and adolescents showing a low polyphenol intake associated to the overall dietary pattern generally poor in fruit and vegetables even if direct comparison among results is difficult due to the lack of energy adjustment of data in the different age subclasses. The main sources of polyphenols identified depending on the country were non-alcoholic beverages (UK, Argentine), fruit (apple, pear), juices, chocolate (in Helena European study [46]).

Extensive research on polyphenols in human studies has shown a potential role of these compounds in the modulation of CVD markers [107]. In the present systematic review, we found an overall inverse association between total polyphenol intake (highest quantile, above 1170 mg/day) and CV risk events and mortality. In addition, an increased protection against T2D events was observed for total polyphenol intake (mean intake of the 4^{th} quartile) higher than 2632 mg/day [61]. However, the results are not univocal and 4 out

of 9 papers reported no association at doses of polyphenols higher than 1200 mg/day or above (>2400 mg/day). These conflicting results could be attributed to the high heterogeneity of the studies in term of selected population characteristic, markers/endpoints measured (i.e. marker of CV risk analyzed), dietary habits (very different between countries), and polyphenol food sources (i.e. tea, coffee, fruits, alcoholic beverages).

Recent evidence from systematic reviews and meta-analyses of cross-sectional and prospective cohort studies seem to suggest that the intake of certain polyphenol classes and subclasses, more than total polyphenols, may reduce the incidence of T2D, CVD events and CVD mortality. However, most of the effects were found when comparing the highest quantiles *versus* the lowest. In fact, we reported a lower risk of CV events for an intake of total flavonoids ranging from 115 to 944 mg/day, an inverse association for T2D with the highest quartile of total flavonoids (585 mg/day), and a low risk of mortality for cardiovascular events and all–cause mortality for the highest quintile of total flavonoid intake (range 360-800 mg/day) [78,80]. These results are in line with observations reported by other authors. For example, McCullough et al [74], showed that a total flavonoid intake above 512 mg/day was inversely associated with fatal events for CVD in men and women. Feliciano and coworkers [108], reported that high consumers (>788 mg/day of total flavonoids) showed an inverse association with CVD events and CVD mortality. Wang and colleagues [109] found a reduced risk of CVD events for doses of flavonoids (including flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones and flavan-3-ols) between 139 and 604 mg/day. Finally, Grosso et al [110] showed that increasing by 100-mg/day flavonoid intake led to a linear decreased risk of 6% and 4% of all-cause and CVD mortality.

As regard the diverse subclasses of polyphenols, several studies have reported a positive effect for flavonols, flavones, flavanones, isoflavones, anthocyanidins and proanthocyanidins. For example, Wedick and coworkers [67], have shown that the highest quintile of anthocyanins (about 22.3 mg/day) and anthocyanin-rich fruit intake (≥ 5 times/week) was associated with a lower

risk of T2D. Conversely, limited evidence is available for lignans. One study performed by Rienks and colleagues [111] showed that high levels of plasma enterolactones (lignan precursors) were associated with a 30% and 45% reduction of all-cause and CVD mortality risk.

Interestingly, in the last years, a growing attention has been devoted to the impact of polyphenols on different health outcomes including for instance renal insufficiency, respiratory function, immune function, and vascular activity. For these outcomes, flavonoids and proantocyanidins have shown an apparent promising beneficial effect. Very recently, another research path has focused on the contribution of polyphenols in the older subject health outcomes. Specifically, the effect on retardation/prevention of some age-related complications such as cognitive decline, frailty and bone fractures has been investigated. On the whole, we have found an overall positive association between high intake of polyphenols and classes/subclasses, and a modulation of different outcomes associated with aging. In particular, total flavonoids and subclasses have been apparently associated with a higher bone mineral density, low risk of bone fractures and macular degeneration, while only total urinary polyphenols, but not dietary polyphenols, have been associated with a low risk of pre-frailty and frailty in older subjects. However, this type of investigation is at early stages thus, further studies have to be performed in order to strength the evidence on the associations found. In addition, since the preliminary observations on protective effects have been found mainly for specific compounds, future studies should be focused on the contribution of subclasses or individual polyphenolic compounds, and even metabolites, instead of total polyphenols.

4. Conclusions

Undoubtedly, polyphenols exert numerous biological activities as reported in a plethora of *in vitro* and *in vivo* studies. In addition, several systematic reviews and meta-analyses of observational and intervention studies have found a reduced risk for numerous chronic diseases. We documented an overall inverse association between polyphenol intake and CV risk events and

mortality, as well as, between polyphenols and other outcomes of health status. However, most of the associations were found for specific polyphenol classes/subclasses as well as markers/endpoints. At present, few and conflicting results are available for total polyphenols thus, as also reported more than 10 years ago [112], it is still difficult to establish a reference and/or prudent intake of total polyphenols, even if we found an approximate mean intake of about 900 mg/day. Some studies suggest an inverse association between high total flavonoid intake (generally higher 500 mg/day) and CV events and/or mortality. However, this value should be considered as a temptative level due to the elevated heterogeneity of the studies and the numerous limitations associated with the evaluation and estimation of polyphenol intake. It is then fundamental to consider that polyphenol intake correspond to differences in dietary behavior and selection of diverse food sources of the same compounds could affect the overall impact differently. Therefore, it is reasonable to argue in terms of dietary patterns more than focusing on single contributions. In this context, polyphenol-rich dietary pattern seems to exert health benefits and should be considered a valid tool for the prevention of numerous chronic diseases.

At the same time, further investigation is highly recommended in order to address the need for: 1) improved dietary assessment methods; 2) standardized and validated analytical procedures for the analysis of polyphenols and related subclasses in foods; 3) implementation of food databases increasing food items and information available on the different polyphenol subclasses; 4) validation of specific polyphenol intake biomarkers. Nevertheless, despite information from observational studies are necessary to identify potential role of diet-related compounds, the availability of well controlled and specifically targeted dietary intervention studies (addressing also dose-response effects) seems to be mandatory to allow the identification of a reference or prudent intake (e.g. in term of health-promoting properties) for food bioactives such as polyphenols, directed to the general population or specific vulnerable groups (e.g. older subjects).

Figure 1. Polyphenol subclasses

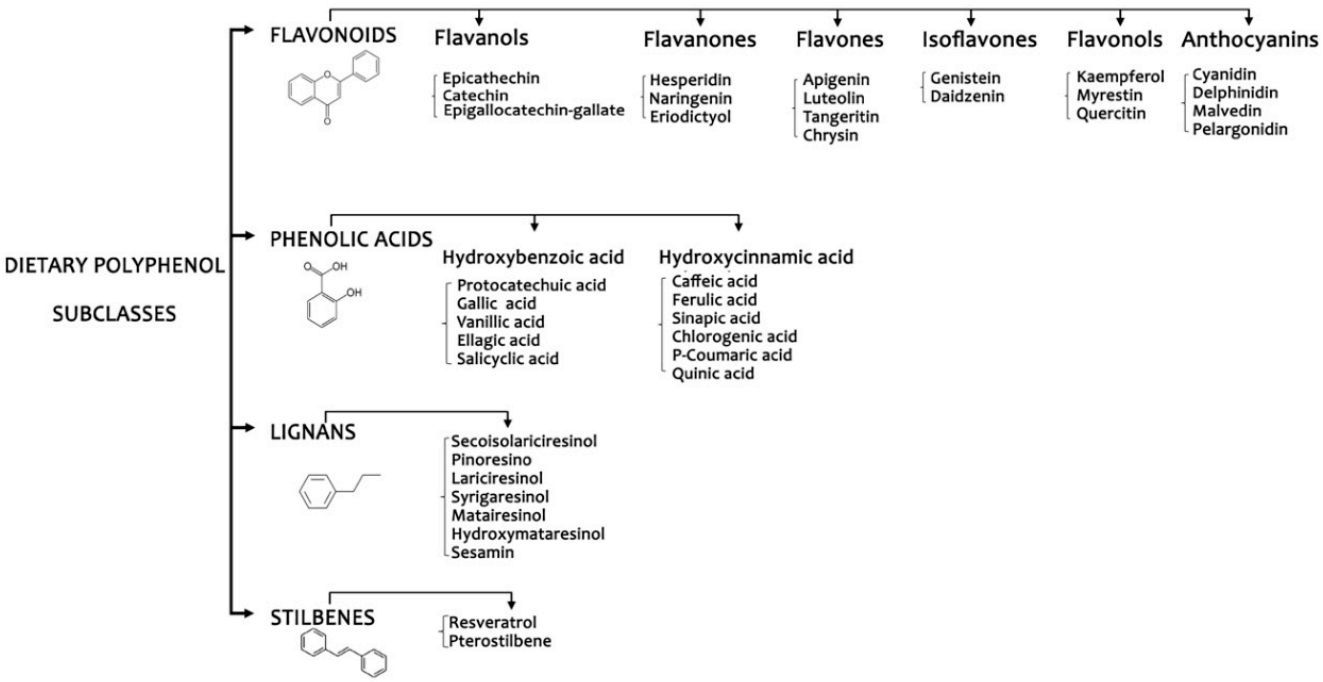


Figure 2. PRISMA Diagram

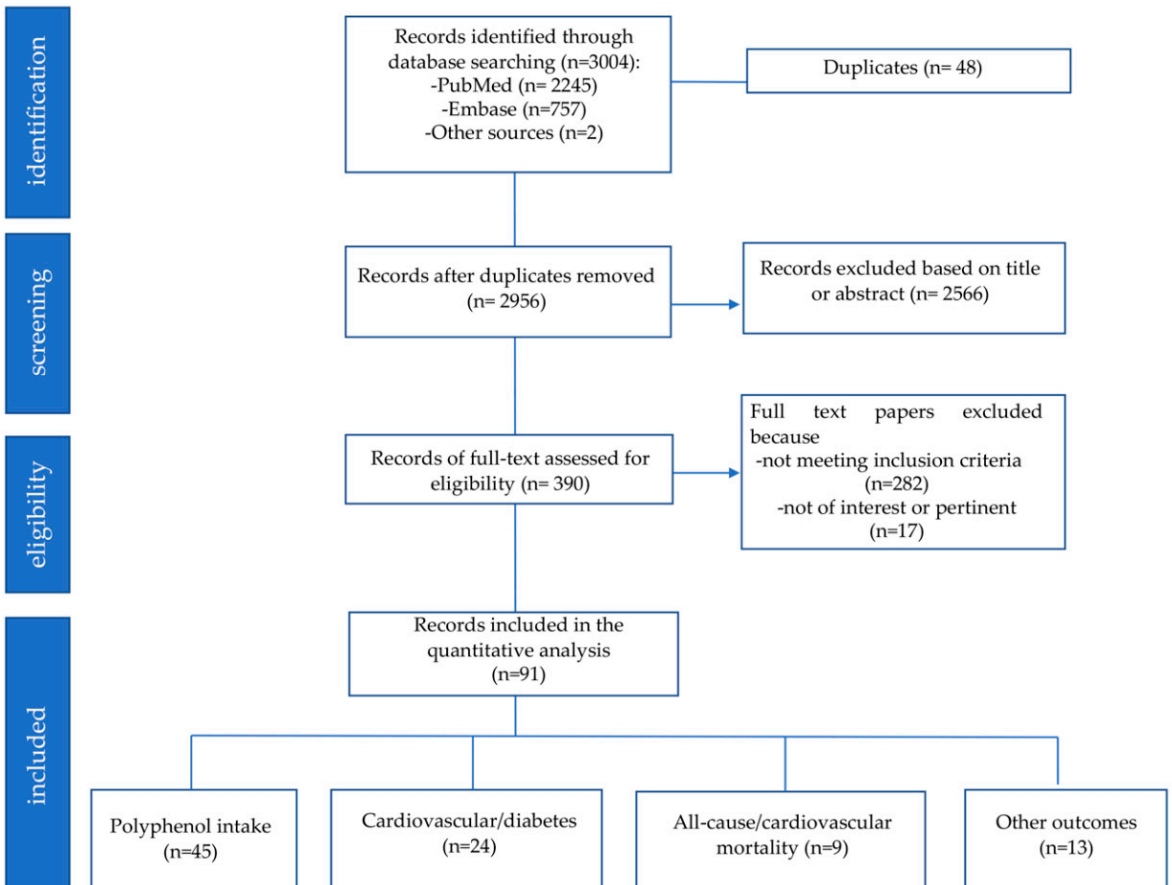
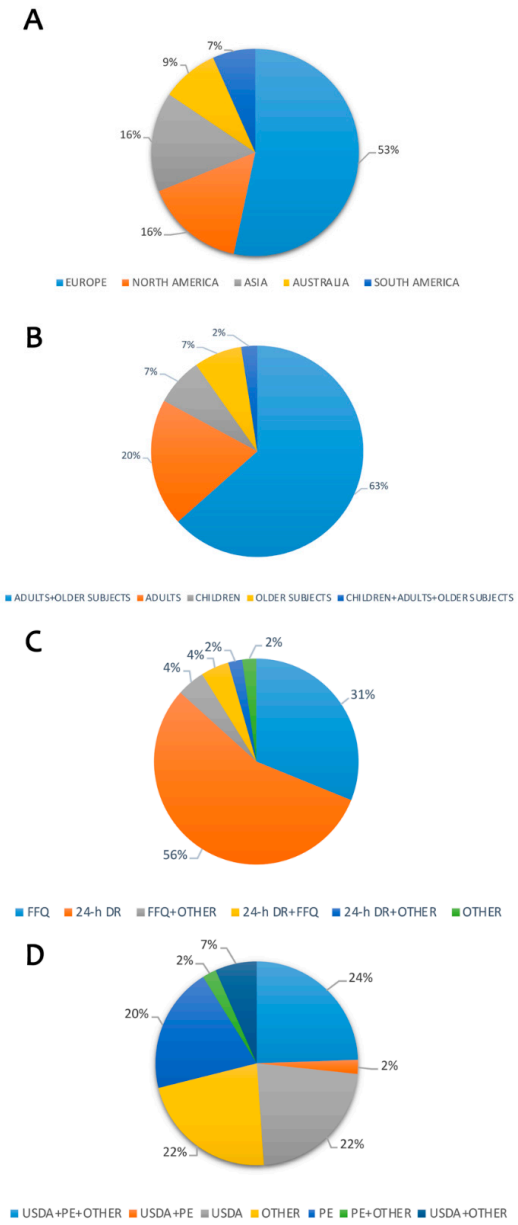
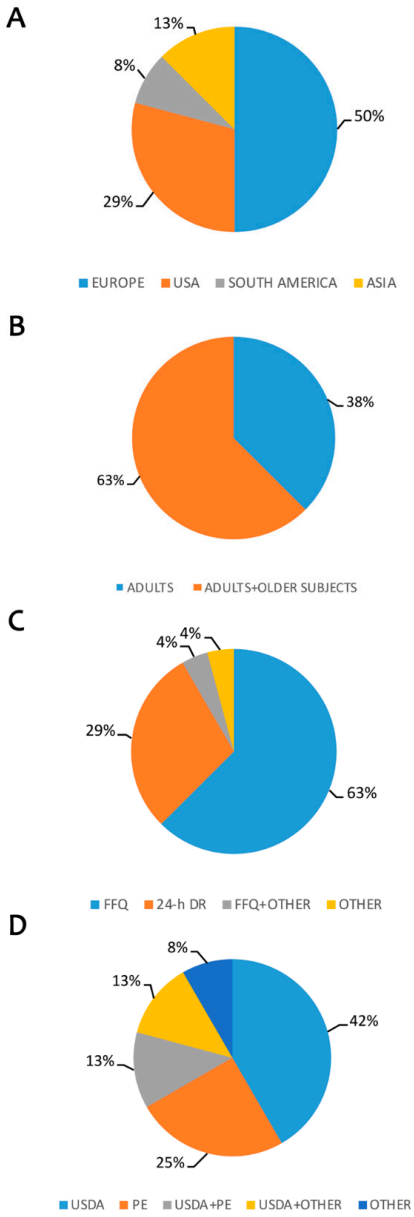


Figure 3. Estimation of polyphenols intake among countries



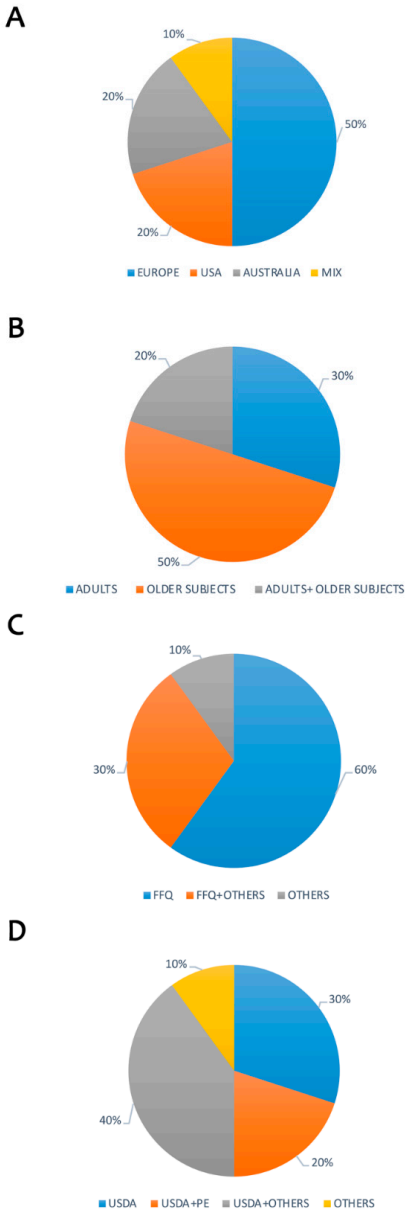
Legend: A) Target population considered; B) Distribution of published data by country; C) Questionnaires used to evaluate food intake; D) Polyphenol database used for evaluation of intake FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer

Figure 4. Estimation of polyphenols intake and risk for cardiovascular diseases and diabetes



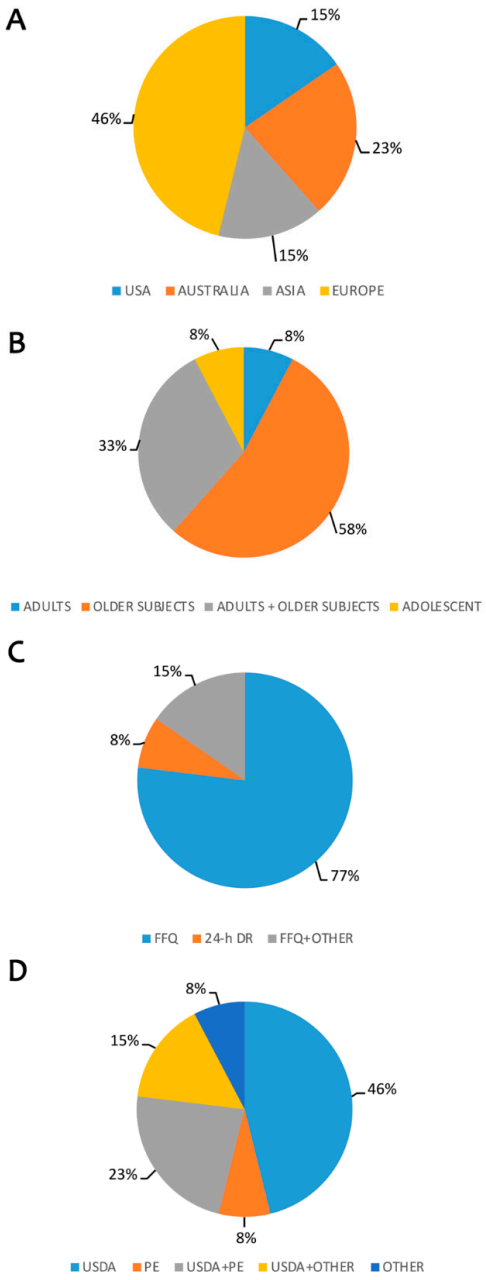
Legend: A) Distribution of published data by country; B) Target population considered; C) Questionnaires used to evaluate food intake; D) Polyphenol database used for evaluation of intake
FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer

Figure 5. Estimation of polyphenols intake, all-cause and cardiovascular mortality risk



Legend: A) Distribution of published data by country; B) Target population considered; C) Questionnaires used to evaluate food intake; D) Polyphenol database used for evaluation of intake
FFQ: Food Frequency Questionnaire; USDA: United States Department of Agriculture; PE: Phenol-Explorer

Figure 6. Estimation of polyphenols intake and other outcomes



Legend: A) Distribution of published data by country; B) Target population considered; C) Questionnaires used to evaluate food intake; D) Polyphenol database used for evaluation of intake
Legend: FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer

Supplementary Materials: no supplemental material provided.

Author Contributions: S.B., M.M. and M.T. performed independently the literature search through scientific databases, reviewed the abstracts, assessed and verified the eligibility of the studies. B.K., B.C. and N.H.L acted as additional independent reviewers, fixed any bias or controversial in the study selection and contents. M.M. and S.B. prepared the tables. M.T. prepared the figures. C.D.B. revised the tables, the figures and wrote the first draft of the manuscript. M.P. and S.G. improved the manuscript. P.R., designed the study, interpreted the results, improved and critically revised the entire manuscript. A.C., R.Z., C.A.L., P.K and A.C. participated to the critical discussion of the results and final revision of the manuscript.

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3.2 Chapter 2

- **Polyphenols and intestinal permeability: rationale and future perspectives**

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- **Exploring the molecular pathways behind the effects of nutrients and dietary polyphenols on gut microbiota and intestinal permeability in aging by metabolomics: novel approaches for future clinical applications.**

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3.2.1 Polyphenols and intestinal permeability: rationale and future perspectives

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Abstract

Increasing evidence links intestinal permeability (IP), a feature of the intestinal barrier (IB), to several pathological or dysfunctional conditions. Several host and environmental factors, including dietary factors, can affect the maintenance of normal IP. In this regard, food bioactives such as polyphenols have been proposed as potential IP modulators even if the mechanisms involved are not fully elucidated yet. The aim of the present paper is to provide a short overview of the main evidence from *in vitro* and *in vivo* studies supporting the role of polyphenols in modulating IP and briefly discuss future perspectives in this research area.

Introduction

Over the last ten years there has been significant research effort to investigate the central role of gut function and properties in the promotion of human health and/or the development of several pathological conditions.

The intestine is the main organ involved in the absorption of nutrients and water and it is the largest area of contact with environmental factors. It contains a large number of specialized immune cells that can coordinate with

defensive responses that prevent or counteract exposure of the host and its immune system to luminal antigens of different origins (e.g. microbial and dietary origin) [1].

