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Definition and critical evaluation of nutritional strategies to achieve the development of optimized healthy and sustainable dietary patterns: from theory to practice

Massimiliano Tucci

R12505

Supervisor: Prof. Patrizia Riso

Ph.D. Dean: Prof. Diego Mora

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Abstract

The concept of sustainable food systems is not new, but nowadays the definition of dietary patterns or recommendations able to improve both health and contribute to the transformation of food systems is strongly advocated. In the current scenario, the recognized intersection of climate change, population growth, and the increasing incidence of chronic diseases represents a global challenge involving also diets and food production. In the last years, scientific evidence accumulated on the definition of optimized diet that can be considered both healthy and sustainable. In 2019 the EAT–Lancet Commission on healthy diets from sustainable food systems addressed these issues and defined plausible range of intake for eight different food categories, as a useful starting point for the definition of locally adapted healthy and sustainable dietary patterns. For this reason, this authoritative proposal has also been defined as the “Planetary health diet”. However, given the complexity of the issues related to diet sustainability, this research field must be considered in its infancy, and several aspects still need to be elucidated. In this context, the present PhD thesis tried to contribute to such research topic thoroughly discussing the available results and providing new data.

Specifically numerous tasks were addressed and described in four chapters.

The first chapter included three sub-tasks devoted to: i) provide an overview of sustainability principles available in the Food-Based Dietary Guidelines (FBDGs); ii) define a methodology to adapt the Planetary health diet to the Italian/Mediterranean context (EAT-IT), evaluating its nutritional adequacy and main associated issues; iii) identify further strategies of diet adaptation for different energy targets addressing also specific nutritional needs.

In the second chapter, the main goal was to evaluate the environmental impact associated with the developed EAT-IT dietary pattern to demonstrate its sustainability. By considering the need of human intervention studies to provide evidence on the role of sustainable diet in the real scenario, in the third chapter an explorative pilot study has been implemented to evaluate the feasibility of the EAT-IT dietary pattern and its effect on nutritional and health related outcomes.

Finally, since the optimization of healthy and sustainable diets in terms of nutrient composition can be extended also to the diverse bioactive compounds abundantly present in plant-based diets, the fourth chapter was focused on the opportunity to include specific bioactive-rich foods, through an evaluation of their potential health benefits, when consumed by a vulnerable group of population. Specifically, in this chapter, a systematic review of *in vitro* and *in vivo* study was carried out to assess and provide knowledge on the effect of plant-based foods (e.g. anthocyanin-rich blueberry) on vascular function in older subjects, and results obtained through an acute *in vivo* study with blueberry were reported and discussed.

The main results from the first task indicated poor alignment of current FBDGs with sustainability principles. In addition, the Planetary health diet, when adapted to the Italian/Mediterranean food context, resulted as an overall balanced plant-based diet, characterized by low amount of meat and dairy and high amount of nuts and legumes. From a nutritional perspective, some critical nutrients, were identified (i.e. vitamin D, iron, and calcium). In the second part of the thesis, it has been demonstrated a low carbon footprint (CF), but not water footprint (WF) of the EAT-IT dietary pattern also underlying that the final levels of impact are strongly influenced by individual dietary choices within the same food category. As regards, the pilot dietary intervention developed in the third chapter, it was found an overall acceptability of the EAT-IT pattern in the young healthy volunteers enrolled. As regards nutritional intake, it was not drastically changed by the intervention, apart from a reduction in total energy intake and an increase of PUFA (in particular W-6) intake.

Finally, the results of the fourth chapter evidenced the contribution of selected plant-based foods, and particularly anthocyanin-rich blueberry, in the modulation of health-related outcomes such as vascular function, with important implications for vulnerable targets (e.g. older subjects). Results from the randomized controlled trial (RCT) demonstrated that a selected anthocyanin-rich cultivar of blueberry could acutely improve arterial reactivity in older subjects and modulate plasma markers of vascular function, but not arterial stiffness and other metabolic and functional markers. Overall results support the importance of the identification of bioactive rich plant-based products, for their potential exploitation in healthy and sustainable diets.

In conclusion, these data indicate that plant-based diets can be nutritionally adequate to satisfy requirements and are associated with lower carbon footprint. However, specific nutrients could result as critical and thus, on one hand, dietary plan should be finely tuned and critically evaluated depending on the target population; on the other hand, future strategies may also involve a discussion on the opportunity to include the formulation of new, fortified or enriched targeted foods. In this context, optimized diets should not only consider adequacy in terms of nutrient intake, but as far as possible, also the inclusion of specific bioactive-rich products able to provide additional health benefits. Finally, although the EAT-IT diet resulted apparently acceptable, further research is needed to assess the long-term impact of such type of plant-based diets on behavioral and related health outcomes.

1. INTRODUCTION

1.1 Why do we need to make our diets more sustainable?

At present, food systems represent one of the main drivers of environmental impact. In this regard, an authoritative document from Food and Agriculture Organization of the United Nations (FAO) and Food Climate Research Network (FCRN) published in 2016 (Gonzalez Fischer and Garnett, 2016) claims that "*current food production is destroying the environment upon which present and future food production depends*". On the same topic, a more recent report by FAO, entitled "The state of the world's land and water resources for food and agriculture (SOLAW 2021)", states that "*current patterns of agricultural intensification are not proving sustainable. Pressures on land and water resources have built to the point where productivity of key agricultural systems is compromised and livelihoods are threatened*".

According to FAO, the ways through which food systems are threatening the environment, are many as reported below (Vermeulen *et al.,* 2012; Godfray *et al.*, 2010; Whitmee *et al.*, 2015; Bar-On *et al.,* 2018; Poore and Nemecek, 2018; Crippa *et al.*, 2021; FAO, 2021):

- food systems currently contribute to a 25-30% share of total anthropogenic greenhouse gas emissions (GHGEs), that are causing current climate change;
- food systems account for 70% of human water use and are the major source of water pollution;
- food systems are the leading causes of deforestation and land use change;
- food systems are the main drivers of biodiversity loss;
- unsustainable fishing practices deplete stocks of species we consume and cause wider disruption to the marine environment.

To understand the reasons why food systems represent a contributor of that magnitude to global environmental impact, it is necessary to consider some relevant facts. In fact, eating represent a physiological need to be satisfied daily, and thus food systems have been, and still are, indispensable to constantly provide enough food to feed the global population, that is currently still growing (United Nations, accessed on 2022).

From a historical point of view, GHGEs increased rapidly starting from 1850, as a consequence of the growth of the world population and the expansion of industrial systems (see Figure 1) (World resources institute, 2014, accessed on 2022; Myers *et al.*, 2017). Global population reached one billion around 1800, and then started to increase more and more rapidly, albeit with different patterns and rates between more and less developed countries, mainly due to the reduction in mortality resulting from the spread of modern hygiene and medicine, and increasing life expectancy (Lutz and Qiang, 2002). Despite this historic growth in global food demand, food systems were successful to keeping the pace, thanks to some agricultural innovations that occurred in the second half of the 19th century. As an example, the development of higher-yielding grain varieties production, the increased use of synthetic fertilizers and pesticides, and mechanization of agricultural labor improved food availability (Foley *et al.*, 2011; Myers *et al.*, 2017). This transition, known as the "Green revolution", saved millions of people from starvation, but was associated with elevated output and pollution: for instance, global fertilizer use is reported to be increased by 500% (over 800% for nitrogen alone) from 1950 (Matson *et al.*, 1997; Tilman *et al.*, 2001; Moritzer, 2008; Myers *et al.*, 2017). It is noteworthy that FAO underlined that the notion of "sustainable diet" was already proposed in the 80s, and thus this concept was already known during the Green revolution but, at that time, feeding an expanding global population (and thus promoting the intensification of agrifood systems) was considered the priority (FAO, 2010). However, during the last decades, it was evident that the CO_2 and other greenhouse gases emitted because of human activities were the main drivers of many changes observed across the atmosphere, ocean, cryosphere, and biosphere accumulated. As a consequence, in 2007 the Fourth Assessment Report of the International Panel on Climate Change (IPCC) firstly concluded that warming of the climate system was unequivocal (see Figure 2), fostering the scientific debate on these issues (Shukla *et al.*, 2019).

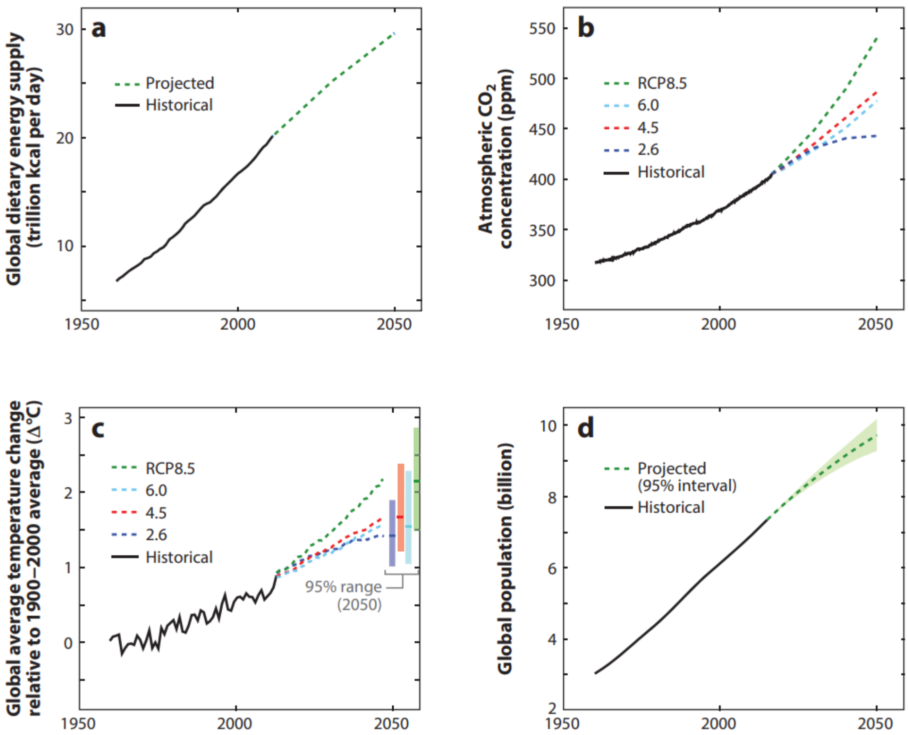

Figure 1 – Relationship between global population growth, global food production, GHGEs increase and climate change, as described by Myers et al. 2017 (Myers *et al.*, 2017). Legend: RCP= representative concentration pathway.

Food systems include the whole processes and the people involved in food production, processing, transport, consumption, or food waste disposal (Herforth *et al.*, 2022), and each of these processes are associated with a certain environmental impact. However, still the highest share of impact is associated with the agriculture, in particular in developing countries (Smith *et al.*, 2008; Foley *et al.*, 2011; Crippa *et al.*, 2021). Recent data from the state of the world's land and water resources for food and agriculture (SOLAW 2021) indicates that, currently, agriculture alone occupies about 38% of Earth's ice-free land surface (47.52 million km^2, of which 15.56 million km^2 for cropland and 32.96 million km^2 for pastures). Emissions from agriculture represent the largest contributor of global anthropogenic greenhouse emission, with a 13% share of the total (7 billion tons of CO2-eq for a total of 54 billion tons), and is a significant contributor to water stress in countries with high levels of this issue, such as Central Asia, the Middle East– Western Asia and Northern Africa (Food Ang agriculture organization Of The United Nation, 2021). The current situation is described as critical, also because it is supposed that global population will rise to 9.7 billion people by 2050 and FAO estimates that agriculture will need to produce almost 50 percent more food by 2030 to achieve the "zero hunger" goal (2021).

Despite the success of food systems, that enabled population growth and improved nutritional status for many, it is clearly reported that food systems are currently governed and managed in a way that need to be improved since they still do (and will) not guarantee sufficient access to food and prevention of malnutrition in the world (Herforth *et al.*, 2022).

The Sustainable Development Goals (SDGs) are a call for action by all countries, defined by the United Nations (UN) in 2015, and consist of 17 goals and 169 targets to promote prosperity while protecting the planet (UN, accessed on 2022b). Sustainable Development Goal 2 (i.e., end hunger, achieve food security and improved nutrition and promote sustainable agriculture) reports that "*A profound change of the global food and agriculture system is needed if we are to nourish today's 815 million hungry and the additional 2 billion people expected by 2050*" (SDG, accessed on 2022). In this regard, current data indicates that world hunger has recently increased, due to the detrimental effects of COVID-19 pandemic, and, in 2020, almost

9.9% of global population resulted undernourished (of which more than a half in Asia and more than one-third in Africa) (FAO, 2021; FAO, IFAD, UNICEF, WFP, 2021). Also, in 2019, 3 billion people could not afford a healthy diet (FAO, 2021; FAO, IFAD, UNICEF, WFP, 2021). At the same time, according to the World Health Organization, in 2016 more than 1.9 billion adults (18 years and older), were overweight and of these over 650 million were obese (WHO, accessed on 2022). Furthermore, it is estimated that one-third of the edible parts of food produced for human consumption, gets lost or wasted globally (equal to about 1.3 billion ton/year) (FAO. 2011).

Given this scenario, a substantial transformation of global food systems has been widely encouraged. In this regards, the EAT-Lancet Commission on healthy diets from sustainable food systems (whose report of 2019 "Food in the Anthropocene" will be further discussed later) advocates the involvement of multiple stakeholders to achieve this goal, from individual consumers to policy makers and all actors in the food supply chain (Willett *et al.*, 2019). In fact, even though the major share of food systems' impact is still represented by agriculture and related land use change, and not strictly dependent on cooking or waste disposal, dietary shift is also to be considered as pivotal to achieve the aims of more sustainable food systems, since *what and how much we eat* directly affects *what and how much is produced* (Gonzalez Fischer and Garnett, 2016).

Diet represents a selection of foods, eaten by an individual, chosen among those made available by the food system. Conversely, the sum of diets creates the overall food demand (see Figure 2) directing and/or shaping the overall food system response (Meybeck and Gitz, 2017). On this topic, the 2017 Report on nutrition and food security by the High Level Panel of Experts on Food Security and Nutrition (HLPE), stated that dietary patterns interact with food systems, not only as an outcome of existing food systems but also as a driver of change for the future ones (HLPE, 2017).

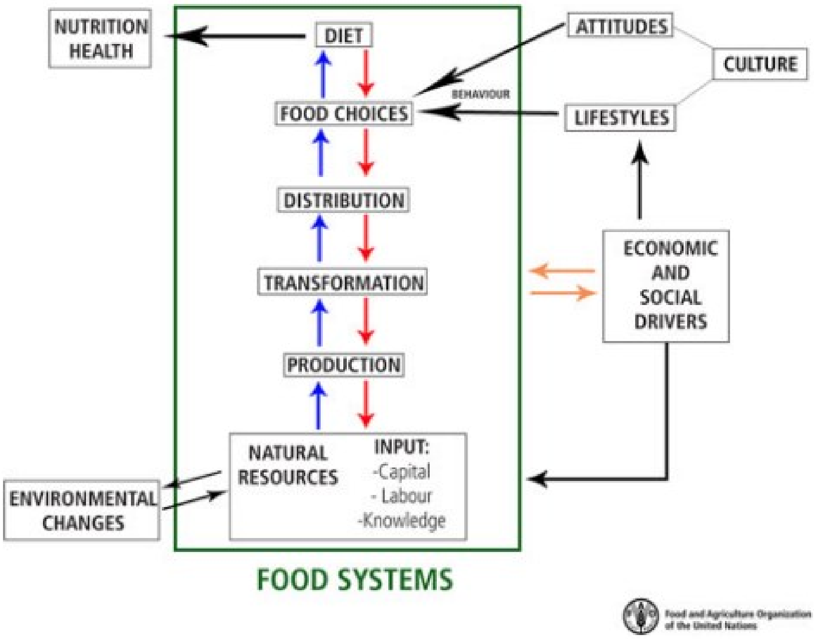

Figure 2 – Food systems and diets (Meybeck and Gitz, 2017). Diets are both a result and a driver of food systems. Therefore, approaching food systems by adopting the perspective of diets can bring operational insights to the issue of the evolution of food systems towards sustainability.

1.2 What do we mean by sustainable diets?

The general concept of sustainability is not new, but it has only recently been applied to environmental policy and food systems. In fact, sustainability started to spread across institutions in 1987, when it was defined in the well-known UN World Commission on Environment and Development (also called "the Brundtland Report" in reference of the principal chairperson of the event) as the "*development that meet the needs of the present generation without compromising the ability of future generations to meet their own needs*" (Keeble, 1988; Kuhlman and Farrington, 2010; Sánchez-Bravo *et al.*, 2020). Later, this concept was adopted by the United Nations in its Agenda for Development in 1997, in which it is stated that sustainable development consists in three interdependent and mutually reinforcing components, i.e., economic development, social development and environmental protection, the three dimensions or “pillars” of sustainability (Purvis, 2019). This representation of sustainability has frequently been used, despite it is also criticized since the distinction between social and economic dimensions is considered unclear and often impossible to make in practice when assessing the impact of a policy on well-being (Kuhlman and Farrington, 2010). In any case, focusing on food and nutrition sustainability, the most referred definition of sustainable diets, accepted as the official one, was agreed in 2010 at the International Scientific Symposium on Biodiversity and Sustainable Diets, organized by Food and Agriculture Organization of the United Nations (FAO) and Biodiversity International (FAO, 2012). This well-established definition claims that: “*Sustainable Diets are those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations. Sustainable diets are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resources”.* This official definition clearly shows that the concept of sustainability is complex since it involves several different dimensions and issues to be addressed. A sustainable diet should in fact:

- be associated with low environmental impact
- be a healthy diet
- contribute to nutrition security
- promote biodiversity
- be considered acceptable by the population
- be affordable.

Achieving at the same time all these objectives is critical not only in relation to the magnitude and complexity of each of them, but also because they are not necessarily synergistic.

1.3 How a sustainable diet looks like?

According to a 2014 discussion paper by Tara Garnett, director of the Food Climate Research Network (FCRN), while it is hard to disagree with the FAO's definition of healthy and sustainable diets, at the same time it is very unclear what such a diet might look like on the plate (Garnett, 2014). This paper also highlights that the definition suggests that these multiple dimensions are synergistic, but instead tradeoffs are inevitable. For instance, meat is generally associated with high environmental impact. However, counter intuitively, since the more of an animal one is prepared to eat, the fewer animals are needed for a given quantity of animal-based product, this may involve that also eating the fattier parts (often added with salt or processed to increase palatability) or the offal should be considered a sustainable behavior. However, this could rise issues from a health perspective.

1.3.1 *Plant-based diets are associated with lower environmental impacts*

It is not an easy task to practically define how to achieve a healthy and sustainable diet. As a starting point for the sustainability dimension, there is a general agreement that plant-based diets are associated with lower environmental impact compared to diets rich in animal foods (Nelson *et al.*, 2016; Lynch *et al.*, 2018). A review of all available articles that used mathematical optimization to define dietary patterns able to improve both health and sustainability, found that all of them were plant-based diets involving a reduction in meat, and particularly ruminant meats, such as beef and lamb (Wilson *et al.*, 2019). The reason why such foods are associated with higher levels of environmental impact is related to the fact that moving up in the trophic chain there is a progressive loss of energy and thus, generally speaking, considerable more inputs are required to obtain animal foods, that are often intensively feed through grain crops (Sabaté and Soret, 2014). According to the USDA, in 2003, the US livestock population consumed more than 7 times as much grain as is consumed directly by the entire American population (Pimentel and Pimentel, 2003). The analysis of the life cycle of foods (Life Cycle Assessment – LCA) is determined by considering the production, processing, transportation, storage, retail, and disposal practices used and allows to estimate the outputs associated with specific food items (Roy *et al.*, 2009). This approach indicated already in 2003 that the production of meat proteins requires 6-17 more land use compared to an identical amount of protein present in a processed soy-based food, as well as 4.4-26 more water use and 6-20 more fossil fuel (Reijnders and Soret, 2003). Recently, more comprehensive analysis of food products have been also conducted. Tilman and Clark collected in 2014 data on 82 types of crops and animal products, allowing the calculation of theoretical diet related GHGEs (Tilman and Clark, 2014). Their results indicated that overall animal products are associated with higher levels of GHGEs for serving, especially considering beef. Intermediate levels of impact were found for fish products, presenting high variability, and other animal foods such as poultry and pork meat, eggs and dairy. Finally, vegetable products are overall associated with lower levels of GHGEs. These data are showed in Figure 3.

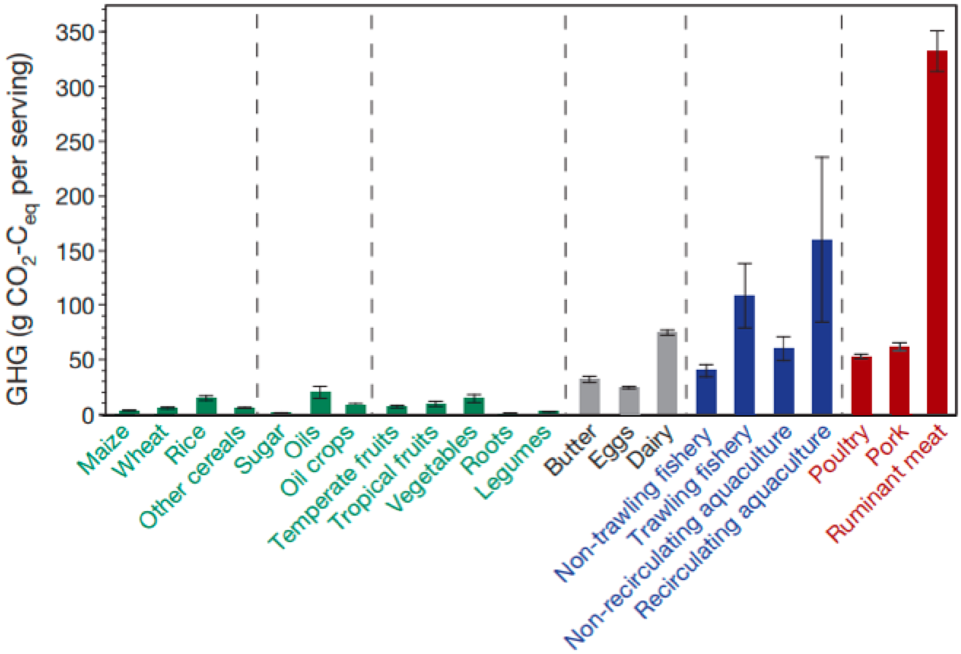

Figure 3 – Lifecycle GHGEs (CO2-Ceq) for 22 different food types. Data derived from an analysis of 555 food production systems (Tilman and Clark, 2014). Legend: GHG= greenhouse gas.

1.3.2 Plant-based diets: intersection with health

According to the elaboration of Tilman and Clark, changes towards healthier and more plant-based diets, such as the Mediterranean, pescatarian or vegetarian diet can have globally significant benefits also in terms of GHGEs, even though they specified that, conversely, minimizing environmental impacts does not necessarily maximize human health (Tilman and Clark, 2014). However, in literature it is also diffusely promoted the concept that a dietary shift to a more plant-based diet can drive not only the transition to more sustainable food systems, but also to a healthier way of eating, with positive effects on population health (Nelson *et al.*, 2016; Hemler and Hu, 2019). For instance, the 2015 work of Barilla Center for Food and Nutrition proposed a visual representation of the relationship between health and environmental impact of different food categories by comparing the Mediterranean pyramid to an environmental pyramid in which food items are disposed in relation to their levels of carbon, water, and ecological footprint, after screening for LCA data in literature (the "Double Pyramid" represented in Figure 4) (Ruini *et al.*, 2015). Data indicates that the carbon footprint of 1 kg of fruit (0.47 kg CO2eq) or vegetables (0.82 kg CO2eq) is 55 and 32 times lower, respectively, than the carbon footprint of red meat (26.17 g CO2eq), or that the water footprint of 1 kg of pasta (1,770 L) is 11 times lower than the water footprint of 1 kg of bovine meat (18,870 L) (Ruini *et al.*, 2015). According to this work, the foods offering the greatest benefits from a nutritional/health viewpoint (such as vegetables, grains, pulses, and fruit) are those with the lowest environmental impact, while the foods that should be consumed in moderation for health reasons, such as red and processed meats, are those with the highest impact, thus supporting the intrinsic sustainability of the Mediterranean (and other similar) diets and the simultaneous benefits of adopting a more plant-based pattern (Ruini *et al.*, 2015).

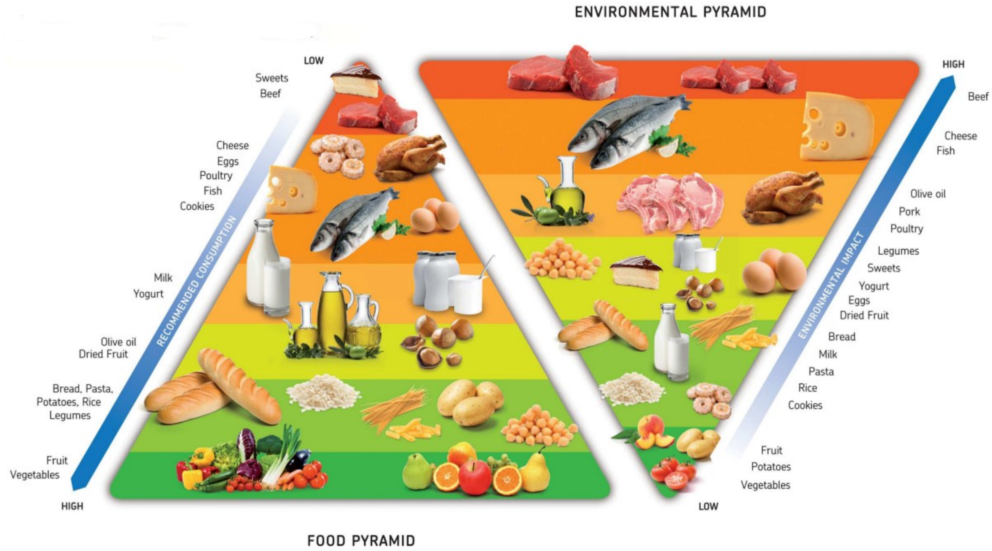

Figure 4 – The Double pyramid for adults, 5th edition, modified from: "Working toward Healthy and Sustainable Diets: The "Double Pyramid Model" Developed by the Barilla Center for Food and Nutrition to Raise Awareness about the Environmental and Nutritional Impact of Foods" (Ruini *et al.*, 2015).

Other works evaluated the link between healthiness and environmental impact of food categories/items. The article by Clark and co-authors (Clark *et al.*, 2019) analyzed how consuming an additional serving per day of 15 food groups is associated with 5 health outcomes in adults (i.e., stroke, diabetes, colorectal cancer, coronary heart disease, and total mortality risk) and 5 aspects of agriculturally driven environmental degradation (i.e., greenhouse gas emission, land use, eutrophication, acidification, and scarcity-weighted water use), considering data from meta-analysis. This work shows that, generally, foods associated with the highest detrimental effect on one of the five health outcomes, tend to affect the others negatively also, and that the same trend occurs for environmental impact. However, there are some exceptions. For instance, nuts are associated with elevated water use, despite low levels of impact for the other considered outcomes, and clearly fish are associated with low land use, despite overall medium-high levels of impact. This analysis indicates that, while red meat (processed or not), is associated with elevated increase in disease risk and environmental impact, other foods can be associated with lower increase in disease risk, but elevated levels of environmental impact (i.e., fish, dairy, eggs, and chicken), or vice versa, increased risk of disease but lower environmental impact (i.e., refined grains and sweetened beverages). These data were summed up in the form of radar plot and are reported below (Figure 5).

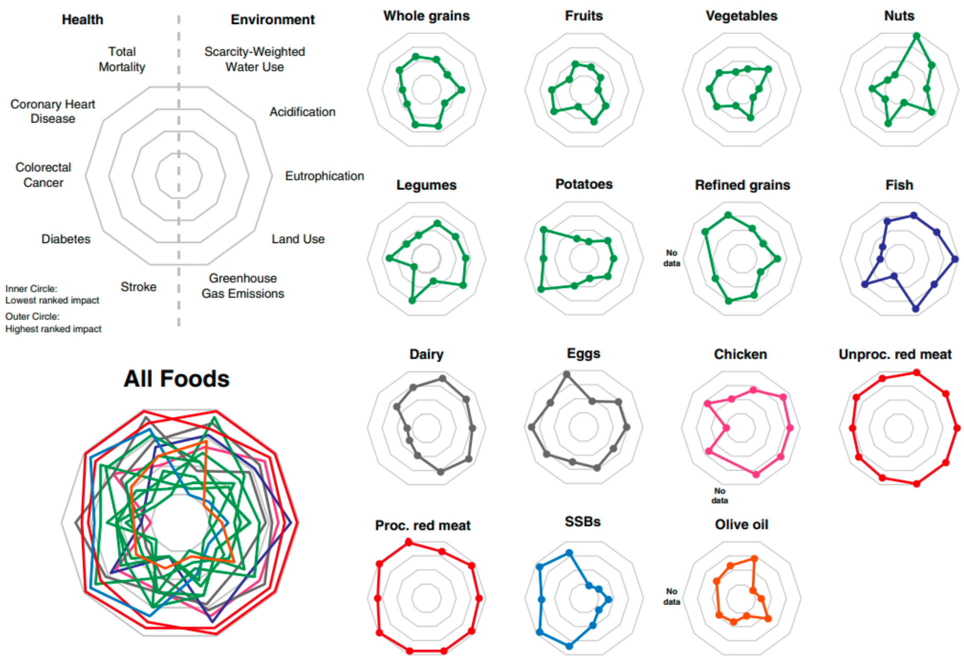

Figure 5 – Radar plots of rank-ordered health and environmental impacts per serving of food consumed per day (Clark *et al.*, 2019). Legend: SSBs: sugar-sweetened beverages.