The definition and specific ontology related to the gut as a complex anatomical and functional system has been widely debated. Bischoff et al [2] defined the intestinal barrier (IB) as a "*functional entity separating the gut lumen from the inner host and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensines, IgA), immunological elements (lymphocytes and innate immune cells), muscular and neurological elements*". Differently, intestinal permeability (IP), which contributes to the regulation of solute and fluid exchange between the lumen and tissues, should refer to a key feature of IB that is measurable as a whole or at a given site (e.g. evaluating specific molecules/factors flux rates). IP evaluation can be used to address a normal/stable or disturbed/compromised permeability related with IB function [2]. In this context, it is fundamental to underline that IB integrity and functionality can be affected also by the characteristics of intestinal microbial ecosystem and mucosal immune system.

From an anatomical point of view, a well-organized monolayer of epithelial cells is required to form a selective permeability system mainly controlled by the transcellular and the paracellular pathways [3].

While the absorption and/or transport of nutrients (i.e. sugars, amino acids, vitamins, fatty acids, minerals) occur through specific transporters or membrane channels (transcellular path) [3], a complex system of junctions

crucial for the transport between adjacent cells (i.e. tight junction (TJ), gap junctions (GJ), adherent junctions (AJ), and desmosomes) constitute the paracellular path [4].

TJs have composite molecular structure consisting of multiple protein complexes (with more than 50 proteins identified) that include a series of transmembrane tetra-span proteins, named occludin, claudins and tricellulin, able to develop fibrils crossing the membranes and creating a connection with adjacent cells proteins. In addition, single span transmembrane proteins are included and are mostly represented by junctional adhesion molecules (JAM, belonging to the immunoglobulin superfamily). The claudin proteins are considered to be the structural pillar of TJ [5]. Specifically, TJ sealing, fundamental to avoid paracellular permeability is provided by claudin-1, -3, -4, -5, and -8, while claudin- 2 can form charge-selective pores. Less information is available for the specific activities of claudins-7, -12, -15 and occludin [6].

The transmembrane proteins strictly interact with the intracellular scaffold proteins such as zonula occludens (ZO-1, ZO-2, ZO-3) and cingulin tight-fitting the actin cytoskeleton. In particular, increased paracellular permeability is activated by perijunctional actomyosin ring contraction induced by myosin light chain kinase (MLCK). In addition, other signalling proteins, including protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) together with phosphorylation are involved in the regulation pathways of assembly, disassembly, and maintenance of TJ specific properties [7]. Finally, adherent

junctions, together with desmosomes and gap junctions located beneath the TJ are involved in the cell-to-cell adhesion and intracellular signalling but seem not to contribute to paracellular permeability [8].

By considering the complex interplay of functions and activities of TJ proteins and signals regulating the fluxes/exchanges of molecules between the lumen and the environment, it is clear that TJ barrier integrity is essential for human health and metabolic homeostasis.

In fact, an impairment or defect in IB function can lead to modest (i.e. sub-clinical) but chronic immune system activation that might contribute to the pathogenesis of intestinal diseases such as inflammatory bowel disease [4], celiac disease [9], intestinal bowel syndrome [10] up to colon cancer [11]. In addition, recent research showed a possible correlation of IB dysfunction with several clinical conditions such as metabolic syndrome, obesity, Non-alcoholic Fatty Liver Disease (NAFLD) [12], diabetes [13] , inflammatory joint diseases [14] but also neurological conditions, such as major depression and degenerative disorders such as Parkinson's disease [15] and multiple sclerosis (MS), involving the central nervous system (CNS) [16].

It is noteworthy that emerging experimental evidence suggests that an alteration of IB function and/or increased IP can actually occur also during aging, thus, potentially representing a further mechanism underpinning the activation of the low-grade systemic inflammation process (also named inflammaging) identified in older subjects [17]. The alterations can take place at different levels of the intestinal barrier: for example, induced by impairment of

the epithelium (physical barrier) and/or of the immune cells/function, or by an alteration of the chemical barrier consisting in the thick mucus layer able to reduce the passage of bacteria through the epithelium (i.e. mucin secretion) or due to an inefficient/inadequate microbial barrier (represented by the commensal “protective” bacteria). In this regard, it has been demonstrated that age-associated microbial dysbiosis can promote IP and consequently inflammation. In addition, dysbiosis is not only an age-associated characteristic but it can be found in different clinical conditions associated with inflammation (e.g. obesity, diabetes, NAFLD).

Thus, intestinal microbiota can be considered a critical regulator of the IP. Gut microorganisms may act directly on IP by affecting tight junction properties and activities and indirectly by modulating inflammation, which is a well-recognized factor promoting IP impairment [18]. Consequently, the manipulation of the complex intestinal microbial ecosystem has been proposed as a novel strategy to restore IP [2].

Diet and IP

An adequate nutritional status is fundamental to maintain normal IB function (being able to affect all the components of IB) and accordingly, malnutrition is associated with increased IP [19]. For example, Guerriero et al [20] showed that a depletion of glutamine, tryptophan and zinc could lead to increased IP.

Overall, it has been demonstrated that dietary patterns are a dominant factor in shaping the intestinal microbiota [21]. Hence, strategies to modify the relative

abundance of specific bacterial groups by means of dietary interventions has been proposed with the aim also to modulate the concentrations of microbial metabolites in the gut e.g. butyrate affecting tight junction integrity but also inhibiting TNF- α release and inflammation [22].

It has been demonstrated that the Western diet, characterized by high-energy and high-fat intake or high fructose consumption, can alter IP by affecting the gut microbiota composition [20]. In addition, this dietary pattern often involves the consumption of food components like specific fatty acids, alcohol, additives, gliadin, chitosan and food processing methods that are known to alter IB physical structure homeostasis and/or commensal microbial homeostasis. On the other hand, a healthy dietary pattern, such as the Mediterranean diet (MD) rich in fruit, vegetables, legumes and unrefined cereals has been suggested to positively affect IP and related conditions [20]. This may be related to an increased production of short chain fatty acids (SCFAs) by gut commensal bacteria following fiber degradation provided by MD dietary pattern. Moreover, plant based dietary patterns including MD are also commonly abundant of bioactive compounds such as polyphenols that have been recently on the spotlight of research for their potential modulatory properties with respect to IP [23].

Rationale for polyphenols contribution to a protective dietary pattern in the context of IP

Polyphenols (PPs) are secondary metabolites of plants, widely distributed in fruits, vegetables and plant-derived foods. A diet rich in fruits, vegetables and plant-based beverages has been estimated to provide about 1 g of polyphenols/day [24], with significant variations depending also on the extent of consumption of beverages rich in polyphenols (tea, wine, coffee, fruit juices). The basic monomer in polyphenols is the phenolic ring. Phenols can be mainly classified into phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (flavons, flavanones, flavanols, flavonols, isoflavones and anthocyanidins), stilbenes (i.e. resveratrol) and lignans. PPs are recognized to be poorly bioavailable, rapidly absorbed and extensively metabolized by gut microbiota [25]. Additional biotransformation can occur in liver and kidney through methylation, glucuronidation and sulfation reactions of phenolic hydroxyl groups [26] or these reasons, the concentration of the native compounds in the blood is low compared to their metabolic derivates (from nanomoles up to micromoles per liter).

PPs and their metabolites are widely studied for their numerous biological activities, including antimicrobial, antiproliferative, antioxidant and anti-inflammatory function 27. These effects are exerted both at intestinal and systemic levels. In particular, PPs may exert their effects by down regulating inflammatory genes (i.e. nuclear factor-kB, NF-kB) and up-regulating cytoprotective and antioxidant genes (i.e. nuclear factor erythroid 2–related

factor 2, Nrf-2). This modulation may bring to a reduction of cytokines production (e.g., IL-8, IL-1β, and TNF-α) and boost the bodies' own antioxidant status (HO-1, SOD, and GPx) 28. Furthermore, recent studies have shown the capacity of PPs to modulate pattern recognition receptors such as Toll-like receptors and nucleotide-binding oligomerization domain proteins, whose activation in epithelial cells may lead to intestinal inflammation. Moreover, PPs seem to be involved in the regulation of epigenetic factors through interaction with the enzymes responsible for DNA methylation and acetylation by reducing intestinal inflammation 29.

Several studies documented the effects of PPs in the modulation of intestinal microbial ecosystem. However, the mechanisms by which these compounds modulate the gut microbiota remain unclear. Some studies report that the interaction between PPs and microbiota may involve interference with enzymatic expression and activity, and modulation of specific pathways related to anti-oxidant and anti-inflammatory activity [30]. In addition, PPs has been proposed to exert a prebiotic effect potentially inhibiting the pathogenic bacteria and stimulating the growth of beneficial microbes [31–33]. In fact, the microbiota can extensively metabolize PPs in numerous derivatives that could affect not only the composition of microbiota but also specific signalling pathways [30]. Another important aspect regards the possible involvement of PPs in the metabolism of colonic products, such as short chain fatty acids (SCFA), sterols (cholesterol and bile acids), and microbial products of non-absorbed proteins which may directly or indirectly counteract or suppress pro-

oxidant and/or pro-inflammatory responses with an overall improvement of gut health [34].

To unravel the complex scenario related with PP-microbiota interaction in vivo, a combination of metabolomic, microbiome and metagenomic approaches are strongly demanded [30].

Finally, in the last few decades, specific research has been devoted to the evaluation of PPs as promising protective factors and regulators of the epithelial homeostasis and intestinal barrier function. In particular, a direct/indirect effect of regulation of tight junction proteins has been investigated.

Mechanisms of polyphenols regulation of IP

At present, the exact mechanisms linking PPs with intestinal epithelial barrier function have not been established yet. Some studies hypothesized a direct/indirect involvement of nuclear factor-κB (NF-κB) signalling in the onset of intestinal permeability. This pathway is recognized as one of the most important mediators of the inflammation; cytokines and interleukins have shown to activate NF-κB and impair the epithelial barrier function by tight junction disassembly. Conversely, PPs have documented to block NF-κB activation by inhibiting IKK (kinase) phosphorylation and/or preventing proteasomal degradation of IκB [35].

Other important factors potentially involved in increasing intestinal permeability are the multiple protein kinases such as mitogen-activated protein kinases (MAPK), phosphoinositide-3-kinases (PI3K)/Akt, protein

kinase C (PKC), tyrosine kinases, myosin light chain kinase (MLCK) and AMP-activated protein kinase (AMPK). Most of them are regulators of fundamental biological processes in epithelial cells, including barrier function, primarily through regulating TJ expression. Some PPs have shown to improve the epithelial barrier function through inhibition of the activation of numerous kinases [28].

In order to ascertain the availability of data supporting the role of PPs on IP, a literature search has been performed using the following terms “intestinal permeability” OR “intestinal barrier” AND “polyphenols” OR “bioactives” OR “phenolics” as keywords in PubMED. The use of the word “polyphenols” as specific keyword consistently reduced the number of results. On the contrary, a more appropriate search with single PP subclasses AND “intestinal permeability” provided a larger number of in vitro and animal studies mainly summarized in **Tables (1-2)** and an apparent lack of human intervention studies.

In vitro studies

The main lines of evidence on the *in vitro* effects of PPs in the modulation of the potential mediators and regulatory pathways involved in the IP are reported in **Table 1.** Most of the studies are performed on Caco-2 cell line [35–54], as a model of the intestinal barrier, followed by T84, HT29/B6 cells (colonic adenocarcinoma cell line) [55–59], IPEC-J2 cells (intestinal porcine enterocytes) and ECV304 cells (human endothelial cell line) [60,61]. The main evidence of

protection are available for berberine, quercetin and catechin tested in a range of concentration between 10 and 200 μM. Other PPs tested included genistein, anthocyanins, resveratrol, theaflavin and mix of PPs. Most the studies have shown an increase in transepithelial electrical resistance (TEER) across a cellular monolayer confirming the integrity and functional permeability of the membranes [35,39–45,49–51,53,54,58,61,62]. In addition, most the PPs tested have shown to increase the expression and/or production of numerous TJ proteins including zonula occludens (ZO)-1, occludin, and the family of claudins whose alteration may result in increased paracellular permeability [37,38,40,49,51–53,59,61]. Finally, some studies have reported the capacity of PP to counteract inflammatory process induced by TNF-α and INF-γ down-regulating the expression of several interleukins such as IL-8 and IL-6 [44,63].

Animal studies

In **Table 2** are reported the effects of polyphenols and polyphenol-rich extracts in the modulation of IP in animal models [40,45,63–70]. Most of the studies were performed in healthy rat models (i.e. Wistar rats, Sprague-Dawley rats) and intestinal permeability was induced by stimuli such high fat diets, mannitol, inflammatory cytokines, or chemicals [45,68,70]. Two studies used mice with IL-10 deficiency in order to test the effect on intestinal permeability [65,66].

The main PPs used were obtained from grape seed extracts (1% GSE; g GSE per g dry food weight) [65,66] and grape seed proanthocyanidin extracts (5-50

mg/kg) [70]. Other studies included berberine (200 mg/kg) [64], (-)-epicatechin (2-20 mg/kg) [45] and epigallocatechin-3-gallate (about 3 mg/ml) [69]. Some studies were performed by testing anthocyanins-rich raspberry extract, polyphenol-rich propolis extract, and oregano essential oil [40,68]. The doses administered ranged from nearly physiological (epicatechin) up to supra- physiological (i.e. berberine). The duration of the intervention varied from few days (3-10 days) up to several weeks (15-16 weeks).

On the whole, the results obtained support an improvement of intestinal permeability following the intervention with PPs and PP-rich extracts. In particular, the studies showed the capacity of PPs to up-regulate some important genes such as AMPK and ERK and down-regulate NF-kB as pathways involved in the inflammation process. In line with the observations reported in the *in vitro* studies, the compounds tested have shown to increase the expression of zonula occludens (ZO)-1, occludin, and several claudins involved in the functioning of tight junctions.

Human studies

The number of human intervention studies with intestinal permeability as primary or secondary outcome increased in the last years as also documented by the number of trials made available and reported in public registers (i.e. ISRCTN, ClinicalTrial.gov).

Most of these studies were performed, or are ongoing, by using probiotics, prebiotic fibers, dietary supplements, and sugars. Only 4 studies seem to have

explored the potential beneficial effects of polyphenols/polyphenol-rich foods on intestinal permeability in humans **(Table 3)** [71–74]. The studies differ in terms of population (overweight/obese, cyclists, older subjects), foods administered (green tea, flavonoid-rich beverage, mix of polyphenol-rich foods), dose of bioactives (650 mg of flavonoids, 750 mg of polyphenols), duration of intervention (from 2 weeks up to 8 weeks), marker of intestinal permeability selected (endotoxin, lactulose:mannitol ratio, zonulin levels). The trials are still ongoing, and the results will be useful to increase understanding on the actual role of polyphenols and polyphenol-rich foods in humans where a large number of factors can interact affecting intestinal permeability.

In this context, the MaPLE project (Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly) has been developed with the aim to test the hypothesis that changing the diet of older subjects with established enhanced IP by increasing their polyphenols consumption can alter IME in a way that is beneficial for IB function, resulting in reduced IP and decreased translocation of inflammogenic bacterial factors from the digestive tract into the bloodstream [74]. To test this hypothesis, a multidisciplinary approach has been used (i) to evaluate the impact of a polyphenol-rich dietary pattern on IB, IP and IME in a target group of older subjects; and (ii) to investigate the possible mechanisms involved in the polyphenol-microbiota-IP interactions through *in vitro and in animal* models.

Findings obtained from our and other studies will be “pivotal” for the development of new and advanced hypothesis and experimental approaches in this complex area of research.

Some considerations on IP assessments in different contexts

IP can be evaluated through numerous methodologies and consequently data obtained can differ among studies. The techniques vary depending on the setting (in vitro, ex-vivo or in vivo models), the models (cells, animals, humans), the markers (i.e. ions, macromolecules, bacteria and bacterial products) but also the compartments (i.e. tissues, blood, urines). The measurement of IP can be performed through *ex vivo* and *in vivo* approaches [75]. An example of ex vivo approach includes the use of an Ussing chamber able to measure the transport of ions and molecules (i.e. nutrients, drugs) across various epithelial tissues by using fresh intestinal tissue. In vivo, the assessment of IP can be performed through permeability assays (i.e. evaluation of ratio lactulose/mannitol, sucralose, sucrose, polyethylene glycols or 52Cr-EDTA in urines), analysis of bacterial related markers (i.e. endotoxin test, EndoCAb, D-lactate, butyrate production), markers of epithelial damage (i.e. citrullin, fatty acid binding protein, cludin-3), and/or other related markers (i.e. faecal calprotectin). Finally, histological approaches measuring for example Globet cell analysis, shedding of epithelium or Paneth cell loss, can be performed [2].

A novel biomarker of IP is zonulin, a protein secreted by enterocytes but also from other type of cells (i.e. epithelial cells), known to be a physiological

modulator and thus to control IP reversibly *via* intercellular TJs [76]. Increased zonulin serum levels have been observed in many gut-related diseases and emerging evidence suggests an increased zonulin level in specific subjects (e.g. older persons) [77] and in different diseases (e.g. diabetes, obesity) [78,79]. The reliability and accuracy of the different markers to assess IP is clearly a fundamental part of the recent discussion and a hot topic considering the increasing demand for non-invasive diagnosis tools [80].

Conclusion and future perspectives

There is increasing demand for non-invasive strategies able to modulate critical regulatory functions for human health such as IP, which can play a role in the pathogenesis of intestinal and systemic diseases. The improvement or manipulation of the diet, for example increasing or reducing specific nutrients and/or including food bioactives such as PPs is recognised as a potential powerful tool to be explored also in the context of IP. From data available PPs activity seems to be plausibly a consequence of multiple mechanisms which may also depend on the type and amount of compounds considered. Recent literature suggests that PPs may modulate IP through a number of direct and indirect effects including the impact on intestinal ecosystem and immune system. Thus, future research should be directed to increase understanding of the diet-microbiota-intestinal permeability axis possibly through the development of well controlled dietary intervention studies. This type of research is still in its infancy by considering the few human studies available.

Finally, by considering the wide discussion in literature on IP evaluation, a further effort is needed to better define the reliability of the already available IP biomarkers and the potential exploitation of new and/or improved candidate biomarkers.

Abbreviations used

IP, intestinal permeability; IB, intestinal barrier; IME, intestinal microbial ecosystem; TJ, tight junction; GJ, gap junction; AJ, adherent junction; JAM, junctional adhesion molecules; ZO, zonula occludens; MLCK, myosin light chain kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; NAFLD, non-alcoholic fatty liver disease; MS, multiple sclerosis; CNS, central nervous system; TNF, tumor necrosis factor; MD, mediterranean diet; SCFAs, short chain fatty acids; PPs, polyphenols; NF- kB, nuclear factor-kB; Nrf-2, nuclear factor erythroid 2–related factor 2; IL, interleukine; HO, oxigenase enzyme; SOD, superoxide dismutase; GPx, glutathione peroxidase; DNA, deoxyribonucleic acid; IKK, ikB-kinase; PI3K, phosphoinositide-3-kinases; AMPK, AMP-activated protein kinase; TEER, transepithelial electrical resistance; INF-γ, interferon-γ; ERK, extracellular regulated kinase; MaPLE, Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly.

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Table 1- Summary of the main in vitro studies highlighting the mechanisms of action of polyphenol compounds in the modulation of barrier integrity and function

Reference	Cells	Stimulation	Polyphenol source and dose	Signaling Pathway	Response/Marker	Effect
Atkinson and Rao 2001 [36]	Caco-2	Acetaldehyde	Genistein (30–300 μM)	↓ tyrosine kinase	TEER, occludin, ZO-1	↑ TEER ↑ occludin ↑ ZO-1
Watson et al., 2004 [55]	T84	IFN-γ	Epigallocatechin gallate (100 μM)	↓STAT-1 ↓MAPK	TEER	↑ TEER
Amasheh et al., 2008 [47]	Caco-2	-	Quercetin (0-200 μM)	↓MLCK, PKC	TEER, occludin, claudin-1, claudin-3, claudin-4, claudin-7	↑ TEER ↑ claudin-4 = claudin-1 = claudin-3 = claudin-7 = occludin
Suzuki and Hara 2009 [48]	Caco-2	-	Quercetin (0-100 μM)	↓ PKCδ	ZO-2, occludin, claudin-1, claudin-4	↑ ZO-2 ↑ occludin

						↑ claudin-1 ↑ claudin-4
Amasheh et al., 2010 [56]	HT29/B6	TNF-α	Berberine (50 μM)	↓ NF-Kb, PI3K/Akt, tyrosine kinase	Claudin-1, claudin-2	↑ claudin 1 ↓ claudin 2
Chuenkitiyanon et al., 2010 [60]	ECV304	H_2O_2	Quercetin (10 μM)	↓p38	ZO-1, occludin	↑ ZO-1 ↑ occludin
Rogoll et al., 2010 [57]	T84	-	(+)-Catechin (10 μM) (-)-epicatechin (10 μM) Quercetins (10 μM) Phloretins (20 μM) D-(-)-quinic acids (10-50 μM) p-coumaric acids (10 μM) caffeic acids (20 μM)	↓Tight junction permeability	TEER, ZO-1, occludin, claudin-4	↑TEER ↑ ZO-1 ↑ occludin ↑ claudin-4

Shin et al., 2011 [62]	HCT-116	-	Anthocyanin mixture (45 µg/mL; delphinidin, cyanidin, petunidin, delphinidin, malvdin, peonidin-3,5-diglucoside, cyanidin, petunidin, peonidin, malvidin-3-glucoside)	↑p38	TEER, claudin-1, claudin-3, claudin-4	↑ TEER ↓ claudin 1 ↓ claudin 3 ↓ claudin 4
Suzuki et al., 2011 [49]	Caco-2	-	Kaempferol (100 µM)	↓Tight junction permeability	TEER, ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4	↑ TEER ↑ occludin ↑ claudin 1 ↑ claudin 3 ↑ claudin 4 ↑ ZO-1 ↑ ZO-2
Noda et al., 2012 [50]	Caco-2	-				

			Chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin (100 μM)	↓Tight junction permeability	TEER, ZO-1, ZO-2, JAM1, claudin-1, claudin-3, claudin-4	↑ TEER (negative effect for chrysin) Effect on tight junction proteins was compound dependent
Amasheh et al., 2012 [58]	HT-29/B6	IFN-γ, TNF-α	Quercetin (200 μM)	↓Tight junction permeability	TEER, claudin-1, claudin-2, claudin-3, claudin-4, claudin-7, occludin	↑ TEER ↓claudin-2 ↓claudin-3 = claudin-1 = claudin-4 =claudin-7 =occludin
Noda et al., 2013 [51]	Caco-2	-	Naringenin (100 μM)	↑Sp1-dependent transcriptional regulation ↓Tight junction permeability	TEER, ZO-1, ZO-2, occludin, JAM-A, claudin-1, claudin-3, claudin-4	↑ TEER ↑claudin-1 ↑claudin-4 ↑occludin = ZO-1

						= JAM-A
Cao et al., 2013 [52]	Caco-2	IFN-γ, TNF-α	Berberine (100 μM)	↓MLCK	Occludin, claudin-1, ZO-1, intestinal permeability	↑ Occludin ↑ claudin-1 ↑ ZO-1 ↓ intestinal permeability
Carrasco-Pozo et al., 2013 [53]	Caco-2	Indomethacin	Mix of quercetin (33μM), resveratrol (438μM), rutin (164μM), epigallocatechin gallate (218μM)	↑epithelial barrier function	TEER, FD4, ZO-1, occludin	↑TEER (no effect with rutin) ↓FD4 (no effect with rutin) ↑ ZO-1 after quercetin ↑ occludin after quercetin

Piegholdt et al., 2014 [54]	Caco-2	TNF-α	Biochanin A (50 μM), prunetin (50 μM)	↓ NF-Kb, ERK, tyrosine kinase	TEER, claudin 1, occludin, ZO-1, E-cadherin	↑ TEER = claudin 1 = ZO-1 = E-cadherin
Park et al., 2015 [37]	Caco-2	-	Theaflavins-3'-0-gallate (20 μM)	↓MLCK	Occludin, claudin-1, ZO-1	↑occludin ↑claudin-1 ↑ZO-1
Contreras et al., 2015 [38]	Caco-2	TNF-α	(-)-Epicatechin (0.5–5 μM)	↓ NF-Kb, p-IKKα, p-IkBα, MLCK	Occludin, ZO-1, claudin-2	↑ZO-1 = occludin =claudin-2
Valenzano et al., 2015 [39]	Caco-2	-	Berberine (50-200 μM) Quercetin (100-400 μM)	↑epithelial barrier function	TEER, claudin-1 claudin-2, claudin-3 claudin-4, claudin-5 claudin-7, occludin tricellulin, D-mannitol	↑ TEER (only berberine) Quercetin (↑claudin 2, claudin-4, claudin-5, ↓tricellulin)

						Berberin (↓claudin-2, D-mannitol)
Ling et al., 2016 [61]	IPEC-J2	Deoxynivalenol	Resveratrol (0-200 μM)	↓p38, ERK, p-JNK	TEER, FD4, Claudin-1, Claudin-3, Claudin-4, Claudin-7, occludin, ZO-1	↑ TEER ↑ occludin ↑ claudin-3 ↑ claudin-4 ↓FD4 = claudin-1 = claudin-7
Wang et al., 2016 [40]	Caco-2	-	Polyphenol-rich propolis extract (25 and 50 μg/mL)	↑AMPK-α, ERK1/2, Akt, p38	ZO-1, occludin	↑ TEER ↑ occludin ↑ ZO-1
Azzini et al., 2016 [41]	Caco-2	-	3 different polyphenol-rich extracts from Chicory (0.2, 1.3, 10, 17, 34, 70 μM)	↑epithelial barrier function	TEER	↑TEER

Luescher et al., 2017 [35]	Caco-2	TNF-α	Xanthohumol (chalcone; 10 μM), isoxanthohumol (prenylflavone; 10 μM)	↓Tight junction permeability	TEER	↑TEER
Cremonini et al., 2017 [42]	Caco-2	TNF-α IFN-γ	cyanidin, delphinidin, malvidin, petunidin, or peonidin- 3-O-glucoside (0.25–1 μM) crowberry extract (1–10 μg/mL) ACN-rich plant extracts (black chokeberry, black kernel rice, wild blueberry, bilberry, crowberry, domesticated blueberry, red grape (5 μg/mL))	↓IKK and p65 phosphorylation	TEER	↑TEER (only cyanidin and delphinidin, and ACN-rich plant extracts)

Rybakovsky et al., 2017 [43]	Caco-2	^{14}C−D-mannitol	Theaflavins (5-20 µg/mL) Quercetin (100-400 µM) Berberine (50-200 µM)	↑ membrane permeability	Claudin-1,claudin-2, Claudin-4, claudin-5	↑TEER (quercetin) ↓Transepithelial Mannitol Permeability (quercetin) ↑ claudin-2 = claudin-1 = claudin-4 = claudin-5
Van Buiten et al., 2018 [44]	Caco-2	-	Decaffeinated green tea polyphenols (0-100 µg/mL)	↓paracellular permeability	TEER, IL-6, IL-8	↑TEER ↓IL-6 ↓IL-8
Li et al., 2018 [63]	MODE-K	LPS	Naringin (50-200 µM)	↓NF-kB, MLCK/MLC	TNF-α, IL-10, IL-6, MLCK, p-MLC/MLC, p-	↓TNF-α ↓ IL-10 ↓ IL-6 ↓ MLCK

					p65/p65, p-IkBα/IkBα	↓ p-MLC/MLC ↓ p-p65/p65 ↓ p-IkBα/IkBα
Cremonini et al., 2018 [45]	Caco-2	TNF-α	(-)-Epicatechin	↑ERK1/2, AMPK, ↓NF-kB	NOX1/NOX4, FITC-dextran transport, TEER	↑TEER ↓FITC ↓NOX1/NOX4
Vazquez-Olivo et al., 2019 [46]	Caco-2	-	4 polyphenol-rich mango extracts (100 µg/mL) Gallic acid (100 µg/mL)	↑ membrane permeability	Papp	↑Improvement of apparent membrane permeability
Nunes et al., 2019 [59]	HT-29	TNF-α, IL-1, IFN-γ	Non-alcoholic polyphenolic red wine extract (catechin, oligomeric procyanidins, anthocyanin, phenolic acids, ethyl cinnamate,	↓ paracellular permeability	Occludin, claudin-5, ZO-1	↑ occludin ↑ claudin-5 ↑ ZO-1

condensed tannin); 200, 400

and 600 µg/mL

Legend:

ACN, anthocyanin; TNF-α, tumor necrosis factor alpha; IL-(1-10), interleukin-(1-10); IFN-γ, interferon gamma; LPS, Lipopolysaccharide; H_2O_2, hydrogen peroxide; NF-kB, nuclear factor-kB; MLCK/MLC, myosin light-chain kinase; ERK1/2, extracellular signal–regulated kinases; AMPK, 5' AMP-activated protein kinase; p38, p38 pathway; JNK, c-Jun N-terminal kinases, p-IKKα, IκB kinase α; TEER, trans-epithelial eletrical resistance; PI3K/Akt, phosphoinositide 3-kinase; PKC, protein kinase C; STAT-1, signal transducer and activator of transcription 1; MAPK, mitogen-activated protein kinases; NOX, nicotinamide adenine dinucleotide oxidase; FITC, fluorescein; FD4, fluorescein isothiocyanate-labeled dextrans; ZO-1, zonula occludens; JAM-A, junctional adhesion molecule-A; Sp-1, specific protein transcription factor-1.

Table 2- Summary of the main evidence from animal models reporting the effects of polyphenols and polyphenol-rich extracts in the modulation of barrier integrity and function

Reference	Animal model	Diet	Polyphenol source and dose	Signaling Pathway	Response/Marker	Main findings
Gu et al., 2011 [64]	Male C57BL/6 mice	BBR *vs* C LPS-stimulation	BBR: berberine (200 mg/kg) C: control diet 7 days	↓MLCK	Intestinal permeability Claudin-1 Claudin-4 Occludin ZO-1	↑ ZO-1 ↑ occludin ↑Claudin-1 ↑Claudin-4 ↓intestinal permeability
Yang et al., 2014 [65]	C57BL/6 (WT) and IL-10-de□cient (IL-10-/-, IL10KO) female mice	GSE *vs* C dextran sulfate sodium-stimulation	GSE: grape seed extract (0 or 1% GSE C: standard rodent diet 16 weeks	↓NF-kB	Claudin-1 Claudin-2	↑claudin-1 ↓claudin-2

Wang et al., 2013 [66]	IL10-deficient mice (IL10KO)	GSE *vs* C dextran sulfate sodium-stimulation	GSE: grape seed extract (0 or 1% GSE C: standard rodent diet 16 weeks	↓AMPK	Claudin-1 Claudin-2	↑claudin-1 ↓claudin-2
Li et al., 2014 [67]	BALB/c mice	ARF *vs* C dextran sulfate sodium-stimulation	ARF: Anthocyanin-rich raspberry extract (20 mg/kg) C: Saline solution 10 days	↓NF-kB ↓MAPKs	Colonic histological architecture	↑ colonic histological architecture
Wei et al., 2015 [68]	Males Wistar rats	OEO vs C Diquat-stimulation	OEO: oregano essential oil (5 or 20 mg/kg BW) C: saline solution 14 days	↓SOD ↓GSH-Px	ZO-1 occludin	↑ ZO-1 ↑ occludin

Wang et al., 2016 [40]	Male Sprague-Dawley rats	PPE *vs* C 2,4,6-trinitrobenzenesulfonic acid stimulation	PPE: Polyphenol-rich propolis extract (0.3% w/w) C: control diet 14 days	↑AMPK ↑ERK	ZO-1 occludin	↑ ZO-1 ↑ occludin
Bitzer et al 2016 [69]	Male CF-1 mice	DSS treatment D(0.5% citric acid) DE (0.5% citric acid and EGCG) C-diet	EGCG: epigallocatechin-3-gallate (3,2 mg/ml) C: control diet 3 days	--	GLP-2 LAC/RHA SUC/ERY	↓ GLP-2 ↓ LAC/RHA ↓ SUC/ERY
Gil-Cardoso et al 2017 [70]	Female Wistar rats	CAF CAF+GSPE C-group	CAF: cafeteria diet CAF+GSPE: (cafeteria diet + grape seed proanthocyanidin extract 5- 50 mg/kg) C: standard diet	--	ZO-1 Occludin Claudin-1 JAM-A	↑ZO-1

			15 weeks CAF 3 weeks CAF+GSPE			
Cremonini et al 2018 [45]	C57BL/6J mice	HF *vs* C HFE20 *vs* CE	CE: (-)-epicatechin (2-20 mg/kg) C: control diet 15 weeks	↑ERK1/2 ↑NF-kB (p65) ↑AMPK	p65 GLP-2 NOX1/NOX4	↑ p65 (HF) ↑ GLP-2 (CE and HFE20) ↑ NOX1/NOX4 (HF)
Li et al 2018 [63]	Male Kunming mice	CLP + vehicle CLP+ NG (30) CLP+ NG (60)	Naringin (30 mg/kg and 60 mg/kg) 24 - 72 h	--	TEM FITC-dextrane D-lactate	↑survival CLP +NG (30-60) ↑IM Impairment CLP + Vehicle CLP↑ FITC-dextrane and D-lactate

	CLP + NG ↓ FITC-dextrane (dose-dependent)

Legend: NF-kB, nuclear factor-kB; MLCK/MLC, myosin light-chain kinase; ERK1/2, extracellular signal–regulated kinases; AMPK, 5' AMP-activated protein kinase; ZO-1, zonula occludens; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; C, control; HF, high fat diet; HFE20, high fat diet + 20 mg/kg epicatechin;

GLP-2, glucagon-like peptide-2; NOX1/NOX 4, NADPH oxidases; CLP, cecal ligation and puncture; NG, naringin; TEM, transmission electron microscopy; CAF, cafeteria diet; GSPE, grape seed proanthocyanidin; ZO, zonula occludens; JAM, junctional adhesion molecule; DSS, dextran sulfate sodium; EGCG, (-)-epigallocatechin-3-gallate; LAC/RHA, lactulose/rhamnose ratio; SUC/ERY, sucralose/erythritol ratio

Table 3- Summary of the ongoing human studies evaluating the effect of polyphenols and polyphenol-rich food on intestinal permeability

Title	**Source**	**Subject number/characterstics Inclusion criteria**	**Study design**	**Intervention**	**Duration of intervention**	**Markers understudy**
Dietary green-tea confection for resolving gut permeability-induced metabolic endotoxemia in obese adults	ClinicalTrials.gov NCT03413735 [71]	40 Overweight/obese (BMI = 28-40 kg/m^2) Fasting glucose < 126 mg/dL Normotensive (blood pressure < 140/90 mmHg) Non-dietary supplement user Non-smoker	Randomized parallel design	Test group: green tea extract (GTE)-rich confection Placebo group: no green tea extract-rich confection Dose: daily (no information about the amount provided in term of polyphenols)	4 weeks	Primary outcome: Endotoxin Secondary outcome: Gut Permeability (Lactulose to Mannitol Ratio, and Sucralose to Erythritol Ratio) Microbiota (Firmicutes to Bacteroidetes Ratio) Calprotectin Green tea polyphenol bioavailability

Effect of flavonoids on gut permeability in cyclists	ClinicalTrials.gov NCT03427879 [72]	22 Male or female of any race or ethnicity between 18 to 49 years of age Competed in a road race or triathlon in past 12 months Free of chronic disease and gut inflammation conditions Train at least 3 times per week, 1 hour at a time on average Willing to prepare and consume provided pre-workout beverage daily	Randomized crossover design	Test group: a high flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin (8.6%), blueberry powder (2.4%), cocoa powder (1.6%), green tea extract (0.1%), whey protein isolate (0.6%) containing approximately 620 mg flavonoids per serving.	2 weeks	Primary outcome: Urinary lactulose:mannitol ratio Plasma intestinal fatty acid binding protein Secondary outcome: Fecal calprotectin Urinary sucralose:mannitol ratio Inflammatory markers (TNF-α, IL-10) Endotoxin

Maintain weight (no more/less than 5 kg change) Willing to avoid consumption of high flavonoid foods/supplements, large dose vitamin and mineral supplements, and NSAIDs or other medications known to affect inflammation during study period	Placebo group: a low flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin (8.6%), placebo blueberry powder (2.4%), alkalized cocoa powder (1.6%), whey protein isolate (0.6%), containing approximately 5mg flavonoids per serving Dose: 330 mL/ day	Other variables related exercise performance

Effect of dietary flavonoids on intestinal microbiota, intestinal inflammation and metabolic syndrome	ClinicalTrials.gov NCT02728570 [73]	30	Overweight/obese (BMI = 25-35 kg/m^2)	Randomized crossover design	<u>Test group:</u> Prepared diet with diet high levels of dietary flavonoids (340 mg of flavonoids/1000 Kcals) with a macronutrient composition of 17% en from protein, 30% en from fat and 53% energy from carbohydrate <u>Control group:</u> Prepared diet with diet high levels of dietary flavonoids (10 mg of	6 weeks	<u>Primary outcome:</u> Fecal calprotectin Serum PCR Serum TNF-α Serum insulin <u>Secondary outcome:</u> Fecal microbiome composition, short chain fatty acids, eosinophil protein X, myeloperoxidase Intestinal permeability by four sugar differential absorption test

flavonoids/1000 Kcals) with a macronutrient composition of 17% en from protein, 30% en from fat and 53% energy from carbohydrate	Serum endotoxin, IL-6, soluble TNFr-2, fasting glucose Calculated Homeostatic Model Assessment-Insulin Resistance Serum C-peptide Plasma lipid profile Blood pressure Other Outcome Measures: Serum resistin, visfatin, adiponectin, leptin

						Body weight
Effect of a polyphenol-rich diet on leaky gut in the elderly	ISRCTN registry ISRCTN10214981 [74]	60 healthy older subjects Age > 60 years old Intestinal Permeability evaluated by Zonulin serum level Adequate nutritional status evaluated with Mini Nutritional Assessment (MNA) score ≥24 Good cognitive status tested with Mini Mental	Randomized crossover design	<u>Test group:</u> habitual diet + polyphenol-rich products (berries and derived products, blood oranges and derived products, pomegranate juice, Renetta apple and purée, green tea and dark chocolate products) <u>Control group:</u> comparable diet without the polyphenol-rich products	8 weeks	<u>Primary outcome:</u> Zonulin serum levels <u>Secondary outcome:</u> Total blood bacterial load Faecal microbiota composition and metabolism Short chain fatty acids and polyphenol-derived metabolites Inflammatory,

	State Evaluation (MMSE) score ≥24 Self-sufficiency assessed with validated tests (e.g. Barthel index - activities of daily living, Tinetti balance assessment)	Dose: three portion of polyphenol-rich food products daily (about 750 mg of polyphenols)	oxidative stress and related markers Endotoxin LPS-BP Metabolomic markers Metabolic and anthropometric markers

Legend: TNF-α, tumor necrosis factor-alpha; TNFr-2: tumor necrosis factor receptor-2; PCR: C-reactive protein; IL-10, interleukin-10; NSAIDs; nonsteroidal anti-inflammatory drugs; BMI, body mass index; LPS-BP, lipopolysaccharide binding protein

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3.2.2 Exploring the molecular pathways behind the effects of nutrients and dietary polyphenols on gut microbiota and intestinal permeability in aging by metabolomics: novel approaches for future clinical applications

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Abstract

The gastrointestinal tract hosts the largest microbial population of the human body, which works in symbiosis with the host to provide several important functions and contributes to the maintenance of host health. The diet is one of the factors that can most affect the gut microbiota, with subsequent consequences on host health. One consequence of changes in microbiota is changes in intestinal permeability (IP); disruption of this latter is related to the development of several diseases and is a frequent condition in older people. Nevertheless, the molecular pathways regulating these effects are still unclear, and a comprehensive understanding of the dietary components that can affect IP is lacking. Metabolomics, that has been widely used to study the transformation of nutrients by intestinal microbiota, could be a suitable approach for this purpose. However, up to now, the research has focused mainly on dietary fibers and tryptophan, while the activity of dietary polyphenols remains almost completely unexplored. Hence, the aim here was to review the most recent literature concerning the application of metabolomics in the study of the correlation between diet-induced alterations of gut microbiota and the effects on intestinal permeability, with a particular focus on the discovery of the molecular pathways involved. An additional aim was to give a perspective on the future research involving dietary polyphenols, given that despite their potential implication for the prevention and treatment of several diseases related to increased intestinal permeability, few studies have been reported to date.

Keywords: metabolomics, gut microbiota, intestinal permeability, nutrients, polyphenols

Introduction

The gastrointestinal tract (GI) is responsible for a wide range of functions, including digestion and absorption of nutrients, water and ions, regulation of host immunity, protection against the ingress of pathogenic microorganism, and the the metabolism and detoxification of xenobiotics. The GI also hosts the largest microbial population of the human body, which works in symbiosis with the host to accomplish these various intestinal functions. Gut bacteria are particularly important for host health, being involved in the synthesis of vitamins, secondary bile acids and neurotransmitters, and playing a direct role in the metabolism and degradation of dietary components and drugs, that can affect their bioavailability and absorption [1]. It has been estimated that over than 1,000 different bacterial species populate the intestinal environment, with a genome comprising 100-fold more genes than those found in human genome [2]. The physiological variations in the small intestine and colon, such as the presence of distinct chemical environments, nutrients and host immune activity allow distinct groups of bacterial species to populate the different regions of the lower gastrointestinal tract [3,4], and this variability becomes even more complex considering the inter-individual variations and the influence of host genetics [5-7]. Nevertheless, most human gut microbiota share a core set of resident bacteria and related microbial genes [8,9]. *Firmicutes, Bacteroidetes* and *Actinobacteria* are the three most abundant phyla, among the over 50 that have been identified by metagenomic approaches [10,11]. A synergistic equilibrium among the different species and the maintenance of a microbial diversity are of crucial importance for health, since the microbiota plays a central role on the proper functioning of the intestinal barrier and maintaining appropriate intestinal permeability (IP), which is directly involved in the development of numerous disorders. In this vein, a low diversity and a scarce abundance of species as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* have been associated with gut disease states, e.g. Crohn's disease [12], type 1, type 2 and gestational diabetes [13-15], celiac disease [16] and obesity [17].

Diet, as a source of macro- and micro-nutrients and other bioactive components, is one of the factors that most can affect the microbiota. Among the dietary constituents, polyphenols have been in the spotlight in recent years, due to their particular physicochemical properties and their potential to directly affect microbiota activity and host health. Polyphenols are secondary metabolites of plants, fruits and vegetables, and major components of commonly consumed foods and beverages such as chocolate, tea and coffee [18-20] which, due to their characteristic (poly)hydroxylated phenyl moieties and the presence of ionizable functional groups on their scaffolds, have a low bioavailability and are scarcely absorbed by the intestine [21,22]. Consequently, they are prone to catabolism by the gut microbiota, which leads to the production of smaller molecular weight (MW) compounds that can be absorbed across the intestinal wall, enter the bloodstream and eventually, undergo further transformation and conjugation in the liver [23,24]. It has been estimated that total polyphenol absorption in the small intestine is around 5%–10%, while the remaining 90%–95% transits to the large intestinal lumen and accumulates in the millimolar range [20]. Hence, microbial polyphenol derivatives could be responsible for the biological effects attributed to their parent compounds, or at least contribute to the overall activity. Catechins from green tea, for example, have been reported to exert antioxidant, anti-inflammatory and anti-tumorigenic activities [25-27]. However, the most representative green tea catechin, (−)-epigallocatechin gallate, is scarcely absorbed from the intestine and is extensively metabolized by gut microbiota [28] to form smaller MW derivatives that not only contribute to the observed bioactivities of green tea, but can also exert higher activity than the parent compound [29]. Polyphenols and their microbial metabolites could also exert antimicrobial and bacteriostatic activities, hence regulating the overgrowth of harmful bacteria on the intestinal and urinary tract epithelia [20,30]. As an example, cranberry (*Vaccinium macrocarpon* Ait.) fruits, rich sources of type-A procyanidins (PAC-A), are known to exert anti-adhesive activity against the uropathogenic bacteria responsible for most of the lower urinary tract

infections, although the mechanisms of action are still unknown and the outcomes of in vitro assays and in vivo clinical trials aimed at reducing urinary tract infections are frequently inconsistent [31]. Recent studies show that, after supplementation with dry cranberry extracts, urine samples of both rats and human volunteers exert effective anti-adhesive activity against uropathogenic *E. coli*, despite their negligible contents of intact PAC-A [32,33]. However, the same urine samples were characterized by high amounts of hydroxyphenyl-valeric acid and hydroxyphenyl-valerolactone derivatives, previously reported as end-products of microbial degradation of flavan-3-ols [34], indicating the important contribution of PAC-A microbial metabolites to the observed bioactivity [32,33]. Finally, the effects of polyphenols on microbiota, inflammation and oxidative stress and their capacity to regulate the synthesis and expression of specific proteins on the intestinal epithelium seem to be part of the mechanisms by which these compounds can regulate the permeability of the intestinal barrier [35], whose alterations are related to the development of several diseases, especially in older subjects.