Overall, there is evidence supporting the idea that transition towards a healthier diet is likely to result also in a less impacting one. However, it is important to consider that claiming that a healthier diet involves a more sustainable way of eating is different than claiming that a less impacting diet is necessarily a healthier one. In the analysis of Seves *et al*. (2017) and in the one of Collins and Fairchild (Collins and Fairchild, 2007), the best optimization of nutritional characteristics of the diet did not correspond to the highest levels of substitution of meat or meat and dairy with plant based foods, despite their lower levels of related-impact. Another analysis from Perignon and co-workers indicated that moderate reduction of GHGEs (≤30 %) were compatible with nutritional adequacy and affordability without adding major food group shifts to those suggested by nutritional recommendations, while higher GHGEs reductions impaired nutritional quality (Perignon *et al.*, 2016).

1.3.3 Environmental impact associated with plant-based diets is affected by actual eating behavior

All the analysis reported in the previous paragraph about the intersection between health and sustainability of diets are affected by the intrinsic limitation of their theoretical approach, since rely on modeling technique and elaboration of probable, but still hypothetical, scenarios.

Data on environmental impacts of plant-based diets based on actual food intake is still scarce. In this regard, a recent systematic review (Jarmul *et al.*, 2019) including all available evidence on the effect of sustainable diets on environmental footprints and human health was conducted. This review is interesting since it reports data from both modelling studies and empirical ones. The results indicated that notable differences exist between modelling studies and the relatively small number of empirical studies. For instance, some modelling studies indicates a possible GHGEs reduction ranging from -67% and -82.3% compared to a habitual diet, while empirical data obtained a considerably lower reduction that ranged from -26.9% to -43.3%. Furthermore, all water use was found to be higher in sustainable diets compared with a "usual" one.

In this regards, it is interesting to consider the results obtained by Rosi *et al*. (2017) who assessed the actual environmental impact of three groups of 51 Italian adults, having an omnivorous, a vegetarian or a vegan diet. The authors assessed subjects' food intake through a 7-days food record and calculated the related impact in terms of carbon, water and ecological footprint using the Barilla Center for Food and Nutrition database (Ruini *et al.*, 2015). The results indicated that vegetarian and vegan diets were indeed associated with lower levels of impact for all the three indicators, in particular for carbon and ecological footprint (e.g., -34,3% and -40.9% for vegetarian and vegan CF respectively), but interestingly the differences among vegetarian and vegan levels of impact were not statistically significant. This was related to the higher consumption of processed plant-based foods in vegans that compensated eggs and dairy consumption of vegetarian subjects. Furthermore, high inter-individual variability within each dietary group was registered, with a few vegetarian or vegan subjects presenting higher diet-associated environmental impact than other omnivorous ones.

The research of Soret *et al*. used observational data from North-America to evaluate differences in GHGEs between vegetarian, semi-vegetarian and non-vegetarian subjects (2014). This analysis of real food intake found a reduction of GHGEs by 21.6% and 29.2% for semi-vegetarian and vegetarian compared to non-vegetarian, respectively.

Despite scarce, these results on empirical data, indicate that in real setting scenarios several factors can affect the final levels of environmental impacts, reducing the magnitude of theoretical modelling study. All together these results further demonstrate the necessity to involve all the stakeholders in the improvement of food systems sustainability, giving that a plausible reduction of global diet related GHGEs of 20-30% means obtaining an absolute small reduction in global emission if global population will rise with a similar or higher trend (unless technology innovation would allow to decrease food production impact).

1.3.4. Critical aspects in the definition of healthy and sustainable diets

Several issues affect the definition of healthy and sustainable diets considering their suggested overlap with the concept of plant-based diets. One of them is related to the scarcity of data on environmental impact of different food items. Recently, the database used for the elaboration of the Double pyramid has been revised and used as a starting point to develop a freely accessible multilevel database of carbon and water footprint of food items, the SU-EATABLE LIFE (SEL) (Petersson *et al.*, 2021). The SEL data indicates that food values can vary greatly within the same category, also depending on the fact that the same foods can be differently processed e.g., frozen, or, in case of fruit and vegetables, they can be grown in heated greenhouse or imported. For instance, cucumber is associated with 0.27 kg of CO2 eq./kg, but this value rises to almost tenfold when considering cucumber grown in heated greenhouse (2.55 kg of CO2 eq./kg), with low levels of uncertainty; this value is higher than the median value of carbon footprint of milk, which correspond to 1.44 CO2 eq./kg.

Very recently, an analysis of the intersection between healthiness and sustainability of 57,000 commercially available products from UK and Ireland was performed by combining the NutriScore, for the nutritional evaluation, with single ingredient sum of environmental impact, estimated through an algorithm (Clark *et al.*, 2022). This work showed that: i) high levels of variability of commercially available food products within the same food category exist, depending on the ingredients and their production system; ii) if reliable information could be made available, then consumers could have been empowered to choose the best option within alternatives, allowing for a more easily implementable diet transition.

Another aspect that needs to be carefully considered is that the levels of GHGEs associated with food production and specific food items are not static and can decrease or increase with time according to changes in production systems and technology. Data clearly indicates that while global GHGEs associated with food production is rising in absolute terms (from over 3.7 Gt CO2 eq in 2000 to almost 4.2 Gt CO2 eq in 2014), this increase does not translate into an equal increase in emission intensity, which in fact dropped from over 0.68 kg CO2 eq/USD 1 of food production in 2000 to less than 0.46 in 2014 (Mrówczyńska-Kamińska *et al.*, 2021). According to this analysis, technological efficiency and the higher yield associated with intensive agriculture allow to decrease the levels of GHGEs intensity ratio for food production (Mrówczyńska-Kamińska *et al.*, 2021). However, although the lower GHGEs intensity ration, the rise in food demands and population growth overall determine an increase in total GHGEs (Mrówczyńska-Kamińska *et al.*, 2021). The same trend has been reported by other authors, that showed an increase by 40% in food production from 1990 (using cereals production as a proxy), but only a 12.5% increase in food systems GHGEs (including land use change), with a more pronounced reduction in developing countries (due to the higher possibility of improve production systems) (Crippa *et al.*, 2021). Another study showed that significant variation exists in environmental impact of the same food items across different countries (e.g. CF of bovine meat from Paraguay and Brazil, were 17 and 5 times higher, respectively, than that of Danish bovine meat, mainly due to land use change and deforestation levels) (Kim *et al.*, 2020).

Finally, also country specific characteristic of diets must be considered: the health and environmental improvement of shifting to a more plant-based diet will result in a healthier and sustainable diet depending on the population food intake. The study of Sugimoto found that differently from Western countries, among Japanese adult population the optimization of nutritional adequacy was linked with an increased levels of GHGEs, due to country-specific food intake characteristics (Sugimoto *et al.*, 2020).

A global modelling analysis with country-level details performed in 2018 by Springmann et al., indicated that qualitative differences exist between the health and environmental benefits that can be attained by dietary changes, and the differences between diet-related environmental impacts and regions (Springmann *et al.*, 2018a). For instance, modelling the effect of dietary shift prioritizing food security and reducing under- and overweight is estimated to result in lower impact for more developed countries, while in increased resource use in low-income countries (Springmann *et al.*, 2018a).

Overall, these considerations and the available data on single foods indicate that, in the estimation of environmental impacts, several critical issues need to be considered when facing the concept of healthy and sustainable diets as summed up point by point below.

- Plant-based foods are generally associated with low carbon and water footprint, but with some exceptions (e.g., nuts are connected with elevated water use and rice cultivation is linked with high methane production) and can be associated with other forms of environmental impact such as fertilizers, pesticides, and plastic pollution, that however lack of standardized procedures to obtain proper indicators (Clark *et al.*, 2019; Petersson *et al.*, 2021).
- Animal foods (and particularly red meat) are associated with the highest levels of environmental impact, but also represent an important source of essential nutrients including omega-3 fatty acids, iron, B vitamins, zinc, and vitamin A. More developed countries are characterized by meat overconsumption, but recommendations of sustainable diets involving the reduction of animal foods consumption can be particularly problematic in low- and middle-income countries that already struggle with nutrition transitions and micronutrient deficiencies (HLPE, 2017; Kim *et al.*, 2020; Leroy and Cofnas, 2020).
- Dietary substitutions are pivotal in determining final health and environmental outcomes (Rosi *et al.*, 2017; Clark *et al.*, 2022).
- Total animal product replacement do not always correspond to higher reduction in environmental impact, compared to partial substitution; in

addition it could have detrimental effect on nutritional profile, and is less easily implementable by consumers compared to small dietary transition (Collins and Fairchild, 2007; Petti *et al.*, 2017; Rosi *et al.,* 2017; Seves *et al.*, 2017; Clark *et al.*, 2022).

- Considerable variability exists in environmental impact within food items of the same food category, in relation to their ingredient composition and production system (Kim *et al.*, 2020; Clark *et al.*, 2022)
- Meat category includes different typologies (e.g., red, or white, processed or not) that are associated with different health and environmental outcomes (Garnett, 2014; Tilman and Clark, 2014; Clark *et al.*, 2019, 2022).
- Since environmental impact data depends on production systems, technological innovation and resource optimization can result in lower levels of impact for the same food (Garnett, 2011; Kim *et al.*, 2020).
- These evaluations do not consider overconsumption and can suggest that a shift to a more sustainable plant-based diet is also likely to lower incidence of overweight, that represent an important burden on health; however overconsumption depends on portion size, and high energy density foods, such as sweet and pastries, are associated with intermediate or low levels of environmental impact (Garnett, 2013).
- Frequently, socio-economical aspects, such as affordability, acceptability, and economic consequences on specific sectors are not considered (HLPE, 2017; Downs *et al.*, 2020).

1.4 Guidelines to promote healthy and sustainable diets

Given the criticalities that were listed in the previous chapter, some scientific-based guidelines are required to enable population to consider a shift to a more healthy and sustainable diet. In the discussion paper by Garnett (Garnett, 2014), the following tentative guidelines were proposed:

- Diversity – a wide variety of foods eaten
- In energy balance
- Based around: tubers and whole grains (but not rice); legumes; fruits and vegetables - particularly those that are field grown and robust
- Dairy products or fortified plant-substitutes eaten in moderation and other calcium- containing foods also consumed
- Meat eaten sparingly – and all animal parts consumed
- Unsalted seeds and nuts included
- Some fish and aquatic products sourced from certified fisheries, although less frequently than advised by the Eatwell Plate
- Limited consumption of sugary and fatty sweets, chocolates, snacks, and beverages
- Tap water in preference to other beverages

In 2019, FAO and the World Health Organization (WHO) developed a list of 16 guiding principles related to sustainable healthy diets, targeted at governments and other stakeholders in policy making and communication (FAO and WHO, 2019). These 16 principles are divided into three categories: health aspects, environmental impact, and sociocultural aspects, and are listed as follow. Specifically, sustainable healthy diets (SHDs):

1. start early in life with early initiation of breastfeeding, exclusive breastfeeding until six months of age and continued breastfeeding until two years and beyond, combined with appropriate complementary feeding;
2. are based on a variety of unprocessed or minimally processed foods, balanced across food groups, while restricting highly processed food and drink products;
3. include wholegrain, legumes, nuts and an abundance and variety of fruits and vegetables;

4. can include moderate amounts of eggs, dairy, poultry and fish and small amounts of red meat;
5. include safe and clean drinking water as the fluid of choice;
6. are adequate (i.e., reaching but not exceeding needs) in energy and nutrients for growth and development and to meet the needs for an active and healthy life across the lifecycle;
7. are consistent with WHO guidelines to reduce the risk of diet-related NCDs, and ensure health and wellbeing for the general population;
8. contain minimal levels, or none if possible, of pathogens, toxins and other agents that can cause foodborne disease;
9. maintain greenhouse gas emissions, water and land use, nitrogen and phosphorus application and chemical pollution within set targets;
10. preserve biodiversity, including that of crops, livestock, forest-derived foods and aquatic genetic resources, and avoid overfishing and overhunting;
11. minimize the use of antibiotics and hormones in food production;
12. minimize the use of plastics and derivatives in food packaging;
13. reduce food loss and waste;
14. are built on and respect local culture, culinary practices, knowledge and consumption patterns, and values the way food is sourced, produced and consumed;
15. are accessible and desirable;
16. avoid adverse gender-related impacts, especially with regard to time allocation (e.g., for buying and preparing food, water and fuel acquisition).

In this document, the FAO and WHO also clearly stated that one of the actions required to make SHD available, accessible, affordable, safe, and desirable is the development of national food-based dietary guidelines (FBDGs) according to these principles. This is a fundamental goal that would be important to achieve.

1.5 The planetary healthy and sustainable diet of the EAT-Lancet Commission

Although the Mediterranean diet has been widely defined as a sustainable diet, this aspect would be intrinsic to its characteristics, since it is characterized by high amounts of olive oil and olives, fruits, vegetables, cereals (mostly unrefined), legumes, and nuts, moderate amounts of fish and dairy products, and low quantities of meat and meat products (Germani *et al.*, 2014; Dernini and Berry, 2015; Coats *et al.*, 2020; Serra-Majem *et al.*, 2020). Otherwise, to date there are not many proposals for diets defined a priori to meet sustainability requirements as identified by scientific evidence.

In this regard, the EAT-Lancet Commission on healthy diets from sustainable food systems in its exhaustive article attempted to give a scientific background to foster two endpoints of the global food systems: final consumption (healthy diets) and production (sustainable food production) (Willett *et al.*, 2019). Related to the first endpoint, there is the definition of a “reference diet”. Briefly, this commission of 37 experts from various fields of nutrition, agriculture, and sustainability, started considering that a limitation that preclude the adoption of strategies to improve food systems is that there is a lack in safety threshold values for different indicators of diet associated environmental impact. The Commission claims that the definition of such indicators should replicate the logic used by IPCC, i.e., the definition of a safety threshold (“scientific targets”) of total greenhouse gas emission to stay within in order to contain the increase in temperature within a certain safe degree (i.e., 1.5 °C). Thus, the EAT-Lancet Commission used already defined threshold values for six different forms of environmental impact (the “planetary boundaries” to which food systems should remain to be considered as moving in “safe operating space” (Rockström *et al.*, 2009)), such as total global amount of cropland use, and calculated reference values of average daily intake for eight different food categories (i.e., whole grain, tubers or starchy vegetables, vegetables, fruits, dairy foods, protein sources, added fats, and added sugars) that allow to remain within these safe thresholds. These reference values ("scientific targets") settled to obtain a 2500 kcal healthy diet, should be further elaborated to be adapted to every different food context around the world, thus the nomenclature of "Planetary health diet" (Selvik and Fullilove, 2020). However, these recommendations can contain food systems within safe operating space for a maximum of 10 billion people.

This authoritative proposal represents the first attempt to circumscribe the characteristic of a healthy and sustainable diet, starting from scientific data.

Given the complex scenario composed by different cultures and local traditions, the necessity of thinking in terms of different dietary patterns, rather than a single, universal and "ideal" diet, has been advocated (HLPE, 2017). However, despite the report from the EAT-Lancet Commission considered this demand, it did not indicate how to achieve it (Blackstone and Conrad, 2020).

Some attempts were made to compare the EAT-Lancet Commission reference diet (ELCRD) to national guidelines and to propose some adjustments. In detail, Lassen et al. (Lassen *et al.*, 2020) proposed a culturally adapted ELCRD for the Denmark population through the adjustment of the energy target and portion size of different food categories to increase compliance with the Danish dietary guidelines. Sharma et al. (Sharma *et al.*, 2020) compared the ELCRD indications with actual food intake of different Indian regions, highlighting critical points and identifying actions to orientate policies. Differently, Blackstone and Conrad (Blackstone and Conrad, 2020) investigated how this pattern differed from American national guidelines, which did not include adjustments related to environmental sustainability. Therefore, the authors conclude that it is necessary to stimulate the development of models inspired by these principles and adapted to different nations. At present, only few comparisons have been made although these country specific re-elaborations and discussions could help improving specific guidelines.

1.6 Healthy and sustainable diets and climate change: a vicious cycle

When analyzing the interrelation among diet, health and sustainability to develop guidelines able to respond to the future needs, it is necessary to remind that malnutrition, in its all forms, *i.e.*, under-, over- and poor nutrition, should be adequately reduced and optimal nutrition targeted in order to maximize human benefits coping with the containment of environmental impact. This goal cannot disregard the consideration of another important impacting variable that is represented by climate change itself. In Figure 6 it is represented the mean temperature variation, registered in the different part of the world, and showing that eastern countries are those facing the higher increases, but also that in Europe variations up to 2.8 °C have been measured.

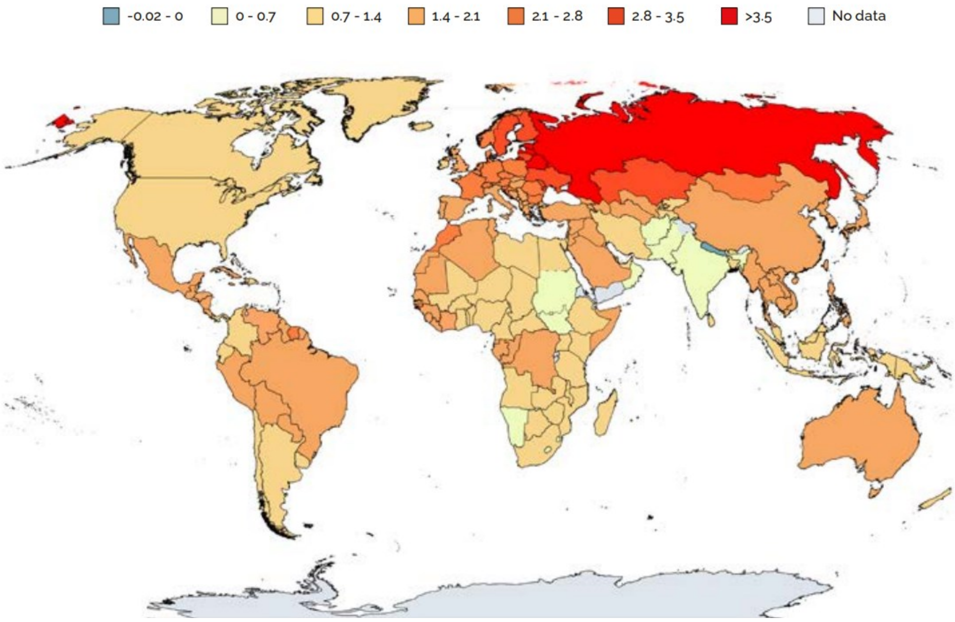

Figure 6 – Increase in mean temperature across different areas of the world, according to FAO (Food and Agriculture Organization of the United Nations (FAO), 2021). Legend: color intensity indicates the mean delta temperature (ΔT) in different geographic areas.

In this context, one of the paradoxes of the relationship between diets and climate change is that food systems are one of the main contributors to GHGEs, contributing to climate change, but at the same time climate change has detrimental effects on food security and on the nutritional quality of foods.

1.6.1 Effects of climate change on food security and safety

Considering food security and food safety, climate change can affect food production in relation to increasing temperature and extreme water weather such as heat, droughts, and floods (Lake *et al.*, 2012). Negative impacts are likely to be greater in regions where climate change combines with factors that increase vulnerability, such less developed countries in which change in rainfall (level, pattern, or variability) is more likely to results in crop failure (Lake *et al.*, 2012). The increased temperature also cause ocean warming, with increased algae development and decreased oxygen content (FAO, 2015). Overall these consequences can affect fish product availability, which represent an important source of protein (up to 70% in different less developed countries) (Thilsted *et al.*, 2014), as well as of n-3 PUFA (EPA and DHA, which do not require bioconversion from ALA, which can instead be found in vegetal food) and micronutrients, such as iron, zinc, vitamin D and B12 (Myers *et al.*, 2017). Other issues could be related to storage and transportations since higher temperature facilitate the production of mycotoxins in crops (Maggiore *et al.*, 2020). Vulnerable groups, including children, women, elderly people, those with existing health problems and disabilities, the poor and marginalized are likely to be disproportionately affected by climate change issues (Fanzo *et al.*, 2017).

1.6.2 Effects of climate change on nutritional quality

Linked to the issue of food security is also the problem of nutritional adequacy and food quality. In fact, there is a solid consensus about the fact that one of the more plausible effects of climate change on nutrient profile of foods will be related to detrimental effects on the nutritional quality of crops. Experimental trials in which crops are grown in open field conditions under elevated CO_2 have revealed that many important food crops (e.g. wheat, barley, rice, potatoes, soy and peas) show 3–17% lower concentrations of protein, iron and zinc (Myers *et al.*, 2014; Blandino *et al.*, 2020). Moreover, also the climate change related increase in the levels of ozone are considered to be responsible for yield loss of maize (up to 5,5%), wheat (up to 15%), and soy (up to 14%) (Avnery *et al.*, 2011). This side effect of climate change on nutrition need to be carefully considered since wheat, rice, and maize represent about 56% of the global dietary energy supply derived from plants (Lake *et al.*, 2012). Population more prone to reach insufficient intake of these nutrients, such as elder people in low-income countries (e.g. India, South Asia, Sub-Saharan Africa, North and Middle East Africa), can be exposed to a relevant risk of inadequate nutrition since their diet rely more than population of developed countries on vegetal food and crops (Fanzo *et al.*, 2017; Smith and Myers, 2018). Reductions in the zinc and iron content of the edible portion of these food crops will increase the risk of zinc and iron deficiencies across these populations and will add to the already considerable burden of disease associated with them (Myers *et al.*, 2014). This issue is also relevant for protein intake in countries (e.g. India) were a large portion of population is considered to be at risk for not reaching protein requirements (Myers *et al.*, 2014; FAO, 2015). In another publication these data has been elaborated to calculate the risk of inadequate intake for these nutrients in the future scenario, highlighting an heterogenous situation in which the less interested countries are the western and developed countries, despite having a high risk scenario in least-developed countries such as Africa and Asia (Smith and

Myers, 2018). Other relevant aspects, but less supported by consistent literature, is the decreased yield of dairy animals (WHO, 2019) and possible reduction of other micronutrients, including calcium, in crops under more severe, but reachable standing current trend, CO2 concentration (Myers *et al.*, 2017). This means that climate change could create differences among the nutritional composition of several important staple foods, and modelling approach to diet optimization will probably have to consider these issues. A recent and extensive analysis by Dave et al. stressed the importance of implementing a holistic approach when addressing the definition of sustainable diets able to achieve adequate food security (Dave *et al.*, 2021). According to their analysis, the failure to consider aspects related to bioavailability in sustainability measurements undermines the complementary role that animal foods play in achieving nutrition security in vulnerable populations (Dave *et al.*, 2021). Other issues are related to current global trend in human nutrition, that is shifting to a more homogeneous diet. In this regard, a slavish adoption of a standardized diet, without considering specific nutritional issues, can lead to a further reduction in biodiversity of crops and breeds, that reduce agricultural resilience, increase the susceptibility of pests and limit variety and nutritional benefits (Garnett, 2013; Dave *et al.*, 2021). Finally, optimizations models to define healthy and sustainable diets are frequently based on LCA, that requires the use of functional units that can be defined in many ways (e.g., considering the impact of serving sizes, the quantity of one or more nutrients or the energy content). A recent document by FAO defined a nutritional LCA study in which the provision of nutrients is considered the core function of food items (McLaren *et al.*, 2021). This document aimed to provide a consensus on an LCA methodology able to properly integrate nutritional value and nutrient quantities, thus supporting the necessity to increase the awareness of the centrality of nutrition in the definition of dietary patterns sustainability (McLaren et al., 2021).

1.7 Further challenges in the development of sustainable diets and possible nutritional perspective

Defining a global sustainable healthy diet is challenging not only because should be adapted to local contexts but also because it should target all different needs including those of vulnerable people with specific nutrient requirements (such as young children, adolescent girls, pregnant and lactating women, the elderly, and ill people), that are widely present within the population (HLPE, 2017). Considering a vulnerable groups significant for the future scenario, the older subjects should be carefully considered since world population is aging rapidly (He *et al*., 2016). In 2012, 562 million of global population were aged 65 and over, mainly concentrated in Europe, but their number is expected to increase to 1.4 billion by 2030 and developing countries are likely to experiment a demographic transition even faster than the one that occurred in more developed ones (Dobriansky *et al.*, 2007; He *et al.*, 2016; UN, 2017). Population aging reflects the achievement of human innovation and governance to allow better life conditions, food security and health: today, living to age 70 or age 80 is no longer a rarity in many parts of the world and this was unthinkable even just a hundred years ago. However, increasing longevity has led to new challenges, including the need to reduce the burden of chronic diseases through the promotion and the achievement of optimal nutritional status (Dobriansky *et al*., 2007; Barkoukis, 2016; Bruins *et al.*, 2019).

These aspects are also of great importance since according to the GBD the principal dietary risk factors correlated with global mortality and disability are i) high sodium consumption ii) low consumption of whole grains iii) low consumption of fruits iii) low consumption of nuts and seeds iv) low consumption of vegetables and, v) low consumption of seafood omega-3 fatty acids (GBD 2017 Diet Collaborators, 2019). It should be noted that it is the low consumption of plant-based foods, that is currently the main dietary problem leading to negative health outcomes, rather than the consumption of red and processed meat, which is at the bottom of the list (see Figure 7). For example, plant-based foods are positively associated with significant health benefits, in relation to cardiovascular health, which is the principle cause of death and disability, in particular in the last decades of life (Joseph *et al.*, 2017; Leong *et al.*, 2017; GBD 2017 Diet Collaborators, 2019; Noale *et al.*, 2020).

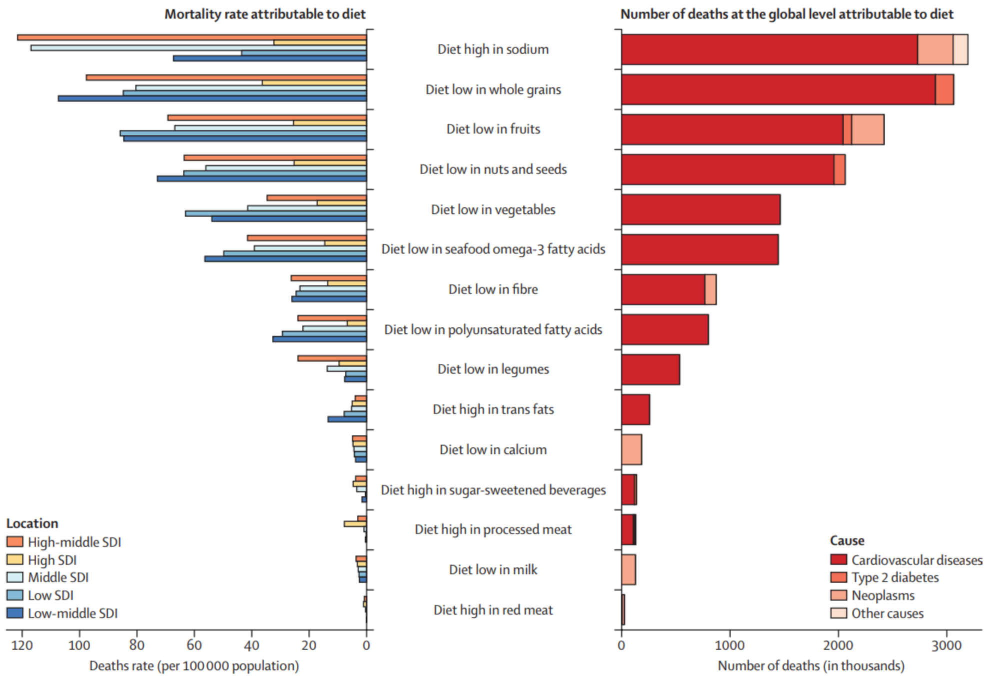

Figure 7 – Number of deaths and age-standardized mortality rate (per 100 000 population) attributable to individual dietary risks at the global and SDI level in 2017 (Afshin *et al.*, 2019). Legend: SDI: Socio-demographic Index.

Extensive dose-response meta-analyses indicate significant disease risk reduction for high intake of fruit and vegetables (Aune *et al.*, 2017; Wallace *et al.*, 2020; Zurbau *et al.*, 2020). These protective effects have been attributed to several nutritional characteristics of these food categories, such as fibers, that can reduce insulin response associated to carbohydrate intake, and decrease total and LDL cholesterol. The high content of vitamins and minerals can improve different metabolic and physical responses (e.g. optimizing energy metabolism, reducing inflammatory responses or normalizing blood pressure) and can exert anti-oxidant activities (Miller *et al.*, 2017), More recently, nutritional research is focusing also in elucidating the important role of the myriad of phytochemicals and bioactive compounds, which are likely to act synergistically through many different biological mechanisms able to reduce the risk of chronic diseases and premature mortality (Perez-Vizcaino and Fraga, 2018; Morand and Tomás-Barberán, 2019). Bioactives are substances that are not essential for maintaining body functions, but that exert health-promoting effects (Fraga and Oteiza, 2018). Thus, currently classifications include among bioactives those found both in animal and plant foods (e.g. peptides and bioactives protein from fish products), but they are more widely diffused among plant-foods, in which they are also called phytochemicals, and include carotenoids, polyphenols (furtherly subdivided among subclasses, i.e., flavonoids, phenolic acids, lignans, and stilbenes), glucosinolates, and fibers, as well as other molecules that still need to be identified (Liu, 2013; Galanakis, 2021). Potential mechanism that have been investigated in relation to their contribution are in particular related to their antioxidant activity and oxidative stress reduction, their interaction with cell membrane, enzymes, receptors, and transcription factors (Fraga *et al.*, 2010; Zhang *et al.*, 2015). Among the mostly investigated bioactives there are polyphenols, with all their subclasses. In particular, in 2019 we conducted a systematic review of the available literature published in the previous 10 years, that evaluated polyphenols intake and their association with mortality, cardiovascular disease, diabetes and other health outcomes (Del Bo' *et al.*, 2019). We found an overall inverse association between polyphenol intake and CV risk events and mortality, as well as between polyphenols and other outcomes of health status (Del Bo' *et al.*, 2019). Despite the limitation related to the estimation of polyphenols intake and the elevated heterogeneity among the revised literature did not allow to define a reference/prudent intake of total polyphenols, data suggested that a polyphenol-rich dietary pattern should be considered a valid tool for the prevention of numerous chronic diseases (Del Bo' *et al.*, 2019). In this regard, it should be considered that the Mediterranean diet, which is listed among the healthy and sustainable diet, is considered to be associated with positive effects on health also in relation to the synergistic effects of its polyphenols together with the other bioactives (Martínez-González *et al.* , 2019). Consequently, plant based sustainable diets should be carefully developed in view of their contribution to the intake of different bioactive compounds.