Many efforts have been made to characterize the microbial community colonizing the human intestine, for which the widespread use of metataxonomics based on 16S rRNA gene profiling and metagenomics (microbiomics) has been particularly important. However, although representing powerful tools for bacterial identification and classification, microbiomics does not allow to obtain information about fluctuations in metabolic activities [1]. To this purpose, metabolomics is the most suitable approach, and numerous reports based on metabolomic analysis have been reported over the last decade [36]. Focusing on the application of metabolomics in the study of diet-microbiota interactions and searching for the keywords “metabolomics AND diet AND microbiota” in PubMed, we found that the number of publications almost doubled from 2014 to 2018, as an index of the popularity that metabolomics gained during the recent years (Fig. 1). Metabolomic approaches have been widely used to study the transformation of nutrients and xenobiotics by intestinal microbiota [37-42], thus allowing the

characterization of hundreds of metabolites derived from macro- and micronutrients and polyphenols coming from fruits and vegetables. In 2009, Jacobs published a first review article regarding the role of colonic microbiota in the degradation of non-digestible food ingredients and their impact on gut health and immunity [43]. For the first time, the importance of metabolomics in the study of the links between the bioconversion of non-digestible food ingredients, their bioavailability and their downstream effects on microbiota composition and host metabolism was recognized [43]. More recently, the use of integrated multi-omics approaches has faxcilitated the study of the molecular interactions between diet and microbiota, and has led to the identification of several metabolites that are produced as a result of microbial metabolism of various dietary constituents. Nevertheless, considering the challenges to study the mutual relationship between gut microbiota and the host, its tight connection with diet, environment and lifestyle, and the still incomplete characterization of the huge microbial metabolome, the path to assess precise and validated metabolites to link the microbial activity to specific effects on health is just starting. In a way to find a clinical relevance of metabolomics data and offer to clinicians a robust tool to predict, prevent and treat several diseases, further progress is necessary.

The aim of this work was to review the most recent literature regarding the application of metabolomics in the study of the interactions between food components and gut microbiota and the effects on IP, with a particular focus on the elucidation of the molecular pathways involved. Since to date the research has mainly focused on the degradation of non-digestible fibers and tryptophan and on the bioactivity of their metabolites, a major part of the work will be dedicated to these important dietary components. Additionally, a perspective on the future research involving the role of dietary polyphenols in modulating the activity and composition of gut microbiota and the effects on IP will be discussed, given that, despite their potential implication in the prevention and treatment of several diseases, few clinical studies have been performed up to now.

The role of microbiota and microbiota-derived dietary metabolites in regulating intestinal permeability

The intestinal wall represents a barrier that selectively transports nutrients, ions and water from the lumen to the bloodstream, via passive and active mechanisms. A layer of epithelial cells constitutes the main physical barrier between the intestinal lumen and the mucosal tissues [44]. Tight junctions (TJ), composed of transmembrane proteins and junctional adhesion molecules that regulate the flow of water, ions and small molecules, seal the paracellular spaces [45]. Several distinct proteins contribute to form the TJ, including mainly occludins and claudins, depending on the tissue and location that interlink within the paracellular space [46]. Although highly cross-linked, the structure of TJ is dynamic, so that it can be 'opened' and 'closed' following specific stimuli [47]. Physiological stimuli could shrink the TJ to prevent the diffusion of toxins, viruses or bacterial fragments to the mucosal layer, while they can open the paracellular space to allow the diffusion of nutrients [48]. For instance, the activation of the sodium dependent glucose transporter led to the opening of TJ and allowed the diffusion of small molecules and peptides with MW < 40,000 Da [49]. On the other hand, the physiological structure and dynamism of TJ could be altered due to pathological states [50], leading to a condition of increased IP, also known as "leaky gut". Celiac disease, inflammatory bowel disease and type I diabetes are three of the principal pathological causes of leaky gut [51], which leads to the permeation of potentially harmful molecules, organisms or microbial fragments from the intestinal lumen to the mucosal layer, inducing a cascade of events that result in immune activation and local or systemic inflammation. Older people are frequently affected by decreased intestinal barrier function and consequently leaky gut [52]. Among the causes, the aging-related decline of immune function (namely immune-senescence), the remodeling of intestinal epithelium and the alterations of gut microbiota composition are thought to be the most important ones [52-54]. As observed in disease-associated increased IP, the dysfunction of the intestinal barrier in older subjects facilitates the diffusion of toxic substances or peptides and

microbial fragments to the mucosal layer and to the bloodstream and the triggering of a systemic inflammatory response [55].

As previously stated, diet plays an important role in the maintenance of the gut barrier integrity and is hence determinant for IP. The short-chain fatty acids (SCFAs), produced by the degradation of dietary fibers by several bacteria in the gut (including *Clostridium, Eubacterium*, and *Butyrivibrio*), have been the most studied microbial catabolites involved in the regulation of IP to date. Among them, butyrate has been identified as a marker of the positive effects of non-digestible dietary fiber consumption on microbiota composition and intestinal permeability. It exerts several activities on the intestinal wall, such as controlling inflammation by altering the expression of pro-inflammatory cytokines [56], preserving the intestinal barrier function by inducing the expression of TJ proteins claudin-1 and claudin-2 [57], and modulating composition of gut microbiota by inhibiting the growth of pathogenic bacteria [58]. Food is the only source of non-digestible carbohydrates, and alterations in diet lead to variations in the production of intestinal butyrate. In aged mice, the increased butyrate production after the consumption of high doses of soluble fiber was associated with an induced expression of the TJ proteins Tjp2 and Ffar2 and to a counterbalance of the age-related microbiota dysbiosis, with a significant amelioration of the increased IP condition typical of older individuals [59]. Similar effects of a high fiber diet were also observed in mice affected by autoimmune hepatitis, characterized by an imbalance of Treg/Th17 cells and increased IP [60]. After dietary intervention, the levels of butyrate were increased in feces, and the expression of TJ proteins ZO-1, occludin and claudin-1 was induced in the ileum, with consequent increased intestinal barrier function and decreased translocation of bacterial components through the intestinal wall [60]. The same effects were also observed in mice treated with sodium butyrate, indicating a direct involvement of this bacterial metabolite in the regulation of IP [60].

Microbial tryptophan metabolites also play an important role in regulating barrier functions and gut microbiota activity. A metabolomic approach allowed

to obtain preliminary elucidations about the role of tryptophan and its microbial and endogenous derivatives in the regulation of immune tolerance toward intestinal microbiota [61]. Starting from these findings, further research has elucidated the role of other microbial-derived tryptophan metabolites in the regulation of gut permeability, by direct effects on epithelial cells. Venkatesh et al. showed that indole-3-propionic acid (IPA), produced by the firmicute *Clostridium sporogenes*, regulates mucosal integrity and intestinal barrier function by activating the pregnane X receptor (PXR) and upregulating junctional protein-coding mRNAs [62]. More recently, Dodd et al. Used an integrated targeted-untargeted approach to identify 12 microbial metabolites derived from the reductive activity of *C. sporogenes* on aromatic amino acids (phenylalanine, tyrosyne and tryptophan), of which nine (lactate, acrylate and propionate derivatives) were reported to accumulate in host plasma [63]. The authors particularly focused on IPA and its effects on gut barrier and the mucosal immune system, and their results supported the findings of Venkatesh and coll. about the PXR-mediated effect on gut permeability [62,63]. A treatment with 20 mg kg^{-1} IPA for four consecutive days was shown to significantly decrease the IP in HFD-fed obese T2D mice [64], which, prior to treatment, were characterized by higher IP and lower circulating IPA levels compared to lean animals. Plasma IPA amounts were also reported to increase in the same obese model 3 months after Roux-en-Y gastric bypass (RYGB) surgery [64], indicating, once again, the direct involvement of gut microbiota in the maintenance of the intestinal barrier functions. Furthermore, results from *in vitro* assays reported by the same authors showed that IPA could reduce the permeability of T84 cell monolayer compromised by pro-inflammatory cytokines [64]. Other metabolites derived from the same degradation pathway of tryptophan, i.e. indole (produced by *Escherichia coli, Clostridium bifermentans, Proteus vulgaris, Paracolobactrum coliforme, Achromobacter liquefaciens*, and *Bacteroides* spp.) [65], indole-3-acetic acid (produced by *C. sporogenes*) and tryptamine (produced by *C. sporogenes* and *Ruminococcus gnavus*) [66], were also reported to exert anti-inflammatory

activity both in the intestinal lumen and in the liver [66,67], and to up-regulate the expression of several proteins involved in the trans-epithelial cells linkage on the intestinal wall, such as tight junction proteins TJP1, TJP3, and TJP4, and gap junction proteins GJE1, GJB3, GJB4, and GJA8, among others [65].

In recent years, polyphenols have been widely considered for their beneficial effects on health and polyphenol-rich diets have been evaluated for the prevention of several chronic diseases, ranging from metabolic disorders to inflammation and cancer. Some studies have also evaluated the consumption of polyphenol-rich food for the prevention of diseases associated to aging, such as cognitive impairment [68] and depression [69], although up to now the reported effects have been inconsistent. However, numerous in vitro and animal studies show that the consumption of polyphenol-rich food could positively affect IP, reinforcing the barrier properties of the intestinal epithelium by direct influence on the synthesis and expression of tight junction proteins [70,71] or by interaction with gut microbiota. As previously described, this latter is directly involved in the metabolic transformation of plant polyphenols and in the production of smaller MW derivatives [72], which in turn contributes to the maintenance of barrier function and drives changes in gut microbiome constituents [73,74], with important effects for host health. However, although several molecular targets of dietary polyphenols and their metabolites on the intestinal epithelium have been elucidated [75], it is unclear how the interaction of the same compounds with gut microbiota leads to beneficial effects on the intestinal barrier, and further efforts are required to fill this gap. Mice fed a high-fat diet supplemented with 4% w/w powdered green tea leaves rich in flavanols showed an increased intestinal population of *Akkermansia* spp. after 22 weeks [76], a bacterium that has been implied in the maintenance of a functional intestinal barrier through the preservation of mucus layer thickness [77]. More recently, Li et al. reported that the consumption of a medium-dose (20 mg/kg per day) of bilberry anthocyanin extract (BAE) promoted the generation of SCFAs (acetic acid, propionic acid and butyric acid) in aging rats, through the regulation of the intestinal microbiota [78]. Specifically, several

starch-utilizing and butyrate-producing bacteria (among whom *Lactobacillus* and *Bacteroides*) were induced by BAE, while harmful species such as *Verrucomicrobia* and *Euryarchaeota* where inhibited. These variations, associated with decreased levels of TNF-α and IL-6 in the colon induced by BAE consumption, contributed to the restoring of the intestinal barrier function typically altered in older individual [78]. Overall, these results indicate that the effects of polyphenols on IP are related to both direct effects on the expression of TJ proteins and to changes induced to the intestinal microbiota, with an increase in the prevalence of species that can preserve barrier functions through the production of active metabolites or by direct action on the mucous layer. Nevertheless, the data supporting these observations are still scarce, and up to now only few compounds (e.g. butyrate) correlating the diet-induced modifications of gut microbiota to the effects on the intestinal integrity and permeability have been discovered.

Conclusion and future perspective

It is well known that a healthy microbiota is associated with good host health, and diet plays a crucial role in regulating this equilibrium. Although the study of the effects of dietary interventions on gut microbiota and IP and investigations of the mechanisms of action have begun only recently, it appears clear that appropriate dietary habits and the regular consumption of vegetables and fruits rich in fibers and polyphenols play an important role in the maintenance of proper intestinal functions. The precursors of SCFAs and of several indole or phenolic derivatives produced by bacterial catabolism in the intestinal lumen, for example, are abundant constituents of both plant-derived foods, as cereals, nuts, fruits and vegetables rich in non-digestible fibers [79], and animal-based foods such as dairy products, eggs and meat, which are rich sources of tryptophan [80]. Thanks to the employment of integrated multi-omics approaches, the involvement of several partners (food components, microbiota and microbial-derived compounds) in the maintenance of the intestinal barrier function and the molecular pathways

behind this activity are being gradually elucidated, although further efforts are required to link specific food components and their metabolites to specific mechanisms of action.

In conclusion, the studies reviewed in this work could be considered as a starting point for further research, with the final goal being identification of precise biomarkers. These biomarkers, once validated for clinical relevance, will be novel instruments available to clinicians for the development of dietary plans aimed at managing and preventing diseases directly linked to increased IP, as chronic inflammation and immunological disorders, which are determinant for the gradual decline of health in older subjects.

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IMAGES

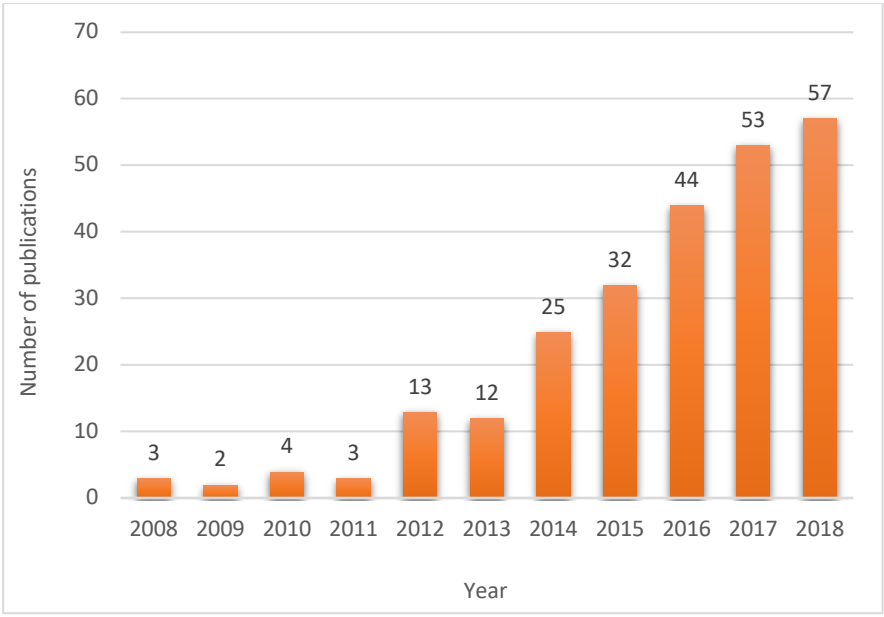
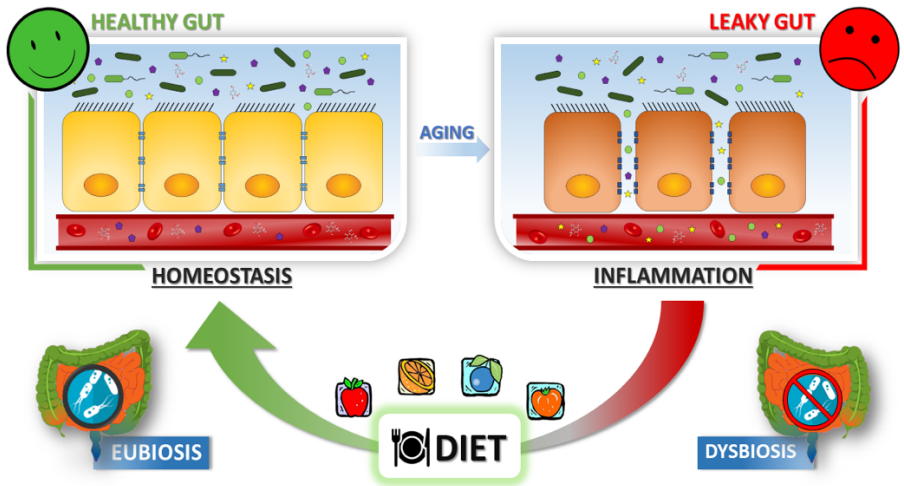


Figure 1. The increase of the scientific literature regarding the use of metabolomics in the study of the interactions between diet and gut microbiota during the last 11 years. Source: PubMed (https://www.ncbi.nlm.nih.gov/pubmed/).

GRAPHICAL ABSTRACT (TOC)



3.3 CHAPTER 3

- **Effect of a polyphenol-rich dietary pattern on intestinal permeability and gut and blood microbiomes in older subjects: Study protocol of the MaPLE randomised controlled trial**
  (pre-print of a submitted paper)

- **The MaPLE randomised control trial: effect on intestinal permeability and related markers**
  (unpublished results – manuscript under submission)

3.3.1 Effect of a polyphenol-rich dietary pattern on intestinal permeability and gut and blood microbiomes in older subjects: Study protocol of the MaPLE randomised controlled trial

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Abstract

During aging, alterations of the intestinal microbial ecosystem can occur contributing to immunosenescence, inflamm-aging and impairment of intestinal barrier function (increased intestinal permeability (IP). In the context of a diet-microbiota-IP axis in older subjects, food bioactives such as polyphenols may play a beneficial modulatory role.

Methods:

MaPLE is a project centered on a randomized, controlled cross-over dietary intervention trial [polyphenol-rich diet (PR-diet) versus control diet (C-diet)] targeted to older people (≥ 60 y) living in a well-controlled setting (i.e. nursing home residences). The 8-week interventions are separated by an 8-week wash-out period. Three small portions per day of selected polyphenol-rich foods are consumed during intervention in substitution of other comparable products within the C-diet. Biological samples are collected before and after each treatment period to evaluate markers related to IP, inflammation, vascular function, oxidative stress, gut and blood microbiomics, metabolomics.

Results and discussion:

Evidence that increasing the consumption of polyphenol-rich food products can positively affect intestinal microbial ecosystem resulting in reduced IP and decreased translocation of inflammogenic bacterial factors into the bloodstream will be provided. The integration of data from gut and blood microbiomics, metabolomics and other IP-related markers will improve the understanding of the beneficial effect of the intervention in the context of polyphenols−microbiota−IP interactions. Finally, findings obtained will provide a proof of concept of the reliability of the dietary intervention, also contributing to future implementations of dietary guidelines directed to IP management in the older and other at-risk subjects. **Trial registration:** ISRCTN10214981.

Keywords: Gut barrier function, leaky gut, flavonoids, phenolics, inflammation, aging, inflamm-aging

Introduction:

Age-associated changes significantly compromise health status and increase the risk of chronic diseases. Within these modifications, recent research has been focusing on those that specifically occur at the gut epithelium level with impact on intestinal immune homeostasis and related systemic responses (1). The maintenance of a functional intestinal barrier (the functional entity separating the gut lumen from the inner host) (2), seems to be of utmost importance to facilitate healthy aging. Nevertheless, no conclusive evidence exists for a direct or causal link between the aging process and intestinal mucosa integrity impairment (3,4).

The intestine acts both as a barrier (to keep harmful substances out of the body) and as a selectively permeable surface that allows the controlled passage of substances from the gut lumen through the gut wall and into the body. This controlled flux across the intestinal wall is known as intestinal permeability (IP) (2). Inappropriate IP (i.e. loss of control of the influx of substances from the gut) has been associated with several disorders and diseases, such as irritable bowel syndrome, inflammatory bowel disease, allergy, colon cancer, obesity, celiac disease, inflammatory joint diseases and neurologic pathologies (e.g. Parkinson's disease) (5–8). In this regard the intestinal microbiota is considered an important factor in the regulation of IP, in fact, gut microorganisms may directly affect IP through tight junction modulation (9) and indirectly by contributing to the up/down regulation of inflammatory processes, which is a key factor in causing impaired IP (10). Consequently, manipulation of the complex intestinal microbial ecosystem (i.e. the microbiota and derived metabolic products) has been proposed as a novel strategy to maintain/improve normal IP function (2).

Increasing evidence suggests that dietary patterns can represent a relevant factor in shaping the intestinal microbiota and modifying the relative abundance of specific bacterial taxa (11–13). Consequently, modulating the

concentrations of health-affecting microbial metabolites in the gut such as butyrate (14,15), has been suggested to preserve tight junction integrity and inhibit TNF-alpha release, thus maintaining appropriate IP condition (16). Nutrients are also essential themselves and malnutrition is associated with increased IP (17).

Older subjects are often characterized by alterations of the intestinal microbial ecosystem (18,19), which may be due to inadequate nutrition, drug treatments and other age-related factors: all of these seem to contribute to immunosenescence and inflamm-aging (18,20).

In the context of a diet-microbiota-IP axis, food bioactives may have a key role in regulating the numerous interconnected processes involved. Particularly, polyphenols exert antioxidant, anti-inflammatory/immunomodulatory properties at intestinal and systemic levels, and there is increasing mechanistic evidence suggesting their potential to modulate IP (21,22). In addition, polyphenols are extensively metabolized by the microbiota and can affect its composition (13,23). The combination of the modulation of intestinal ecology by polyphenols and the effect on derived microbial metabolites has been shown to improve inflammatory markers (24). Taken together, these data support findings obtained from observational studies in older subjects suggesting that a high polyphenol diet is associated with favorable health outcomes (25). But, well-controlled intervention studies are still lacking (21).

Methods/design

Objective and hypothesis

The aim of the MaPLE project (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) is to evaluate the hypothesis that an increased intake of polyphenol-rich foods can reduce IP and lower inflammogenic bacterial factors in the bloodstream promoting an overall protective/beneficial metabolic phenotype in older subjects. Three approaches have been taken; the main study, a dietary intervention

randomized controlled trial described here, combined with pre-clinical studies in an animal model of aging to test the impact of the polyphenol-rich diet on IP associated markers, and also in cultured human intestinal cells (caco-2) to investigate the capacity of single polyphenols to modulate IP.

MaPLE Trial: protocol and study design

The MaPLE RCT is a single-blind randomised, controlled, cross-over dietary intervention trial [polyphenol-rich diet (PR-diet) versus control diet (C-diet)] in older people (≥ 60 y) living in a well-controlled setting. Each intervention period consists of 8 weeks and is separated by an 8-week wash-out period in which participants consume their habitual diet to avoid carry-over effects.

The PR-diet and C-diet were developed to provide adequate and comparable levels of energy and nutrients. The PR-diet was achieved by replacing three portions per day of low polyphenol foods/beverages with specific polyphenol-rich foods/beverages (as detailed below). During the study, subjects are asked to fast overnight before each scheduled time-point of blood, urine and feces collection. In addition, daily menus and weighted food records (WFRs) are collected throughout the trial. An overview of the study design is represented in **Fig. 1 and 2**.

Trial status

The intervention has been completed; samples analysis and data elaboration is ongoing.

Location

The intervention has been performed at Civitas Vitae (OIC Foundation, Padua, Italy) which hosts a large number of older people living in residential care buildings or in independent residences located in the same area, depending on individual willingness and level of disabilities. The OIC Foundation provides several facilities including a dedicated area for meal preparation. This allows to collect accurate information with regard the

composition of the diets from the recipes used for each of the foods in the meals delivered daily to the participants. We were able to accurately assess food intake using weighed food records in the intervention study.

Participant enrollment

Before recruitment, a meeting with the medical staff and nurses' coordinators at OIC Foundation took place in order to present and widely discuss the aim, methodologies and technical aspects related to the development and the management of the MaPLE trial. After this meeting, several formal presentations of the project aim and some general information on the intervention planned were organized at OIC Foundation for the hosts and their families. Finally, an accurate evaluation of the hosts characteristics was performed in collaboration with the physicians/geriatricians and nurses' coordinators to pre-select based on the verification of the main inclusion and exclusion criteria (see below) and to identify plausible candidates for the study. Subjects who were interested in participating in the study signed an informed consent reporting all the information on the dietary intervention, the analysis and protocols that they were asked to undertake/follow.

More specifically, volunteers were selected according to the inclusion and exclusion criteria reported below:

Inclusion criteria:

- Age ≥ 60 years
- Adequate nutritional status evaluated with Mini Nutritional Assessment (MNA), score≥24
- Good cognitive status tested with Mini Mental State Examination (MMSE), score≥24
- Self-sufficiency assessed with validated tests (e.g. Barthel index – activities of daily living, score ≥60)
- Increased intestinal permeability evaluated by serum zonulin level

Exclusion criteria:

- Celiac disease
- Severe liver disease with cirrhosis
- Severe renal insufficiency (dialysis)
- Presence of severe Chronic Obstructive Pulmonary Disease (COPD; oxygen therapy for many hours a day) or severe cardiovascular disease (heart failure class III or IV NYHA - New York Heart Association)
- Antibiotic treatment in the last month
- Malignant tumor that required treatment in the previous 2 years

Each subject enrolled has been assigned to an ID number. The encoding of samples is hidden to both the investigators and the participants. All clinical and personal data, including the biological samples, of the subjects involved in the study are collected and stored anonymously.

Polyphenol-rich dietary protocol

In order to define the polyphenol-rich dietary protocol, an initial estimation of nutrient and total polyphenol intake was performed through the analysis of the daily menu provided at the OIC Foundation.

Subsequently, an identification of the specific polyphenol-rich food products to be included in the diet was carried out in order to consider not only the amount and contribution of the different polyphenols but also the food preparation in order to ensure their bioavailability. In addition, an evaluation of conditions to enable optimal texture (e.g. considering the use of purees instead of the whole product) and an assessment of the product acceptability by the target population was also undertaken.

The polyphenol-rich dietary protocol (PR-diet) was finally developed by including in the C-diet 3 portions per day of the following selected polyphenol-rich foods: berries and related products, blood orange, pomegranate, green tea, Renetta apple, and dark chocolate.

A schematic plan of the type and serving sizes of polyphenol-rich products consumed daily in the PR-diet is shown in **Fig. 3**. The MaPLE polyphenol-rich foods provided a mean of 724 mg/day of total polyphenols as estimated by Folin-Ciocalteu analysis (26). In addition, the PR-diet and C-diet were kept comparable in terms of energy intake and nutrient composition, and to achieve this, polyphenol-rich products were substitute for other comparable products (e.g. foods used for snack or breakfast) and this continued across the entire period of intervention.

Information on potential adverse effects

Even though no reports of adverse effects due to a polyphenol-rich diet had been registered or reported in the literature, subjects were advised to annotate and communicate any adverse symptom perceived during the intervention period. Since green tea was selected within the polyphenol-rich food sources to be used in the intervention study, there was a comprehensive discussion to define the dose to use. Green tea extract is a rich source of epigallocatechin-3-gallate (EGCG) known for many different protective effects; however, the intake of very high doses of EGCG/green tea extracts as supplements has been reported to cause liver toxicity. Recently, it has been proposed an EGCG upper level (UL) based on human intervention studies of 300 mg EGCG/day in healthy adults (27). The proposed UL based on an ADI derived from animal toxicity data was 322 mg EGCG/day in a 70 kg adult. These values are applicable to the oral exposure under fed conditions, and consistent with those published by France (28) and Italy (29). In MaPLE, the dietary intervention provided 200 mg of green tea powder (i.e. 120 mg total polyphenol including about 100 mg EGCG) 2 times per week. This quantity was regarded as very likely to be safe taking into account the target population and the contribution of other food sources containing EGCC.

Assessment of food intake

Food intake before (enrollment phase) and during the intervention periods was recorded through the evaluation of OIC Foundation daily menus and the use of WFRs. The daily menus, covering different seasons, were analyzed to quantify nutrient and polyphenol content. Moreover, the day before each time-point, a WFR was completed and both nutrient and polyphenol intake were estimated. At least 3-WFRs were completed during each intervention period. Daily menus and WFRs were assessed using MetaDieta® (Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) to estimate energy and nutrient intake. Total polyphenol estimation was performed by using the Phenol Explorer database (phenol-explorer.eu) to provide estimates of polyphenol concentrations in each food, and where there were no useful values, using our proprietary data or values obtained from the literature. Total polyphenol content of the foods was estimated directly using the Folin-Ciocalteau method (30).

Biological sampling

Blood, urine and fecal samples were collected at each time-point as defined in Fig.1. For blood drawing, a specific vacutainer was used. Urine and fecal samples were collected using specific containers designed for this purpose. An aliquot of each collected blood sample was immediately stored at -80°C for microbiomic analyses. The remaining blood was processed by centrifugation and then serum and peripheral blood mononuclear cell (PBMC) fractions were obtained, divided into aliquots and stored at 80°C. Urine and fecal sample were divided into aliquots, and all human tissue samples were stored at -80°C until analysis.

In addition, a brush was used to collect an oral mucosal sample from each participant for further evaluation. The brush with the collected tissue was stored in a cryovial containing a buffered saline solution, which was immediately frozen.

Outcome measurements

The primary selected outcome of the study was zonulin as an IP marker, whereas other IP related markers (e.g. CD14, calprotectin), inflammatory markers (CRP, TNF-α, IL-6), oxidative stress and vascular function markers (DNA damage, VCAM-1, ICAM-1), metabolomics, taxonomic (16rRNA) and microbiota composition, were included as secondary outcomes to support and validate our study hypothesis.

Anthropometric measurements

Body weight, height and BMI calculation were assessed at the beginning and the end of each intervention period following the international guidelines of Lohman et al. (31).

Blood pressure

Each participant was monitored at the beginning and the end of each intervention period measuring both systolic and diastolic pressure obtained in a resting, seated position following the validated JNC 7 guidelines (32).

Metabolic and functional markers

At enrollment and at each time-point, metabolic and functional parameters (i.e. glucose, insulin, lipid profile, liver and renal function) were assessed by a standardized validated protocol, using an automatic biochemical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). Low density lipoprotein cholesterol (LDL-C) concentration was estimated using the Friedewald formula (33), while non-high-density lipoprotein cholesterol (non-high-density lipoprotein-cholesterol, HDL-C) was calculated by subtracting HDL-C from total cholesterol (TC). The HOMA-Index and Cockroft-Gault index were calculated according to the relevant formula (34,35).

Intestinal Permeability evaluation

Intestinal permeability was evaluated by quantifying serum zonulin concentrations. Human zonulin is a protein (i.e. prehaptoglobin-2) released by enterocytes able to promote the activation of the signaling transduction pathway that cause tight junction protein disassembly enabling potential bacterial factor translocation (36). In this study, zonulin serum levels were quantified using the Immunodiagnostik® ELISA kit (Bensheim, Germany) with samples collected in the selection phase and at the beginning and the end of each intervention period. Subjects selection based on IP was performed by considering reference values reported in the manufacturer's instructions and data published on different target groups (37–39). Other IP related markers, such as CD14 and fecal calprotectin, were also quantified to support the primary outcome.

Inflammatory markers

The concentrations of several markers related to inflammatory processes were quantified using specific ELISA kits (R&D Systems, Biotechne, Abingdon, UK). CRP (DCRP00), IL-6 (HS600B), TNF-α (HSTA00E) were quantified in serum at the beginning and the end of each intervention periods.

Vascular function markers

In order to assess vascular function, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were quantified in serum samples at each intervention time point using an ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy).

Oxidative stress marker (Comet assay)

The levels of endogenous and oxidatively-induced DNA damage, as markers of oxidative stress, were assessed in peripheral blood mononuclear cells (PBMCs) by the comet assay. The samples are collected before and after each intervention period. Levels of endogenous DNA damage were assessed using a specific enzyme (formamidopyrimidine DNA glycosylase, FPG

sensitive sites) that can be used to detect 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. Oxidatively-induced DNA damage was measured by treating the cells with hydrogen peroxide and by evaluating the capacity of cells to counteract an oxidative insult. Both Comet assay protocols have been previously described by Del Bo' et al (32).

Blood bacterial load and taxonomic profiling

Bacterial DNA quantification and sequencing reactions were performed by Vaiomer SAS (Labège, France) using optimized blood-specific techniques as described earlier (40–43). Specifically, DNA was extracted from 100 µl of whole blood and quantified by quantitative PCR targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene with primers EUBF 5'-TCCTACGGGAGGCAGCAGT-3' and EUBR 5' -GGACTACCAGGGTATCTAATCCTGTT-3' (44). The results are reported as 16S rRNA gene copies per ng of total DNA and per µl of blood. DNA from whole blood was also used for 16S rRNA gene taxonomic profiling using MiSeq Illumina® technology (2 x 300 paired-end MiSeq kit V3, set to encompass 467-bp amplicon) as previously described (42,43). To determine bacterial community profiles, the bar-coded Illumina paired reads were demultiplexed, then single read sequences were trimmed and paired for each sample independently into longer fragments; nonspecific amplicons (<350 bases or >500 bases) were removed and remaining sequences clustered into OTU using FROGS v1.4.0 (45) with default parameters; a taxonomic assignment was finally performed against the Silva 128 Parc database. Bioinformatics analysis of the sequencing data was performed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline.

Fecal Microbiota composition

All of the following steps were performed in-house at QIB. Fecal samples were weighed into Lysing Matrix E bead beating tubes (MPBio, Santa Ana, CA,

USA) and extraction was completed according to the manufacturer's protocol for the FastDNA™ SPIN Kit for Soil (MPBio) but extending the bead beating time to 3x60s. DNA was quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad CA, USA), normalized to 5ng/µl and the V3/V4 region of the 16S rRNA was amplified using the primers detailed below. Sequencing was performed using a 600 cycle MiSeq v3 reagent kit (Illumina, San Diego, CA, USA) giving approximately 100,000 reads per sample.

Bioinformatic analysis was conducted using VSEARCH (46); reads were merged, and primer sequences trimmed. Reads were dereplicated and singletons removed. Prior to Chimera removal, reads were clustered at 98% similarity, de novo Chimera removal was performed using the UCHIME algorithm (47) and the OTU table and sequences were prepared. Data was subsequently analyzed using the phyloseq package in R (48).

Primers:

16S 341F – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S 806R – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Metabolomics

Urine samples collected prior and after each intervention period were subjected to targeted metabolomics analysis by applying the Quantitative Dietary Fingerprinting approach recently developed by González-Domínguez et al. (49) with the aim of monitoring metabolite alterations derived from the polyphenol-rich diet and to associate these changes with improvements in clinical and biochemical outcome measurements (e.g. IP evaluated through zonulin levels, inflammatory and oxidative stress markers, blood bacterial load). To this end, urine samples were treated by solid phase extraction (SPE) and subsequently analyzed by reversed-phase ultra-high-performance liquid

chromatography coupled to tandem mass spectrometry (RP-UHPLC-MS/MS) to obtain a comprehensive assessment of the urinary food metabolome, with the simultaneous quantitative determination of about 350 dietary derived metabolites. Complementarily, plasma samples are also analyzed using a modification of the previously described targeted metabolomics approach, adapted to deal with the chemical complexity of blood samples (high content of proteins and lipids) and to enlarge the metabolomic coverage. This novel method is based on a similar RP-UHPLC-MS/MS instrumental configuration that enables the simultaneous measurement of both food intake biomarkers and endogenous metabolites from multiple chemical classes (ca. 1000 metabolites), including amino acids and derivatives, biogenic amines, carbohydrates, organic and fatty acids, vitamins and various lipid classes (e.g. acylcarnitines, steroid hormones, bile acids), among others. To expand the method coverage towards the high-polarity low molecular weight metabolome, an orthogonal hydrophilic interaction liquid chromatography (HILIC) procedure was also applied, covering a broad range of polar metabolites (ca. 300 metabolites), comprising common and acetylated amino acids and microbiota derivatives, low molecular weight organic acids (including short chain fatty acids and related compounds) and carbohydrates (e.g. sugars, conjugates and advanced glycation end products).

Sample size, randomisation, and statistics

According to data literature (38,50) it was estimated that at least 50 subjects were required to demonstrate an IP reduction of 30% with 80% power and 0.05 significance and considering a 15% drop-out rate. Subjects were randomly divided by using a computer random number generator. The randomisation and allocation were performed by a person not involved in the trial and blinded to the participants, investigators/health care providers and researchers involved in samples analysis. Statistical analyses were performed by means of R statistic software version 3.4.2. Particularly, the following statistical elaborations will be performed to identify significant differences

between treatments: (i) the analysis of variance (ANOVA) with repeated measures, (ii) Wilcoxon paired data test, (iii) Linear Mixed Model (LMM) analysis. In addition, regression and correlation analyses (Spearman and Kendal test) are carried out to highlight associations between blood microbiomic data, fecal bacterial profiling data, and physiological and biochemical data. When appropriate, a post-hoc p-value adjustment is performed using the Hochberg-Benjamin correction. Significance is set at $P \leq 0.05$; significance in the range $0.05 < P < 0.10$ is accepted as trend. Potential gender differences will be also considered in all the analyses.

Discussion

There is growing evidence of a link between IP impairment and increased inflammation (2). Since aging is characterized by low grade systemic inflammation it is possible that an increase in IP may induce the activation of inflammatory pathways and the immune system caused by the translocation of intestinal microbes, toxins, and/or nutritional components from the gut lumen through the epithelium and into the bloodstream (51). While there is preliminary mechanistic evidence obtained in animal models on the complex interaction between age-associate microbial dysbiosis, IP and inflammation (5), the properties of the human intestinal barrier, in the context of the ageing process, has not been fully investigated (4). The dietary pattern and the intestinal microbial ecosystem homeostasis have been addressed as potential key points for the development of strategies to enable healthy aging. The manipulation and/or improvement of the diet by increasing the consumption of food bioactives (e.g. polyphenols) or specific nutrients is recognized as a potential powerful tool to be explored also in the context of IP. However, human intervention studies are still very scarce, and most of these performed using probiotics, prebiotic fibers and dietary supplements (21).

For these reasons, the ongoing MaPLE project has been planned in order to test the hypothesis that changing the diet of older subjects with enhanced IP by increasing their polyphenols consumption can alter the intestinal microbial

ecosystem in a way that is beneficial for IB function, resulting in reduced IP and decreased translocation of inflammogenic bacterial factors from the digestive tract into the bloodstream.

The development and management of well-controlled and adequately balanced dietary intervention studies is not an easy task and it becomes even more difficult when the target population is older subjects. Consequently, the first task of the project was dedicated to the optimization of the trial in order to overcome the possible problems related to compliance with the dietary instructions and to other relevant potential confounding factors (e.g. periods of illness or the use of drugs that may be relevant in this target group). For this reason, the MaPLE RCT was planned in a residential area for older people, since it provided a favourable and controlled environment in which it was possible to optimize and standardize most of the important experimental conditions. For example, since outcome data from dietary intervention studies are prone to being affected by individual differences in diets and lifestyle behaviour over time (e.g. during the two eight week periods of dietary intervention), we were able to ensure both strict compliance with the dietary intervention and a consistent dietary pattern among participants by including the polyphenol-rich products in their usual meals provided by the residential home. In addition, the selection of polyphenol-rich foods was based on three important considerations: (i) That the types of foods selected were largely universally liked, (ii) that the texture of the selected products was suitable for older subjects (e.g. with dentition challenges), and (iii) that the portion of food would reliably provide a high dose of polyphenols. In addition, weighed food intake was also assessed to provide us with data to allow accurate estimates of actual nutrient and polyphenol intake in the two periods of treatment (PR- and C- diet). This allowed a high degree of control and substantially reduced between treatments differences.

As regard the primary outcome, serum zonulin concentrations were used as the marker of IP because of the low reliability and applicability of the multi-sugar test in the older population (i.e. due to a high rate of incontinence

amongst the elderly participants and the need for adherence to a strict dietary protocol before the test)(52).

It is also noteworthy that the MaPLE RCT is testing, for the first time, the hypothesis that a dietary intervention may modulate quantitatively the bacterial DNA in bloodstream and qualitatively the blood microbiota composition. This should provide further evidence of the impact of the dietary intervention on IP being potentially associated with a reduction in translocation of bacterial factors. Other objectives of the MaPLE RCT are to integrate microbiota profiling data with inflammation and metabolomics data to improve understanding on the impact of the dietary intervention. In addition, the inter-individual response to the treatment will be investigated and food metabolite profiling data will be exploited for the identification of a set of potential biomarkers with relevance in the context of preventing or treating impaired IP. Finally, results will be pivotal for the development of new dietary approaches and guidelines for managing IP related conditions in the complex context of healthy aging.

Abbreviations

IP, intestinal permeability; MaPLE, Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly; PR-diet, polyphenol-rich diet; C-diet, control diet; WFRs, weighted food records; MNA, mini nutritional assessment; MMSE, mini mental state examination; COPD, chronic obstructive pulmonary disease; NYHA, new York heart association; EGCG, epigallocatechin-3-gallate; UL, upper level; PBMCs, peripheral blood mononuclear cells; LDL-C, lipoprotein cholesterol; HDL-C, non-high density lipoprotein-cholesterol; TC, total cholesterol; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxiguanosine; QIIME, quantitative insights into microbial ecology; SPE, solid phase extraction; HILIC, orthogonal hydrophilic interaction liquid chromatography; ANOVA, analysis of variance; LMM, linear mixed model.

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Availability of data and materials

At the end of the project, the datasets generated during the study will be made freely available in the Dataverse repository, https://dataverse.unimi.it/dataverse/JPI-MaPLE.

Author’s contributions

MaPLE project includes three research units: (i) University of Milan, Italy, coordinated by Patrizia Riso (PR) and Simone Guglielmetti (SG), who are the principal investigators of the project, (ii) University of Barcelona, Spain, coordinated by Cristina Andres-Lacueva (CAL), and (iii) Quadram Institute, UK, coordinated by Paul A Kroon (PAK). PR and SG are responsible for the trial conception and design. AC contributed to the development of study protocol for clinical and ethical aspects and critically revise the results from a clinical perspective. CAL and PAK contributed to the definition of polyphenol-rich dietary intervention and markers selection. SB and CDB helped in the management of the intervention study and performed the sampling and analysis of markers related to IP, vascular function and oxidative stress. SG and GG are responsible of blood microbiomics analysis and statistical elaboration of metagenomic data. RGD and GP performed the metabolomics analysis coordinated by CAL. NHL and RZR contributed to the estimation of polyphenol intake together with SB. BK and MSW contributed to microbiota composition and inflammatory marker analysis coordinated by PAK who is responsible of polyphenol metabolism evaluation and mechanistic studies. MP provided support for the critical evaluation of nutrient composition of dietary protocol and elaboration of results on food intake. PR, SG, SB and

CDB drafted the manuscript; all the authors critically revised the draft and approved the final version.

Ethics approval and consent to participate

The Ethics Committee of the Università degli Studi di Milano approved the study protocol (15/02/2016; ref: 6/16/CE_15.02.16_Verbale_All-7). All participants gave their written informed consent to use samples and data collected from MaPLE prior to participating in the study.

Competing interests

The authors declared that they have no competing interests to declare.

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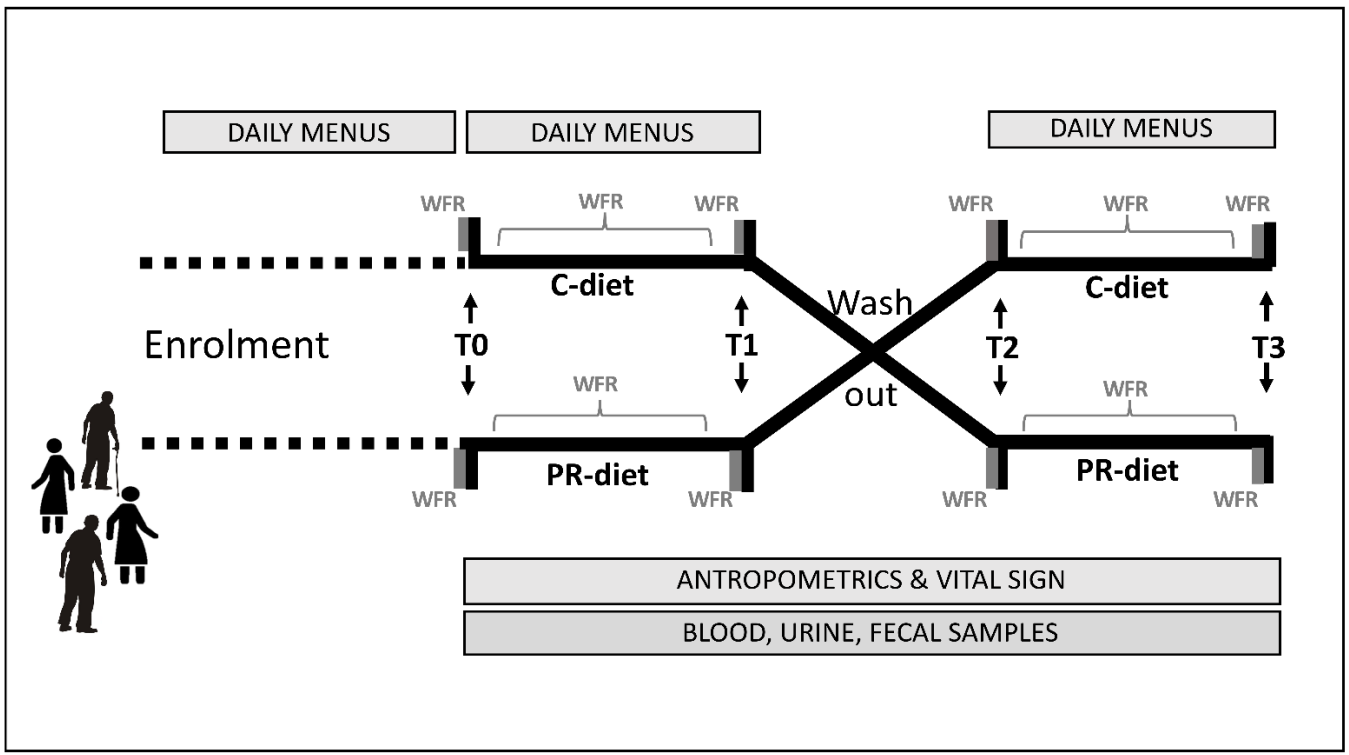
Figure,titles and legends

Fig. 1 Study design: Schematic representation of the study workflow



WFR = weighed food records;

T0, T1, T2, T3 = time of intervention; C-diet = control diet PR-diet = polyphenol-rich diet

Fig. 2 – Standard protocol items: recommendations for interventional trials (SPIRIT) figure

	ENROLLMENT	T0	T1	T2	T3
Eligibility screening	X				
Informed consent	X				
Intestinal Permeability test	X				
Daily menu evaluation	X				
Polyphenol intake	X				
PHYSICAL ASSESSMENT AND VITAL SIGN					
Anthropometrics		X	X	X	X
Blood pressure		X	X	X	X
DIETARY ASSESSMENTS					
Daily menu evaluation		X	X	X	X
Weighted Food Record		X	X	X	X
BIOCHEMICAL ASSESSMENTS					
Intestinal Permeability test		X	X	X	X
Inflammatory markers		X	X	X	X
Vascular function markers		X	X	X	X
Oxidative stress markers		X	X	X	X
Metabolomics		X	X	X	X
Microbiota composition		X	X	X	X
Blood microbiomics		X	X	X	X

Fig. 3 Daily plan of MaPLE polyphenol-rich food products: 3 portions per day are scheduled

WEEK	POLYPHENOL-RICH FOOD PRODUCTS (SERVINGS)		
MONDAY	Chocolate callets (10 g)	Renetta puree (100 g)	Pomegranate juice (125 ml)
TUESDAY	Blood Orange (200 ml)	Renetta Apple (150 g)	Berry puree (100 g)
WEDNESDAY	Cocoa powder° (2 g)	Renetta Apple (150 g)	Blueberry (120 g)
TUESDAY	Green tea* (200 ml)	Chocolate callets (10 g)	Renetta Apple (150 g)
FRIDAY	Blood Orange (200 ml)	Pomegranate juice (125 ml)	Blueberry (120 g)
SATURDAY	Chocolate callets (10 g)	Berry puree (100 g)	Renetta puree (100 g)
SUNDAY	Green tea (200 ml)	Blood Orange fruit (110 g)	Renetta Apple (150 g)

Legend: Chocolate powder was dissolved in hot milk or water; *Green tea was prepared by solubilization of 200 mg of green tea extract in 200 ml of hot water. $^{+}$Renetta apple puree was prepared in controlled conditions and stored at -18°C

3.3.2 The MaPLE randomised control trial: effect on intestinal permeability and related markers

Abstract

Altered intestinal permeability causing bacterial translocation may be responsible of the increase of inflammatory processes in the older subjects. Specific dietary patterns, contributing to a higher intake of food bioactives, may be candidate strategies to improve well-being in the older subjects. The MaPLE trial has been developed with the aim to test the hypothesis that a polyphenol rich diet can exert beneficial effects on IP and related markers. The results obtained seem to support the beneficial effect of the dietary approach in the improvement of IP as demonstrated by the reduced serum zonulin levels whose baseline concentrations has been also associated with bacterial DNAmia abundance in the target subjects.