Considering the specific effects of bioactives and bioactive-rich foods; e.g., anthocyanins result strongly related to health effects on vascular systems (Speciale *et al.*, 2014; Reis *et al.*, 2016; Krga and Milenkovic, 2019), it has been suggested that a possible strategy to optimize body function and overall health, in particular among specific targets of population such as vulnerable people, could consist in the exploitation of biodiversity (e.g., wisely choosing the best crop varieties) or in the development of new functional or optimized traditional foods with more pronounced health benefits (Morand and Tomás-Barberán, 2019).

In this regard, nutritional research is particularly active in the elucidation of: i) variations in their concentration within foods (which is influenced by several factors including climate change), ii) the impact of food matrix on absorption, and iii) the role of the microbiota on their metabolism (Fraga and Croft, 2019).

Within this context, it is interesting to consider that the EAT-Lancet report endorses fruit and vegetables consumption, being a plant-based diet, however, do not address the difference in composition and characteristic of phytochemicals within its reference diet, thus opening a possible research space aimed at optimizing this model, not only in relation to different energy and nutrient targets, but also in relation to specific recommendations including bioactives compounds within the paradigm to enable targets groups of population to optimizing their diet by choosing among selected plant-based foods able to provide targeted health benefits.

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2. AIMS AND OBJECTIVES

The aim of this PhD thesis is to analyze, discuss, and provide scientific evidence on the concept of sustainable diets, as part of the MIND FoodS HUB project.

In this regard, the objective of the general project is the creation, in the context of Milano INnovation District (MIND), of both an infrastructure and a skill-hub for the development of an innovative concept related to the identification, production, and sustainable transformation of plant-based products and derivatives with nutritional and health benefits. The main outcomes are related to providing strategies, methodologies, technologies, as well as new or improved products, to promote healthy and sustainable food systems and dietary patterns.

Specifically, the main research tasks addressed in this PhD thesis have been organized into 4 chapters, and the different objectives are described below.

CHAPTER 1. Different activities have been performed in order to: i) explore the degree of alignment of current food-based dietary guidelines (FBDGs) to guiding principles of sustainable healthy diets, ii) develop a sustainable Mediterranean-based dietary pattern in line with the EAT-Lancet Commission reference diet, adapted to the Italian food habits (EAT-IT), settled on 2500 kcal, and evaluate its nutritional adequacy in relation to the nutrient and energy reference recommendation and to a dietary pattern based on the Italian FBDG, iii) identify a procedure of adaptation of the EAT-IT dietary pattern to different energy targets, also considering the inclusion of selected bioactive-rich foods while maintaining nutritional adequacy and feasibility.

CHAPTER 2. Based on previous outcomes, the subsequent activities focused on the evaluation of the actual environmental impact of the developed EAT-IT dietary pattern, through the identification of the most contributing foods and the estimation of the effect of different food choices on the final environmental outcomes.

CHAPTER 3. To move from theory to practice, the third part of the research was devoted to the setting of a pilot human intervention trial to investigate the actual feasibility of the EAT-IT dietary pattern, as well as its effect on eating behavior, nutritional status and metabolic/functional markers through a 6-week crossover study.

CHAPTER 4. The optimization of healthy and sustainable diets involves also increasing the availability of evidence on the protective role of food products in relation to their bioactive components, abundantly present in plant-based diets. In this context, different activities have been performed in order to: i) analyze the literature and provide data on the vasoactive and protective properties of plant-based foods ii) evaluate the potential exploitation of well targeted *in vivo* studies for vulnerable segments/sections of the population, such as the older subjects; ii) based on previous findings, verify the hypothesis that the intake of an anthocyanin-rich blueberry cultivar can acutely improve endothelial function in older subjects, through a randomized, controlled, crossover trial.

3. RESULTS CHAPTERS

3.1 Chapter 1

- **Principles of sustainable healthy diets in worldwide dietary guidelines: efforts so far and future perspectives.**
  (Post print – published paper: doi: https://doi.org/10.3390/nu13061827)

- **An Italian-Mediterranean Dietary Pattern Developed Based on the EAT-Lancet Reference Diet (EAT-IT): A Nutritional Evaluation.**
  (Post print – published paper: doi: 10.3390/foods10030558)

- **Development of an approach to adapt the EAT-IT dietary pattern to different energy targets: assessment of potential nutritional issues associated to a 2000 kcal dietary plan.**
  (Unpublished results)

Review

3.1.1. Principles of sustainable healthy diets in worldwide dietary guidelines: efforts so far and future perspectives

Daniela Martini[1*], Massimiliano Tucci[1], James Bradfield[2], Antonio Di Giorgio[1], Mirko Marino[1], Cristian Del Bo'[1], Marisa Porrini[1*], Patrizia Riso[1]

[1]Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133 Milan, Italy

[2]NNEdPro Global Centre for Nutrition and Health, Cowley Road, Cambridge, CB4 0WS, UK

*Correspondence: daniela.martini@unimi.it; marisa.porrini@unimi.it

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Abstract: Food choices and eating behaviors have a large impact on both human and planetary health. Recently, the Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization have developed a list of 16 guiding principles to achieve sustainable healthy diets (SHDs). They proposed that development of food-based dietary guidelines (FBDGs) should be a core element in the implementation of these SHDs in each country. The objective of this review is to explore the degree of alignment of current FBDGs to these guiding principles. A total of 43 FBDGs, written or translated into English, were collected from the online repository developed by the FAO and were analyzed for their adherence to each of the guiding principles. Results were stratified for period of publication and geographical macro-area. Overall, there were high levels of inclusion of the factors related to health outcomes, especially in the more recent FBDGs. Conversely, environmental impact and socio-cultural aspects of diet were considered less frequently, especially in the older FBDGs. These results highlight the importance of revising FBDGs, especially to include emerging topics which represent the areas with the highest scope for improvement in the future

versions of FBDGs. Replication of the present study in the coming years will be worthwhile to monitor improvements in the adherence of global FBDGs to the guiding principles of SHDs. The attainments of such goal could promote a more rapid transition towards SHDs also highlighting pivotal research trajectories to increase adoption and evaluate impact on food system.

Keywords: food-based dietary guidelines; healthy diets; sustainability; environmental impact

1. Introduction

The Global Burden of Disease Study 2017 estimated that, in the adult population (older than 25 years), 22% of total deaths (11 million total) and 15% of disability-adjusted life-years (255 million total) are attributable to dietary risk factors (Afshin *et al.*, 2019). While it is well-established that food choice and eating behaviors represent two important determinants of human health, they may also have an impact on the health of the planet at large (Springmann *et al.*, 2018; Clark *et al.*, 2019). Related is the increasing evidence indicating that food systems have a significant impact on the environment, given they are responsible for almost 30% of total greenhouse gas emission in developed countries (Vermeulen *et al.*, 2012; Crippa *et al.*, 2021). Furthermore around 40% of the Earth's total land surface is currently used for agricultural practices while it also accounts for approximately 70% of total freshwater use (Smith *et al.*, 2008; Whitmee *et al.*, 2015). As global populations continue to grow, it is estimated that the impact of diet on the environment will likely see an associated rise (Tilman and Clark, Whitmee *et al.*, 2015). Thus, the measure of how healthy a diet is must be viewed through a double lens of human and planetary health, with the latter a considerable challenge (Clark *et al.*, 2019; FAO and WHO, 2019; Willett *et al.*, 2019).

Nutritionally inadequate diets represent a major driver of current climate change and will likely also exacerbate malnutrition, food insecurity and hunger, increasing the disease burden attributable to food availability and nutrition (FAO and WHO, 2019; Kim *et al.*, 2020). Undernutrition, obesity, climate change and fresh water depletion have been described as interconnected global challenges, with food systems an important underlying driver (Lake *et al.*, 2012). Thus, the definition and the implementation of healthy and sustainable diets are pivotal steps to tackle these critical issues. This has been recognized on a global level, with the concept of sustainability significantly represented in the United Nations (UN) Sustainable Development Goals (SDGs) (2020). The SDGs are a call for action by all countries, defined by UN in 2015, and consisting in 17 goals and 169 targets to promote prosperity while protecting the planet (UN 2015).

Recently, the Food and Agricultural Organization (FAO) of the UN and the World Health Organization (WHO) have developed a list of 16 guiding principles related to sustainable healthy diets (SHD), targeted at governments and other stakeholders in policy-making and communications, to address the implementation of these issues (FAO and WHO, 2019). These 16 principles are divided into three categories: health aspects, environmental impact and sociocultural aspects. In this document the FAO and WHO also clearly state that one of the actions required to make SHD available, accessible, affordable, safe and desirable, is the development of national food-based dietary guidelines (FBDGs) according to these principles.

FBDGs are an attempt to condense a large body of scientific evidence, translating the relations between dietary patterns and health into specific, culturally appropriate, and actionable recommendations (Montagnese *et al.*, 2015; Herforth *et al.*, 2019; Carrillo-Álvarez *et al.*, 2020). Since FBDGs are primarily intended for consumer information and education, they should be simple to understand and implement (Brown *et al.*, 2011). The development of national/regional FBDGs is promoted given the potential to improve human health alongside economically advantageous outcomes (Van't Erve*et al.*, 2017). For FBDGs to be effective, they must be nationally- or even regionallyspecific, making use of local data on consumption, dietary habits, customs and disease burden (Albert *et al.*, 2007). For this reason, each region or country must create tailor-made FBDGs to provide appropriate advice according to the needs of that particular population (FAO/WHO, 1992; Albert, 2007).

In 1992, during the International Conference on Nutrition, the FAO and the WHO encouraged governments to develop FBDGs as an important action to promote appropriate diets and healthy lifestyles (FAO/WHO, 1998). Subsequently, in 1995, FAO and WHO elaborated and published the first general process for developing FBDGs (EFSA, 2010), which still remains a point of reference (Brown *et al.*, 2011). Later, in 2010, the European Food safety Authority (EFSA) provided further guidance on developing FBDGs for the diverse populations of Europe, following an approach consisting of 7 steps (Gonzalez Fischer and Garnett, 2016). In 2005 the WHO reported that 75 countries, 33 of which were in Europe, had developed FBDGs (Albert *et al.*, 2007). FBDGs must be periodically updated to account for advancements in nutritional research or changes in the needs of populations (CREA, 2018). For example, Italy, recently published the latest edition of its FBDGs (December 2018), dedicating an entire chapter to sustainability (Duchin, 2008). Thus, it is worth considering if the Italian, and other worldwide FBDGs, make reference to the principles of healthy and sustainable diets, as recently defined by FAO and WHO.

Based on these observations, the aim of this review was to explore if Italian and other available FBDGs considered the 16 guiding principles for SHDs. This evaluation will allow (i) investigation of the current efforts made by different countries in fostering the adoption of healthy and sustainable diets, and (ii) identification of possible gaps that should be considered in the next revisions of worldwide FBDGs.

2. Materials and Methods

2.1. Selection and collection of dietary guidelines

In this study, we selected FBDGs of different countries using the online repository developed by FAO (Food and Agriculture Organization of the United Nations (FAO), no date), where the majority of available world FBDGs can be reviewed. This repository was created in 2014 and since then it has been continually updated to include every new revision (or first edition) of a country's FBDG. In this repository the countries are divided into geographical macro-areas (i.e. Africa, Asia and Pacific, Near East, Europe, Latin America and the Caribbean, and North America).

The FBDG of each country listed by the FAO was collected from the repository however a further check for updates not listed was also completed. The search was performed in October 2020 and was updated in February 2021.

For this analysis, the only inclusion criterion was the availability of an English version of the FBDGs. An FBDG that was not in English but provided an English summary of the FBDG was also accepted. Conversely, exclusion criteria were: (i) FBDGs constituted only of a one-page informative poster or brochure; (ii) FBDGs only available in a language other than English (iii) FBDGs addressing only the diets of children. For each country, only the most recent version of the FBDGs was considered. In cases where multiple versions existed for use by the general population and professionals/policy makers, only the former was considered. Finally, for each selected FBDG, only the guidelines addressing the generally healthy population were included in the final evaluation, except for guiding principle n° 1, which specifically refers to infants.

2.4. Data collection from FBDG and comparison with the guiding principles for sustainable healthy diets

For each selected FBDG, the following information was collected: official name of the guideline, country, macro-area, year of the most recent revision and key messages. In case of reprint, the date of first publication of the FBDG was used for the analysis.

To assess the levels of adherence of each FBDG included in the analysis with the 16 guiding principles defined by FAO and WHO, each FBDG was searched for information or key messages matching with the relative guiding principles, listed in Table 1.

More specifically, key messages were defined for each guiding principle, considering the three main classes of guiding principles for SDHs proposed by the FAO and the WHO: (i) regarding health aspects; (ii) regarding environmental impact; (iii) regarding sociocultural aspects.

For each FBDG, the key messages were analyzed for adherence with the 16 principles included in these three classes of principles of SHD. This analysis was performed by two independent reviewers (M.T., and A.D.G.). When needed, disagreement between reviewers was solved through consultation with a third reviewer (D.M.) to reach a consensus.

Results were then summarized, stratifying by macro-areas and by year of publication. When describing time of publication, three strata were used: i) those published up to 2010; ii) those published between 2011 and 2015; iii) those published since 2016. The macro-areas were represented by Africa, Asia and Pacific, Near East, Europe, Latin America and the Caribbean, and North America as reported in the online repository by FAO (Food and Agriculture Organization of the United Nations (FAO), no date).

However, due to the high variability in the number of countries for each macro-area, North America and Latin America were grouped together, as well as Asia and the Pacific, and the Near East. Thus, macro-areas were finally grouped as: i) Africa; ii) Asia and Pacific, and Near East; iii) Europe; iv) North and Latin America.

3. Results

3.1. Dietary guidelines: geographical distribution and period of publication

The number and the period of publication of the FBDGs included in the present evaluation are reported in Figure 1.

A total of 43 FBDGs were available in English on the FAO online repository. Fourteen FBDGs (33%) were developed in the most recent category (since 2016), while the majority were less recent: eighteen (42%) were developed between 2011-2015 and eleven (26%) were developed before 2010. Regarding the geographical macro-area, most FBDGs were published by European countries (14 FBDGs), followed by Near East, Asia and Pacific (n=16, including Australia and New Zealand), North and Latin America (n=9) and Africa (n=5).

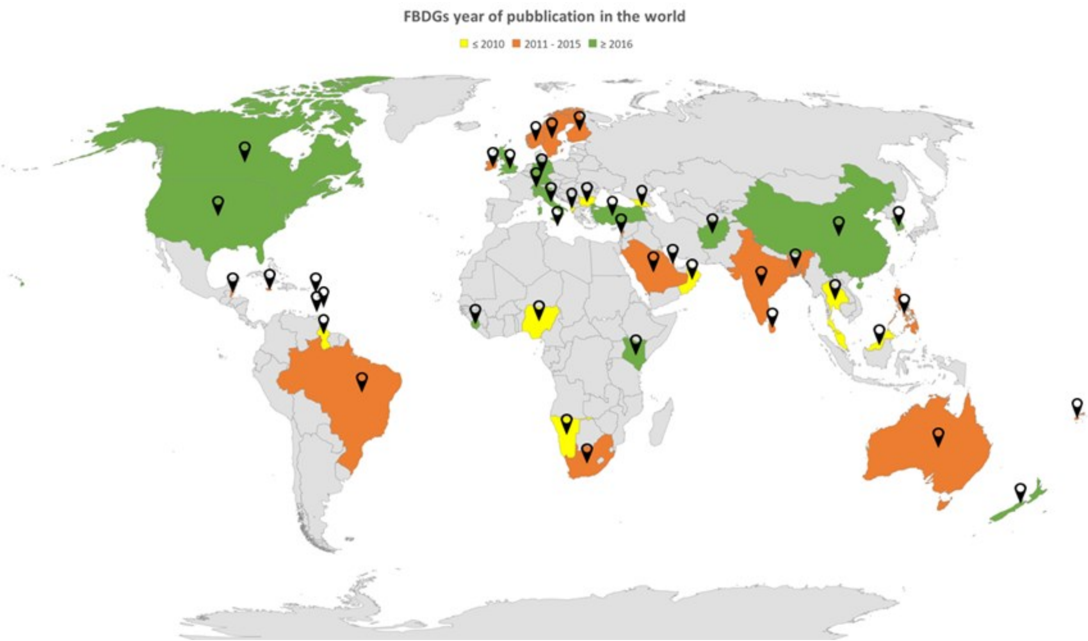

Figure 1 - Geographical distribution and period of publication of the dietary guidelines. Legend: yellow: up to 2010; orange: 2011-2015; green: after 2016.

3.2 Compliance of total FBDGs to the guiding principles for sustainable healthy diets

Guiding principles for SHDs and the compliance of the available FBDGs are presented in Table 1. Analysis revealed a high level of compliance from FBDGs with health-related guiding principles, but a critically low level of compliance with principles related to environmental impact and sociocultural aspects. More specifically, the guiding principles related to health aspects (i.e. principles 1-8) were included a minimum of 56% of the time for principle n° 1, up to a maximum of 95% and 98%, for principles n° 2 and 3, respectively. Conversely, aspects related to the environmental impact (i.e. principles 9-13) were included at far lower rates, ranging from 5% (principle n°11) to 23% for the principle n° 13. Finally, we found low levels of compliance with guiding principles related to socio-cultural aspects (i.e. principles 13-16), ranging from a minimum of 7% for principle n° 16 to a maximum of 53% for principle n° 14.

Category	Number	Recommendation	N° of FBDGs including the principle	% (*n* = 43)
Health aspects	1)	Sustainable healthy diets start early in life with early initiation of breastfeeding, exclusive breastfeeding until six months of age, and continued breastfeeding until two years and beyond, combined with appropriate complementary feeding.	24	56%
	2)	Sustainable healthy diets are based on a variety of unprocessed or minimally processed foods, balanced across food groups, while restricting highly processed food and drink products.	41	95%
	3)	Sustainable healthy diets include wholegrain, legumes, nuts and an abundance and variety of fruits and vegetables.	42	98%
	4)	Sustainable healthy diets can include moderate amounts of eggs, dairy, poultry and fish; and small amounts of red meat.	32	74%
	5)	Sustainable healthy diets include safe and clean drinking water as the fluid of choice.	33	77%
	6)	Sustainable healthy diets are adequate (i.e. reaching but not exceeding needs) in energy and nutrients for growth and development, and to meet the needs for an active and healthy life across the lifecycle.	29	67%

	7)	Sustainable healthy diets are consistent with WHO guidelines to reduce the risk of diet related NCDs, and ensure health and wellbeing for the general population.	31	72%
	8)	Sustainable healthy diets contain minimal levels, or none if possible, of pathogens, toxins and other agents that can cause foodborne disease.	29	67%
Environmental impact	9)	Sustainable healthy diets maintain greenhouse gas emissions, water and land use, nitrogen and phosphorus application and chemical pollution within set targets.	8	19%
	10)	Sustainable healthy diets preserve biodiversity, including that of crops, livestock, forest-derived foods and aquatic genetic resources, and avoid overfishing and overhunting.	4	9%
	11)	Sustainable healthy diets minimize the use of antibiotics and hormones in food production.	2	5%
	12)	Sustainable healthy diets minimize the use of plastics and derivatives 11 in food packaging.	3	7%
	13)	Sustainable healthy diets reduce food loss and waste.	10	23%

	14)	Sustainable healthy diets are built on and respect local culture, culinary practices, knowledge and consumption patterns, and values on the way food is sourced, produced and consumed.	23	53%
Sociocultural aspects	15)	Sustainable healthy diets are accessible and desirable.	17	40%
	16)	Sustainable healthy diets avoid adverse gender-related impacts, especially with regard to time allocation (e.g. for buying and preparing food, water and fuel acquisition).	3	7%

Table 1 - Guiding principles for healthy and sustainable diets. Legend: FBDGs= food based dietary guidelines; WHO= World Health Organization; NCDs= non-communicable diseases.

3.3 Compliance of FBDGs with guiding principles for sustainable healthy diets, by publication time and geographical macro-area

3.3.1 Health aspects

The inclusion of the guiding principles for SHD related to health aspect (i.e. principles 1-8), by period of publication and geographical macro-area, is summarized in Figure 2.

Regarding differences based on time of publication, these guiding principles were more widely addressed in the more recent FBDGs (Fig. 2A). All principles were most often considered in the FBDGs produced between 2011-2015 and those produced since 2016. For instance, principle n°2 was included in 100% of FBDGs published between 2011-2015 and since 2016, and 82% in the period before 2010. The only exception was for guiding principle n° 8, related to food safety and hygiene (i.e. SDHs contain minimal levels, or none, if possible, of pathogens, toxins and other agents that can cause foodborne disease) that was most often considered in the FBDG published before 2010 (73%) compared to those of the other two time periods (67%).

Regarding geographical macro-areas (Fig. 2B), a high variability was observed among the rate of inclusion of the guiding principles in the 4 different macro-areas. The lowest rate of inclusion was in the American FBDGs (on average 58% of inclusion) and the highest in the ones from the "Near East, Asia and the Pacific" area (84%). Intriguingly, principle n°1 relating to breastfeeding, was considered in 75% and 80% of the FBDGs published in African and Asia and Pacific, and Near East, respectively, but only in the 38% and 33% of the European and American versions, respectively. Conversely, only 2 American FBDGs (22%) considered principle n°6 that was included in the 92% and 75% of the European Asiatic FBDGs and in those from Asia, Pacific, and Near East, respectively.

Figure 2. - Rate of inclusion of the guiding principles for sustainable healthy diets related to health aspects by the 43 FBDGs included in the evaluation, divided by period of publication (A) and geographical macro-area (B).

3.3.2 Environmental impact

The inclusion of the guiding principles for SHD related to environmental impact (i.e. principles 9-13) by period of publication and geographical macro-area, is summarized in Figure 3.

In short, these guiding principles were more widely considered in the more recent FBDGs (i.e. between 2011-2015 and since 2016) compared to the older versions (Fig. 3A). In fact, none of the FBDGs published before 2010 included in this analysis considered these 5 aspects, while they were considered on average by 19% and 14% of the FBDGs published between 2011-2015 and since 2016, respectively. The principles included in the lowest number of FBDGs were principles n°11 (included in 11% and 0% of FBDGs published between 2011-2015 and since 2016, respectively) and n°12 (11% and 7% in the two time periods, respectively). Conversely, among principles related to environmental impact, n°13 was considered in the highest number of FBDGs (22% and 43% in the two time periods, respectively) followed by principle n°9 (33% and 14% respectively).

Dividing results by geographical macro-areas (Fig. 3B), African FBDGs showed the lowest rate of inclusion, with only one country including one of the 5 guiding principles. Conversely, the highest rate of inclusion was observed in the European ones, on average considered in the 20% of FBDGs.

Figure 3 - Rate of inclusion of the guiding principles for sustainable healthy diets related to environmental impact by the 43 FBDGs included in the evaluation, divided by period of publication (A) and geographical macro-area (B).

3.3.3 Socio-cultural aspects

The inclusion of the guiding principles for SHD on socio-cultural aspects by period of publication and geographical macro-area, is summarized in Figure 4.

In general, socio-cultural aspects (principles 14-16) were considered in few FBDGs, in a manner similar to what observed for environmental impacts. Percentages of FBDGs including these principles ranged from 7% for principle n°16 to 53% for n°14. Like environmental aspects, these principles were mostly considered in FBDGs published between 2011-2015 and since 2016, compared to those published before 2010 (Fig. 4A). For instance, principle n°14 was considered in only 27% of FBDGs published before 2010, but in the 61% and 64% of those published between 2011-2015 and since 2016, respectively.

In relation to geographical distribution, the lowest rate of inclusion was observed in the European countries where on average the guiding principles were considered in the 15% of the FBDGs (ranging from 0% of the principle n°16 to 23% for principle n°14) while American and African FBDGs showed the highest rate (52% and 47%, respectively) (Fig. 4B).

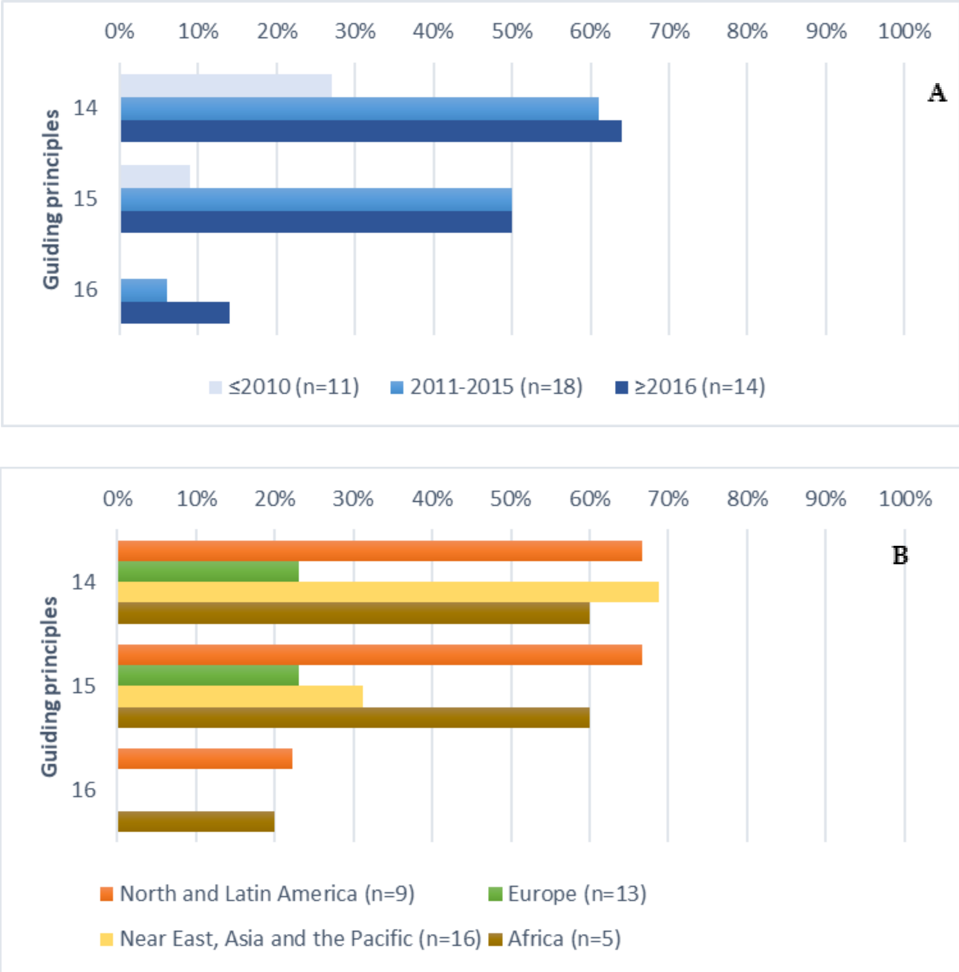

Figure 4 - Rate of inclusion of the guiding principles for SHD related to socio-cultural aspects by the 43 FBDGs included in the evaluation, divided by period of publication (A) and geographical macro-area (B).

4. Discussion

The FAO and WHO have previously suggested that the development of FBDGs may represent a useful action for the implementation of SHDs, taking into consideration the increasing evidence supporting the relationship between nutrition, human health and environmental impact (Duchin, 2008; FAO, 2010; Johnston *et al.*, 2014). Accordingly, it is interesting to investigate previous efforts made to include sustainability and socio-cultural issues in FBDGs, to ultimately highlight the main gaps which exist that should be addressed in the future revisions.

In the present review, we aimed to provide an overview of worldwide FBDGs, investigating the extent to which FBDGs address the guiding principles for SHDs proposed by the FAO and WHO. The analysis of key messages within the 43 FBDGs included in the present evaluation highlighted that, as expected, the principles related to the health aspects of the diet were considered by a large majority of FBDGs. As already observed, this is likely because disease prevention and nutrient recommendations were considered the most pressing issues in the establishment of FBDGs in the past decades (Bechthold *et al.*, 2018).

Interestingly, there was greater adherence with all principles in the most recent FBDGs, highlighting the importance of revising these documents, taking into consideration new scientific evidence, social developments and emerging topics that may affect dietary behavior.

Despite overall high levels of compliance of FBGDs with health aspects, guiding principle n°1, relating to breastfeeding and complementary feeding, was included in only 56% of total FBDGs. These were mostly by African countries and countries of the “Asia and Pacific, and Near East” macro-area. This point could represent a possible gap in the current FBDGs that should be included in the future revisions. Poor knowledge about breastfeeding factors contributes to the low rates of exclusive breastfeeding in many countries around the world and FBDGs may represent a viable tool to facilitate a change in practice. In turn, this may help to achieve the goal of having at least 50% of children exclusively breastfed for the first six months of life (WHO/UNICEF, 2012).

Intriguingly, the principle related to adequate energy intake was represented differently in the various macro-areas. Developed countries focused more on highlighting the importance of not exceeding energy intake and maintaining a healthy body weight, while developing countries tended to focus more on the prevention of undernutrition or nutritional deficiencies. However, it is noteworthy that despite the prevalence of undernutrition and the scale of the problem in developing countries, the prevalence of obesity is increasing and, in some countries, it has reached levels similar to that of developed countries (Abdullah, 2015). Thus, the co-existing double burden of over- and undernutrition should be paid more attention in the FBDGs, especially for countries affected.