Introduction

As previously reported during ageing an enhanced IP could contribute to the onset of a chronic low-grade inflammation that has been previously defined as inflamm-aging (Franceschi et al, 2010). This condition can be responsible of the development of several age-related diseases including metabolic syndrome, obesity, diabetes and cardiovascular diseases. Gut microbiota seems to play a central role in driving inflammation, as it can release several inflammatory products, and contribute to the (dys)regulation of intestinal permeability (IP) (Nagpal et al, 2018). In this regard, it is reported that gut microbiota may act directly on IP by affecting tight junctions' assembly and dis-assembly and/or indirectly by modulating inflammation thus, the manipulation of the intestinal ecosystem has been proposed as a potential novel strategy to improve IP (Bishoff et al. 2010). Such manipulation may be obtained through the exploitation of improved non-invasive dietary approaches able to shape gut microbiota and related metabolic activities. We

have recently showed that bioactive compounds such as polyphenols may positively affect IP. In fact, polyphenols biological functions include both their antioxidant and anti-inflammatory properties, and their immunomodulatory activity both at instestinal and systemic levels (Bernardi et al, 2019).

Despite the exact molecular mechanisms are not completely revealed (Peron et al, 2019), polyphenols may directly and/or indirectly partecipate at different levels of the intestinal barrier by regulating tight junctions' function, the production of numerous inflammatory cytokines and the activation of antioxidant genes (Bernardi et al.,2019). Furthermore, polyphenols undergo extensive modifications by microbiota with an impact on the overall microbial ecosystem.

Consequently, in the overall assumption of a diet-microbiota-IP axis in the older subjects, the development of a polyphenol-rich dietary pattern specifically targeted to these critical subjects, may represent a potential strategy to positively affect the microbiota composition and improve IP and related conditions.

As previously reported (Bernardi et al., 2019), human intervention studies aimed at investigating the role of dietary factors and, more specifically polyphenols, in the modulation of IP are still lacking. Within this context, the MaPLE trial has been developed with the aim to verify the hypothesis that by increasing the intake of polyphenol-rich foods in the older subjects it is possible to improve IP and a set of numerous potentially related markers. To this aim, a randomized, controlled trial has been developed and detailed information about the conception of the study protocol has been previously reported in the present thesis (Guglielmetti et al., 2019, submitted).

Materials and Methods

MaPLE project (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) has been developed to test the hypothesis that a polyphenol-rich diet can modulate IP and inflammogenic bacterial factors in the older subjects.

Setting of the intervention

The intervention has been carried out at Civitas Vitae (OIC Foundation, Padua, Italy, Fig. 1) which consists of residential care or independent residences where older subjects can live depending on their needs and/or conditions (e.g. self-sufficiency). The presence of a dedicated area for meal preparation and a good team of professional staff was essential to better monitor the diet, also through the evaluation of menus and ingredients used for recipes delivered. Moreover, it has been possible to register weighted food diaries (WFR) and to facilitate the compliance to the dietary instructions during the intervention periods.

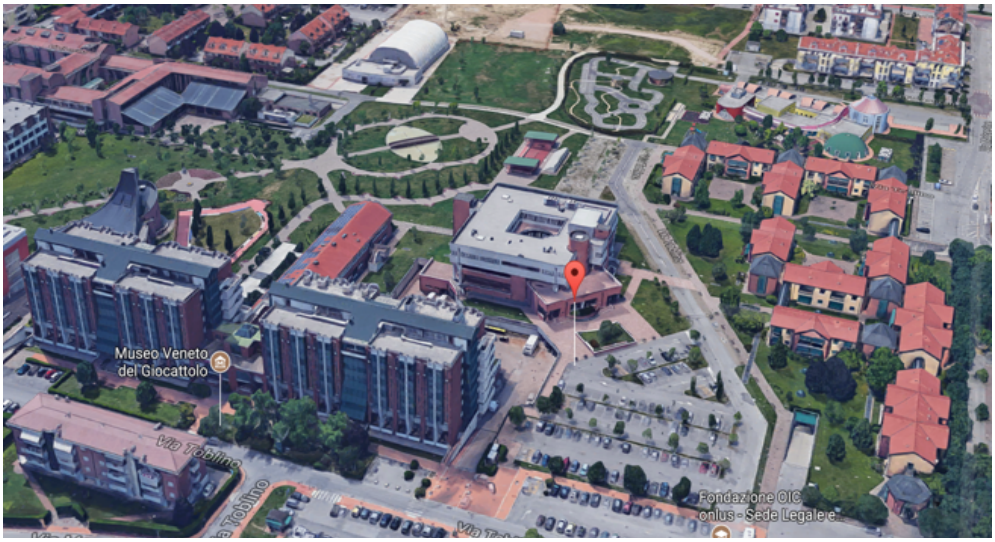


Fig. 1 MaPLE – setting – OIC Foundation, Padua

Subjects recruitment

Prior to set up the intervention, several meetings have been organized with the medical staff and nurses' coordinators at OIC Foundation to discuss methodologies and technical aspects, thus to pre-select hosts on the basis of their characteristics and clinical conditions according to the inclusion and exclusion criteria reported below.

Inclusion criteria: subjects had to be ≥ 60 years old, with an adequate nutritional status evaluated with Mini Nutritional Assessment (score ≥ 24), a

good cognitive status (Mini Mental State Examination score ≥ 24), be self-sufficient (Barthel index – activities of daily living, score ≥ 60) and with an increased intestinal permeability (IP) evaluated through zonulin serum level. *Exclusion criteria***:** subjects reporting celiac disease, severe condition or disorders such as cirrhosis, renal insufficiency (dialysis) and/or presence of severe Chronic Obstructive Pulmonary Disease (COPD; oxygen therapy for many hours a day) or severe cardiovascular disease (heart failure class III or IV NYHA - New York Heart Association). Moreover, subjects with malignant tumor that required treatment in the previous 2 years have been excluded as well as those under antibiotic treatment in the last month before the intervention period.

Subjects selected signed an informed consent reporting all the information on the dietary intervention, the analysis and protocols that they were asked to follow.

Study design

A single-blind, randomized, controlled, cross-over dietary intervention trial [polyphenol-rich diet (PR-diet) versus control diet (C-diet)] in selected older subjects (≥ 60 y) living in a well-controlled setting has been developed. Each intervention period lasted 8 weeks and has been separated by an 8-week wash-out period with a free-diet to avoid any carry-over effects of the intervention. At each timepoints of the study, anthropometrical data, vital signs, blood, urine and fecal samples have been collected. In addition, daily menus and weighted food records (WFRs) have been collected prior and during the overall intervention as previously reported in the submitted paper by Guglielmetti et al. 2019.

Development of the polyphenol-rich diet (PR-diet).

In order to set up the intervention and develop the PR-diet, an initial evaluation of the menu provided by OIC Foundation have been performed through the

use of Metadieta® software for the nutrient/energy composition and through phenol-explorer database for the total polyphenol content (Table 1).

The PR-diet has been achieved by replacing three portions per day of low polyphenol foods/beverages with specific selected polyphenol-rich foods/beverages such as berries and related products, blood orange, pomegranate, green tea, Renetta apple, and dark chocolate. The products provided a mean of 724 mg/day of total polyphenols as previously reported in the present thesis (manuscript submitted by Guglielmetti et al 2019). On the whole, the PR-diet has been developed to roughly double the polyphenol intake with respect to the C-diet and to provide adequate and comparable levels in terms of energy and nutrients.

Table 1: Energy and nutrient profile of mean daily menu provided by OIC Foundation

Nutritional factors	**Mean ± SD**
Energy (Kcal)	1996 ± 78.5
Protein (% of energy)	20.2 ± 2.2
Total Carbohydrates (% of energy)	46.2 ±1.8
Total Lipids (% of energy)	33.8 ± 2.0
SFA (% of energy)	8.9 ± 1.0
MUFA (% of energy)	17.2 ± 1.0
PUFA (% of energy)	3.9 ± 1.3
ω-3 (% of energy)	0.7 ± 0.3
ω-6 (% of energy)	3.2 ± 1.2
Fiber (g/1000 Kcal)	10.9 ± 1.1
Total Protein (g)	100.2 ± 8.3
Total lipids (g)	74.7 ± 6.3
SFA (g)	19.6 ± 2.7
Cholesterol (mg)	247.7 ± 125.1
MUFA (g)	38.1 ± 2.9
ω-3 (g)	1.5 ± 0.8
ω-6 (g)	7.04 ± 2.5
Total Fiber (g)	21.7 ± 2.6
Vitamin E (mg)	15.3 ± 2.4
Vitamin C (mg)	238.9 ± 22.5
Vitamin B1 (mg)	1.4 ± 0.2
Vitamin B6 (mg)	2.3 ± 0.2
Folates (µg)	378.4 ± 130.2
Vitamin B12 (µg)	6.4 ± 4.8
Total polyphenols (mg)	828.7 ± 79.1

Data represents mean daily intake

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Evaluation of food intake and polyphenol intake

At each time point of the study and during the intervention periods the menu provided in the different seasons have been evaluated through Metadieta® software to assess the energy intake and nutrient composition. Moreover, to better estimate the actual food intake, at least 3 weighted food records

(WFRs) have been registered during the PR-diet and C-diet intervention periods.

In addition, for both the menus and WFRs, an estimation of total polyphenol intake has been performed through the use of Phenol-Explorer database (phenol-explorer.eu) or specific literature for those ingredients not available in the database. These evaluations allowed also to better monitor the compliance to the intervention.

Anthropometrical and vital sign evaluations

Before and after each time points of the intervention, weight, height and blood pressure have been registered according to international guidelines. Moreover, BMI has been calculated according to the formula – weight (kg)/height $(m)^2$.

Biological sampling and analysis

After an overnight fast, before and after each intervention period, blood samples have been drawn in vacutainer containing silicon gel for serum separation. Serum have been obtained through centrifugation (1400 g x 15min, 4°C), thus, splitted in few aliquots into specific vials and stored at -80°C until analysis. Several metabolic and functional parameters have been evaluated through validated methods. In particular, glucose, insulin, lipid profile, liver and renal function (i.e. Aspartate aminotransferase, alanine aminotransferase, creatinine) have been analyzed through a standardized routine-use automatic biochemical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). In addition, low density lipoprotein cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (non-high-density lipoprotein-cholesterol, HDL-C) levels have been estimated by using the Friedewald formula (ref) and by subtracting HDL-C from total cholesterol (TC). In addition, Homa-Index and Cockroft-Gault index were calculated according to the formula previously defined in literature (Drinka et al., 1989).

Evaluation of IP through zonulin serum level

To test the zonulin serum level, the Immunodiagnostik® ELISA kit (Bensheim, Germany) have been used. The assay is based on a competitive Elisa method consisting in the addition to each sample (including standard and control samples) of a biotinylated zonulin tracer (at first step) and the use of a pre-coated 96 well plate with polyclonal anti-zonulin antibody. The peroxidase-labelled streptavidin addition is used to bind the biotinylate zonulin tracer. After the reaction, the plate reader TECAN Infinite F200 (Tecan Group Ltd. Mannedorf, Switzerland) has been used to read the fluorescence (at 450 nm); concentration of serum zonulin level have been calculated by a 4PL standard curve as reported by the manufacturer.

Evaluation of vascular markers

Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), have been quantified through ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy). After competitive treatment with antibodies and fluorophore, fluorescence of plates has been read by absorbance through the TECAN plate reader previously mentioned. A 4PL logarithmic scale have been used to calculate serum concentration. A specific dilution factor has been multiplied if needed.

Statistics

Statistical analysis of data has been performed by means of *R statistic software version 3.4.2.* Data have been tested for normality through Shapiro-Wilks test. To identify significant differences between treatments an ANOVA for repeated measures design or Wilcoxon paired data test has been performed. The least significant difference (LSD) test with p 0.05 as level of statistical significance has been used as post hoc analysis to further evaluate differences among means. In addition, an elaboration by considering the distribution of zonulin serum level at baseline has been carried out. Specifically, this was obtained by stratifying subjects with zonulin serum levels

≤ median value (LSZ group) and > medial level (HSZ group). The regression and correlation analyses (Spearman and Kendal test) have been carried out to highlight associations between zonulin levels (high vs low) and physiological and biochemical parameters. Potential gender differences have been also considered in all the analysis.

Significance have been set at $p \leq 0.05$. P values in the range $0.05 < p < 0.10$ have been considered as trend.

Results

Subjects recruitment and selection workflow

A total of 491 older subjects were assessed for eligibility to participate to the trial. 349 subjects were excluded since they did not meet the inclusion criteria due to the severe clinical status (e.g. MMSE very low, drug treatment) evaluated by physicians at OIC or because not willing to participate due to personal reasons. A total of 72 subjects were further screened for serum zonulin levels and on this basis 3 subjects were excluded while difficulties in *drawing blood* was the exclusion criteria for other 3 subjects.

Sixty-six subjects (27 Male 39 Female) have been finally enrolled in the trial in the period between January-May 2017. The whole cross-over intervention study has been completed by November 2017. A schematic representation of the study diagram, reporting all information from the recruitment to the end of the study are reported in Fig. 2.

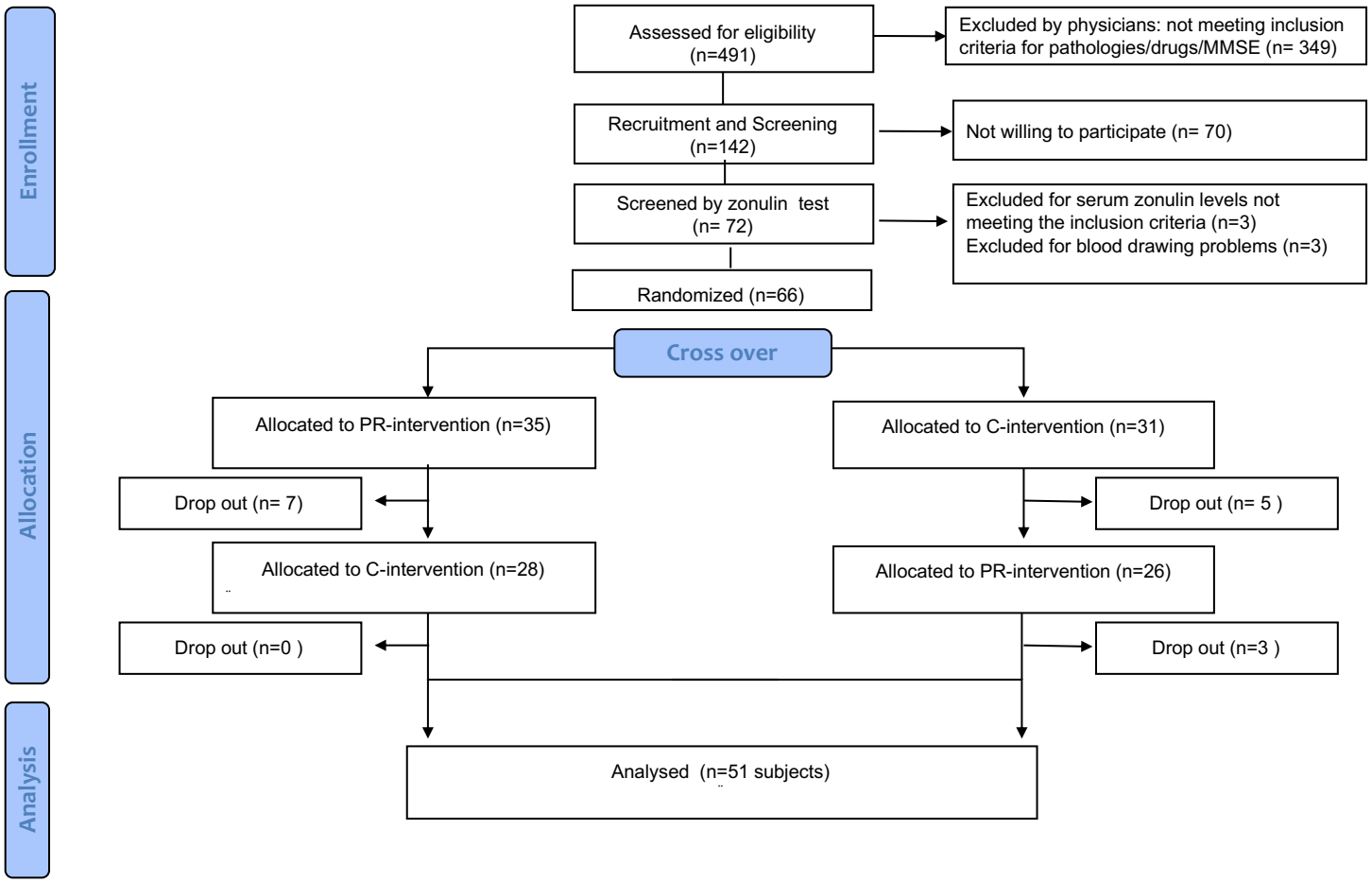


Fig. 2 - Consort diagram

Baseline characteristics of the study population

In Table 2 are reported data on anthropometrical, physical and biochemical characteristics at baseline of the subjects (n= 51) participating to the study. Age ranged from 60 to 98 years, with a mean age of 78.0 ± 10.3 years. Body mass index (BMI) was generally in the normal range except for a subject with a BMI higher than 40 kg/m^2. Conversely, a high inter-individual variability has been observed for glucose and total cholesterol levels [(IQR 86;113) – (IQR 167;242)].

Table 2 – Baseline characteristics of subjects

Variables	Median (IQR)	Mean (SD)
Age (y)	77 (70;87)	78.0 ± 10.3
Body weight (kg)	73.6 (62;82)	73.0 ± 13.8
BMI (kg/m^2)	25.7 (22.5;30.5)	26.7 ± 5.5
SBP (mm Hg)	125 (120;130)	125.6 ±10.8
DBP (mm Hg)	75 (70;80)	75.8 ± 10.0
Glucose (mg/dL)	95 (86;113)	113.5 ± 67.2
Creatinine (mg/dL)	0.87 (0.62;1.05)	0.9 ± 0.29
Uric Acid (mg/dl)	5.10 (4.20;6.60)	5.5 ± 1.76
TC (mg/dL)	194 (167;242)	196.3 ± 50.1
HDL-C (mg/dL)	45 (37;55)	46.5 ± 14.9
LDL-C (mg/dL)	120 (85;146)	120.5 ± 36.7
TC/HDL-C (ratio)	4.18 (3.54;5.43)	4.45 ± 1.17
LDL/HDL-C (ratio)	2.57 (2.08;3.45)	2.72 ± 0.76
TG (mg/dL)	117 (89;169)	146.1 ± 93.4
AST (U/L)	17 (13;22)	17.8 ± 5.7
ALT (U/L)	11 (8;19)	13.4 ± 7.2
GGT (U/L)	23 (17;46)	38.1 ± 39.0
Insuline uU/mL	6.20 (4.70;9.20)	8.4 ± 6.4
HOMA index	1.55 (1.15;2.50)	2.9 ± 5.4
Cockcroft-Gault index	69.4 (53.7;82.5)	74.6 ± 40.3
Zonulin (ng/mL)	40 (34.5;49.2)	42.2 ± 11.8
sVCAM-1 (ng/mL)	967.9 (628;1327.1)	1239 ± 1683
sICAM-1 (ng/mL)	51.4 (43.9;65.4)	55.6 ± 20.5

All data are presented as median and interquartile range (IQR) and as mean ± standard deviation (SD);

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA, homeostasis model assessment; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1.

As regard the dietary behavior of the 51 subjects who completed the experimentation in Table 3 are reported data on energy and nutrient intake at baseline. On the whole, data show an overall intake able to cover nutritional requirements for most nutrients for this target group of population. Notably, a modest energy intake was observed with introduction of less fiber and PUFA compared to the recommendations. These results can be partially due to the wide preference of volunteers for dishes of the traditional cuisine and a reduced selection of vegetables.