Despite differences among countries, our analysis indicates that most FBDGs recommend adherence to plant-based dietary patterns, high in wholegrains, fruit and vegetables, nuts, and legumes, and with a low to moderate amounts of animal products like meat. As already observed by Herforth and colleagues (2019), the consumption of a variety of foods, with differing proportions of the various food groups, and the reduction of some specific nutrients (e.g. sugar and salt) appears nearly universal across guidelines, despite not all countries stating so explicitly.

Overall, this advice is largely comparable to the Mediterranean dietary pattern (Serra-Majem *et al.*, 2020), which has been reported also to have a lower environmental impact compared to the average current diet of the Italian population (Germani *et al.*, 2014; Coats *et al.*, 2020).

In comparison to health aspects, few FBDGs included instructions around environmental impacts of diet, with the lowest rates in Africa. These results are in line with those recently observed by Berthold and coworkers who have found that sustainability was considered only in the Swedish FBDGs while the other 33 dietary guidelines analyzed did not discuss the topic (Bechthold *et al.*, 2018). However, it is noteworthy that some revised versions of FBDGs have been developed in more recent years, including the last revision of the Italian FBDG that dedicated an entire chapter to aspects related to sustainability (e.g. reduction of food waste) (CREA, 2018). Overall, the poor alignment with environmental improvements may be related to the fact that some environmental principles are more easily implementable at a population level (e.g. the reduced use of plastic), compared to others (e.g. minimal use of antibiotics in food production) that require policy change.

Among the various environmental factors, guiding principle n° 13 related to food waste and losses, was included most frequently (23% of total FBDGs). It is estimated that food waste and losses account for about 15% of the total environmental impact of food production in Europe (Scherhaufer *et al.*, 2018), representing an associated economic loss for all actors throughout food supply chains. The majority of food waste occurs during production (73%) (Scherhaufer *et al.*, 2018), but since food waste also significantly occurs at individual and household levels, further efforts should be made to align FBDGs with this principle. Furthermore, reducing food waste is an additional target of the SDGs which have been agreed to by countries around the world (United Nations, no date).

We also found that overall, FBDGs do not align well with sociocultural aspects of food and diet, despite occurring more frequently than aspects related to the environmental impact. Several socio-cultural aspects can affect food choices and dietary behaviors. For instance, these include accessibility to shops and food sources, depending on resources such as transport and geographical location (Donkin *et al.*, 2000). Such factors influence food availability and their affordability. Indeed, it is reported that low-income groups tend to consume poorer quality diets, low in fruits and vegetables (Sanchez-Villegas *et al.*, 2003). However, it has also been reported that a healthy and environmentally sustainable diet can be affordable for groups of various economic prosperity, while these issues were strictly related to cultural and knowledge barriers (Dibsdall *et al.*, 2003; Reynolds *et al.*, 2019). In this regard, guiding principle n° 15, related to accessibility and desirability, has been included more widely in Africa, America and Near East, Asia and the Pacific rather than in Europe.

This is likely because there were more FBGDs from middle/low-income countries in this macro-area rather than in Europe, which emphasized affordability recommendation (e.g. “cultivate your own vegetable garden”). Other important determinants of food choices are related to the social setting and context (Feunekes *et al.*, 1998). Gender has been described as an important factor, depending on the general culture scenario, for instance with gender being the strongest determinant of time spent cooking (Riegelhaupt *et al.*, 2010). Gender equality is considered essential for a world free from hunger, malnutrition and poverty (Food and Agriculture Organization of the United Nations (FAO), 2020), thus the low level of alignment of FBDGs for this principle (addressed in only 7% of total FBDGs) shows that avoid adverse gender-related impacts, especially with regard to time allocation, should be hopefully considered in the next revisions of the FBDGs.

Overall, the FBDGs included in this analysis do not align well with the principles laid out by the FAO and WHO. This is true for all categories, especially those factors related to the environmental impact of diet and these factors represent the main areas that countries should see to improve in subsequent revisions of the FBDGs, especially considering that current FBDGs are not in line with a set of global environmental targets related to climate change and environmental resource use (Springmann *et al.*, 2020).

Our study has some limitations worth noting. First, we considered only FBDGs developed or translated in the English language, thus we cannot exclude the possibility that FBDGs available in other languages could have included the guiding principles to a greater degree. Secondly, we evaluated the presence of key messages within FBDGs to assess the alignment with the associated guiding principles, not considering the level of detail provided in the dietary recommendations.

However, our study has also several strengths. First of all, in contrast to other studies, we considered FBDGs from all over the world and not only from one continent or geographical area (Montagnese *et al.*, 2015; Coats *et al.*, 2019; Oliveira *et al.*, 2019). Additionally, we focused on several categories of factors related to health and sustainable diets, to provide a clear overview of the efforts made so far by the various countries included in the analysis. Furthermore, we stratified FBDGs to consider both time of publication and geographical macro-area. This approach allowed us to perform a comprehensive comparison of alignment of the FBDGs and to better elucidate which factors are the main drivers influencing the rate of inclusion of the different principles for SHDs.

5. Conclusions

In conclusion, the present review highlights that current FBDGs are poorly aligned with the guiding principles for SDHs, as proposed by FAO and WHO. This is especially true for factors related to socio-cultural aspects and environmental impacts of the diets. In the coming years, it would be useful to replicate this approach to monitor improvements in the alignment of FBDGs. It would also be worth investigating the effectiveness of these FBDGs in improving knowledge among the general population on this topic, as well as in improving dietary behaviours. In this regard, the availability of up to date FBDGs could help promoting a more rapid transition towards a more sustainable food system also supporting the development of multidisciplinary research enabling both the improvement – in terms of environmental and nutritional sustainability - of the food chain from production to consumption, and investigating how to increase adoption of healthy diets and evaluate their impact.

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Article

3.1.2 An Italian-Mediterranean Dietary Pattern Developed Based on the EAT-Lancet Reference Diet (EAT-IT): A Nutritional Evaluation

Massimiliano Tucci[1,†], Daniela Martini[1,†], Cristian Del Bo'[1], Mirko Marino[1], Alberto Battezzati[1,2], Simona Bertoli[1,2], Marisa Porrini[1] and Patrizia Riso[1,*]

[1]Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133 Milan, Italy; massimiliano.tucci@unimi.it (M.T.); daniela.martini@unimi.it (D.M.); cristian.delbo@unimi.it (C.D.B.); mirko.marino@unimi.it (M.M.); alberto.battezzati@unimi.it (A.B.); simona.bertoli@unimi.it (S.B.); marisa.porrini@unimi.it (M.P.).

[2]International Center for the Assessment of Nutritional Status (ICANS), Università degli Studi di Milano, 20133 Milan, Italy

[*]Correspondence: patrizia.riso@unimi.it

[†]Both authors contributed equally to this work.

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Abstract: There is an urgent need to promote healthy and sustainable diets that are tailored to the preferences and cultures of different populations. The present study aimed to (i) define a Mediterranean dietary pattern in line with the EAT-Lancet Commission reference diet (ELCRD), based on 2500 kcal/day and adapted to the Italian food habits (EAT-IT); (ii) develop a mid/long-term dietary plan based on EAT-IT and a dietary plan based on the Italian Dietary Guidelines (IDG); (iii) compare the two dietary plans in terms of portions, frequencies of consumption, and nutritional adequacy based on the nutrient and energy recommendations for the Italian adult population. The main differences between the two plans were related to the higher amount of fruit and vegetables in the IDG compared to the EAT-IT, while the EAT-IT plan was higher in nuts and legumes, which represent the main protein sources in the ELCRD. Differences in the protein sources, especially milk and derivatives, and for cereal-based foods, were also found. Dietary plans were comparable for most nutrients, except for higher energy from lipids and vegetal protein, a higher amount of fiber, and lower levels of calcium that were evidenced for the EAT-IT dietary plan compared to the IDG-based one. In conclusion, the analysis of the EAT-IT demonstrated certain nutritional issues. It remains to be determined whether this may represent a health concern in further studies aimed at investigating the feasibility of sustainable dietary patterns.

Keywords: healthy and sustainable diet; planetary healthy diet; nutrition; sustainability; nutritional adequacy; environmental impact; Mediterranean diet; dietary guidelines

1. Introduction

A large body of evidence demonstrates the role of poor diet on malnutrition and the potential impact of a suboptimal diet on mortality and morbidity for noncommunicable diseases. The Global Burden of Disease Study 2017 estimated that, in the adult population (older than 25 years), 22% of total deaths (11 million total) and 15% of disability-adjusted life-years (255 million total) across 195 different countries are attributable to dietary risks, such as the low intake of whole grain, fruits, nuts, vegetables, and omega-3 fatty acids and the excessive intake of sodium (Afshin *et al.*, 2019). In addition to the effect of a poor diet on human health, the growing degradation of natural resources led to an increased interest in evaluating the impact of food choices (and today's dietary guidelines) on planetary health (Meybeck *et al.*, 2017; FAO 2008; FAO 2017). Food systems indeed account for a large part of land and water use and of greenhouse gas emissions due to agriculture but also to processing, packaging, refrigeration, transport, retail, catering, domestic food management, and waste disposal (landfills) (FAO 2017; Pelletier *et al*., 2011; Vermeulen *et al*., 2012). In this scenario, there is an urgent need to promote healthy diets that at the same time have a low environmental impact, are socio-culturally acceptable, and are economically accessible, as highlighted by the Food and Agriculture Organization of the United Nations (FAO), which defines sustainable diets as "those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations. Sustainable diets are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resources" (FAO 2010). A large body of evidence suggests moving toward dietary plant-based diets that are effective in improving human health, and at the same time, in reducing environmental impacts (Springmann *et al*., 2018). However, it is noteworthy that this does not necessarily mean a shift to vegetarian and/or vegan diets, which some studies have shown to be nutritionally inadequate (Seves *et al*., 2017) and not always related to a lower ecological footprint compared to other diets that include modest amounts of animal-based foods (Collins *et al*., 2007). The report of the EAT-Lancet Commission on healthy diets from sustainable food systems (Willet *et al*., 2019) aimed to establish global targets that are useful for defining a safe operating space for food systems, enabling them to assess which diets and food production practices can help to ensure that the United Nations (UN) Sustainable Development Goals (2020) and Paris Agreement are achieved. The EAT-Lancet Commission Reference Diet (ELCRD) (see Appendix A) is mainly characterized by whole grains, vegetables, legumes, nuts, unsaturated oils, low amounts of seafood and poultry, and low or no red meat, processed meat, added sugar, refined grains, or starchy vegetables. Research has estimated that a shift from the current global diet to this healthy diet could prevent ≈24% of the total deaths from 2017 (Wang *et al*., 2019). A peculiarity of this report is the description of a universal healthy reference diet that was developed with the aim to provide a basis for estimating the health and environmental effects of adopting an alternative diet relative to the standard current diets. According to the Commission, this pattern allows for the flexible, global application of specific criteria within a safe operating space, with foods and amounts tailored to the preferences and cultures of different populations. For this reason, it has been referred to as a “Planetary health diet” since it can and should be adapted to develop meals that are consistent with food cultures and cuisines of the different countries, maintaining both healthiness and environmental sustainability (Willet *et al.*, 2019). Previous studies have developed applications of the ELCRD with the specific purpose to compare different dietary patterns. In detail, Lassen *et al*. (2020) proposed a culturally adapted ELCRD for the Denmark population through the adjustment of the energy target and portion size of different food categories to increase compliance with the Danish dietary guidelines. Sharma *et al*. (2020) compared the ELCRD indications for the food intake of rural and urban households, as well as poor and rich households, of different Indian regions, highlighting critical points and identifying actions to orientate policies. Differently, Blackstone and Conrad (2020) investigated how this pattern differs from American national guidelines, which currently do not include adjustments related to environmental sustainability. The authors highlighted that, despite some similarities between the EAT-Lancet and American dietary guidelines, where the latter recommend higher amounts of fruit, starchy vegetables, red meat, and discretionary calories and lower amounts of nuts, seeds, and whole grains compared to the ELCRD. To the best of our knowledge, no similar studies have been performed in Mediterranean countries, where, as reported by the EAT-Lancet Commission, the typical dietary pattern, i.e., the Mediterranean diet, has some features in common with the ELCRD, being a dietary plant-based diet with low red meat but high total fat intake mainly due to olive oil (D’Alessandro *et al*., 2019). Based on these premises, the present work pursued the following three objectives: (i) to develop a Mediterranean-based dietary pattern in line with the EAT-Lancet Commission reference diet, which was adapted by considering the Italian food habits and culture; (ii) to translate this dietary pattern into an example of a feasible and sustainable mid/long-term dietary plan that is able to cover nutrient requirements; (iii) to develop a similar dietary plan that is in line with the Italian Dietary Guidelines (IDG) (CREA, 2018); (iv) to compare the two dietary plans in terms of portions, frequencies of consumption, and overall nutritional adequacy for the Italian adult population.

2. Materials and Methods

The main steps that were taken to adapt the ELRCD-based dietary plan to Italian eating habits and to compare it with an IDG-based dietary plan are shown in Figure 1 and described in detail below. Overall, the process was divided into four main phases:

- Definition of the ELCRD adapted dietary pattern (named EAT-IT).
- Development of a mid/long-term dietary plan based on the EAT-IT dietary pattern.
- Definition of another dietary plan in line with IDG, which was useful as a basis for comparing nutritional intakes.
- Comparison of the EAT-IT-based and IDG-based dietary plans in terms of serving size, frequencies of consumption, and nutritional adequacy.

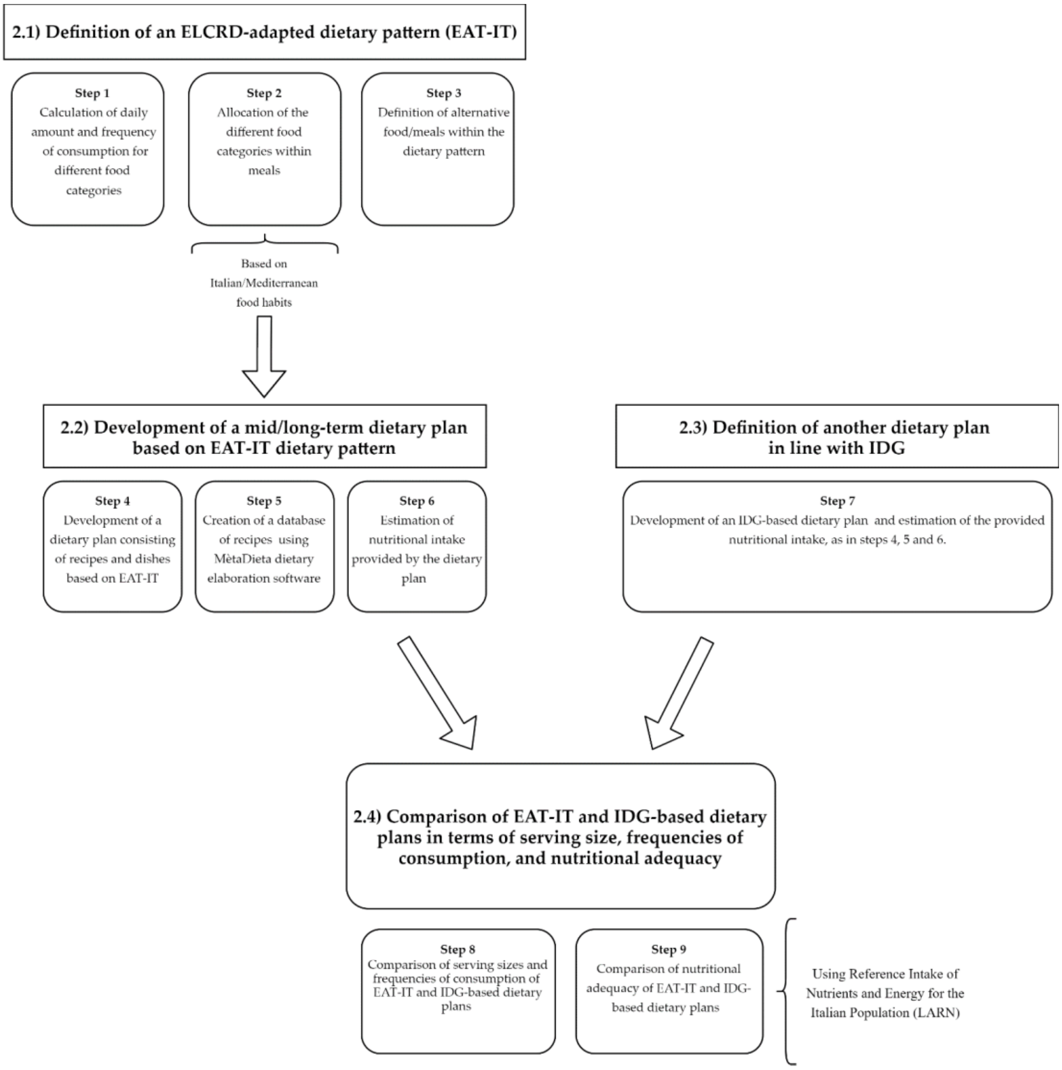

Figure 1 – Overview of the protocol used for the development and analysis of the dietary pattern based on the EAT-Lancet Commission Reference Diet (ELCRD) that was adapted by considering the Mediterranean/Italian food habits (EAT-IT). IDG: Italian dietary Guidelines.

2.1. Phase 1: Definition of the EAT-IT-Based Dietary Plan

This first phase consisted of three steps that were devoted to the definition of an ELCRD-adapted dietary pattern based on Mediterranean food habits. The

ELCRD provides the daily intake for eight different food categories (whole grain, tubers or starchy vegetables, vegetables, fruits, dairy foods, protein sources, added fats, and added sugars), which are expressed as both grams/day and kilocalories/day considering a diet of 2500 kcal/day (Willet *et al.*, 2019), while no information regarding the frequencies of consumption or other indications related to the meal preparation are provided. For these reasons, with the aim to develop a mid/long-term dietary pattern, the daily intakes were converted to weekly amounts (step 1) that were expressed as grams. Data were used to calculate feasible weekly frequencies of consumption, while the intake expressed as kilocalories was used to develop isocaloric alternatives within the same food category. Once we calculated the portion and the frequency of consumption for the eight categories, foods were allocated into different meals (step 2). This was performed in line with the indications from the Mediterranean food pyramid, which, for instance, includes cereal-based products and fruits and vegetables at every main meal. The final step (step 3) consisted of the development of a dietary pattern, putting together the meals designed in step 2 but providing alternatives that would allow for following this dietary pattern for the long term. The resulting scheme was named EAT-IT, as it was rearranged based on the Italian/Mediterranean food habits and in line with the indications from the EAT-Lancet Commission.

2.2. Phase 2: Simulation of an EAT-IT-Based Dietary Plan

The second phase included three steps that were devoted to the development of a dietary plan, which was based on the dietary pattern planned as described in Section 2.1 and considering 2500 kcal/day as the target energy intake. First, recipes and dishes, based on traditional and generally consumed recipes that are eaten by the Italian population, were constructed based on the EAT-IT scheme and considering an adequate number of alternatives/substitutions with the purpose to cover a mid-to-long period (i.e., several months/a season) (step 4). For instance, for protein sources, several recipes were used to offer an adequate number of alternatives, with the aim to increase the variability of the dietary plan and to simulate a situation in which the consumers can choose different dishes based on their food preferences and ingredient availability. The number of alternatives proposed was greater for food categories with higher frequencies of consumption in traditional Italian meals. These recipes were then used to create a database that was elaborated upon using software for nutritional assessment (MètaDieta professional 4.1.1 METEDA Srl–Roma, Italy) to simulate a monthly dietary plan that is consistent with Italian habits (step 5). Specifically, five meals per day were considered (breakfast, lunch, dinner, and mid-morning and midafternoon snacks), including a "typical" Italian breakfast (Marangoni *et al*., 2009) made of a cereal-based food, a dairy product, and jam or juice, as well as snacks (Marangoni *et al*., 2019) consisting of nuts. Once developed, the dietary plan was analyzed for the nutritional characteristics in terms of energy and macro- and micronutrients, and finally provided as daily amounts in kilocalories, grams, milligrams, or micrograms, depending on the nutritional component (step 6).

2.3. Phase 3: Simulation of a Dietary Plan in Line with IDG

This third phase of the process was aimed at defining a dietary plan that was developed based on the IDG, similar to what was performed in Section 2.2. Toward this aim, in step 7, we performed an elaboration of a mid/long-term dietary plan following the same procedure that was used for elaborating the EAT-IT plan.

2.4. Phase 4: Comparison of the Portions, Frequencies of Consumption, and Nutritional Adequacy of the EAT-IT- and IDG-Based Dietary Plans

The final phase of this process was devoted to the comparison of the newly developed IDG-based dietary plan with the EAT-IT plan in terms of the serving sizes, frequencies, and nutritional adequacy. Specifically, in step 8, the EAT-IT serving sizes and frequencies of consumption were compared with those proposed in the last edition of the IDG and referring to the target energy intake of 2500 kcal. Finally, the nutritional adequacy of the two developed dietary plans was analyzed (step 9). The comparison was made by considering the nutritional adequacy of the two dietary plans, i.e., their ability to reach the target intakes and to cover the reference values for energy and macro- and micronutrients as defined for Italian adults with 2500 kcal as the recommended energy intake (both men and women), using the Levels of Reference Intake of Nutrients and Energy for the Italian Population (LARN) as a reference (SINU, 2014). In detail, data were compared with reference intakes (RIs, for carbohydrates and lipids). The average requirements (ARs) and the population reference intake (PRI) were instead used when available (i.e., for protein, all vitamins except vitamin E, and all minerals except sodium, chlorine, and potassium). The AR represents the level of nutrient intake sufficient to satisfy the needs of 50% of healthy subjects of a specific group of a population while the PRI refers to the amount needed to cover 97.5% of the population, thus providing important information for nutritional assessment. For the micronutrients for which AR was not available, adequate intake (AI) was considered (vitamin E, sodium, chlorine, and potassium). When available (i.e., for saturated fatty acids (SFA), cholesterol, sugar, and fiber), the suggested dietary target (SDT) was also taken into account for nutritional evaluation.

3. Results

3.1. Definition of the EAT-IT Dietary Pattern and Simulation of a Consistent Dietary Plan

As described in the Materials and Methods section (see Section 2.1), step 1 was devoted to the calculation of the daily or weekly servings for the different foods based on the ELCRD model. These portions, expressed as grams/week, were then used to calculate feasible weekly frequencies of consumption. For instance, for the subcategory "eggs" (with a daily intake of 13 g/day based on the ELCRD), 91 g/week were calculated, which corresponded to about two eggs of 50 g per week. In addition, the food portions were expressed in kilocalories/week to develop isocaloric alternatives within the same food category: for instance, 250 mL of whole milk was considered equivalent to 330 mL of semi-skimmed milk. In step 2, the different foods were allocated in the daily meals (i.e., breakfast, lunch, dinner, and snacks) according to the Mediterranean food pyramid (D'Alessandro *et al.*, 2019), as summarized in Tables 1 and 2, in which meal compositions and possible alternatives are reported. By using combinations of such meals, the EAT-IT dietary pattern was developed (step 3) to be adherent to the ELCRD indication but adapted to the Italian dietary habits and recipes, and sufficiently varied to be followed for a relatively long period. In detail, the single meals were organized as follows: (1) breakfast included a source of whole grains (e.g., oat flakes or wholemeal rusks), milk or derivatives, and added sugars (e.g., jam or fruit juice); (2) lunch and dinner were composed of a source of whole grains (e.g., brown rice, corn, or wholemeal pasta) or starchy vegetables (i.e., potatoes), a protein source (e.g., legumes, chicken and poultry, beef, lamb and pork, or fish), vegetables, fats (mainly extra virgin olive oil), and a portion of fruit at the end of each meal; (3) snacks (twice a day) consisting of a portion of nuts. Once we defined the EAT-IT dietary pattern, a complete dietary plan with recipes and alternatives was developed to simulate a real-life diet with meals and preparations that were chosen to include all foods in the defined servings and frequencies but remaining consistent with the Italian culinary tradition (steps 4 and 5). For example, to facilitate the use of large amounts of legumes, traditional recipes already present in Italian food habits, such as "pasta e fagioli" (i.e., pasta with "borlotti" beans) or "riso e bisi" (rice with peas), were included (data not shown). The newly developed dietary plan was finally evaluated in terms of the nutritional characteristics, as described below (Section 3.3).

Table 1. Reference scheme for breakfast and snacks.

(1A) Breakfast		
Food Category	**Portion Size**	**Possible Alternatives**
(a) Dairy	155 kcal	250 mL whole milk 330 mL semi-skimmed milk 230 g whole milk yogurt 350 g semi-skimmed yogurt 20 g butter
(b) Cereal	170 kcal	45 g cornflakes 45 g rusks (5 slices) 75 g whole bread
(c) Sugars*	120 kcal	50 g jam 220 mL fruit juice
(1B) Snack		
Food Category	**Portion Size**	**Possible Alternatives**
Nuts	290 kcal	Almonds—50 g Peanuts—50 g Pistachios—50 g Walnuts—40 g

Breakfast (1A) is composed of a portion of (a) dairy, (b) cereals, and (c) sugars. Snacks (1B) are composed of a portion of nuts. The amount of nuts indicated in the 1B scheme for snacks can be eaten as a single snack or on two occasions per day, halving the total amount indicated in the table for each occasion. * The EAT-Lancet Commission reference diet indicates 25 g of sugars per day, which was considered as “free sugars” that is “all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer, plus sugars naturally present in honey, syrups, and fruit juices,” as indicated by the World Health Organization (WHO).

Table 2. Reference scheme for meals.

(a) Whole Grains			
Lunch (400 kcal)		Dinner (275 kcal)	
Food	**Theoretical Portion**	**Food**	**Theoretical Portion**
Brown rice (364 kcal/100 g)	120 g	Brown rice (364 kcal/100 g)	80 g
Spelt (335 kcal/100 g)	120 g	Spelt (335 kcal/100 g)	80 g
Whole pasta (355 kcal/100 g)	120 g	Whole pasta (355 kcal/100 g)	85 g
Corn (365 kcal/100 g)	110 g	Corn (365 kcal/100 g)	80 g
Common bread (275 kcal/100 g)	180 g	Common bread (275 kcal/100 g)	120 g
		Potatoes (78 kcal/100 g)	325 g

(b) Protein Sources				
Food	**Energy to Create Harmonized Portion**	**Theoretical Average Energetic Density**	**Weekly Consumption**	**Theoretical Portion**
Beef, lamb, and pork	215 kcal	214 kcal/100 g	1 time	100 g
Chicken and other poultry	215 kcal	214 kcal/100 g	2 times	100 g
Eggs	160 kcal	146 kcal/100 g	1 time (2 eggs)	125 g
Fish	150 kcal	143 kcal/100 g	2 times	105 g
Legumes (dried)	245 kcal	379 kcal/100 g	8 times	65 g

Others	
(c) Vegetables	Approximately 40 kcal (150 g/meal)
(d) Oil and seasoning	Approximately 25 g/meal mainly from food sources rich in unsaturated fatty acids, such as extra-virgin olive oil
(e) Fruit	Approximately 60 kcal (100 g/meal)

Meals (lunch and dinner are composed of a portion of (a) whole grains, (b) protein sources, (c) vegetables, (d) oil and seasoning, and (e) fruit. The main meals, namely, lunch and dinner, include (i) a portion of cereals or potatoes, (ii) a portion of a protein source in relation to their calculated frequencies of consumption, (iii) a portion of vegetables, (iv) oil and seasoning, and (v) a portion of fruit to end the meal. This table has no prescriptive value; thus, lunch and dinner can be interchanged. This table can be theoretically used to compose complete dishes (e.g., "pasta al ragù" + fruit) or a meal composed of a single portion of each component (omelet with spinach, whole bread, and fruit).

3.2. Comparison of the Serving Sizes and Frequencies of Consumption between the Italian Dietary Guidelines and the EAT-IT Dietary Patterns

As described in Section 2.4, the indications provided by the last edition of the Italian Dietary Guidelines (CREA, 2018) in terms of both serving sizes and frequencies of consumption were used to develop a parallel dietary plan (step 7, data not shown). This IDG-based dietary plan was compared with the EAT-IT dietary plan, as reported in Table 3.

Interestingly, one of the main differences between the two dietary patterns was related to the amount of fruits and vegetables provided, which was lower in the EAT-IT compared to the IDG dietary plans (overall 200 g/day vs 450 g/day for fresh fruits and overall 300 g/day vs up to 600 g/day of fresh vegetables, respectively). Large differences were also observed for protein sources, with a lower amount of chicken meat in the EAT-IT, compared to the

IDG (overall 200 g/week vs 300 g/week), as well as fish (200 g/week vs 300 g/week), eggs (four medium eggs of 50 g/week vs two medium eggs), and milk and derivatives (overall 375 mL/day of milk or yogurt vs 250 mL/day). The Italian guidelines suggest three portions of cheese/week, while cheese is only included as an alternative for milk in the EAT-IT.

Conversely, compared to the IDG, the EAT-IT provided a higher quantity of legumes (eight portions/week vs three portions of 150 g of fresh legumes or 50 g of dried legumes/week) and nuts (45 g day vs 2.5 portions of 30 g), while red meat was com- parable (100 g/week). The comparison for “cereals and derivatives” was tricky since the indications are different in the two schemes, for example, with the Italian guidelines suggesting 4.5 portions of bread/day (for a total of 225 g/day) and one portion/day of 120 g of pasta, rice, corn, and other cereals, while the EAT-IT potentially allowed for bread up to a maximum of 300 g/day or cereals twice a day (for a maximum daily amount of 190 g).

The IDG also includes indications related to the intake of sweet bakery products, which are not specifically included in the EAT-IT dietary pattern. Therefore, a comparison was not easy due to the substantial differences in the nature of the indications provided for this category. Finally, an important difference between the two dietary plans was related to further indications provided by the IDG that were not present in the EAT-IT, e.g., suggestions of equivalences for dried fruit and portion and frequencies of consumption for leaf salads, preserved fish, and water.