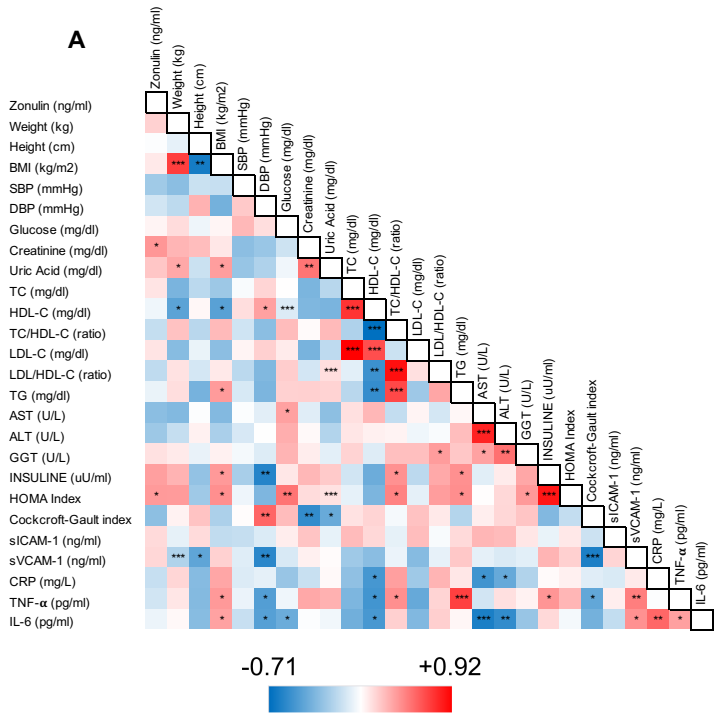
Table 3 – Dietary intake in older subjects at baseline

Variables	Median (IQR)	Mean (SD)
Energy (Kcal)	1587 (1526;1665)	1582 ± 107
Total carbohydrates (% of energy)	50.8 (48;51.9)	50 ± 2.7
Proteins (% of energy)	17.9 (17.3;18.6)	18 ± 0.8
Protein animals (% of energy)	*67.6 (66.1;69.7)*	*67.7 ± 3.9*
Protein vegetables (% of energy)	*32.2 (30.1;33.3)*	*31.9 ± 3.6*
Total lipids (% of energy)	31.5 (30.3;34.2)	32 ± 2.3
SFA (% of energy)	8.5 (7.7;9.0)	8.6 ± 1.5
MUFA (% of energy)	16 (15.4;16.9)	16.3 ± 20.2
PUFA (% of energy)	3.2 (3.0;3.3)	3.2 ± 6.1
ω-3 (% of energy)	0.6 (0.59;0.64)	0.6 ± 0.2
ω-6 (% of energy)	2.5 (2.3;2.6)	2.5 ± 0.4
Total Fiber (g/1000 kcal)	11.1 (10.5;12.1)	11.2 ± 1.2
Cholesterol (mg)	217.3 (187.3;228.2)	207.6 ± 30.3
Total carbohydrates (g)	209.7 (196.8;224.8)	210.7 ± 21.5
Glucose (g)	79.4 (66.8;90.7)	81.0 ± 15.2
Proteins (g)	71.2 (68.7;73.2)	70.3 ± 4.0
Animals proteins (g)	*48.1 (45.0;50.0)*	*47.6 ± 3.9*
Vegetable proteins (g)	*22.7 (22;23.4)*	*22.4 ± 2.7*
Total lipids (g)	55.6 (53.2;57.6)	56.2 ± 4.9
SFA (g)	15.0 (13.0;16.0)	15.2 ± 3.0
MUFA (g)	28.5 (27.1;29.2)	28.7 ± 2.4
PUFA (g)	5.6 (5.3;5.9)	5.7 ± 0.7
Total ω-3 (g)	1.1 (1.0;1.1)	1.1 ± 0.3
Total ω-6 (g)	4.3 (4.2;4.7)	4.5 ± 0.6
Fiber (g/day)	17.6 (16.4;19.7)	17.8 ± 2.4
Calcium (mg)	829.6 (705.4; 887.5)	803.5 ± 135.5
Iron (mg)	9.3 (8.8;10.0)	9.4 ± 0.9
Vitamin B_{12} (µg)	4.3 (3.7;4.3)	4.2 ± 1.0
Vitamin C (mg)	99.4 (72.4;143.6)	111.8 ± 56.1
Vitamin E (mg)	11.8 (11.2;12.2)	11.4 ± 2.9
Vitamin B_1 (mg)	0.7 (0.7;0.9)	0.8 ± 0.2
Folates (µg)	313.6 (280.4;346.0)	301.6 ± 73.4
Vitamin B_6 (mg)	1.5 (1.4;1.6)	1.5 ± 0.3

All data are presented as median and interquartile range (IQR) and as mean ± standard deviation (SD);
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Analysis of subjects characteristics based on Higher (HSZ) *or* Lower serum zonulin level (LSZ) at baseline

In Fig 3 are reported the results of the correlation between serum zonulin levels (HSZ or LSZ) and all the markers under study. In HSZ subjects, zonulin levels were positively correlated with HOMA index (p = 0.041) and creatinine (p = 0.026). However, the correlation with creatinine was not confirmed after adjustment for the Cockcroft-Gault formula. No significant correlation has been reported in the LSZ group of subjects.



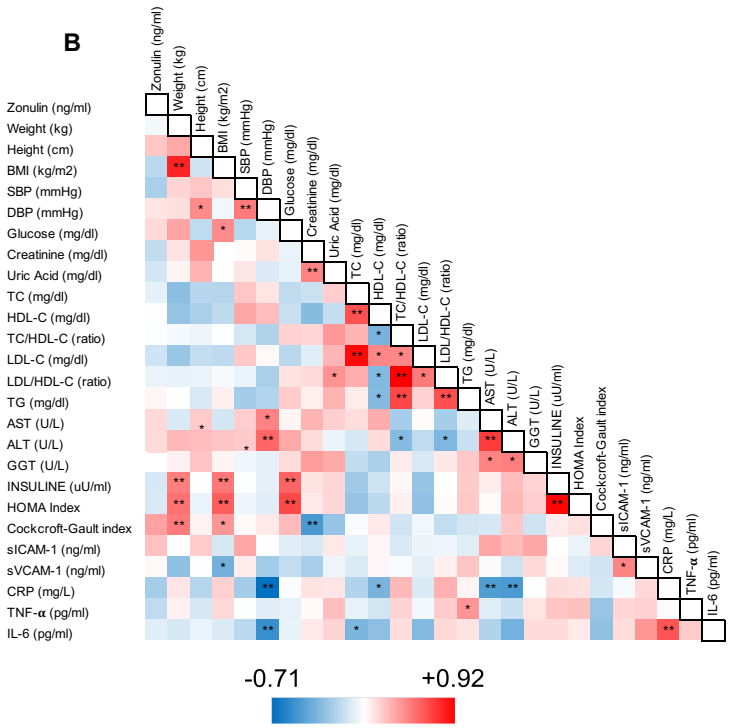


Fig. 3 – Correlations between the different markers at baseline in a) HSZ subjects (serum zonulin levels > median) and b) LSZ subjects (serum zonulin levels ≤ median)
The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Nutritional and polyphenol composition of the standard menus provided at OIC Foundation

MaPLE intervention was carried out over three seasons, in Table 4 are reported the nutritional and polyphenol composition of the standard menus provided by OIC staff during winter, spring and summer. The information obtained from the analysis of the menus were used to define the nutritional composition and to estimate the polyphenol intake of the participants. The three menus were comparable in terms of energy intake (mean value of 1976.4 ± 9.2 Kcal) and macronutrients distribution. In addition, the amount of polyphenols was comparable, apart from an apparent increase during the summer season.

Table 4 – Nutritional and polyphenol composition of the menus provided during the intervention study

Variables	Winter menù	Spring menù	Summer menù	Menùs mean
Energy (Kcal)	1888.6 ± 102	2012.4 ± 175.7	2028.3 ± 66.2	1976,4 ± 9,2
Total Carbohydrates (% of energy)	47.3 ± 3.2	46.4 ± 4.7	46.5 ± 3	46,7 ± 0,1
Protein (% of energy)	18.7 ± 2.5	20 ± 2.3	19.6 ± 2.8	19,4 ± 0,2
Protein animals (% of energy)	11.1 ± 2.8	13.4 ± 2.8	12.8 ± 2.5	12.4 ± 0.3
Protein vegetables (% of energy)	6.2 ± 0.9	6.2 ± 1.1	6.4 ± 0.8	6.,3 ± 0.1
Total Lipids (% of energy)	34.1 ± 4.2	33.7 ± 4.1	34 ± 4.6	33.9 ± 0.2
SFA (% of energy)	8.7 ± 1.3	8.9 ± 2	8.6 ± 1.7	8.7 ± 0.2
MUFA (% of energy)	17.9 ± 3.3	16.9 ± 2.4	17.7 ± 2.2	17.5 ± 0.5
PUFA (% of energy)	3.7 ± 0.8	3.8 ± 0.7	3.9 ± 1.4	3.8 ± 0.1
ω-3 (% of energy)	0.7 ± 0.4	0.7 ± 0.4	0.7 ± 0.4	0.7 ± 0
ω-6 (% of energy)	3 ± 0.9	3 ± 0.8	3.1 ± 1.3	3 ± 0.1
Total Fiber (g/1000 Kcal)	12.1 ± 2.1	11.6 ± 2.4	12.3 ± 1.7	12 ± 0.4
Cholesterol (mg)	264.3 ± 90.7	358.4 ± 133.9	287.6 ± 122.5	303.4 ± 40.9
Proteins (g)	88.1 ± 14.3	100.9 ± 19.6	98.8 ± 11.3	95,9 ± 1,2
Protein animals (g)	56 ± 13.8	68.3 ± 20.5	64.8 ± 10.9	63 ± 2
Protein vegetables (g)	31.3 ± 4.1	30.9 ± 4.2	32.6 ± 4	31.6 ± 1
Total lipids (g)	71.2 ± 7.8	75.3 ± 12.5	76.5 ± 12.3	74.3 ± 0.7
SFA (g)	18.3 ± 3.3	19.8 ± 4.6	19.4 ± 4.4	19.2 ± 0.2
MUFA (g)	37.3 ± 5.8	38 ± 7.1	40.1 ± 5.8	38.5 ± 1.2
PUFA (g)	7.7 ± 1.6	8.5 ± 2.1	8.7 ± 3,4	8.3 ± 0.1

Total ω-3 (g)	1.4 ± 0.8	1.5 ± 0.9	1.5 ± 0.9	1.5 ± 0
Total ω-6 (g)	6.2 ± 1.8	6.7 ± 2.1	7.1 ± 3	6.7 ± 0.2
Fiber (g/day)	22.9 ± 4.2	23.2 ± 4.4	24.8 ± 2.9	23.6 ± 0.9
Calcium (mg)	642.8 ± 253.9	665.8 ± 174.9	637.7 ± 112.4	648.8 ± 16.2
Iron (mg)	11.9 ± 2.1	14.2 ± 2.9	12.1 ± 1	12.7 ± 1.2
Vitamin B12 (mcg)	4.8 ± 2.2	5.3 ± 2.3	6.3 ± 5.1	5.5 ± 0.6
Vitamin C (mg)	224.8 ± 33.4	232.5 ± 28.2	242.4 ± 45.2	233.2 ± 5.7
Vitamin E (mg)	13.7 ± 1.9	15 ± 3.2	15.5 ± 2.4	14.7 ± 0.3
Vitamin B1 (mg)	1.4 ± 0.4	1.6 ± 0.4	1.5 ± 0.4	1.5 ± 0.1
Folates (mcg)	342.4 ± 78.1	376.8 ± 138.2	339.6 ± 69.9	352.9 ± 21.5
Vitamin B6 (mg)	2.3 ± 0.6	2.7 ± 0.7	2.5 ± 0.4	2,5 ± 0,1
Total polyphenols (mg)	727.1 ± 95.6	753.7 ± 47.2	826.3 ± 114.8	769.0 ± 51.3

Data are reported as mean ± SD
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Effect of intervention on food and polyphenol intake

In Table 5 are presented data on food and polyphenol intake obtained from weighted food diaries during PR and C-diet. Overall, the subjects well accepted and easily consumed all the PR-products provided daily and no adverse effects have been reported. Compliance to the dietary intervention was high thank also to the daily monitor of the subjects. The energy intake estimated from the WFRs during the PR and C-diet was about 20% less compared to the energy calculated from the menus (Table 4). During the trial, energy intake did not differ in the two periods of intervention (PR-diet vs C-diet) while slight differences from a nutritional point of view were found for carbohydrates, animal proteins, total lipids, MUFA, PUFA and ω-6 fatty acids, fiber, iron, calcium and vitamin C (Table 5). In particular, carbohydrates, fiber and vitamin C were higher in the PR-diet with respect to the C-diet, while animal proteins, total lipids, MUFA, PUFA, ω-6, iron and calcium were higher in the C-diet as calculated from available food databases used to calculate nutrient composition.

Regarding polyphenol intake, the amounts introduced during the C-diet were comparable with those estimated from the three menus (Table 4). The consumption of PR-products significantly increased the intake of polyphenols by about 70% compared to C-diet (Table 5).

Table 5 – Effect of intervention on food and polyphenol intake

Variables	PR- diet	C diet	P value
Energy (Kcal)	1537 ± 183	1559 ± 153	0.365
Total carbohydrates (% of energy)	47.2 ± 5.4	45.2 ± 5.2	**0.016**
Protein (% of energy)	17.7 ± 1.8	18.0 ± 1.9	0.185
Protein animals (% of energy)	66.5 ± 8.2	68.9 ± 7.3	**0.013**
Protein vegetables (% of energy)	27.3 ± 6.5	28.7 ± 6.8	0.100
Total lipids (% of energy)	34.9 ± 4.7	36.9 ± 4.7	**0.012**
SFA (% of energy)	11.3 ± 2.3	11.8 ± 2.5	0.179
MUFA (% of energy)	15.2 ± 2.8	16.4 ± 2.7	**0.012**
PUFA (% of energy)	3.2 ± 0.8	4.0 ± 1.5	**<0.001**
ω-3 (% of energy)	*0.6 ± 0.2*	*0.6 ± 0.2*	*0.291*
ω-6 (% of energy)	*2.6 ± 0.7*	*3.4 ± 1.3*	***<0.001***
Total Fiber (g//1000 kcal)	11.4 ± 1.8	10.5 ± 1.8	0.005
Cholesterol (mg)	216.3 ± 62.2	210.8 ± 67.0	0.587
Total carbohydrates (g)	188.6 ± 24.2	184.2 ± 27.0	0.286
Proteins (g)	66.7 ± 10.5	68.9 ± 8.7	0.063
Protein animals (g)	45.0 ± 9.8	48.0 ± 8.7	**0.003**
Protein vegetables (g)	17.7 ± 3.8	19.3 ± 3.7	**0.001**
Total lipids (g)	59.1 ± 13.3	63.1 ± 11.3	**0.040**
SFA (g)	19.2 ± 5.5	20.3 ± 5.3	0.209
MUFA (g)	26.0 ± 5.5	28.6 ± 6.0	**0.004**
PUFA (g)	5.6 ± 2.0	6.9 ± 2.6	**<0.001**
Total ω-3 (g)	1.0 ± 0.4	1.1 ± 0.4	0.315
Total ω-6 (g)	4.5 ± 1.7	5.7 ± 2.3	**<0.01**
Fiber (g/day)	17.4 ± 3.3	16.4 ± 3.2	**0.006**

Calcium (mg)	736.9 ± 207.7	875.0 ± 233.2	**<0.001**
Iron (mg)	8.5 ± 1.7	9.2 ± 1.6	**0.003**
Vitamin B12 (µg)	6.2 ± 6.5	5.4 ± 6.3	0.537
Vitamin C (mg)	128.8 ± 47.2	111.7 ± 40.1	**0.012**
Vitamin E (mg)	8.5 ± 2.2	8.9 ± 2.3	0.366
Vitamin B1 (mg)	0.9 ± 0.2	0.9 ± 0.2	0.123
Folates (µg)	233.3 ± 66.0	250.8 ± 72.7	0.126
Vitamin B6 (mg)	1.4 ± 0.3	1.5 ± 0.3	0.079
Total Polyphenols (mg/day)	1391.2 ± 188.1	812.3 ± 193.1	**<0.001**

All data are expressed as mean ± standard deviation (SD);
PR, polyphenol-rich diet; C, control diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Effect of the intervention on anthropometrical, physical, biochemical and functional parameters

In Table 6 are reported the results on anthropometrical, physical, biochemical and functional parameters evaluated before and after each treatment.
ANOVA revealed a treatment *x* time interaction for diastolic blood pressure (p = 0.028) and uric acid (p=0.034). Post hoc analysis (LSD test) documented a significant decrease (p=0.034) following control diet for uric acid and a trend of reduction for diastolic blood pressure following PR-diet intervention.
Regarding lipid profile, a time effect (p=0.039) was observed for total cholesterol showing a tendency towards a reduction in both interventions. For the other lipid parameters, including the different calculated ratios, no significant effect was observed. Finally, no differences were observed for anthropometrical parameters, markers of vascular function, glucose metabolism as well as markers related to liver and renal function.
In Table 7 and 8 the results obtained on the markers under study are reported by considering gender. In the group of males (Table 7), ANOVA revealed a significant effect of time for total cholesterol (p=0.003), LDL-C (p=0.020) and the ratio total cholesterol/HDL-C (p=0.031) documenting a significant reduction (LSD test) following PR-diet but not control diet. Regarding females (Table 8), a treatment x time interaction has been observed for systolic blood pressure (p= 0.042); in particular, post-hoc analysis documented a significant reduction (p=0.01) after PR-diet, but not C-diet. No effect was reported for the other variables under study.

Table 6- Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical and functional characteristics in the whole group of subjects

Variables (n = 51)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
Body weight (kg)	73.4 ± 14.4	73.7 ± 14.6	72.8 ± 13.7	72.8 ± 13.7	0.043	0.824	0.320
BMI (kg/m^2)	26.6 ± 5.6	26.9 ± 5.7	26.7 ± 5.4	26.6 ± 5.6	0.090	0.777	0.733
SBP (mmHg)	127.2 ± 12.7	124.5 ± 14.6	126.5 ± 9.8	126.2 ± 10.4	0.749	0.107	0.234
DBP (mmHg)	76.0 ± 10.3	73.8 ± 9.4	75.5 ± 6.8	76.9 ± 7.5	0.198	0.628	**0.028**
Glucose (mg/dL)	114.4 ± 68.2	107.4 ± 42.8	108.6 ± 42.3	105.7 ± 38.2	0.163	0.096	0.360
Creatinine (mg/dL)	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.386	0.220	0.422
Uric Acid (mg/dL)	5.5 ± 1.8	5.67 ± 1.7	5.8 ± 1.9	5.5 ± 1.7	0.793	0.361	**0.034**
TC (mg/dL)	194.2 ± 51.0	189.5 ± 49.7	191.6 ± 49.1	188.1 ± 50.9	0.411	**0.039**	0.700
HDL (mg/dL)	47.1 ± 14.6	46.6 ± 14.0	46.9 ± 14.9	46.9 ± 15.6	0.876	0.607	0.695
LDL (mg/dL)	119.3 ± 36.6	115.4 ± 33.8	116.4 ± 35.3	114.1 ± 36.9	0.321	0.054	0.646
TC/HDL (ratio)	4.4 ± 1.3	4.2 ± 1.1	4.3 ± 1.1	4.2 ± 1.1	0.610	0.107	0.511
LDL/HDL-C (ratio)	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.8	0.426	0.238	0.775
TG (mg/dL)	140.2 ± 86.9	136.9 ± 76.3	141.6 ±91.7	135.6 ± 92.9	0.992	0.285	0.781
AST (U/L)	17.7 ± 5.4	17.4 ± 5.2	17.7 ± 5.3	17.9 ± 5.3	0.632	0.840	0.509
ALT (U/L)	13.7 ± 7.2	13.2 ± 6.6	13.5 ± 6.8	13.9 ± 6.5	0.656	0.831	0.382
GGT (U/L)	38.7 ± 31.9	37.1 ± 30.6	38.8 ± 39.6	36.8 ± 29.0	0.954	0.354	0.903
Insuline (uU/mL)	8.3 ± 6.6	7.2 ± 3.6	8.4 ± 6.7	7.3 ± 4.4	0.467	0.068	0.639
HOMA index	2.9 ± 5.5	2.0 ± 1.9	2.7 ± 4.6	2.1 ± 2.2	0.153	0.145	0.810
CG index	72.7 ± 36.0	74.8 ± 40.5	74.2 ± 40.8	74.6 ± 38.7	0.494	0.189	0.449
sVCAM-1 (ng/mL)	980.4 ± 527.8	1037.4 ± 683.9	1319.9 ± 1713.2	1094.4 ± 703.0	0.095	0.462	0.197
sICAM-1 (ng/mL)	54.9 ± 20.5	59.9 ± 28.8	57.9 ± 23.8	55.7 ± 22.8	0.665	0.352	0.600

All data are expressed as mean ± standard deviation (SD)

PR, polyphenol-rich diet; C, control diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; GOT/AST, aspartate aminotransferase; GPT/ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; CG, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1
T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction.