Table 3. Comparison between the suggested portions in the Italian dietary guidelines for healthy eating (for a 2500 kcal diet) and the EAT-IT dietary plan (i.e., the ELCRD tailored to consider Italian food habits), which was developed based on the planetary health diet.

		Italian Guidelines	EAT-IT Dietary Pattern
Food Group	**Food Subcategory**	**Daily or Weekly Portion**	
Cereals and derivatives	Bread	4.5 portions/day of 50 g (225 g/day)	≠ Max daily amount of whole grain bread of about 375 g
	Pasta, rice, corn, spelt, and barley	1.5 portions/day of 80 g (120 g/day)	≠ Max daily amount of about 200 g
	* Bread substitutes (rusks, crackers, and breadsticks)	1 portion/week of 30 g (30 g/week)	≠ About 45 g of rusks (five slices) can be eaten at breakfast
	* Sweet bakery products (brioche, croissants, and biscuits)	2 portions/week of 50 g for croissants or cake or 30 g/week for biscuits (100 or 60 g/week)	≠ Sweet products can be eaten at breakfast and are indicated as “sugars and other sweeteners”
	* Breakfast cereals	2 portions/week of 30 g (60 g/week)	≠ About 45 g of breakfast cereals can be eaten at breakfast

Tubers	Potatoes	2 portions/week of 200 g (400 g/week)	↓ 1 portion/week of 325 g (325 g/week)
× Fruits	Fresh fruits	3 portions/day of 150 g (450 g/day)	↓ 200 g/day
	Dried fruits	3 portions/day of 30 g (90 g/day)	n.s
× Vegetables	Fresh vegetables	3 portions/day of 200 g (600 g/day)	↓ 300 g/day
	Leaf salad	3 portions/day of 80 g (240 g/day)	n.s.
Meat	* Red meat (beef, pork, and sheep meat)	1 portion/week of 100 g (100 g/week)	Beef, lamb, or pork—100 g/week (100 g/week)
	White meat (chicken, turkey, or rabbit)	3 portions/week of 100 g (300 g/week)	↓ Chicken and other poultry—2 portions of 100 g/week (200 g/week)
Fishery	Fish (including mollusks and crustaceans)	3 portions/week of 150 g (450 g/week)	↓ Fish—2 portions/week of 105 g (210 g/week)
	* Preserved fish (e.g., canned tuna)	1 portion/week of 50 g (50 g/week)	n.s.
Egg	Egg	4 medium eggs/week (200 g/week)	↓ 1 portion/week of 2 medium eggs (125 g/week)

× Legumes	Fresh legumes or canned	3 portions/week of 150 g (450 g/week)	↑ 8 portions/week of 65 g of dried legumes—about 200 g of fresh legumes (520 g or 1560 g/week)
	Dried legumes	3 portions/week of 50 g (150 g/week)	
× Milk and derivatives	Milk	3 portions/day of 125 mL (375 mL/day)	↓ 1 portion/day of 250 mL of milk or other isocaloric equivalences of milk derivatives (e.g., yogurt, butter, etc.) (250 mL/day)
	Yogurt and other fermented milk	3 portions/day of 125 g (375 mL/day)	
	Cheese (fat <25% and less than 300 kcal/100 g)	3 portions/week of 100 g (300 g/week)	
	Cheese (fat >25% and more than 300 kcal/100 g)	3 portions/week of 50 g (150 g/week)	
× Fats and seasoning	Vegetable oil (e.g., extra virgin olive oil and seed oil)	4 portions/day of 10 mL (40 mL/day)	↑ 50 g/day of added fats, preferably from dietary plant sources. Butter is excluded because it is already included in the milk and derivatives food category
	Butter and other animal fats	4 portions/day of 10 g (40 g/day)	

Nuts and seed	Walnuts, peanuts, almonds, seeds, etc.	2.5 portions/week of 30 g (75 g/week)	↑ 40–50 g/day
Water	Water	At least 10 glasses of 200 mL/day (2 L/day)	n.s.

Legend: n.s.: not specified. ×: the portions reported for the food included in that category are alternatives and not additive (e.g., for "fruits," 150 g of fresh fruit OR 30 g of dried fruit); *: subcategory for which it is possible to have a lower frequency of consumption and increasing the consumption of other foods from the same category, according to the Italian dietary guidelines (IDG). ≠: food category with different recommendations between the IDG and EAT-IT but not clearly definable in terms of whether the amount is higher, equal, or lower. ↑↓ higher or lower recommendations, respectively, in the EAT-IT dietary pattern compared to the IDG.

3.3. Assessment of the Nutritional Adequacy of the EAT-IT- and IDG-Based Dietary Plans

In this last part of the process, the energies, and nutrient intakes of the two developed dietary plans (i.e., the EAT-IT- and IDG-based dietary plans) were analyzed to assess their nutritional characteristics. In detail, the energy and macro- and micronutrients provided by the two dietary plans were compared with the Italian recommendations (LARN, 2014) developed by the Italian Society of Human Nutrition (SINU) to verify the ability of these dietary patterns to satisfy the nutritional requirements, considering an energy target of 2500 kcal. Table 4 shows the comparison of the macronutrient composition and distribution of the two dietary patterns. The overall higher amount of lipids provided by the EAT-IT dietary plan (36.2%) compared to the IDG-based dietary plan accounted for 30.3% of the daily energy on average. The difference in the lipids intake appeared to be mainly due to the monounsaturated fatty acids (MUFA), which were considerably higher in the EAT-IT-based dietary plan (19.6%) relative to the IDG plan (14.9%). The high amount of lipids in the EAT-IT plan was accompanied by a relatively low amount of carbohydrates (about 48%), which was slightly above the lower threshold set at 45% of energy by LARN. In the IDG dietary plan, carbohydrates provided about 54% of energy (including energy from fiber).

Table 4. Comparison between the macronutrients provided by the IDG and EAT-IT dietary plans for a 2500 kcal diet.

Macronutrient Intake				
Nutrient	**IDG**	**EAT-IT**		**LARN (adults)**
Energy	2500	2500	Kcal	
Protein	97.7	97.6	g	AR 0.71 g/kg × die (PRI 0.9 g/kg × die)
Energy protein/total energy	15.6	15.6	%	12–18% En †
Animal protein	47.6	35.6	g	
Animal protein/total protein	48.7	36.5	%	
Vegetal protein	50.2	62.0	g	
Vegetal protein/total protein	51.3	63.5	%	
Lipids	84.3	100.6	g	
Energy lipids/total energy	30.3	**36.2 ***	%	RI 20–35% En
SFA	23.2	19.3	g	
Energy SFA/total energy	8.4	7.0	%	SDT < 10% En
MUFA	41.3	54.5	g	
Energy MUFA/total energy	14.9	19.6	%	
PUFA	10.3	17.4	g	
Energy PUFA/total energy	**3.7 ***	6.3	%	RI 5–10% En
Total ω-6	8.7	15.0	g	
Energy ω-6/total energy	**3.1 ***	5.4	%	RI 4–8% En
Total ω-3	1.8	2.1	g	
Energy ω-3/total energy	0.6	0.8	%	RI 0.5–2.0% En
Cholesterol	248.7	165.4	mg	SDT < 300 mg
Carbohydrates	317.3	276.8	g	
Energy carbohydrates/total energy ¥	53.9	47.8	%	RI 45–60% En
Sugars ×	111.5	85.3	g	
Energy sugars/total energy	**17.8 ***	13.6	%	SDT < 15% En
Total fiber	39.1	44.1	g	SDT > 25 g/die
Total fiber/1000 kcal	15.6	**17.7 ***	g	RI 12.6–16.7 g/1000 kcal
Energy total fiber/total energy	3.1	3.5	%	

Legend: AR: average requirement; EAT-IT: dietary pattern based on the EAT-Lancet Commission Reference Diet with adaptations for the Italian population; En: energy; IDG: Italian Dietary Guidelines; LARN: Reference Intake of Nutrients and Energy for the Italian Population; MUFA: monounsaturated fatty acids; PRI: population reference intake; RI: reference intake; SDT: standard dietary target; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids. *: Deviations from the reference requirements; †: range of energy from protein considered as an acceptable level of consumption (not an RI itself) in the LARN; ¥: energy from carbohydrates includes energy from fiber; ×: sugars contained in foods, including added sugars, sugars naturally occurring in milk, fruit, and vegetables, as reported in the LARN.

Regarding the total fiber, the amount was higher in the EAT-IT dietary plan relative to the IDG and slightly higher than the range reported in the LARN (12.6–16.7 g/1000 kcal). Despite these differences, both dietary plans were verified to cover the requirements for the majority of the nutrients, including some of the most critical ones, such as omega-3 essential fatty acids (0.6% and 0.8% of energy for the IDG and EAT-IT, respectively) and protein was comparable between the two dietary patterns (97.6 and 97.7 g, respectively). Energy from omega-6 fatty acids was within the range (RI = 4–8% of energy) for the EAT-IT plan (6.3%) and just slightly below the RI for the IDG (3.7%). Notably, both dietary plans provided low amounts of cholesterol (less than the 300 mg/day suggested by the LARN), where the cholesterol level was markedly lower in the EAT-IT-based dietary plan (165.4 mg) compared with the IDG plan (248.7 mg).

Considering vitamins (Table 5), no nutritional inadequacy emerged from the analysis of the two dietary plans, except for vitamin D, which was found to be lower than the average requirement indicated by the LARN (10 μg) in both the IDG and EAT-IT dietary plans (2.3 and 1.9 μg, respectively).

Table 5. Comparison between the vitamins provided by the IDG and EAT-IT dietary plans for a 2500 kcal diet.

Vitamin (Vit.) Intake					
Nutrient	IDG	EAT-IT		LARN (Adults 18–59 Years)	
				AR	PRI or AI §
Vit. A (retinol eq.)	2400	1500	μg	Male 500 μg (female 0.4 mg)	PRI male 700 μg (female 600 μg)
Vit. D (cholecalciferol, ergocalciferol)	**2.3 ***	**1.9 ***	μg	10 μg	PRI 15 μg
Vit. E (tocopherols, tocotrienols)	17.1	21.6	mg		AI male 13 mg (female 12 mg)
Vit. B1 (thiamine)	1.4	2.5	mg	Male 1 mg (female 0.9 mg)	PRI male 1.2 mg (female 1.1 mg)
Vit. B2 (riboflavin)	2.4	1.6	mg	Male 1.3 mg (female 1.1 mg)	PRI male 1.6 mg (female 1.3 mg)
Vit. B3 (niacin)	23.0	26.0	mg	14 mg	PRI 18 mg
Vit. B6 (pyridoxine)	2.8	3.2	mg	1.1 mg	PRI 1.3 mg
Vit. B9 (folic acid)	617.5	433.7	μg	320 μg	PRI 400 μg
Vit. B12 (cyanocobalamin)	4.3	3.3	μg	2 μg	PRI 2.4 μg
Vit. C (ascorbic acid)	250.9	175.5	mg	Male 75 mg (female 60 mg)	PRI male 105 mg (female 85 mg)

Legend: IDG: Italian Dietary Guidelines; EAT-IT: dietary pattern based on the EAT-Lancet Commission Reference Diet with adaptations for the Italian population; LARN: Reference Intake Levels of Nutrients and Energy for the Italian Population; AR: average requirement; PRI: population reference intake; AI: adequate intake; §: AI was obtained from the average intakes observed in the apparently healthy population free from deficiencies. It was used as a substitute for AR and PRI when these indicators could not be formulated based on available scientific evidence. *: The level of intake for the respective nutrient was inadequate to satisfy the nutritional requirements.

For other vitamins, such as vitamin K (phylloquinone and menaquinone), B5 (pantothenic acid), and B8 (biotin), a thorough evaluation could not be carried out due to the presence of many missing data for these vitamins in the food composition databases used (i.e., the Food Composition Database for Epidemiological Studies in Italy (IEO) and the Council for Agricultural Research and Economics (CREA, 2020) food composition database). Regarding the mineral intake, the comparison of the dietary plans revealed wide differences in the calcium levels provided by the two dietary plans. Specifically, the EAT-IT based dietary plan was less prone to satisfy the average requirement defined by the LARN, providing, on average, 680 mg/day (Table 6). The overall iron was found to be adequate in both the IDG- (17.9 mg) and EAT-IT-based dietary plans (22.1 mg), even when compared to the PRI (18 mg for female and 10 mg for male) reported by the LARN, and with the higher amount provided by the EAT-IT (≈22 mg), despite the overall lower amount of meat (considering both white and red meat) when compared to the IDG. All other assessed minerals (magnesium, phosphorus, potassium, and zinc) were adequate in both dietary plans. Sodium was found to be lower (about 827 mg) in the EAT-IT dietary plan compared to the AI suggested by the LARN (1500 mg) and close to the SDT (2000 mg) for IDG (≈2017 mg), while chlorine was found to be lower than the AI (2300 mg) in both the IDG- and EAT-IT-based dietary plans (1217 and 531 mg, respectively). Regarding the values of sodium and chlorine, discretionary salt was not included in the evaluation. As already reported for vitamins, the adequacy of certain minerals, such as copper, iodine, manganese, and selenium, was not assessed due to missing data in the database.

Table 6. Comparison between minerals provided by the IDG and EAT-IT dietary plans for a 2500 kcal diet.

Mineral intake						
Nutrient	**IDG**	**EAT-IT**		**LARN (Adults 18-59 Years)**		
				AR	**PRI or AI §**	**SDT**
Calcium	1079.1	**675.6 ***	mg	800 mg	PRI 1000 mg	
Sodium	**2070.3 ***	826.9	mg		AI 1500 mg	<2000 mg
Chlorine	1217.0	531.0	mg		AI 2300 mg	<3000 mg
Iron	17.9	22.1	mg	Male 7 mg (female 10 mg)	PRI male 10 mg (female 18 mg)	
Magnesium	356.2	491.4	mg	170 mg	PRI 240 mg	
Phosphorus	1851.4	1867.0	mg	580 mg	PRI 700 mg	
Potassium	4939.2	4609.5	mg		AI 3900 mg	
Zinc	14.8	15.9	mg	Male 10 mg (female 8 mg)	PRI male 12 mg (female 9 mg)	

Legend: AI: adequate intake; AR: average requirement; EAT-IT: dietary pattern based on the EAT-Lancet Commission Reference Diet with adaptations for the Italian population; IDG: Italian Dietary Guidelines; LARN: Reference Intake Levels of Nutrients and Energy for the Italian Population; PRI: population reference intake. §: AI was obtained from the average intakes observed in an apparently healthy population free from manifest deficiencies. It was used as a substitute for AR and PRI when these indicators could not be calculated based on available scientific evidence. *: The level of intake for the respective nutrient was inadequate to satisfy the nutritional requirements.

4. Discussion

In this study, we developed a practical application of the EAT-Lancet Reference Diet into a dietary plan that is consistent with Italian/Mediterranean food habits. Indeed, the sustainability of diets represents a crucial issue for the future (Downs *et al*., 2020), as healthy and sustainable diets should be both adequate for satisfying nutritional requirements and respectful of local traditions and cultures (FAO, 2010). In this context, the last revision of the IDG (CREA, 2018), which was developed by the CREA Food and Nutrition Research Centre, has provided dedicated information on this specific issue (i.e., "How to ensure a varied, safe, healthy and sustainable diet"), highlighting how an adequate consumption of the different food groups of the Italian tradition, including limited amounts of animal products, can positively impact both on humans' and the planet's health. The Planetary health diet is based on whole grains, legumes, nuts, fruit, and vegetables and includes a limited amount of dairy, meat, and other animal sources of protein and fats. The current literature is certainly not complete, but diets including reduced amounts of meat and dairy are indicated by many studies as both having a lower impact on the environment and being nutritionally adequate (Seves *et al*., 2017; Kim *et al*., 2020). Collins and Fairchild (2007) calculated that, based on the food consumption of the Cardiff population, the lowest environmental impact was obtained with a partial substitution of food with a high ecological footprint, while a typical vegetarian diet was associated with a lower reduction in the ecological footprint and lower nutritional adequacy. Seves *et al*. (2017) performed similar calculations based on the food consumption of the Dutch population, reporting that a vegan diet (all meat and dairy replaced with plant-based foods) was associated with the highest reduction in greenhouse gas emissions and land use but was nutritionally inadequate, while a 30% replacement, despite having a less marked reduction on the environmental impact, resulted in the best performance in terms of nutritional adequacy. These considerations underline the importance of better evaluating the pros and cons of modifications in the traditional diet and the need for a better understanding of the possible nutritional and functional impacts of revised, sustainable dietary patterns. The adaptability and scalability of the ELCRD have been investigated from different points of view in studies performed in different countries, including the USA (Blackstone *et al*., 2020), India (Sharma *et al*., 2020), and Denmark (Lassen *et al*., 2020). Considering the Italian scenario, Ferrari *et al*. (2020) calculated the daily portion of foods that could minimize gas emissions in an optimized Italian diet, while maintaining, as much as possible, an adequate nutritional intake. Conversely, no studies were found that previously developed a Mediterranean-Italian dietary pattern based on the EAT-Lancet reference diet. We performed a nutritional adequacy assessment on two examples of dietary plans (one from the EAT-IT dietary pattern and one based on the IDG), which highlighted some potential issues related to the frequency of consumption of some foods/food classes and/or the intake of specific nutrients. For instance, the differences in the levels of energy from carbohydrates reflected slight differences in the serving sizes of cereals, particularly during breakfast. Indeed, the IDG includes specific recommendations regarding the consumption of biscuits, pastries, or other cereal-based products at breakfast, making this meal rich in carbohydrates sources, while the EAT-IT pattern includes a lower amount of whole meal cereals. Conversely, the high amount of energy from lipids and the high amount of fiber in the EAT-IT dietary plan likely reflected the large intake of plant oil, nuts, and legumes, which represented the main differences between the two dietary patterns analyzed. Legumes and nuts are used in EAT-IT as an alternative for other protein sources, as reflected by the lower amount of total meat, fish, eggs, and dairy in the EAT-IT compared to the IDG. Regarding the nutritional adequacy of the two developed dietary plans, there was a higher fat intake promoted through the EAT-IT diet compared to the IDG one, where this was slightly higher than the LARN reference intake (i.e., 20–35% En). Despite being quite high, this value does not exceed the SDT for SFA and mainly consisted of MUFA because extra virgin olive was the primary source of fat in both dietary plans. An extensive review of randomized controlled trials demonstrated that dietary MUFA (20–25% of the total energy) prevented or ameliorated cardiovascular disease by modulating several biological parameters, such as the lipids profile, blood pressure, and insulin sensitivity (Gillingham *et al.* 2011). The EAT-IT-based dietary plan also resulted in higher PUFA compared to the IDG dietary plan due to the higher amount of nuts. Other differences were observed for micronutrients. The level of calcium intake apparently provided by the EAT-IT dietary plan (about 675 mg) was low relative to the IDG plan (>1 g) and compared to the average requirements for adults (i.e., 800 mg/day). The intake of calcium represents a critical issue, considering that the mean daily intake from food in adults, according to the last available Italian National Food Consumption Survey INRAN-SCAI 2005-06 (Sette *et al.*, 2011), is already lower than recommended (i.e., the average intake estimated: 756 mg for males and 697 mg for females). The amount of calcium in the EAT-IT dietary plan was in line with estimates provided by the EAT-Lancet Commission, who indicated a theoretical value of 718 mg of calcium from the Reference Diet (Willet *et al.*, 2019). The differences between the two dietary plans were likely due to the higher amount of milk and cheese included in the IDG compared to the EATIT dietary plan. This issue was also highlighted by Lassen *et al.* (2020), who adapted the ELCRD to reach the indications of their Nordic Nutrition Recommendations (2012) of 1 g by increasing the portion of dairy foods and cheese. However, the calculations performed did not consider the calcium content in water, which could significantly contribute to the intake, reducing the risk of inadequacy with respect to the calcium requirements (Galan *et al.*, 2002). The calcium content in tap water may largely vary; thus, it is difficult to evaluate the contribution of water to the total calcium intake (Cormick *et al.*, 2020). Considering an average value of 60 mg/L for Italian tap waters (Ministry of Health), 2 L provides about 120 mg of highly bioavailable calcium, which still seems to be insufficient to reach the PRI value. Thus, an adequate availability and choice of calcium-rich sources (including mineral waters and/or fortified food products) could be pivotal to avoid the long-term effects of deficiencies of at-risk nutrients, such as calcium, particularly in vulnerable subjects (e.g., women or, in general, subjects with restrictive or less varied eating habits). Moreover, specific guidelines would be fundamental to allow for reaching dietary recommendations depending on the type of source considered. Regarding vitamin D, both menus were very low (≈2 μg) as sources of this important micronutrient, which has impacts on numerous body functions. These values are in line with the levels of intake according to INRAN-SCAI 2005-06 (Sette *et al*., 2011), which indicated a median intake of 1.9 μg for males and 1.5 μg for females in Italy. In addition, the major source for vitamin D is the endogenous synthesis that takes place in the skin, while food sources of vitamin D, such as fatty fish, mushrooms, and eggs, typically play only a minor role in the total contribution [(Pilz *et al*., 2018; Cardwell *et al*., 2018). However, considering specific members of the population have little or no exposure to sunlight or have a diminished synthesis capacity (e.g., older subjects) (Remelli *et al*., 2019), the resulting intake of vitamin D represents a problem for which different strategies could be useful, such as the use of well-designed and targeted fortified foods or novel foods (Pilz *et al*., 2018). Intriguingly, iron was slightly higher in the EAT-IT dietary plan when compared with the IDG one. This could be explained by considering that, while the amount of red meat was comparable between the two dietary plans, the intake of legumes and nuts was higher in the EAT-IT compared to the IDG. However, iron bioavailability largely depends on the food source and the type of iron. The heme iron present in meat generally shows the highest bioavailability, and, conversely, different types of iron (nonheme) and the co-presence of phytate in plant-based food diets could reduce the bioavailability (Hurrell *et al*., 2010; Cocking *et al*., 2020). Conversely, the presence of reducing agents (i.e., vitamin C) should increase the bioavailability (Cook *et al*., 2001). Another apparent difference between the nutritional profile of these two dietary plans is related to sodium and chlorine, which were higher in the IDG dietary plan when compared with the EAT-IT plan. However, in both dietary plans, added salt was not included. The higher amount of sodium and chlorine in the IDG dietary plan could be explained by considering the higher amount of foods containing salt (e.g., bread and cheese) that was suggested by the IDG compared to the EAT-IT. The level of sodium in the IDG dietary plan was slightly above the SDT (without considering added salt) and highlights the importance of policies for reducing the amount of salt contained in foods, which represent the main source of salt (up to 70–75% of the total intake) in Europe (EFSA 2006). Indeed, the actual intake for the Italian population is acknowledged to be largely higher than recommended by the World Health Organization (WHO) (<5 g day) with an average estimated salt intake of almost 11 g for men and 8.5 for women (Donfrancesco *et al.*, 2013; WHO, 2014). Overall, the comparison between the micronutrients provided by the two dietary plans and dietary recommendations often highlighted that the amount could not always cover the needs of the overall population (i.e., the PRI referring to 97.5% of the population). These findings suggest a potential future need for strategies to improve the nutritional characteristics of foods and diets to enable coverage of the nutrient needs of specific target groups. The strategies may include the formulation of new products that are enriched with specific compounds, for instance, by the selection of new cultivars of vegetable products with increased nutritional benefits or the exploitation of novel foods to define new dietary models that are optimized to cover eventual inadequacies. While the simulation of possible dietary patterns with potential benefits in terms of sustainability and human health is a challenging approach with both pros and cons, the present study showed some strengths. For example, the dietary plans were developed by considering the Italian dietary habits, tradition, and culture, as well as in terms of meal composition and distribution throughout the day, with the aim to increase the final acceptability of a future dietary plan. The newly developed dietary pattern was developed to consider several alternatives such that the diet can potentially be used in the medium-long term (e.g., months) due to the feasibility of the dietary pattern. Regarding limitations, the first was the impossibility of performing an extensive and accurate comparison with dietary recommendations due to missing/unavailable data for certain micronutrients in the food databases, as previously reported. In particular, the micronutrients for which it was not possible to assess nutritional intake included vitamin B5, B8, and K, as well as copper, iodine, manganese, and selenium, which were also not considered in the previously cited Italian National Food Consumption Survey INRAN-SCAI 2005-06 (Sette *et al*., 2011). Even considering other databases, such as the USDA, some micronutrients (i.e., vitamin B8 and iodine) are still not complete. This issue indicates the importance of developing more complete food composition databases to better evaluate the ability of dietary plans to cover nutritional requirements (Sacco *et al*., 2013). Therefore, future efforts should be focused in this direction to allow for more accurate nutritional assessments, especially considering diets that can be critical for certain nutrients in the overall population or specific target groups (Sacco *et al*., 2013; Raiten *et al*., 2020). Despite this, deficiencies are unlikely for almost all of the abovementioned micronutrients that have incomplete data in the databases, except for iodine, for which almost 45% of the European population showed insufficient intake (SINU, 2014). These data support the importance of policies that have been established to increase the consumption of iodine-enriched salt and its utilization as an ingredient in food (Cook *et al*., 2001). Second, in the present study, we considered several isoenergetic food alternatives that mimic practical dietary plans that include generally different possible consumer food/meal choices (e.g., whole milk and semi-skimmed milk as alternatives for breakfast) that may differ in nutritional composition. In these cases, we considered the average composition, but differences between foods may also impact the final nutrient intake. This further underlines the importance of making dietary choices that are not only focused on energy content but also defining targeted alternatives that are able to cover potential inadequacies. Last, a thorough evaluation of the effect of processing and cooking on the nutrient content was not performed in a systematic way, despite these factors being able to affect the nutritional compositions of dishes. The recommended portion sizes and frequencies of consumption for the different food groups as proposed in the EAT-IT are quite far from the current dietary behavior of the Italian population. In detail, one of the main differences regards the amount of legumes and nuts that are consumed in a lower amount in the Italian population compared to EAT-IT, while meat, eggs, dairy products, animal fat, and tropical oils are consumed in higher amounts (Leclercq *et al*., 2009). These wide differences could make it difficult to adhere to the EAT-IT dietary pattern developed; thus, further efforts should be done to enable better compliance and acceptability of the model. Among these efforts, the development of specific food-based dietary guidelines and policies might help consumers with understanding how to tailor their dietary habits in order to achieve a more sustainable dietary pattern that is, at the same time, respectful of local culture and tradition. In this regard, it is worth noting that a new version of the Mediterranean pyramid has recently been proposed, which recommends consuming legumes and nuts every day (Serra-Majem *et al*., 2020). The possibility of improving the adoption of eating habits in consumers in line with these guidelines could also be facilitated by implementing attractive recipes and new products that are able to include such foods, and to target different consumer groups, such as the younger consumers who are very often those that are less adherent to the Mediterranean and healthy dietary patterns. In this scenario, the availability of fortified products or alternative sources may become a good option above all in those consumers who are less prone to adapting to new dietary patterns and foods or more strict guidelines. 5. Conclusions Overall, the newly developed dietary pattern represents a possible practical elaboration of the data indicated by Willet *et al*. (2019), but it should not be considered as strict or prescriptive. The defined dietary pattern is intended for adult subjects having a 2500 kcal daily energy need, but adjustments for other energy targets or population groups (e.g., older adults) should be made. Therefore, further efforts are needed to define the concrete feasibility of such patterns in relation to the food habits of the population, considering that making changes that are too large from their usual diet can preclude many people from adopting such diets (Collins *et al*., 2007). Finally, there is a clear need for the validation of these newly developed dietary patterns within the context of proper real-life studies to better elucidate their feasibility, affordability, and their beneficial health effects.

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Appendix A

Glossary of the main terms used.

Name	Definition
ELCRD (EAT-Lancet Commission Reference Diet)	Proposal included in a recent report from the EAT-Lancet Commission, consisting of indications of consumption for different food categories within which it is possible to have a simultaneously sustainable and healthy diet. This set of indications can be theoretically adapted to all food contexts, and for this reason, is sometimes indicated as a "planetary diet"
EAT-IT dietary pattern	Proposal for a local adaptation of the ELCRD to the Italian/Mediterranean food context, which is organized in a schematic quali-quantitative representation that is feasible for mid/long-term utilization
EAT-IT-based dietary plan	Simulation of the application of the EAT-IT dietary pattern in which a long-term menu made of recipes and dishes that are consistent with EAT-IT was designed
IDG (Italian Dietary Guidelines)	Official national dietary guidelines for the Italian population and was recently revised in their latest edition (2018)
IDG based dietary plan	Simulation of a long-term menu that is made of recipes and dishes that are consistent with the indications provided by the IDG
LARN (Reference Intake of Nutrients and Energy for the Italian Population)	Consensus document that includes reference values for the intakes of nutrients and energy to cover the nutritional requirements of different target groups in the Italian population

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3.1.3 Development of an approach to adapt the EAT-IT dietary pattern to different energy targets: assessment of potential nutritional issues associated to a 2000 kcal dietary plan.

Abstract:

There is an urgent need to promote healthy and sustainable diets tailored to the preferences and cultures of different populations. We have developed a Mediterranean-adapted Planetary health diet for Italian population (EAT-IT) based on 2500 kcal, but further efforts should be made to develop dietary patterns for other energy targets or tailored to the specific needs. Aim of this study were i) to define a rationale procedure of adaptation of the EAT-IT dietary pattern to different energy targets, ii) verify the results of this process, through the assessment of nutritional adequacy and updated compliance with the Italian Dietary Guidelines (IDG) of a 2000 kcal adapted EAT-IT dietary pattern. The pattern was properly adjusted by reducing the amount of specific food categories in a non-proportional way, to increase feasibility safeguarding other constraints. Results indicate that EAT-IT pattern can be adjusted to better address Italian food habits and dietary requirements for general population with a 2000 kcal energy target. Macronutrient distribution resulted in line with dietary recommendations (e.g., energy from lipids < 35%), but calcium and iron content may represent examples of major nutritional issues of this pattern. This could be critical in specific target groups (i.e., vegetarian, vegan, older subjects, pregnant and lactating women). The eventual need of fortified foods, supplements of critical nutrients and/or specific recommendations to enhance the positive effects of this pattern should be carefully investigated.