Table 7 - Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical biochemical and functional markers in male subjects

Males (n = 22)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
Body weight (kg)	73.4 ± 14.4	73.7 ± 16.6	72.8 ± 13.7	72.6 ± 13.9	0.251	0.446	0.137
BMI (kg/m^2)	26.9 ± 5.7	26.9 ± 5.7	26.7 ± 5.4	26.6 ± 5.6	0.519	0.854	0.699
SBP (mmHg)	127.2 ±12.7	124.5 ± 14.6	126.5 ± 9.8	126.2 ± 10.4	0.536	0.432	0.600
DBP (mmHg)	76.0 ± 10.3	73.8 ± 9.4	75.5 ± 6.8	76.9 ± 7.5	0.558	0.683	0.073
Glucose (mg/dL)	110.8 ± 48.2	105 ± 31.6	102.5 ± 25.3	102.8 ± 20.4	0.295	0.358	0.317
Creatinine (mg/dL)	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	0.733	0.308	0.460
Uric Acid (mg/dL)	5.7 ± 1.4	5.9 ± 1.4	6.0 ± 1.5	6.0 ± 1.5	0.203	0.521	0.259
TC (mg/dL)	187.4 ± 45.9	173.4 ± 39.4	184.3 ± 46.2	179.7 ± 40.9	0.677	**0.003**	0.106
HDL-C (mg/dL)	44.4 ± 10.6	43.5 ± 10	44.5 ± 11.5	44.5 ± 11.5	0.597	0.616	0.522
LDL-C (mg/dL)	116.6 ± 33.1	107.7 ± 28.5	112.3 ± 34	109.8 ± 30.6	0.714	**0.020**	0.954
TC/HDL-C (ratio)	4.33 ± 1.07	4.07 ± 0.80	4.26 ± 0.95	4.19 ± 1.05	0.829	**0.039**	0.284
LDL/HDL-C (ratio)	2.69 ± 0.73	2.53 ± 0.63	2.57 ± 0.63	2.55 ± 0.69	0.549	0.110	0.129
TG (mg/dL)	128.2 ± 67.7	115.9 ± 55.4	140.2 ± 85.5	130.2 ± 96	0.282	0.082	0.893

AST (U/L)	18.2 ± 5	17.5 ± 4.3	18.8 ± 5	19.6 ± 5.9	**0.042**	0.933	0.220
ALT (U/L)	14.3 ± 5.6	13.8 ± 5.9	15.2 ± 7.3	16.1 ± 7.4	0.074	0.729	0.387
GGT (U/L)	46.7 ± 30	42 ± 30.8	41.7 ± 30.4	43.8 ± 31.1	0.590	0.610	0.131
Insuline (uU/mL)	7.5 ± 3.3	6.9 ± 2.5	7.2 ± 4	7.2 ± 4	0.872	0.509	0.498
HOMA Index	2.1 ± 1.1	1.8 ± 1.2	1.9 ± 1.2	1.8 ± 1.0	0.453	0.274	0.588
CG index	77.2 ± 39.8	82.4 ± 48.4	80.1 ± 49.4	79.1 ± 45.7	0.880	0.153	0.091
sVCAM-1 (ng/mL)	921.6 ± 569.6	957.8 ± 824.6	939.0 ± 653.2	888.8 ± 547.8	0.737	0.920	0.724
sICAM-1 (ng/mL)	56.6 ± 22.9	60.0 ± 33.7	60.4 ± 27.7	57.9 ± 26.2	0.695	0.886	0.305

All data are expressed as mean ± standard deviation (SD)
PR, polyphenol-rich diet; C, control diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; GOT/AST, aspartate aminotransferase; GPT/ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; CG, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1;
T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction

Table 8 - Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical and functional markers in male subjects in female subjects

Females (n = 29)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
Body weight (kg)	73.4 ± 14.4	73.7 ± 14.6	72.8 ± 13.7	72.6 ± 13.9	0.086	0.563	0.864
BMI (kg/m^2)	26.9 ± 5.7	26.9 ± 5.7	26.7 ± 5.4	26.6 ± 5.6	0.079	0.827	0.959
SBP (mmHg)	127.2 ± 12.7	124.5 ± 14.6	126.5 ± 9.8	126.2 ± 10.4	0.089	0.165	**0.042**
DBP (mmHg)	76.0 ± 10.3	73.8 ± 9.4	75.5 ± 6.8	76.9 ± 7.5	0.236	0.774	0.183
Glucose (mg/dL)	117.1 ± 80.9	109.3 ± 50.2	113.2 ± 60.2	107.9 ± 47.8	0.375	0.167	0.698
Creatinine (mg/dL)	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.4	0.8 ± 0.3	0.211	0.481	0.134
Uric Acid (mg/dL)	5.5 ± 2.0	5.5 ± 1.9	5.6 ± 2.2	5.2 ± 1.8	0.390	0.184	0.079
TC (mg/dL)	200.7 ± 54.7	201.7 ± 53.8	197.1 ± 51.5	194.5 ± 57.3	0.183	0.787	0.645
HDL-C (mg/dL)	49.2 ± 16.9	48.9 ± 16.2	48.9 ± 17	48.8 ± 18.1	0.764	0.813	0.945
LDL-C (mg/dL)	121.3 ± 39.5	121.3 ± 36.8	119.6 ± 36.5	117.4 ± 41.2	0.341	0.608	0.691
TC/HDL (ratio)	4.4 ± 1.2	4.4 ± 1.2	4.3 ± 1.2	4.3 ± 1.2	0.261	0.957	0.712
HDL/LDL-C (ratio)	2.6 ± 0.7	2.6 ± 0.8	2.6 ± 0.8	2.6 ± 0.8	0.614	0.999	0.234
TG (mg/dL)	149.3 ± 99.3	152.8 ± 86.5	142.8 ± 97.6	139.7 ± 92.0	**0.030**	0.973	0.593
AST (U/L)	17.4 ± 5.7	17.4 ± 5.9	16.8 ± 5.5	16.5 ± 4.4	0.180	0.731	0.732
ALT (U/L)	13.3 ± 8.2	12.7 ± 7.2	12.3 ± 6.1	12.2 ± 5.2	0.304	0.596	0.709
GGT (U/L)	32.7 ± 32.5	33.4 ± 30.6	36.7 ± 45.8	31.4 ± 26.7	0.621	0.449	0.257
Insuline (uU/mL)	8.8 ± 8.2	7.4 ± 4.3	9.3 ± 8.1	7.4 ± 4.8	0.711	0.092	0.785
HOMA Index	3.5 ± 7.2	2.2 ± 2.3	3.3 ± 6	2.3 ± 2.7	0.790	0.181	0.593

CG index	69.3 ± 33.2	69 ± 32.9	69.8 ± 33.1	71.1 ± 32.9	0.287	0.663	0.550
sVCAM-1 (ng/mL)	1025.1 ± 499.2	1097.8 ± 562.6	1609.0 ± 2172.6	1250.3 ± 773.9	0.066	0.467	0.208
sICAM-1 (ng/mL)	56.6 ± 18.7	59.9 ± 25.0	55.9 ± 20.7	54.1 ± 20.1	0.336	0.200	0.121

All data are expressed as mean ± standard deviation (SD)
PR, polyphenol-rich diet; C, control diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; GOT/AST, aspartate aminotransferase; GPT/ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; CG, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1;
T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction

Effect of intervention on serum zonulin levels

In Table 9 are reported the results on serum zonulin levels before and after each treatment period for the whole group of subjects and divided for gender. On the whole, ANOVA revealed an effect of the treatment ($p=0.008$) and a treatment x time interaction ($p=0.025$). In particular, post-hoc analysis reported a significant decrease in serum zonulin levels after PR-diet but not C-diet.

By considering possible differences of impact depending on gender, no effect was showed for males, while an effect of the treatment ($p=0.004$) and treatment x time interaction ($p=0.010$) was reported for females ($p=0.033$, LSD test) with increased levels of zonulin after C-diet compared to PR-diet.

Table 9- Effect of 8-week intervention with PR-diet and C-diet on serum zonulin levels in the all group of subjects and in each sex group

Zonulin (ng/mL)	Before PR-diet	After PR-diet	Before C-diet	After C-diet	P for T	P for t	P for T *x* t
All subjects (n= 51)	41.9 ± 10.4	39.0 ± 8.9*	42.8 ± 10.9	44.3 ± 5.1	**0.008**	0.462	**0.025**
Male (n = 22)	43.1 ± 12.1	39.7 ± 8.1	43.5 ± 12.1	42.4 ± 15.2	0.409	0.141	0.497
Female (n= 29)	41.0 ± 9.0	38.5 ± 9.5	42.3 ± 10.1	45.8 ± 10.0*	**0.004**	0.694	**0.010**

All data are expressed as mean ± standard deviation (SD);
PR, polyphenol-rich diet; C, control diet; T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction
* Significantly different from each other time points ($p < 0.05$)

Discussion:

In this study, we have tested the hypothesis that a polyphenol-rich dietary patterns can positively affect intestinal permeability and modulate markers related to health status in the older subjects. To this aim we defined polyphenol diet by considering the basal total polyphenol intake in the target population and trying to double the intake in order to reach amounts in the highest quantile of intake identified in previous observational studies where older subjects were included or specifically considered (Rabassa, et al., 2015;Tresserra-Rimbau, et al 2016; Del Bo' et al, 2019).

Data on food intake are generally obtained through Food Frequency Questionnaires (Naska, et al. 2017); this could be recognized as a bias for estimation of bioactive compounds due to the scarce precision on ingredients/recipes used and amount of food consumed. In the present study, we moved over this kind of bias by using daily weighted food records that were also compared to the menu provided within the residential care setting. This approach allowed to underline an about 20% lower energy intake with respect to the standard menu but an almost comparable intake of total polyphenols in C-diet with respect to the standard menu (about 800 mg/day). These latter results seem in line with what we have previously documented in a recent review by Del Bo' et., al (2019).

The most important observation related to the present study concerns the potential effect of the PR dietary intervention on zonulin serum level considered as primary endpoint and marker of IP. Zonulin, also known as prehaptoglobin-2, is a 47-kDa protein produced mainly by epithelial cells (e.g. in the gut) which is able to reversibly modulate paracellular permeability (Fasano et al., 2012). A few studies have reported associations between the results obtained through the most common and validated IP test (based on sugar urine excretion evaluation following standardised multi-sugar intake) and serum zonulin levels (e.g. Weght et al., 2019).

For this reason, in the present work we tried also to increase evidence on serum zonulin level as IP marker. Increased level of serum zonulin has been reported by Ganda Mall et al, (2018) in older subjects with altered IP condition, moreover, Sapone et al, (2006) reported association between increased serum zonulin level and altered IP condition in type 1 diabetic subjects.
Interestingly, we documented a significant associations of zonulin with HOMA index in subjects classified in the HSZ group (defined on the basis of the median serum zonulin level at baseline) but not in the LSZ suggesting an important contribution of zonulin in discriminating subject's characteristics as evidenced in other studies obtained in subjects with metabolic dysregulation.

Results obtained through the present randomised well controlled cross-over intervention study demonstrates a significant decrease of serum zonulin levels ($p = 0.025$) by considering the overall population under study. When divided by gender, statistical significance persisted in female ($p = 0.010$) but not in male subject even if the same trend of reduction has been reported. It is noteworthy that in this case the reduced sample size could, at least in part, justify the lack of significance, thus only a further evaluation with an increased number of male and female volunteers could help demonstrate an actual different response.
Nevertheless, the reduced zonulin levels following the PR-diet registered in our study could be considered an important result demonstrating also the reliability of non-invasive dietary interventions as potential strategies to improve IP in the older population likely contributing to reduce the associated pathological and/or dysmetabolic conditions.
The effect of polyphenols and polyphenol-rich dietary patterns has been evaluated in several observational and interventional studies documenting a potential beneficial effect on age-related disease (Roman et al., 2019). For example, Grosso et al., (2016) supported the hypothesis that polyphenols may exert beneficial effects on certain age-related clinical conditions such as metabolic syndrome showing a significant inverse association with polyphenol

intake. Poti et al.,(2017) reported in a review the results about the potential beneficial effect of long term consumption of polyphenols on neurodegenerative diseases (i.e Alzheimer's disease, Parkinson, etc).

The aging process is also characterized by a physiological alteration of blood vessels and consequently of the endothelial function and blood pressure. In particular, systolic hypertension is very common in older subjects aged ≥ 65 years representing a major risk factor for CVD and strokes (Chrysant et al., 2018). Several studies have reported a potential protective effect of polyphenols and polyphenol-rich foods against hypertension. In our experimental conditions, the intervention with a polyphenol-rich dietary pattern positively affected blood pressure. In particular, a trend towards a reduction for diastolic blood pressure have been observed in the whole group of subjects while a significant reduction in systolic blood pressure has been observed in the group of females following PR-diet but not C-diet. These data could be considered in line with what reported by the TOSCA study (Vitale et al., 2006) where they found significant lower mean of both diastolic and systolic blood pressure in the highest tertile of total polyphenol intake with respect to the lower tertile. The same result on systolic and diastolic blood pressure has been documented in the Spanish cohort of the PREDIMED study (Medina-Remon et al., 2015).

Regarding the effect of age on endothelial functionality, it is widely recognized that vascular oxidative stress increases with age as a consequence of the production of reactive oxygen species without an adequate compensatory increase in antioxidant defences (Herrera et al., 2010). Several studies reported that the production of adhesion molecules such as VCAM-1 are an age-dependent parameter and different observations found that older subjects showed elevated levels of ICAM-1 and VCAM-1 with respect to young individuals. Polyphenols have been reported to play an important role in the modulation of endothelial function; in particular, they have been demonstrated to counteract reactive oxygen species, induce the production of numerous vasodilators and reduce the release of several adhesion molecules such as

VCAM-1 and ICAM-1 (Barona, et al. 2012). In the present study, we documented that the intake of polyphenol-rich foods failed to modulate the production of adhesion molecules. These results are in contrast with those reported by other authors in a different target group of population. For example, Lehtonen and colleagues (2011) have shown that 5 weeks of intervention with different berries (sea buckthorn and bilberry) reduced VCAM-1 and ICAM-1 serum concentrations in overweight and obese subjects. Barona et al., (2012) documented that 30-day of grape powder consumption reduced serum levels of VCAM-1 and ICAM-1 in men with metabolic syndrome. Aging is also found to be related to deregulation in lipids and glucose metabolism (Chia et al., 2018) Dietary polyphenols have shown to play an important role in the control of glucose homeostasis, insulin sensitivity and lipid metabolism. In the present study, we found that the intervention with polyphenol-rich foods failed to affect glucose and lipid parameters, apart from a reduction trend in total and LDL-cholesterol. These results are in line with those found by other authors following the intervention with different polyphenols and polyphenol-rich foods such as: tea extracts (Araya-Quintanilla et al., 2919;Zhao et al., 2015; Li et al., 2019) ,orange juice/hesperidin (Mohammadi et al., 2019), pomegranate (Huang et al., 2017; Sahebkar et al., 2016) and different fruit juices (Murphy et al., 2017) but in contrast with those that found an effect on glucose and/or lipid metabolism following the consumption of cocoa products, dark chocolate, flavan-3-ols (Hooper et al., 2012; Jia et al., 2010; Tokede et al., 2011), and berries (Huang et al., 2016.

In conclusion, findings from the present study will contribute to the understanding of the role of diet in the modulation of IP and its evaluation in the older subjects. In addition, the results obtained, together with those that will be gathered through the metabolomics and microbiomics approaches foreseen in the MaPLE project, will be fundamental to better characterize individual response and, likely, pose the basis for the future development of

improved dietary guidelines to promote a healthy phenotype in the older population during the complex process of ageing.

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4. GENERAL CONCLUSION

In conclusion, through this PhD thesis it has been possible to provide further knowledge and understanding on the potential benefits of polyphenols-rich dietary patterns with specific regard to the modulation of intestinal permeability and, likely, age-related conditions. An updated overview has been also provided on the availability and reliability of methods to evaluate food and polyphenol intake and to study intestinal permeability.

In the first chapter of the thesis, a systematic review on polyphenol intake tools and methods and, where possible, the main results found on the association among polyphenol intake and specific endpoints of disease risk, have been been performed.

Results showed that, although a large amount of data support the beneficial effect of polyphenols on numerous health outcomes, it is still difficult to define a reference or prudent level of intake for this class of compounds. However, the revision performed on a large number of datasets considering polyphenol intake has documented a mean intake of about 900 mg/day in the overall population. Nevertheless, high heterogeneity of data from the literature have been found due to different database sources and in certain case, lack of data on total polyphenols or subclasses. In this regard, many studies reported an association only for specific class of compounds (e.g. flavonoids). Geographic locations, socio economic status and also gender differences make it difficult to generalize the observed results. Further investigation and effort are required to reduce the uncertainty of several measurements and to promote databases implementations in order to fulfill the gap still present in this research area.

In the second chapters of the present thesis, the interaction between polyphenols and intestinal permeability have been deeply investigated through a wide revision of the *in vitro, in vivo (animal models)* and human studies available in literature. Particular attention has been focused on novel

biomarkers for the evaluation of IP such as zonulin serum levels and multi-omics approaches (e.g. metabolomics).

Data provided by the recent literature documented that specific PPs could modulate IP through both direct and indirect pathways including their impact on intestinal ecosystem and immune system. Well controlled dietary intervention studies are required to increase knowledge about the complex diet-microbiota-intestinal permeability axis.

In the third chapter a randomized controlled dietary intervention study has been developed, with the important aim to test the hypothesis that a polyphenol-rich dietary pattern can exert beneficial effects on intestinal permeability and age-related conditions in older subjects.

Results obtained seem to support zonulin as a possible biomarker of IP in this target groups. The polyphenol rich dietary pattern developed showed to affect significantly IP since a reduction of zonulin have been observed. In addition, an improvement of other general health related markers such as blood pressure have been shown. A high compliance to the dietary instruction and the intake of the polyphenol-rich products have been confirmed, according to the study results. This enforces the plausibility and reliability of this kind of intervention strategies in the older subjects.

5. IMPLICATIONS AND FUTURE DIRECTIONS

There is demand for novel and non-pharmacological strategies able to improve well-being during the ageing process and the manipulation of the diet has been recognized as a potential powerful tool. This concept can be applied also to the treatment of IP critically involved in many different physiological and metabolic responses.

For instance, dietary patterns with increased or reduced intake of specific nutrients and/or food bioactives such as PPs have been recognized as promising strategies based mainly on results from observational or mechanistic studies. However, as previously documented, this type of research is still in its infancy by considering the scarce human studies which are available, thus, it is of utmost importance to increase the number of well controlled dietary intervention studies on the same or different group of populations where IP could be a fundamental target.

In this scenario, the MaPLE trial has increased evidence on the impact of a polyphenol-rich dietary pattern in the modulation of IP. In addition, it can be considered at present one of the few human intervention studies in which serum zonulin concentrations have been analysed in the older subjects as marker of IP since we found a low reliability and applicability of the defined gold standard method (i.e. multi-sugar test) in this target population. Finally, it has been explored the association of zonulin serum levels with the bacterial DNA in the bloodstream demonstrating a positive correlation (Gargari, et al.,2019). These data are not present in literature and may be also pivotal for the future definition of a panel of biomarkers for the evaluation of IP that, once validated for clinical relevance, could be useful for clinicians but also for nutrition experts interested in the development of preventive/protective dietary approaches.

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Gargari G, Taverniti V, Del Bo' C, Bernardi S, Andrès-Lacueva C,Kroon P.A., et al. (submitted 2019).Blood bacterial DNAemia is associated with serum zonulin levels in older subjects. Microbiome.

6. APPENDICES

Published papers:

- **Bernardi S,** Del Bo' C, Marino M, Gargari G, Cherubini A, Andres-Lacueva C, et al. Polyphenols and intestinal permeability: rationale and future perspectives. J Agric Food Chem 2019:https://doi.org/10.1021/acs.jafc.9b02283.

- Del Bo' C, **Bernardi S**, Marino M, Porrini M, Tucci M, Guglielmetti S, et al. Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern? Nutrients 2019;11:1355.

- Peron G, Hidalgo-Liberona N, González-Domínguez R, Garcia-Aloy M, Guglielmetti S, **Bernardi S**, et al. Exploring the Molecular Pathways behind the Effects of Nutrients and Dietary Polyphenols on Gut Microbiota and Intestinal Permeability: A Perspective on the Potential of Metabolomics and Future Clinical Applications. J Agric Food Chem 2019:doi: 10.1021/acs.jafc.9b01687. doi:10.1021/acs.jafc.9b01687.

Submitted papers:

- Guglielmetti S, **Bernardi S**, Del Bo' C, Cherubini A, Porrini M, Gargari G, et al. Effect of a polyphenol-rich dietary pattern on intestinal permeability and gut and blood microbiomes in older subjects: Study protocol of the MaPLE randomised controlled trial. *BMC geriatrics (submitted 2019)*

- Gargari G, Taverniti V, Del Bo' C, **Bernardi S**, Andrès-Lacueva C,Kroon P.A., et al. Blood bacterial DNAemia is associated with serum zonulin levels in older subjects. *Microbiome*
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Poster presentations:

- **S Bernardi,** O Parodi, G Mellone, F Giugni, F Vozzi, P Riso, *Can mobile application manage dietary behaviour in the older population? The DoReMi project approach.* XXXVII SINU 2016 Congress, Bologna, Italy

- **S. Bernardi***, Evidence for the protective effect of a polyphenol rich diet in the elderly with intestinal permeability: rationale and perspectives.* 22ndWorkshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Free University of Bozen, Bozen, September 20th-22nd,2017

- **S. Bernardi**, S. Guglielmetti, C. Del Bo', M. Marino; G. Gargari, A. Cherubini, C. Andres-Lacueva, N. Hidalgo, P. Casas- Agustench, M. Winterbone, A. Narbad, P. Kroon, P. Riso, *A randomised controlled trial to assess the impact of a polyphenol-enriched diet on intestinal permeability in the elderly: The MaPLE study,* ICPH 2017, Quebec city

- **S. Bernardi**, S. Guglielmetti, C. Del Bo'; G. Gargari, A. Cherubini, C. Andres-Lacueva, P. Kroon, P. Riso,*Valutazione della permeabilità intestinale in un gruppo di soggetti anziani nell'ambito del progetto MaPLE*, SINU 2017, Torino, Italy

- C Del Bo', **S Bernardi**, S Guglielmetti, G Gargari, A Cherubini, C Andres-Lacueva, P Kroon, P Riso, *Evaluation of intestinal permeability in a group of older subjects participating to the MaPLE randomised controlled trial,* Foodomics 2018, Cesena, Italy

- **S Bernardi**, N Hidalgo Liberona , C Del Bo', S Guglielmetti , G Gargari , A Cherubini , P Kroon , C Andres-Lacueva , P Riso, *Estimation of the intake of bioactive compounds in older subjects in a nursing home setting - the MaPLE project*, FBHC2018, Lisbona, Portugal

- ***S.*** **Bernardi**, S. Guglielmetti, G. Gargari, A. Cherubini, C. Andres-Lacueva, N.H. Liberona, P. Casas-Agustench, P. Kroon, P. Riso, *Valutazione dell'assunzione di polifenoli nei soggetti anziani del progetto MaPLE,* SINU2018, Napoli, Italia

- P Riso, S Guglielmetti, **S Bernardi**, G Gargari, A Cherubini, P Kroon, C Andres-Lacueva*, Rationale of MaPLE project focused on intestinal permeability in the older subjects, Poland 2018*

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- A Cherubini, **S Bernardi**, C Del Bo', S Guglielmetti, G Gargari, P Kroon, B.Kirkup, B Carrieri, N Hidalgo Liberona, G Peron, R Gonzalez Dominguez, C Andres-Lacueva, P Riso, *Role of a polyphenol-rich dietary pattern in the modulation of-Intestinal Permeability and related markers in the older subjects*. EuGMS2019, Krakov, Poland

- **S Bernardi**, C Del Bo', S Guglielmetti, A Cherubini, P Kroon, B Kirkup, N Hidalgo Liberona, G Peron, R Gonzalez Dominguez, C Andres-Lacueva, P Riso, *Intestinal permeability modulation through a polyphenol-rich dietary pattern in the older subjects. MaPLE project outcomes and perspectives,* FENS 2019, Dublin, Ireland
  (e-poster presentation)

Accepted abstracts

- P Riso, **S Bernardi**, C Del Bo', P Kroon, B Kirkup, A Cherubini, N Hidalgo Liberona, G Peron, R González-Dominguez, C Andrés-Lacueva, S Guglielmetti, *MaPLE study: reliability and significance of a polyphenol-rich dietary pattern for the improvement of intestinal permeability in the older subjects,* ICPH 2019, Kobe, Japan
  (poster presentation)

Participation to summer schools:

- INJOY EIT Health Summer School - University of Barcelona – 26th June – 4th July 2018 on Nutrition, Healthy Ageing, Business and Innovation.

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Let me say, it’s not the end of a story, it’s a new begin!