1. Introduction

We recently developed an Italian-Mediterranean adapted dietary pattern based on the EAT-Lancet Commission proposal (the EAT-IT dietary pattern). The EAT-IT dietary pattern settled to 2500 kcal has been initially undergone to nutritional adequacy assessment (Tucci *et al.*, 2021). This energy target is the one chosen by the EAT-Lancet Commission for its original proposal since it is close to the global average per capita energy intake and was considered an adequate base value that would fit the higher share of population energy requirements at global level (Willett *et al.*, 2019). However, it is important to consider that a large group of the population have a lower energy target both for individual characteristics and for the sedentary lifestyle and/or need to reduce food intake and body weight. On this topic, high body mass index (BMI) is one of the main rick factors for attributable deaths and disability, mainly in relation to cardiovascular diseases and diabetes, according to the more recent available data from the Global Burden of Disease Study (GBD 2019 Risk Factors Collaborators, 2020). Furthermore, data from dedicated group of the GBD re-analyzed in 2017 all reliable data on overweight and found that presence of overweight and obesity are increasing worldwide, and that overweight alone was responsible for almost 40% of deaths associated with high BMI (GBD 2015 Obesity Collaborators, 2017). According to the most recent assessment, Italian prevalence of overweight (including obesity) stands at 46.1%, with higher levels in the older subjects (Sbaccia *et al.*, 2020). Given this background, the assessment of the actual scalability of the Planetary health diet to different energy targets without compromising (and possibly enhancing) feasibility, nutritional adequacy, and respect of environmental constraints represent an important task. Thus, this study aims to: i) define a rationale procedure of adaptation of the EAT-IT dietary pattern to different energy targets, ii) verify the results of this process, through the assessment of nutritional adequacy and updated compliance with the Italian Dietary Guidelines (IDG) of a 2000 kcal adapted EAT-IT dietary pattern.

2. Materials and methods

2.1 Definition of the EAT-IT dietary pattern

A sustainable Italian-Mediterranean dietary pattern (EAT-IT) providing 2500 kcal was developed as already reported (Tucci *et al.*, 2021). Briefly, the EAT-Lancet Commission on Healthy diet from sustainable food systems provided the optimal average daily intake for eight different food categories (whole grain, tubers or starchy vegetables, vegetables, fruits, dairy foods, protein sources, added fats, and added sugars) to maintain diets within a safe operating space for diet-associated environmental impact, while promoting good nutrition and health. These levels of intake are expressed as both g/day and kcal/day, considering a diet of 2500 kcal/day. We utilized and rearranged these data, calculating feasible portion sizes and weekly frequencies of consumption for these food categories, and allocating them into different meals according to Italian/Mediterranean food habits. Finally, we have summarized these elaborations, to obtain a dietary scheme that can be easily used for the management of meals (Tucci *et al.*, 2021).

2.2. Definition of an iterative process to adapt the EAT-IT pattern to different energy targets

Due to the high risk of obtaining borderline or low values of critical nutrients (e.g., calcium, iron), it was not possible to adapt the EAT-IT dietary pattern to energy targets other from 2500 kcal, just reducing food portions (e.g., -25% to reach an energy target of 2000 kcal). Furthermore it was considered methodologically not correct to adapt the pattern to lower energy targets addressing specific food categories since i) the aim of the EAT-Lancet commission is to define practical dietary guidelines to promote adequate nutrition and low environmental impacts, ii) authoritative data indicates that the original 2500 kcal portions are already below the threshold of safe operating space for environmental impact (Springmann *et al.*, 2020). Thus, any reduction to reach lower energy targets can be considered as adequate and gives space to adaptations that can also: i) safeguard nutritional requirements, through a less pronounced reduction of food categories providing critical nutrients (i.e., meat and dairy), ii) increase acceptability and practical feasibility through a higher reduction of food categories for which it is expected a consumption far from the usual one of the reference population (i.e., nuts and legumes, in this case the Italian one).

To achieve these adaptations, an ad hoc spreadsheet was specifically settled. This spreadsheet was edited to allow the re-definition of the EAT-IT scheme for the management of meals, in relation to the energy targets defined for each food category. The theoretical constraints preclude increasing any category, but only reducing them (even if in not necessarily in a proportional way). The practical constraints, on the other hand, relate to the maintenance of consumption frequencies and realistic portion sizes. Furthermore, given that these food categories are generic (e.g. vegetables), further adaptation can involve the inclusion of specific food items with different bioactives able to improve the final nutritional profile providing additional health benefits.

2.3 Assessment of the nutritional adequacy of a 2000 kcal adapted EAT-IT dietary pattern and comparison with the IDG related pattern

A proposal for a 2000 kcal EAT-IT dietary pattern, as an example of the iterative process of adaptation, was defined as follow: i) an initial 20% reduction of each food categories was applied except for vegetables, fruits, dairy and some protein sources (i.e., chicken and other poultry, eggs, fish, legumes), ii) to reach 2000 kcal, a further reduction of legumes and nuts was applied (final reduction = 38%), iii) the final portions were re-allocated into the different meals, and for protein sources, the frequencies of consumption where re-defined to assure feasible portions. This calculation can be replicated (in an automatized way through the developed calculator spreadsheet) for the definition of any other, even intermediate, energy target.

2.4 Comparison with IDG derived plan and nutritional adequacy assessment

To evaluate whether the adaptation developed reached the aims of obtaining a more suitable pattern, a comparison of this newly developed pattern and the IDG in terms of portion size and frequencies of consumption was performed. For the evaluation of the nutritional adequacy of the EAT-IT dietary pattern, two different mid/long term dietary plans, comprehensive of different recipes and alternatives for every meal, were developed to obtain a realistic evaluation of the nutritional profile of the two different dietary plans. These patterns, one consistent with the EAT-IT dietary plan, and the other one with the IDG, as a control were elaborated using a software (MètaDieta professional 4.1.1 METEDA Srl–Roma, Italy) and then, the resulting nutritional composition was compared with the Italian recommendations for macro- and micronutrients (SINU, 2014).

3. Results

3.1 Reference scheme for meal management of a 2000 kcal adapted EAT-IT dietary plan

The newly developed reference scheme is presented in Table 1 and 2. As for the 2500 kcal version, it consists of three main meals, including breakfast, and a snack with nuts. For lunch and dinner, it is possible to freely compose meals using the Table 2. For breakfast, the main difference is related to the lower levels of cereals and sugars. The amount of nuts for snack results lower than the previous elaboration. For Table 2, the main differences are related to wholegrains, legumes, and oils.

Table 1. Reference scheme for breakfast and snacks.

(1A) Breakfast		
Food category	Portion Size	Possible alternatives
a) Dairy	155 kcal	250 mL whole milk 330 mL semi-skimmed milk 230 g whole milk yogurt 350 g semi-skimmed yogurt 20 g butter
b) Cereal	130 kcal	45 g cornflakes 45 g rusks (5 slices) 75 g whole bread
c) Sugars	90 kcal	50 g jam 220 mL fruit juice
(1B) Snack		
Food Category	Portion Size	Practical Examples (Edible Part)
Nuts	180 kcal	Almonds—50 g Peanuts—50 g Pistachios—50 g Walnuts—40 g

Breakfast (1A) is composed of a portion of (a) dairy, (b) cereals, and (c) sugars. Snacks (1B) are composed of a portion of nuts. The amount of nuts indicated in the 1B scheme for snacks can be eaten as a single snack or on two occasions per day, halving the total amount indicated in the table for each occasion. * The EAT-Lancet Commission reference diet indicates 25 g of sugars per day, which was considered as “free sugars” that is “all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer, plus sugars naturally present in honey, syrups, and fruit juices,” as indicated by the World Health Organization (WHO).

Table 2. Reference scheme for meals.

(a) Whole grain			
Lunch (330 kcal)		Dinner (220 kcal)	
Food	Theoretical portion	Food	Theroretical Portion
Brown rice (364 kcal/100 g)	100 g	Brown rice (364 kcal/100 g)	65 g
Spelt (335 kcal/100 g)	100 g	Spelt (335 kcal/100 g)	70 g
Whole pasta (355 kcal/100 g)	100 g	Whole pasta (355 kcal/100 g)	65 g
Corn (365 kcal/100 g)	95 g	Corn (365 kcal/100 g)	60 g
Common bread (275 kcal/100 g)	150 g	Common bread (275 kcal/100 g)	100 g
		Potatoes (78 kcal/100 g)	260 g

(b) Protein sources				
Food	Energy to create harmonized portion	Theoretical Average Energetic Density	Weekly Consumption	Theoretical Portion
Beef, lamb, and pork	215 kcal	214 kcal/100 g	1 time	100 g
Chicken and other poultry	215 kcal	214 kcal/100 g	2 times	100 g
Eggs	70 kcal	146 kcal/100 g	2 times (1 egg)	70 g
Fish	145 kcal	143 kcal/100 g	2 times	100 g
Legumes (dried)	180 kcal	379 kcal/100 g	7 times	50 g

Others	
(c) Vegetables	Approximately 40 kcal (150 g/meal)
(d) Oil and seasoning	Approximately 20 g/meal mainly from food sources rich in unsaturated fatty acids, such as extra-virgin olive oil
(e) Fruit	Approximately 60 kcal (100 g/meal)

Meals (lunch and dinner are composed of a portion of a) whole grains, b) protein sources, c) vegetables, d) oil and seasoning, and e) fruit. The main meals, lunch, and dinner, include: i) a portion of cereals or potatoes; ii) a portion of a protein source, in relation to their calculated frequencies of consumption; iii) a portion of vegetables; iv) oil and seasoning and v) a portion of fruit to end the meal. This table has no prescriptive value; thus, lunch and dinner can be interchanged. This table can be theoretically used to compose complete dishes (e.g., “pasta al ragù” + fruit) or meal composed of a single portion of each component (omelet with spinach, whole bread, and fruit).

3.2 Comparison of serving sizes and frequencies of consumption with IDG

The Italian Dietary Guidelines provide servings sizes and frequencies of consumption for three exemplificative energy targets, i.e., 2500, 2000, and 1500 kcal. Thus, it is possible to compare this new adaptation of the EAT-IT dietary pattern with the 2000 kcal recommendation of the IDG. Respect to the previous elaboration this new adaptation perfectly matches the quantities of red and white meat suggested by the IDG. However, although this adaptation has already taken into consideration, the non-proportional reduction of certain categories compared to others to reduce the gap previously found with the Italian guidelines, legumes, nuts, and dairy products still represent the main differences with the IDG.

Table 3. Comparison between the suggested portions in the Italian dietary guidelines for healthy eating for a 2000 kcal diet and the 2000 kcal adapted EAT-IT dietary plan.

Italian guidelines			**EAT-IT dietary scheme**
Food group	**Food subcategory**	**Daily or week portion**	
Cereals and derivatives	Bread	3 ½ portion of 50 g (175 g/day)	≠ Max daily amount of wholegrain bread of 300 g
	Pasta, rice, corn, spelled, barley	1½ portion of 80 g (120 g/day)	≠ Max daily amount of 170 g
	*Bread substitutes (rusks, crackers, breadsticks)	**1 portion of 30 g (30g/week)**	≠ About 40 g of rusks (5 slices) can be eaten at breakfast
	*Sweet bakery products (brioche, croissants, biscuits)	**2 portions of 50 g for croissants or cake or 30 g for biscuits (100 or 60 g/week)**	≠ Sweet products can be eaten at breakfast and are indicated in EAT-IT scheme as a "sugars and other sweeteners"
	*Breakfast cereals	**2 portions of 30 g (60 g/week)**	≠ About 35 g of breakfast cereals can be eaten at breakfast
Tubers	Potatoes	**2 portions of 200 g (400g/week)**	**↓ 1 portion of 260 g (260 g/week)**
×Fruits	Fresh fruits	3 portions of 150 g (450g/day)	↓ 200 g/day
	Dried fruits	3 portions of 30 g (90g/day)	n.s.
×Vegetables	Fresh vegetables	2 ½ portions of 200 g (500g/day)	↓ 300 g/day
	Leaf salad	2 ½ portions of 80 g (200g/day)	n.s.
Meat	*Red meat (beef, pork, sheep meat)	**1 portion of 100 g (100 g/week)**	**= Beef, lamb, or pork 100 g/week (100 g/week)**
	White meat (chicken, turkey, or rabbit)	**2 portions of 100 g (200 g/week)**	**= Chicken and other poultry 2 portion of 100g (200 g/week)**
Fishery	Fish (including mollusks and crustaceans)	**2 portions of 150 g (300 g/week)**	**↓ Fish 2 portion of 100 g (200 g/week)**
	*Preserved fish (i.e., canned tuna etc.)	**1 portion of 50 g (50 g/week)**	n.s.

Egg	Egg	**3 medium egg of 50 g (150 g/week)**	**↓ 2 portions of 1 medium egg (2 eggs/week)**
×Legumes	Fresh legumes or canned	**3 portions of 150 g (450 g/week)**	**↑ 7 portions of 150 g or 50 g of dried legumes (1050 g or 350 g/week)**
	Dried legumes	**3 portions of 50 g (150 g/week)**	
×Milk and derivatives	Milk	3 portions of 125 mL (375 mL/day)	↓ 1 portion of 250 mL of milk or other isocaloric equivalences of milk derivatives (e.g., yogurt, butter) (250 mL/day)
	Yogurt and other fermented milk	3 portions of 125 g (375 mL/day)	
	Cheese (fat < 25% and less then 300kcal/100g)	**3 portions of 100 g (300 g/week)**	
	Cheese (fat > 25% and more then 300kcal/100g)	**3 portions of 50 g (150 g/week)**	
×Fats and seasoning	Vegetables oil (e.g., extra virgin olive oil, seeds oil)	3 portions of 10 mL (30 mL/day)	↑ 40 g/day of added fats, preferably from plant sources. Butter is excluded because is already included in milk and derivatives food category
	Butter and other animal fats	3 portions of 10 g (30 g/day)	
Nuts and seed	Walnuts, peanuts, almonds, seeds etc.	**2 portions of 30 g (60 g/week)**	↑ 30 g/day
Water	Water	At least 8 glasses of 200 mL (2 L/day)	n.s.

Legend: n.s.: not specified. ×: the portions reported for the food included in that category are alternatives and not additive (e.g., for “fruits,” 150 g of fresh fruit OR 30 g of dried fruit); *: subcategory for which it is possible to have a lower frequency of consumption and increasing the consumption of other foods from the same category, according to the Italian dietary guidelines (IDG). ≠: food category with different recommendations between the IDG and EAT-IT but not clearly definable in terms of whether the amount is higher, equal, or lower. ↑↓: higher or lower recommendations, respectively, in the EAT-IT dietary pattern compared to the IDG.

3.3 Nutritional adequacy assessment

To assess whether this adaptation was successful in limiting the exacerbation of the issues previously found, a new simulation was elaborated. The nutritional profile for macronutrients is reported in Table 4 and indicates a more balanced dietary pattern, compared to the previous proposal and none of the nutrients resulted outside reference values apart from the amount of fibers expressed on 1000 kcal intake, which resulted slightly above the reference intake.

Table 4. Comparison between the macronutrients provided by the EAT-IT dietary plans for a 2000 kcal diet and the recommended levels of assumption for nutrients and energy for the Italian population (LARN).

Nutrient	EAT-IT 2000 kcal		LARN (adults)
Energy	2000	kcal	
Protein	82.6	g	PRI 0,9 g/kg×die
Energy Protein/total energy	16.5	%	12–18% En †
Lipids	75.1	g	
Energy Lipids/total energy	33.9	%	RI 20-35% En
SFA	14.4	g	
Energy SFA/total energy	6.5	%	SDT <10% En
MFA	40.8	g	
Energy MFA/total energy	18.4	%	
PUFA	12.7	g	
Energy PUFA/total energy	5.7	%	RI 5-10% En
Total W6	10.9	g	
Energy W6/total energy	4.9	%	RI 4-8% En
Total W3	1.6	g	

Energy W3/total energy	0.7	%	RI 0,5-2,0% En
Cholesterol	149.3	mg	SDT <300 mg
Carbohydrates	230.3	g	
Energy carbohydrates/total energy (4 kcal/g) ¥	49.5	%	RI 45-60% En
Sugars	80	g	
Energy sugars/total energy (4 kcal/g)	15	%	SDT <15% En
Total fibres	35	g	SDT > 25 g/die
Total fibres/1000kcal	**17.5***	g	RI 12,6-16,7 g/1000 kcal

Legend: AR: average requirement; En: energy; LARN: Reference Intake of Nutrients and Energy for the Italian Population; MUFA: monounsaturated fatty acids; PRI: population reference intake; RI: reference intake; SDT: standard dietary target; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids. *: Deviations from the reference requirements; †: range of energy from protein considered as an acceptable level of consumption (not an RI itself) in the LARN; ¥: energy from carbohydrates includes energy from fiber; ×: sugars contained in foods, including added sugars, sugars naturally occurring in milk, fruit, and vegetables, as reported in the LARN.

Table 5 reports the vitamin content. No nutritional inadequacy emerged from the analysis of this adaptation, except for vitamin D, which was found to be lower than the average requirement indicated by the LARN.

Table 5. Comparison between the vitamins provided by the EAT-IT dietary plan for a 2000 kcal diet and the recommended levels of assumption for nutrients and energy for the Italian population (LARN).

Nutrient	**EAT-IT**		**LARN (adults)**
Vit. A (retinol eq.)	1.4	mg	PRI male 0,7 mg (female 0,6 mg)
Vit. D	**1.8***	µg	PRI 15 µg
Vit. E	17	mg	AI male 13 mg (female 12 mg)
Vit. B1 (thiamine)	2	mg	PRI male 1.2 mg (female 1,1 mg)
Vit. B2 (riboflavin)	1.6	mg	PRI male 1.6 mg (female 1,3 mg)
Vit. B3 (niacin)	22	mg	PRI 18 mg
Vit. B6 (pyridoxine)	2.7	mg	PRI 1,3 mg
Vit. B9 (folic acid)	409	µg	PRI 400 µg
Vit. B12 (cyanocobalamin)	3.5	µg	PRI 2.4 µg
Vit. C	174	mg	PRI male 105 mg (female 85 mg)

Legend: AI: adequate intake; LARN: Reference Intake Levels of Nutrients and Energy for the Italian Population; PRI: population reference intake. AI was obtained from the average intakes observed in an apparently healthy population free from manifest deficiencies. It was used as a substitute for AR and PRI when these indicators could not be calculated based on available scientific evidence. *: The level of intake for the respective nutrient was inadequate to satisfy the nutritional requirements.

Finally, Table 6 reports the content of minerals, indicating that the levels of calcium, despite low compared to the recommendations, were not less than those provided by the original 2500 kcal version. However, iron levels resulted slightly below the recommended levels for female.

Table 6. Comparison between the minerals provided by the EAT-IT dietary plan for a 2000 kcal diet and the recommended levels of assumption for nutrients and energy for the Italian population (LARN).

Nutrient	**EAT-IT**		**LARN (adults)**
Calcium (Ca)	**704***	mg	PRI 1000 mg
Sodium (Na)	781	mg	AI 1500 mg SDT <2000 mg
Chlorine (Cl)	659	mg	AI 2300 mg SDT <3000 mg
Iron (Fe)	**17.4***	mg	PRI male 10 mg (female 18 mg)
Magnesium (Mg)	384	mg	PRI 240 mg
Phosphorus (P)	1593	mg	PRI 700 mg
Potassium (K)	4080	mg	AI 3900 mg
Zinc (Zn)	13.2	mg	PRI male 12 mg (female 9 mg)

Legend: AI: adequate intake; AR: average requirement; LARN: Reference Intake Levels of Nutrients and Energy for the Italian Population; PRI: population reference intake. AI was obtained from the average intakes observed in an apparently healthy population free from manifest deficiencies. It was used as a substitute for AR and PRI when these indicators could not be calculated based on available scientific evidence. *: The level of intake for the respective nutrient was inadequate to satisfy the nutritional requirements.

4. Discussion

This study aimed at defining a procedure of adaptation of the EAT-IT dietary pattern to different energy targets compared to the original proposal of 2500 kcal. The adaptation of a diet to individual dietary requirements is pivotal for reaching practical implementation and guarantee improvement in nutritional status and diet-related health outcomes. The characteristics of the Planetary health diet, on which the EAT-IT dietary plan is based, were defined based on available literature on the intersections between health and environmental impact outcomes (Willett *et al.*, 2019; Martini *et al.*, 2021). Thus, when adapting the dietary pattern, certain constraints need to be considered. Our previous analysis of the nutritional adequacy of the EAT-IT dietary pattern based on 2500 kcal showed some critical issues such as the one related to calcium content (675 mg/day, excluding water contribution) (Tucci *et al.*, 2021). Thus, simply reducing proportionally all portion sizes to fit different energy targets (i.e., -20% to achieve 2000 kcal) would result in a worse nutritional profile of the dietary pattern.

In this regard, we assessed the nutritional characteristics resulted from an adapted EAT-IT dietary plan based on 2000 kcal, obtained through the proportional reduction of all food categories, apart from critical foods having low environmental impact and widely suggested for achieving a healthy diet, also in relation to their content in bioactives compounds (i.e., fruit and vegetables), or providing fundamental nutrients and already present in low amounts (i.e., bovine meat), while legumes and nuts were reduced in a more consistent way. The resulting analysis showed that the nutrients below nutritional requirements, as indicated by the Levels of Reference Intake of Nutrients and Energy for the Italian Population (SINU, 2014), are limited to calcium and vitamin D, while iron is just below the threshold for female subjects. Iron levels instead can be more critical since low iron availability is frequent in the case of monotonous plant-based diet (Zimmermann and Hurrell, 2007). Iron deficiency can lead to important health consequences and involve substantial social costs (Pasricha *et al.*, 2021). Thus, fortified foods are sometimes suggested since iron deficiency is one of the most commonly observed nutritional deficiencies and clinical studies have shown that the consumption of iron-fortified foods is one of the most effective methods for its prevention (Man *et al.*, 2022). Also, specific indications, such as to differentiate as much as possible dietary choice within every food category or recommend the habitual use of fortified foods and periodical routine blood test to verify proper nutritional status can be provided to target groups of population in order to handle such dietary proposals in the most advantageous way (Craig *et al.*, 2021).

Other issues potentially affecting feasibility of the EAT-IT dietary pattern emerged from the comparison of portion sizes and frequencies of consumption identified with respect to that proposed in the IDG (Tucci *et al.*, 2021). Weekly amount of legumes and nuts included in the EAT-IT dietary plan resulted more than threefold the weekly amount suggested by the IDG, and far away from the Italian legumes consumption, which is very low on average (Leclercq *et al.*, 2009; CREA, 2018). Thus, non-proportional reduction of the different food categories is not only necessary to safeguard nutritional adequacy but can also help in achieving a more implementable and feasible dietary pattern. The comparison with IDG recommendation based on 2000 kcal intake showed that this adaptation resulted in a more superimposable pattern, with lesser differences than the previous proposal of 2500 kcal. The same red and white meat portions are now considered, while the main differences remain related to dairy foods (still lower for the EAT-IT dietary pattern), legumes and nuts (still higher in the EAT-IT dietary pattern).

From a more general point of view, not only nutritional adequacy of new sustainable patterns should be considered but also their feasibility and potential adoption by the general population. This is very important when the type and frequency of consumption of foods are significantly modified with respect to the actual consumption, since this can affect eating behavior and acceptability of a dietary plan (Vitale *et al.* 2021).

Considering determinants of food intake, these include several factors, such as the presence of highly palatable foods, cultural and social pressures, cognitive-affective and physiological factors, including those related to satiety (Leng *et al.*, 2017). Thus, to improve the actual feasibility of the EAT-IT dietary pattern these factors should be also considered in the future, and dietary components modulated to allow small transitions rather than large ones. Small transitions are in fact considered to be more easily implementable by population, compared to large changes that can preclude large share of population from adopting more healthy and sustainable diets (Collins and Fairchild, 2007; Clark *et al.*, 2022). Also, given that lower energy values can be associated with lower satiating capacity, in particular when used for weight reduction, the maintenance of the highest levels of fruits and vegetables, as well as lean fish and meat can also be helpful due to the health benefits of these food categories, but also to increase satiety levels (Johnstone, 2015; Pace and Crowe, 2016; Fardet and Richonnet, 2020; Aaseth *et al.*, 2021). In this context, the EAT-IT dietary plan could have advantages in terms of determinants for the regulation of food intake, but it could be critical in the degree of acceptability for the Italian population. This need to be carefully studied.

Another consideration is related to environmental constraints. As previously reported, the Planetary health diet can theoretically allow to feed a global population of ten billion, allowing food systems to remain within the trajectories of a safe operating space (Rockström *et al.*, 2009; Rockström *et al.*, 2016; Willett *et al.*, 2019). The mean diet-associated environmental impacts at individual level can be used as a further constraint to be considered for the evaluation of proper methods of dietary adaptation. In this analysis we considered that decreasing in a non-proportional way different portion sizes, but without increasing the weekly amount of any of them, and just operating reductions, would necessarily result in lower levels of diet-associated impact. However, if properly quantified, data on the average levels of impact would allow to define specific maximal threshold (that can be considered as an “individual level safe operating space”) allowing a more comprehensive adaptation. For instance, it would be possible to suggest higher portion of foods associated with high environmental impact, but useful for delivering important nutrients, when considering diets of subjects with specific nutritional needs. Finally, the EAT-Lancet commission endorses vegetable foods consumption, which can exert different health promoting effects in relation to their nutritional characteristics (e.g., vitamins and other bioactives) and thus the *ad hoc* inclusion of specific vegetable foods could be exploited to improve the capability of the EAT-Lancet dietary pattern to implement the benefits for specific target groups of population. However evidence in this regard is still limited.

In conclusion, we developed a procedure of adaptation of the EAT-IT dietary plan to different energy targets. The strength of this procedure is that represents a simple approach to adapt the pattern to any energy intake and nutritional need, by modulating the different food categories, allowing space to also recommend consumption of specific food items able to improve final nutritional profile and provide additional health benefits for specific target groups of population. However, it should be considered that while any decrease in portion sizes would correspond to lower diet-related environmental impact, an increase in portions size, such as for athletes and very active people, remains to be debated. However, theoretically, a large share of population would fit the lower energy intake version of the EAT-IT diet, giving margins for higher diet-related impact for those with higher energy requirements (e.g. the most physically active subjects).

Shifting from theory to practice, actual feasibility of energy balanced EAT-IT dietary patterns and effects on nutritional and health status should be addressed with well-designed *in vivo* studies, considering potential issues related with the most critical nutrients. On this topic, the eventual need of fortified foods, supplements of critical nutrients and/or specific recommendations to enhance the positive effects of this pattern should be carefully investigated with further ad hoc research.

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3.2 Chapter 2

- **The environmental impact of an Italian-Mediterranean dietary pattern based on the EAT-Lancet reference diet (EAT-IT).**
  (Pre-print of a submitted article)

Article

3.2 The environmental impact of an Italian-Mediterranean dietary pattern based on the EAT-Lancet reference diet (EAT-IT)

Massimiliano Tucci [1,†], Daniela Martini [1,†,*], Mirko Marino [1], Cristian Del Bo' [1], Valentina Vinelli [1], Paola Biscotti [1], Carlotta Parisi [1], Ramona De Amicis [1,2], Alberto Battezzati [1,2], Simona Bertoli [1,2], Marisa Porrini [1] and Patrizia Riso [1]

[1]Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133 Milan, Italy; massimiliano.tucci@unimi.it (M.T.); daniela.martini@unimi.it (D.M.); mirko.marino@unimi.it (M.M.); cristian.delbo@unimi.it (C.D.B.); valentina.vinelli@unimi.it (V.V.), paola.biscotti@unimi.it (P.B.); carlotta.parisi@studenti.unimi.it (C.P.); ramona.deamicis@unimi.it (R.D.A.); alberto.battezzati@unimi.it (A.B.); simona.bertoli@unimi.it (S.B.); marisa.porrini@unimi.it (M.P.)

[2]International Center for the Assessment of Nutritional Status (ICANS), Università degli Studi di Milano, 20133 Milan, Italy

* Correspondence: daniela.martini@unimi.it

† Both authors contributed equally to this work

Abstract: The definition of healthy and sustainable diet is nowadays considered pivotal, but data related to environmental outcomes are still debated. In this study, we compared the carbon (CF) and water footprints (WF) of an Italian-Mediterranean (EAT-IT) dietary pattern designed on the "Planetary diet", with a pattern based on the Italian dietary guidelines (IDG). The influence of different food categories and food choices on environmental impact was assessed. To this aim, weekly dietary patterns were developed, considering food categories and related portions and frequencies of consumption. Results show that the EAT-IT dietary pattern, compared to the IDG, had a significantly lower CF (2.82±1.07 and 3.74±0.92 kg CO2/day, respectively) but not WF. Protein-rich foods were the main contributors to CF and WF in both dietary patterns. The increased substitution of frozen instead of fresh foods, imported instead of local fruits, greenhouse-grown instead of seasonal vegetables, and processed legume-based foods instead of unprocessed legumes caused an increasing worsening of the CF in both patterns, but with different magnitudes. Our analysis indicated that the EAT-IT dietary pattern can be considered sustainable for CF, but individual choices are likely to largely affect the final environmental outcomes.

Keywords: sustainable healthy diet; planetary healthy diet; nutrition; sustainability; environmental impact; Mediterranean diet; dietary guidelines

1. Introduction

It is well known that diet is crucial for the promotion of human health. In 2017, 11 million deaths and 255 million disability-adjusted life-years were estimated to be attributable to dietary risk factors and in particular to high sodium intake and low consumption of whole grains, fruit and vegetables and nuts (Afshin *et al.*, 2019).

More recently, greater consideration is being given to the impact that diet and the entire food system can exert on the health of the planet, depending on how actual eating behavior and food choices affect production. Indeed, the food system has been shown to account for over a third of global human-caused greenhouse gas emissions (Crippa *et al.*, 2021; Mrówczyńska-Kamińska *et al.*, 2021), half of the land use and over two-thirds of freshwater use (Whitmee *et al.*, 2015; FAO, 2021). For this reason, the food system is considered one of the principal drivers of climate change, which in turn impacts on foods through decreasing food quantity, biodiversity, and nutrient content (Fanzo *et al.*, 2017; Fanzo and Davis, 2019).

It is therefore quite clear that there is a challenge of to develop and provide to the global population, which is constantly growing, diets that are healthy, with a low environmental impact but, at the same time, are socio-culturally acceptable and economically accessible for all (FAO, 2010; Gonzalez Fischer and Garnett, 2016). In this scenario, a global modelling analysis has been applied to the evaluation of the different dietary patterns identified for more than 150 countries, combining nutrient levels, diet-related and weight-related chronic disease mortality, and environmental impacts. Overall, the results obtained demonstrated that plant-based dietary patterns, aligned with the current evidence on healthy eating, can generally lead to a simultaneous reduction of both health and environmental impacts in all regions, despite differences among countries in terms of resource use (Springmann *et al*., 2018a).

With the intention to develop a global healthy and sustainable diet (i.e., "win-win" approach), the EAT-Lancet Commission developed a "planetary" healthy reference diet for an intake of 2500 kcal/day (Willett *et al*., 2019) characterized by a high consumption of whole grains, vegetables, legumes, nuts, unsaturated oils, with low amounts of seafood and poultry, and low or no added sugar, refined grains, or starchy vegetables and red meat, particularly processed meats which generally have the highest environmental impact. The impact of food and diets on the environment can be calculated thanks to the use of different indicators (van Dooren *at al*. 2018). However, only few of them have standardized methodologies, among which are the carbon footprint (CF) and the water footprint (WF) (Petersson *et al*., 2021).

This global reference diet aims to confer both improved health and environmental benefits thanks to the definition of planetary boundaries (e.g., cropland use, biodiversity loss, water use, greenhouse-gas emissions, and nitrogen and phosphorus pollution) that will allow a reduction of environmental degradation caused by food production. The shift from the current global diet to this planetary diet has been estimated to be able to prevent approximately

11 million deaths per year (Willet *et al*., 2019) and aims to provide healthy diets for a global population of about 10 billion people by 2050 while remaining within a safe operating space for the food system (Rockström *et al*., 2016; Willett *et al*., 2019).

The "Planetary health diet" should be adapted in the different countries, taking into consideration food cultures and traditions, and also to improve adherence to the dietary pattern. With this intention, the researchers have developed applications of the Planetary health diet in different contexts (Lassen *et al*., 2020) or have evaluated how current dietary habits differ from this reference diet (Blackstone and Conrad, 2020; Sharma *et al*., 2020).

We have recently developed a Mediterranean dietary pattern in line with the EAT-Lancet Commission reference diet (EAT-IT), based on 2500 kcal/day and adapted to Italian food habits, as published elsewhere (Tucci *et al*., 2021). Briefly, this adaptation was made by translating the EAT-Lancet recommendations into a more practical dietary scheme, allocating the different portion sizes and frequencies of consumption within different meals according to Italian dietary habits. The nutritional adequacy of this dietary pattern was compared the current Italian Dietary Guidelines (IDG) (Tucci *et al*., 2021), showing that the EAT-IT dietary pattern was higher in nuts and legumes compared to the one based on the IDG. Some potential critical issues emerged, mainly related to the low levels of calcium in the EAT-IT dietary plan compared to the IDG-based one.

The aims of the present study are to i) estimate the environmental impact of the EAT-IT dietary plan in comparison with the IDG-based one; ii) elucidate which are the main food categories contributing to the environmental impact of the two dietary plans; iii) estimate the variation of the environmental impact of the diet when different types of foods are selected.

2. Materials and Methods

2.1. Environmental impact of foods

The environmental impact of the foods included in the dietary patterns was calculated by using a recently developed multilevel carbon and water footprint dataset of food commodities which assigns footprint values with related uncertainties to food items, starting from peer-reviewed articles and grey literature (Petersson *et al*., 2021). Data related to carbon footprint (CF, expressed as kg CO2 equivalents/kg or L of product) and water footprint (WF, expressed as L of water/kg or L of product), accounting for greenhouse gas emissions and the consumption of water resources, respectively, were retrieved from the dataset.

Only food items showing both WF and CF values were extracted from the dataset and specifically selected to be used in the dietary plans, as described below.

2.2. Development of dietary pattern and dietary plan

The methodology used for developing the EAT-IT dietary pattern and the mid/long-term dietary plan based on the EAT-IT dietary pattern have been described elsewhere (Tucci *et al*., 2021).

Briefly, we firstly defined the EAT-IT-based dietary pattern considering the daily intake provided in the Planetary healthy diet for the eight different food categories defined within their report (i.e., whole grains, tubers or starchy vegetables, vegetables, fruits, dairy foods, protein sources, added fats, and added sugars) (Willett *et al.*, 2019) and which were converted to weekly amounts (grams/week). These amounts of foods were then allocated into different meals considering the main characteristics of the Mediterranean diet (e.g., sweet breakfast, cereal-based products, fruits, and vegetables at every main meal) and providing alternatives for the different meals in order to allow the long-term feasibility of this dietary pattern.

Once the dietary pattern was defined, a dietary plan of 2500 kcal/day was developed with recipes and dishes based on food habits of the Italian population, and including five meals per day (breakfast, lunch, dinner, and mid-morning and mid-afternoon snacks).

Similarly to the dietary plan based on EAT-IT, a dietary plan based on the IDG was also developed, following the same procedure described above (Tucci *et al., 2021)*.

2.3 Evaluation of the environmental impact of the dietary patterns

In order to calculate an average daily environmental impact, foods (within the same category) were allocated to the different days of the week, respecting the portions and frequencies of consumption of foods in the two dietary patterns. For this purpose, the RANDOM function of Excel was used. This function allows the generation of a random number sequence, that was then associated to the different food items for their random allocation. The mean daily CF (kg CO2 eq./day) and WF (L/day), were calculated for each food category and for the whole dietary patterns.

2.4. Analysis of scenarios depending on food choices

To investigate the impact of different food choices on CF and WF, four different case-studies were selected: i) use of frozen instead of fresh foods (i.e., bread, fish products, and beans); ii) use of imported instead of local fruits; iii) use of greenhouse-grown instead of seasonal vegetables; iv) use of processed instead of unprocessed legume-based foods.

For each scenario, increasing levels of substitution (from 25% to 100%) were simulated in order to investigate the impact of increasing use of these products on CF and WF compared to the EAT-IT and IDG dietary patterns in which 0% use of these products was assumed.

2.5. Statistical analysis

Data on CF and WF are expressed as mean ± standard deviation, except for variations in the different scenarios that are expressed as delta percentages compared to the EAT-IT and IDG dietary patterns described above.

Differences in the total CF and WF and in the relative footprints from the different food categories were analyzed by using the t-test. Statistical analysis was performed by using Student's t-test. The significance level was fixed at $p < 0.05$.

3. Results

3.1 Environmental impact of the two dietary patterns

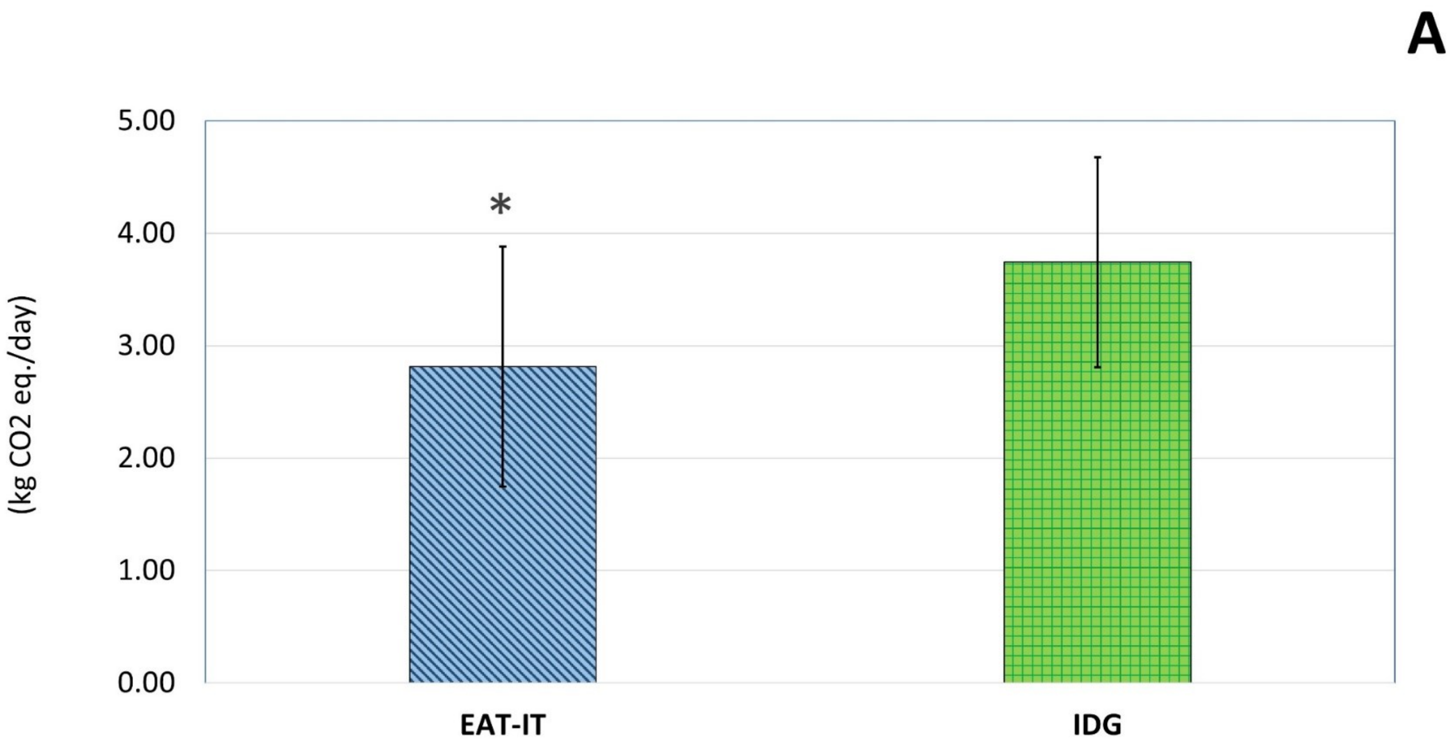
3.1.1 Carbon footprint

The environmental impact of the EAT-IT and IDG-based dietary patterns in terms of CF is reported in Figure 1. In detail, Figure 1A shows significantly lower CF for EAT-IT compared to IDG-based dietary pattern (2.82±1.07 and 3.74±0.92 kg CO_2 equivalents/day, respectively; $p<0.05$).

The higher CF in the IDG dietary pattern was likely due to the significantly higher intake of total whole grains and tubers (0.45±0.08 vs 0.30±0.06 kg CO_2 eq./day, respectively) and total dairy foods (0.85±0.43 vs 0.35±0.07 kg CO_2 eq./day, respectively), while only a significantly higher intake, and thus CF, of total vegetables was observed in the EAT-IT compared to IDG dietary pattern (0.32±0.13 vs 0.14±0.06 kg CO_2 eq./day, respectively) (Figure 1B).

Analyzing the CF in terms of percentage contribution by the different food groups, Figure 1C shows that protein sources were the main contributors in both dietary patterns (36% and 30% in the EAT-IT and IDG-based dietary pattern, respectively). The two dietary patterns were also similar for the % contribution of other food groups such as total whole grains and tubers (11% and 12%, respectively), total fruits (5%), and total oils and seasonings (5%

and 7%, respectively). Conversely, they largely differed mainly for dairy foods (13% and 23% in the EAT-IT and IDG-based dietary patterns, respectively) and total sugars (25% and 16%, respectively).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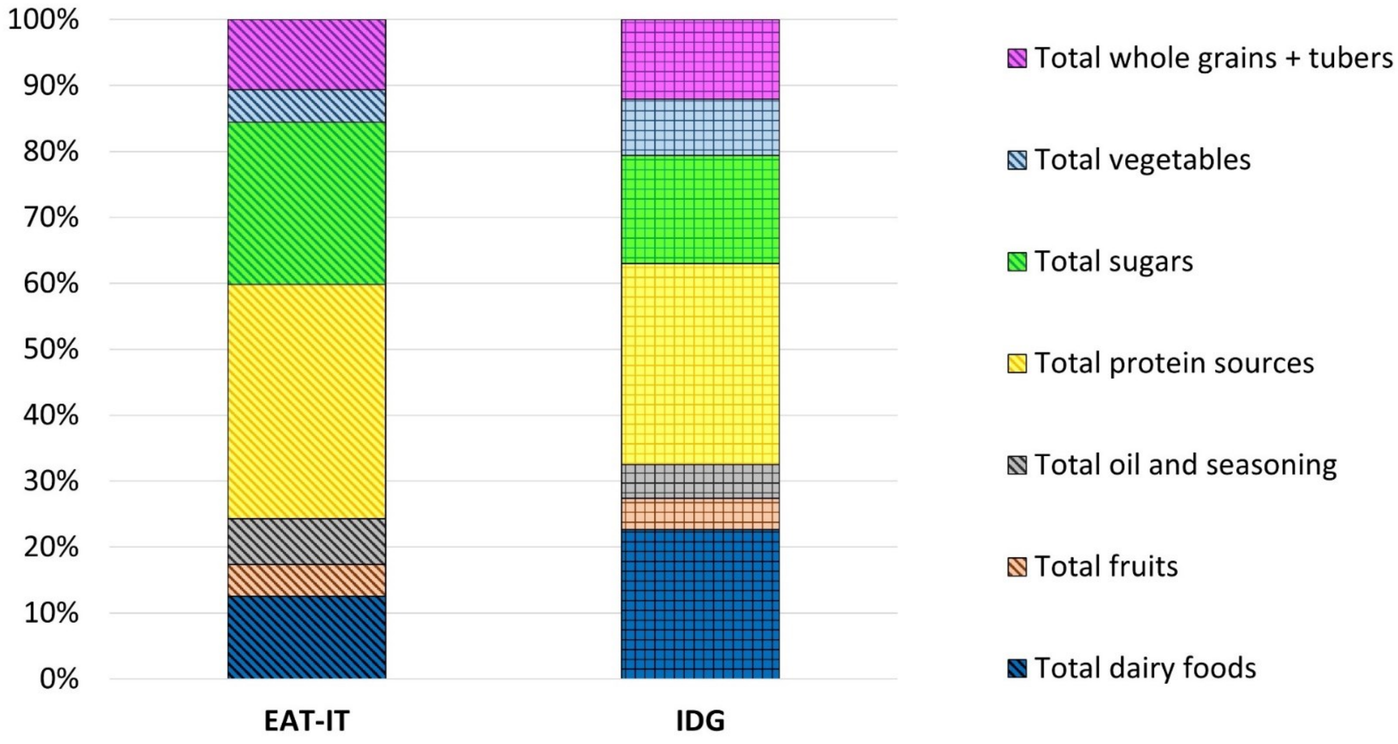
1.00
0.00
(kg CO2 eq./day)
*
EAT-IT
IDG

B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EAT-IT
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Total whole grains + tubers
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Total protein sources
Total oil and seasoning
Total fruits
Total dairy foods

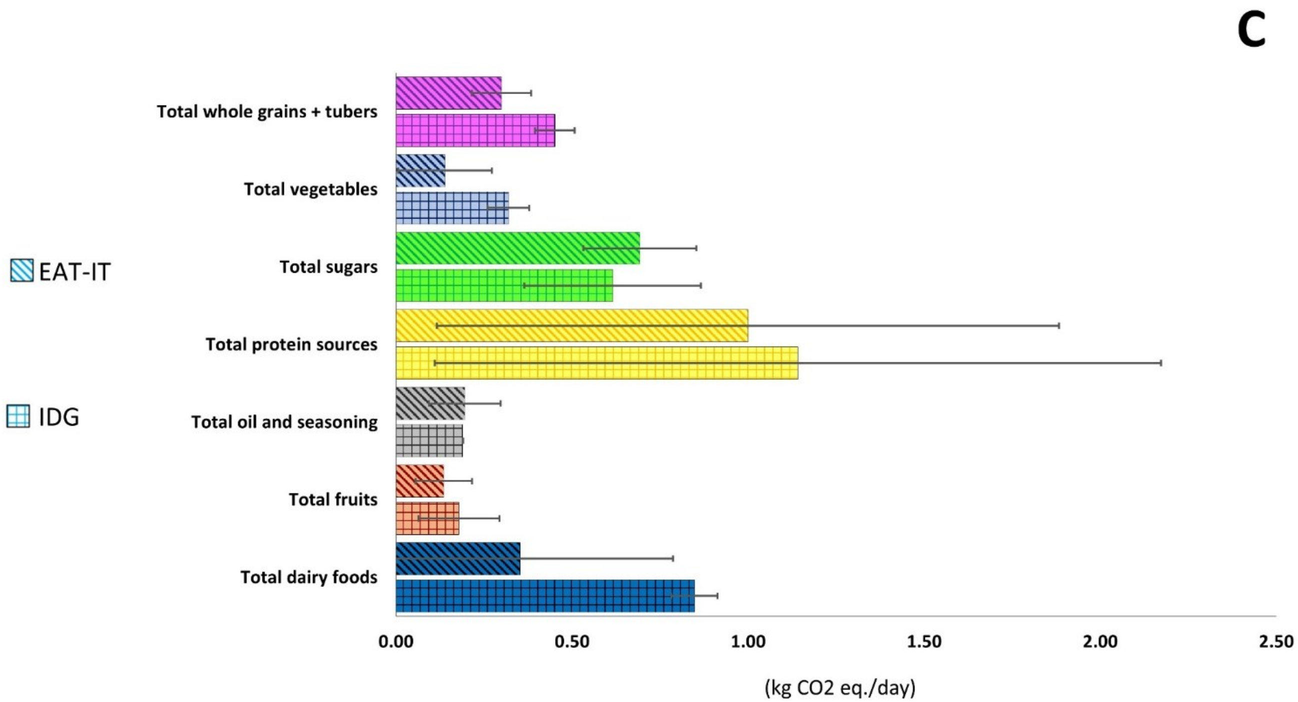


Figure 1 – Total (a) carbon footprint associated with EAT-IT and IDG dietary patterns and relative (b) and percentage (c) contribution of the different food groups. Legend: EAT-IT: EAT-Lancet Commission reference diet (adapted to the Italian food habits); IDG: Italian dietary guidelines; CF: carbon footprint.

3.1.2 Water footprint

Figure 2 shows the WF of the two dietary patterns. WF did not differ between the EAT-IT and IDG-based dietary pattern, being 4189 ± 533 and 4254 ± 662 L/day, respectively (Figure 2A).

Despite the lack of significance between the two dietary patterns, the contribution of the food groups largely varied in the two diets. A higher impact on WF of protein sources and total oils and seasonings was observed in the EAT-IT (Figure 2B), while total fruit and vegetables, dairy foods, and wholegrains and tubers mainly contributed to the impact of the IDG dietary pattern.

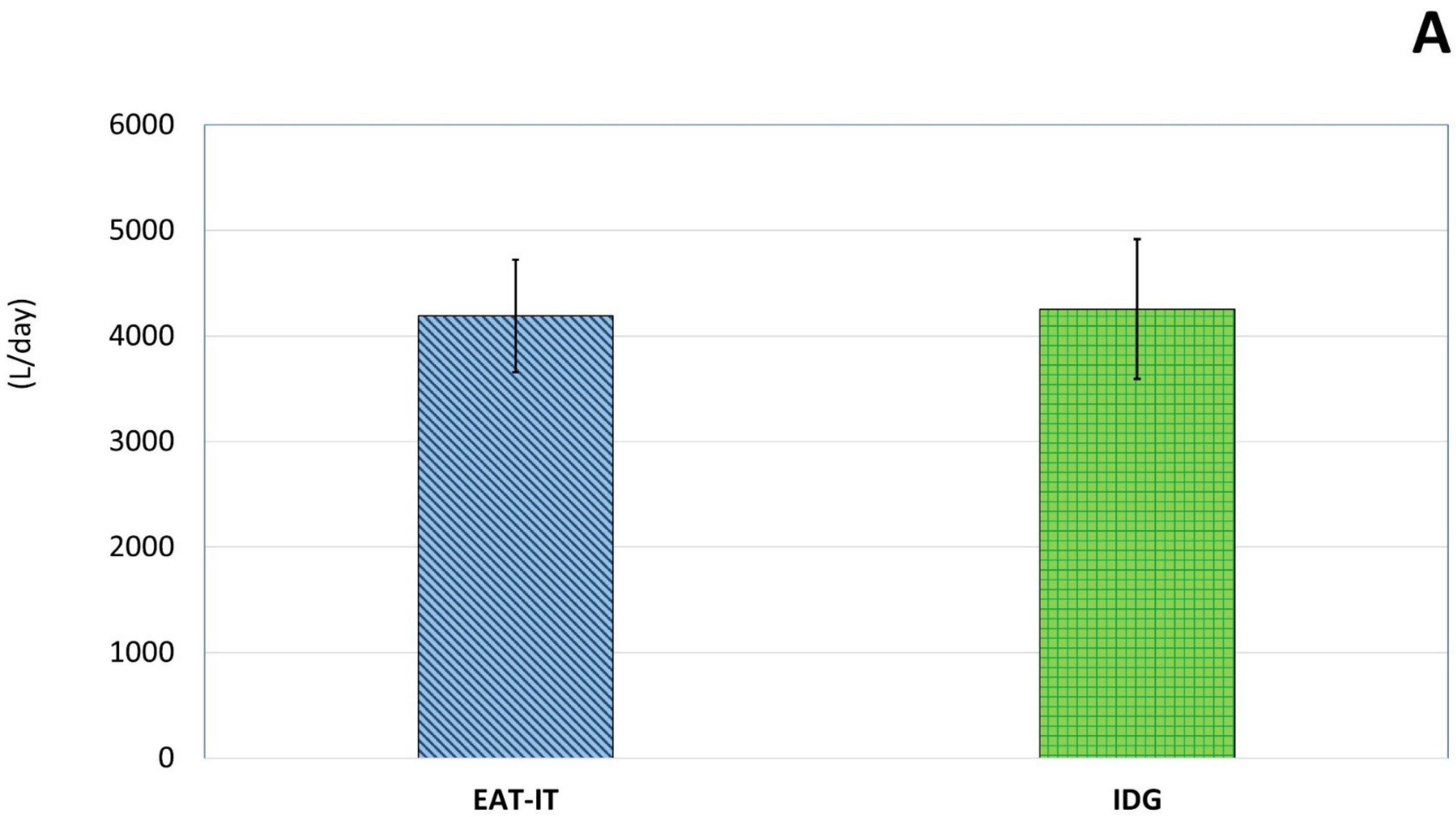
As shown in Figure 2C, considering the percentage contribution of the food groups, protein sources were the main contributors in both dietary patterns but varied from 45% in the EAT-IT to 30% in the IDG-based dietary pattern. The dietary patterns also differed for the contribution of total oils and seasonings (23% and 11%, respectively) and total dairy foods (8% and 19 %, respectively), but also total fruits (8% and 12%) and vegetables (4% and 8%).

3.2 Analysis of scenarios based on different food choices: case-studies

Figure 3 reports the impact of different food choices on CF in four different case-studies. Results show that the gradual increase in the substitution (from 25% to 100%) of frozen instead of fresh foods (Figure 3A), imported instead of local fruits (Figure 3B), grown in greenhouse instead of seasonal vegetables (Figure 3C) and processed instead of unprocessed protein-rich foods (Figure 3D) cause an increasing worsening of the CF compared to the related EAT-IT and IDG dietary patterns described in Section 3.1, in which a 0% of this substitution was assumed.

In details, the substitution of frozen instead of fresh foods from 25% to 100% causes an increase in CF from 2.8 to 8.2% and from 1.8 to 5% in the EAT-IT and IDG dietary patterns, respectively (Figure 3A), the consumption of imported instead of local fruits from 1.1 to 3.9% and from 1 to 4.5%, while the use of grown in greenhouse instead of seasonal vegetables from 9 to 34% and from 12 to 39% (Figure 3C). Finally, the consumption of processed instead of unprocessed protein-rich foods causes an increased CF from 3.4 to 10% and from 1.6 to 3.1% in the EAT-IT and IDG dietary patterns, respectively (Figure 3D).

Conversely to CF, WF in the four case-studies was almost not affected by these substitutions (data not shown).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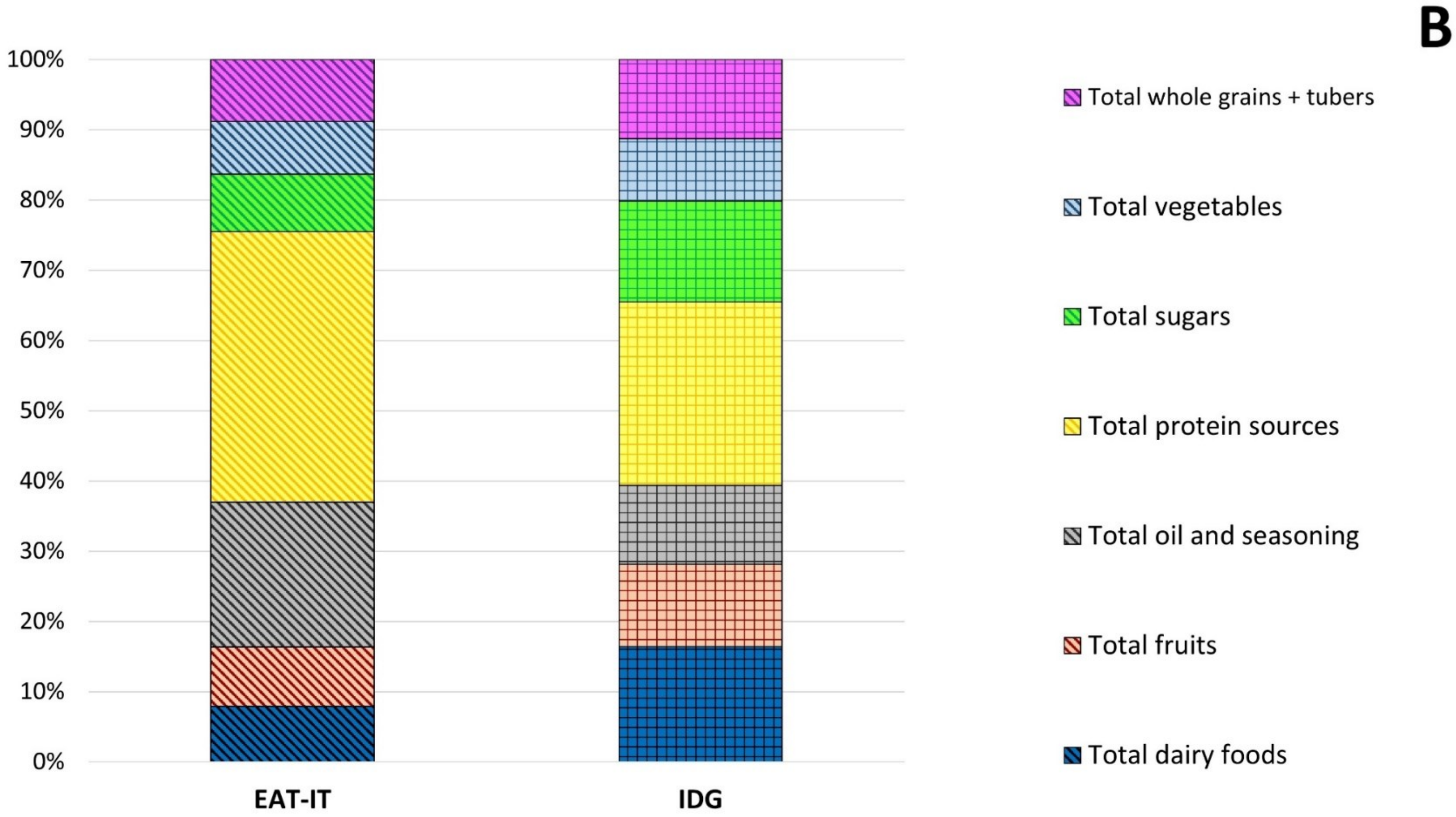
IDG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Total vegetables
Total sugars
Total protein sources
Total oil and seasoning
Total fruits
Total dairy foods

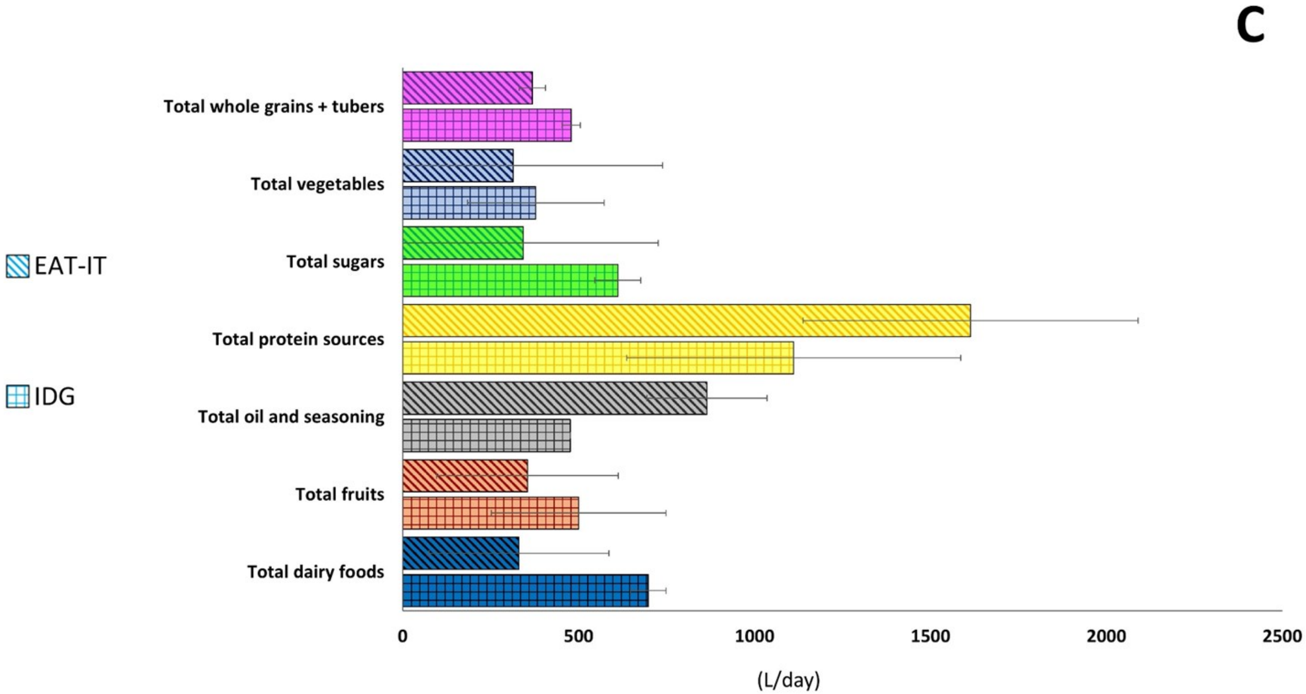

Figure 2 – Total (a) water footprint associated with EAT-IT and IDG dietary patterns and relative (b) and percentage contribution (c) of the different food categories. Legend: EAT-IT: EAT-Lancet Commission reference diet (adapted to the Italian food habits); IDG: Italian dietary guidelines; WF: water footprint.

3.2 Analysis of scenarios based on different food choices: case-studies

Figure 3 reports the impact of different food choices on CF in four different case-studies. Results show that the gradual increase in the substitution (from 25% to 100%) of frozen instead of fresh foods (Figure 3A), imported instead of local fruits (Figure 3B), greenhouse-grown instead of seasonal vegetables (Figure 3C) and processed legume-based foods instead of unprocessed legumes (Figure 3D) caused an increasing worsening of the CF compared to the related EAT-IT and IDG dietary patterns described in Section 3.1, in which 0% of this substitution was assumed.

In detail, the substitution of frozen instead of fresh foods from 25% to 100% causes an increase in CF from 2.8 to 8.2% and from 1.8 to 5% in EAT-IT and IDG dietary patterns, respectively (Figure 3A), the consumption of imported instead of local fruits from 1.1 to 3.9% and from 1 to 4.5%, while the use of greenhouse-grown instead of seasonal vegetables led to rises from 9 to 34% and from 12 to 39% (Figure 3C). Finally, the consumption of processed legume-based foods (e.g., soy burgers) instead of unprocessed legumes causes an increased CF from 3.4 to 10% and from 1.6 to 3.1% in EAT-IT and IDG dietary patterns, respectively (Figure 3D)

Conversely to CF, WF in the four case-studies was barely affected by these substitutions (data not shown).

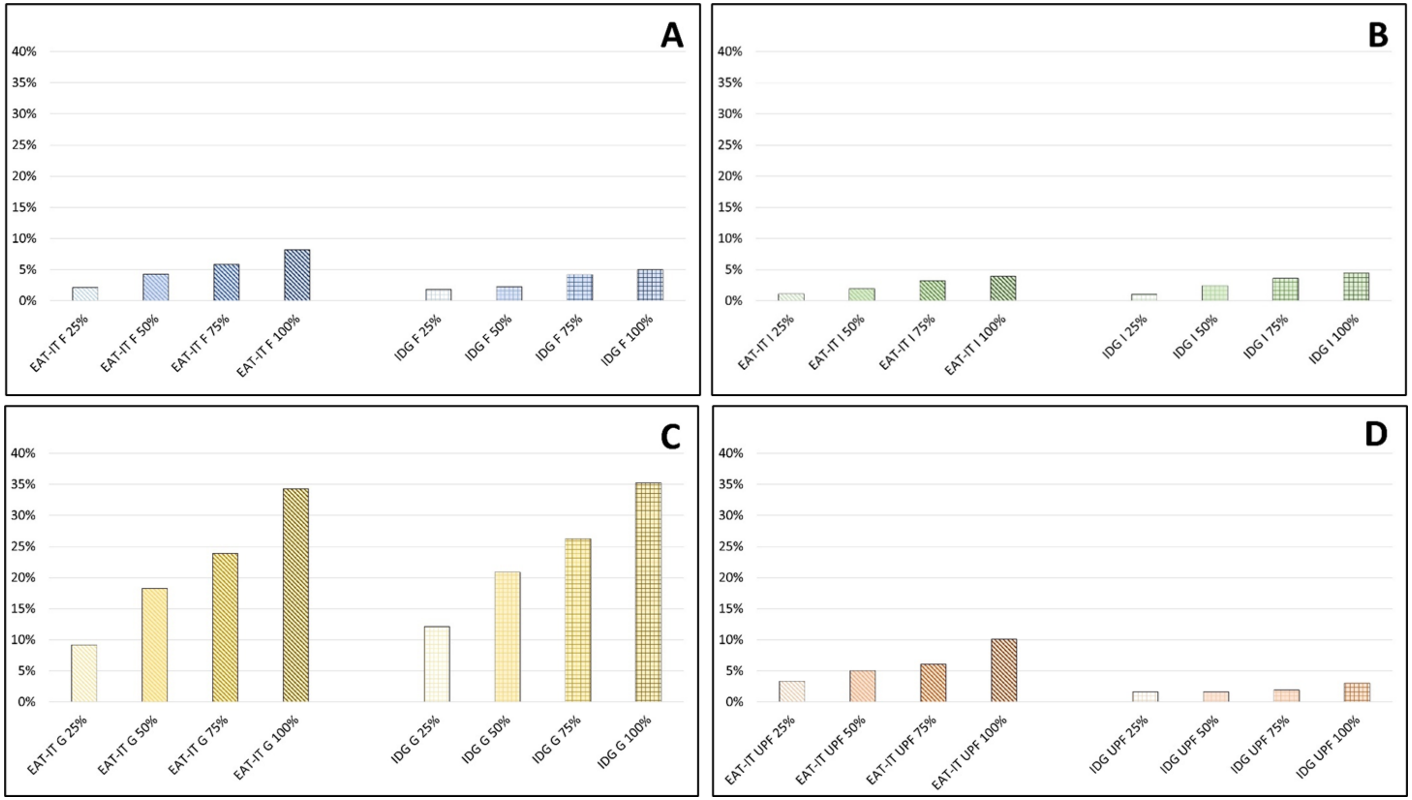

Figure 3 – Impact of different food choices on CF and WF case-studies with a gradual increase in the substitution of frozen instead of fresh foods (a), imported instead of local fruits (b), greenhouse-grown instead of seasonal vegetables (c) and processed legume-based foods instead of unprocessed legumes (d). Data are expressed as delta percentage compared to the EAT-IT and IDG dietary patterns.

4. Discussion

The following study investigated the theoretical carbon and water footprints associated with the EAT-IT dietary pattern, developed based on the planetary diet but adapted to the Italian tradition. The results highlight that this dietary pattern generates a lower CF but not WF compared to a pattern developed based on the IDG. This is not surprising since a recent systematic review that analyzed the environmental outcomes of both empirical and modelling studies on sustainable diets reported an increase in water use (+13.8%) when shifting from baseline to 'sustainable diets' (Jarmul *et al*., 2019). This rep-resents a critical aspect of the definition of healthy and sustainable diets since indicates that the optimization of one indicator of environmental impact does not automatically correspond to general optimization, and trade-offs are considered likely (Garnett, 2014). Few data are available about the average levels of water footprint of the Italian population. Rosi et al. (2017), documented that the food intake of a group of 51 Italian omnivorous adults corresponded to a diet-related water footprint of 3141 L/day. This value differs from those calculated by using our models. In fact, we have found that water footprints for the EAT-IT dietary pattern and IDG were higher compared to the value found by Rosi and colleagues (33.4% and 35.4% for EAT-IT and IDG, respectively). However, data could not be completely compared, due to the different databases used for the assessments.

Considering greenhouse gas emissions, the IDG dietary pattern was associated with a higher CF compared to the EAT-IT: however, it is noteworthy that both diets were more favorable compared to the current dietary habits of the Italian population. In fact, according to a recent analysis based on a national food consumption survey, an average CF of 4 kg CO2 eq/day was found (Ferrari *et al*., 2020). Indeed, it has been recently observed that the current consumption of legumes and nuts should be almost doubled to meet the targets proposed by the EAT–Lancet Commission, whereas the consumption of meat, eggs, dairy products, animal fat, tropical oils, and sugars should be largely reduced (Vitale *et al.*, 2021). The differences observed in CF in the IDG dietary pattern, compared to the EAT-IT one, are probably due to the higher inclusion of animal-based products (in particular eggs, white meat, milk and derivatives), since they are generally characterized by higher gas emissions compared to plant-based foods (Poore and Nemecek, 2018; Clark *et al.*, 2019). These results agree with those by Rosi et al. (2017) who found that omnivorous food choices generated higher carbon footprints than vegetarian and vegan diets. Despite these higher footprints in the omnivorous diets, authors found a high inter-individual variability, with a few vegetarian and vegan subjects' diets showing higher environmental impact than those of some omnivores. Therefore, regardless of the robust and positive effects of plant-based diets on environmental health, results support the role of individual dietary habits in determining the environmental impact of the diet. This is also clearly shown in the present study, in which the large variation (i.e., in terms of standard deviation of CF) within the same food category reflects the effect of single food choices on environmental impact. It should be considered that dietary choice is the result of numerous determinants, and attitudes towards sustainability have been reported to be influenced by gender, location of residence (region and urban vs. non-urban), social class, age, and education [27, 28]. For instance, a German nutritional survey on meat consumption carried out in 2019 found that non-meat consumers do not substitute meat with fish, eggs, or milk products but rather with soya products and other plant-based foods. Thus, the evaluation of the magnitude of dietary choices among different food categories or within different food items within the same one is worthy of further study (Koch *et al.*, 2019).

A recent study conducted by Clark and coworkers (Clark *et al.*, 2022) has estimated for the first time the environmental impact of more than 57,000 food products (including foods belonging to the same food category), commercially available in UK and Ireland. This analysis found that the difference between a most and a least sustainably sourced product (5th and 95th percentile impact, respectively) can be large, depending on ingredient composition (Clark *et al.*, 2022). Our work corroborates these findings, since diet-related environmental impacts resulted in significant influences at a food item level, in relation to its production systems and not just the food category to which it belongs (Kim *et al.*, 2020). This is further shown by the elaboration of different scenarios, such as the substitution of unprocessed legumes with processed legumes-based foods (e.g., soy burgers), or of local with imported fruits, that can further worsen the environmental impact of the diet, in terms of CF. Interestingly, we found lower exacerbating effects on CF for frozen and im-ported fruits, reasonably in relation to i) the limited data on frozen products, that did not allow us to find substitute food items in all food categories, ii) the lower weekly amount of total fruits, compared to vegetables. These results support the consumption of seasonal vegetables as a principle of sustainability for plant-based diets, as suggested by the Food and Agriculture Organization of the United Nations (FAO)/ Food Climate Research Network (2016). However, food product seasonality was not included among the 16 guiding principles of sustainable healthy diets proposed in 2019 by FAO/ World Health Organization (2019). Furthermore, a comprehensive review of all available English-translated food-based dietary guidelines indicated that few of them involved environmental sustainability recommendations and the ones that addressed this issue focused on the advice to consuming foods produced locally (Martini *et al.*, 2021). Given that the inclusion of sustainability criteria within food-based dietary guidelines has been widely advocated (Gonzalez Fischer and Garnett, 2016; Magni *et al.*, 2017; Bechthold *et al.*, 2018; Springmann *et al.*, 2018b, 2020), if our own and previous results are corroborated by other assessments, then a more explicit reference to the seasonality of vegetable products within food-based dietary guidelines could represent a simple and profitable message to be delivered to the population. Another consideration is that despite the four different scenarios which were elaborated separately, their effect on di-et-related environmental outcomes can also be additive. The observed increase in final CF when unprocessed legumes were progressively substituted with processed legume-based products was particularly pronounced in the EAT-IT dietary pattern (+0.28 kg of CO2 eq./day for 100% substitution), due to the higher presence of legumes in comparison with IDG. However, it should be clear that, according to data provided by the database used, 0.28 kg of CO2 eq. corresponds to a portion of just 10.9 g of beef, or 49.0 g of pork. Thus, our analysis aimed to assess the magnitude of food selections among different food items within the same food category on final environmental impact, finding a non-negligible effect on final CF, but largely corroborated the wider literature supporting the lower CF as-sociated with more plant-based diets, in comparison to more meat-based ones (Tilman and Clark, 2014; Chai *et al*., 2019). The present study has some strengths worth noting. To the best of our knowledge, this is the first study investigating the CF and WF of a dietary model developed based on the planetary diet but adapted to a specific socio-cultural context. Moreover, this study matches perfectly with a previous study in which the nutritional characteristics of the EAT-IT dietary pattern were compared to the IDG one (Tucci *et al*., 2021), both in their nutritional and environmental aspects, important domains of sustainable healthy diets. However, there are also some limitations. Some of them are strictly related to the use of databases which, although extensive, do not include all food items, and for many of them, provide data related to a high level of uncertainty. Another intrinsic limitation of such databases is the lack of country-specific data reflecting variability across different national food production systems. Moreover, our choice of including only items with both CF and WF values in the database further limited the variety of single items (e.g., types of fruit) included in the model, mainly due to the scarcity of data regarding WF. These observations support the need for improving such databases, increasing and updating their records to allow more precise assessments. Again, in the present study we investigated the environmental impacts only in terms of CF and WF. However, although these indicators are the two most well-known ones, many other indicators could be used, such as land use and energy use (van Dooren *et al.*, 2018).

Overall, these results suggest the importance of considering both nutritional and environmental aspects of the diet, with economic and socio-cultural aspects which cannot be neglected to favor the transition towards sustainable healthy diets (Perignon *et al.*, 2016; FAO, IFAD, UNICEF, WFP and WHO, 2021). In this regard, despite the urged necessity to favor the transition towards sustainable food systems, literature supports the importance of considering social–cultural aspects and country-specific food habits. since a gradual transition to a sustainable diet is likely to be more easily implementable by the population, and is likely to arise from a general shift towards a healthier lifestyle and social environment (Collins and Fairchild, 2007; Koch *et al.*, 2019; Downs *et al.*, 2020; Kim *et al.,* 2020; Clark *et al.*, 2022).

The importance of a holistic approach to contextualize the environmental impacts of different diets has recently been advocated (Dave *et al.*, 2021). For instance, given the different bioavailability of compounds in plant-based foods and animal-source foods, it has been recommended that future research should give more consideration to the inclusion of bioavailability assessment within nutrition metrics. This will be important to predict and understand the potential positive/negative impact of a major or minor bioavailability of a specific nutrient/non-nutrient on human health (in particular when considering vulnerable groups of populations) which in turn allows better comprehension of the different dimensions of sustainability (HLPE, 2020; Dave *et al.*, 2021; Leroy *et al.*, 2022; Smith *et al.*, 2022). In this scenario, Ferrari *et al.* (2020) tried to optimize a healthy and sustainable dietary model with low gas emission, satisfying dietary requirements and taking into consideration current Italian food consumption patterns, but they could not find any suitable diet fulfilling the iron constraint for females, which remained below the dietary reference intake in the optimized model, supporting the importance of a holistic approach when developing these dietary models.

In conclusion, our analysis integrates the previous evaluation of the nutritional adequacy of a plant-based dietary pattern based on the Planetary diet and adapted to the Italian-Mediterranean food context (EAT-IT dietary pattern). Our results demonstrated a lower CF of this pattern compared to an IDG one, but not reduced levels of WF, thus supporting the concept that optimization of one indicator of environmental impact does not necessarily allow the optimization of all other indicators. Furthermore, we found that individual food choices can markedly influence final diet-related environmental outcomes, showing that limiting animal food products may not be enough to achieve the goal of a low CF-diet. In particular, the worst scenario occurred when large shares of vegetables were substituted with vegetables grown in heated greenhouse and when legumes were substituted with processed legume-based foods, such as soy burgers. Eating fruit and vegetables according to seasonality can represent a viable message to be included in sustainable diet recommendations. Overall, our analysis corroborates the wide scientific literature reporting the lower CF associated with plant-based diets, in comparison with the ones based on a higher consumption of animal foods. Finally, a holistic approach to the evaluation of environmentally optimized diets should be carefully considered to safeguard all the dimensions of a sustainable healthy diet.

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3.3 Chapter 3

- **Effect of a sustainable dietary pattern (EAT-IT) on markers of nutritional status and dietary behaviour in a group of healthy, young subjects: a pilot study.**
  (Unpublished results)

3.3 Effect of a sustainable dietary pattern (EAT-IT) on markers of nutritional status and dietary behaviour in a group of healthy, young subjects: a pilot study

Abstract: Data on healthy and sustainable diets currently derives mainly from modelling study while evidence obtained from intervention studies is lacking. An explorative pilot RCT evaluating the feasibility and effectiveness of the healthy and sustainable dietary pattern previously developed based on the Planetary health diet but adapted for the Italian population (EAT-IT) has been settled. We enrolled 10 healthy subjects and randomly allocated them to follow for 8 weeks the EAT-IT dietary pattern (n= 5) or a Control diet including dietary recommendation based on the Italian Dietary Guidelines (n= 5). Nutrient intake was assessed through weighted food records. Blood samples were collected at the beginning and at the end of the intervention to evaluate health-related parameters. Subjects were characterized for their adherence to the Mediterranean diet and their acceptability of the EAT-IT plan, assessed through validated questionnaires. Overall, biochemical parameters were found to be in the normal range and subjects presented an intermediate adherence to Mediterranean diet. We found that the EAT-IT intervention resulted mainly in increased PUFA, specifically ω-6 intake, compared to the Control group ($p = 0.02$), but small effects were found for other nutrients intake. Interventions did not modify health related outcomes, even if a decrease in haemoglobin and haematocrit in male subjects within the Control group was found. Acceptability of the EAT-IT dietary pattern resulted medium/high. Interestingly, subjects allocated in the EAT-IT group significantly decreased their energy intake (p for time = 0.03) and this could reflect an increased difficulty in sticking with the diet. Independently from intervention, we found low levels of intake for several micronutrients, including iron, calcium, and vitamin D.

1. Introduction

As already reported, the definition and validation of healthy and sustainable dietary patterns represent a "win to win" approach in the fight against the increase in the incidence of chronic diseases (and malnutrition) and in the mitigation of the environmental impact connected to global food systems (Rockström *et al*., 2016; Fanzo *et al.*, 2017; FAO and WHO, 2019). In this regard, we have elaborated the theoretical proposal for the adaptation of the EAT-Lancet Planetary health diet in the Italian-Mediterranean food context and evaluated its theoretical nutritional adequacy (Tucci *et al.*, 2021) as reported in chapter 3.1. Briefly, we found an overall balanced nutritional profile with few exceptions, but quite far from the current consumption of the Italian population in relation to the amount of meat and dairy products (very low in the EAT-IT model) and of legumes (abundant in the EAT-IT model).

Considering these theoretical aspects, which would indicate the possibility of implementing the model with selected foods able improve its nutritional profile and increase adoption with possible health and environmental related benefits, only recently appeared in the literature studies examining the *in vivo* effect of several healthy and sustainable diets. For example, the study by Päivärinta and colleagues (2020) evaluated the effect on nutritional intakes and lipid profile of two different more sustainable plant-based diet proposals based on the Nordic food model, in a group of 136 healthy omnivorous adults aged between 20 and 69 years. Another recent observational study (Laine *et al.*, 2021) linked the level of adherence to the "Planetary health diet" pattern with mortality from various chronic diseases and with environmental impact parameters starting from the data collected within the EPIC study, which followed a cohort of more than 400,000 subjects from 10 different European countries. The study concluded that this diet can be considered healthy and sustainable and estimated that a higher adherence of the European population to the planetary dietary pattern, would be associated to a reduction in the risk of mortality from various chronic diseases and the decrease of greenhouse gases associated with food systems of up to 50%.

However, no study has specifically addressed the evaluation of the actual potential benefits of the "Planetary health diet" and its adaptation through a dietary intervention study, nor it has been taken into consideration the evaluation of the acceptability of such type of dietary pattern. However, the level of satisfaction and acceptability of a diet represent a fundamental element in determining its concrete reliability, adoptability and the related degree of compliance (Shay, 2008; Moore *at al*., 2015). It is also worth to note that according to FAO's definition, acceptability represent a necessary condition to achieve diet sustainability (FAO, 2010). Given this background, the aim of this study is to evaluate the feasibility of an 8-week dietary intervention (EAT-IT dietary pattern) and its effect on overall food and nutrient intake, nutritional status, as well as metabolic/physiological markers through a pilot study involving a small group of healthy young adults.

2. Materials and methods

2.1 Study overview

This study was developed as a single single-blind, randomized controlled trial, presenting a crossover design. Subjects that expressed their willingness to take part in the study were screened for their eligibility and then requested to record their food intake on a 7-days weighed food records, which was used to assess their dietary habits. Subjects enrolled were also requested to undergo a baseline characterization, with routine laboratory analysis. Then, they were randomly allocated to the EAT-IT dietary pattern or a Control diet. Diets were personalized based on individual energy and nutrient requirements as estimated by their anthropometric characteristics and considering their food intake data and lifestyle. Each arm of the study involved an 8-week period of dietary intervention (EAT-IT or Control), followed by a wash-out period of 8 weeks, in which participants could eat freely, followed by another 8-week period of diet (EAT-IT or Control) following the cross-over design.

At baseline, subjects were also requested to fulfill some questionnaires to assess their adherence to the Mediterranean diet, as well as their knowledge on nutrition and sustainability and their behavior. At the beginning and at the end of each of the two interventions, blood samples were collected, and subjects provided a 24-h urine tank and a fecal sample. In addition, volunteers were instructed to fulfill a 4-day weighed food record. Finally, subjects in the EAT-IT arm were also required to fulfill a validated questionnaire on the acceptability of the diet. Since the trial is not completed (i.e. second part of the cross-over and new recruitments are still ongoing), in this chapter only preliminary data obtained after the first treatment intervention will be presented.

2.2 Study participants

For this study subjects have mainly been recruited from the students of the University of Milan. Candidate volunteers had to have at least 18 years and both sexes have been considered. Exclusion criteria were i) use of drugs that can interfere with the analysis, ii) vegetarian or vegan diet iii) presence of conditions that require specific diets or treatments (e.g., celiac disease) or that could be negatively affected by the dietary treatments (e.g., irritable bowel syndrome). In general, any doubt regarding the eligibility of a possible participant was defined and discussed with medical personnel involved in the research.

The enrollment was carried out through advertisements by using emails, social network, bulletin board, and by word of mouth.

Demographic characteristics and basal biochemistry analysis of recruited subjects at baseline are reported in Table 1.

2.3 Dietary habits assessment

Enrolled subjects were initially requested to record their food intake on a 7-day weighted food diary, using an already developed template, but specifically adjusted for this study. During the trial, additional 4-day weighed food records were requested. The data collected were used to evaluate their actual food intake, compliance with the dietary indications during intervention, and the evaluation of changes in their nutrient intake at the end of the intervention. All food diaries were individually elaborated through a software (MètaDieta professional 4.1.1 METEDA Srl–Roma, Italy), to estimate the correspondent energy and nutrient intake.

2.4 Dietary interventions

For this study, subjects on the Control diet received personalized dietary guidelines, based on the Italian Food-Based Dietary Guidelines while those on the EAT-IT diet those based on the Mediterranean-adapted healthy and sustainable diet. Subjects were randomly allocated within these two study arms through a computer random number generator. The EAT-IT dietary pattern as previously reported includes three main meals (breakfast, lunch and dinner) + 1-2 snacks based on nuts. Breakfast includes a portion of milk or derivatives, a portion of whole grains (wholemeal bread, wholemeal rusks, oats, or corn flakes) and a portion of jam or fruit juice. Lunch and dinner are based on a portion of whole grains (wholemeal rice or pasta, spelled, wholemeal bread, etc.) or potatoes, a portion of protein food (beef, pork, or lamb; poultry, eggs, fish, or legumes) according to appropriate frequency of consumption, vegetables, oil, and fruit.

Both dietary patterns were isocaloric with respect to the estimated energy requirements of participants and personalized as much as possible based on individual dietary habits. Participants were also regularly provided with some food products aimed at facilitating adherence to the EAT-IT dietary pattern. Such periodic supplies consisted of commercial products such as pasta, bread, legumes, vegetables, fruit, and milk.

2.4 Anthropometric measure and routine laboratory characteristics

Subjects enrolled were assessed for their anthropo-plicometric measurements (height, weight, waist circumference, and measure of body composition through plicometry, according to standard and validated reference methods (Durnin and Womersley, 1974) by trained dieticians at the International Center for the Assessment of Nutritional Status (ICANS) of DeFENS. Fasting blood samples were also collected for the assessment of fasting glucose, lipid profile and blood count. Briefly, volunteers arrived fasted at the university facilities (ICANS) and blood samples were taken via venipuncture by trained personnel and drawn into a 2,7 ml tube containing ethylenediaminetetraacetic acid (EDTA) for the blood count, and a 7 ml tube without anticoagulant for serum collection used to evaluate glucose and lipid profile. To obtain serum, the tube was left to coagulate for 30 minutes and then centrifuged for 15 minutes at 2300 rpm at 4 °C.

2.5 Adherence to Mediterranean diet

The level of adherence to the Mediterranean diet was evaluated at baseline using the validated MEDI-LITE questionnaire (Sofi *et al.*, 2017). MEDI-LITE is a literature-based questionnaire, that was formulated computing data from all available cohort studies on the association between Mediterranean diet and health status and validated comparing it with the MedDietScore (MDS). The MEDI-LITE questionnaire is composed of 9 food groups for which 3 different daily or weekly consumption are reported and can be used to discriminate different behavior at subject level. By answering to the 9 questions of the questionnaire a score ranging from 0 to 18 is obtained. The cut-off to discriminate between non adherents from adherents to the Mediterranean diet is a score of 8.5.

2.7 Levels of acceptability

Acceptability of the EAT-IT dietary pattern was assessed through the questionnaire created by Barnard *et al*. (2000). This questionnaire consists of 8 questions with a 7-point response scales (e.g. How well do you like the foods that you have been eating in the past 2 week? 1 = "extremely good," 7 = "extremely unappealing"). This questionnaire was validated and showed adequate test-retest reliability.

2.8 Sample size

For this trial, 10 subjects have been initially involved since it represents a small pilot-study developed within a larger dietary intervention to assess the optimal conditions to ascertain the feasibility of a healthy and sustainable diet. Among others, data from this pilot study will be used also to assess proper sample size for the main study and optimize guidelines for ensuring compliance of the participants.

2.9 Statistical analysis

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Only complete data obtained have been considered for the elaborations. To analyze differences between the intervention groups in all continuous variables at baseline, as well as differences in nutrient intakes both at baseline and at the end of the intervention, a two-way analysis of variance (ANOVA) for repeated measures was used, considering the treatment as independent variable (EAT-It dietary pattern vs IDG) and time (before and after the intervention) as dependent variable. In addition, time x treatment interaction was evaluated. When appropriate, a post-hoc p-value adjustment have been performed using the Hochberg-Benjamin correction. Significance has been set at $p < 0.05$. Significance in the range $0.05 < p < 0.10$ is indicated as trend.

3. Results

3.1 Study population

Baseline characteristics of the 10 enrolled subjects are reported in Table 1. All volunteers were young adults (maximum age of 30 years), and their education level was high (bachelor or master's degree). Subjects were all normal weight per height as indicated by their BMI. Also, the other biochemical parameters, C-reactive protein, liver, and renal function resulted within normal levels. Considering blood cell count, values were in the normal range, even if borderline values were observed for females.

Table 1. Baseline characteristics of the 10 enrolled subjects

Variables (n= 10)	**Mean ± ST**
Age (years)	25.5 ± 2.4 (23 to 30 years)
Gender (n, %)	
Female	6 (60)
Male	4 (40)
Education (n, %)	
Basic education (primary)	0
Intermediate education (secondary)	0
High education (university level)	10 (100)
n household component	2.1 ± 1.2
Height (m)	1.70 ± 0.10
BMI	21.6 ± 1.6
Cholesterol (mg/dl)	
Total	180 ± 34
LDL	96 ± 33
HDL	64 ± 17
Total/HDL ratio	3.0 ± 1.1
Triglycerides (mg/dl)	106 ± 31
Fasting glucose	93 ± 7
Haemoglobin - Hb (g/dL)	
Female (12-16)	12.6 ± 0.9
Male (14-18)	15.2 ± 1.1
Haematocrit	
Female (38-45)	38.3% ± 2.4
Male (41-51)	44.7% ± 2.8
MCV	
Female (80-96)	80.8 ± 10.7
Male (80-96)	86.4 ± 2.2
C-Reactive Protein (PCR) (mg/dL)	1.7 ± 2.6
ALT (U/L)	15.4 ± 4.8
GGT (U/L)	19.0 ± 8.6
GOT (U/L)	17.5 ± 3.8

Azotaemia (mg/dL)	31.5 ± 6.2
Uric acid (mg/dL)	1.3 ± 1.5

Values are presented as mean ± SD, except for gender and education, which are presented as total number and percentage value (n, %). Legend: ALT= Alanine transaminase; GGT= Gamma-glutamyl transferase; GOT= glutamic oxaloacetic transaminase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MCV: Mean Corpuscular Volume. For blood count parenthesis reports normal range values differentiated for both sexes.

3.2 Adherence to Mediterranean diet

Table 2 reports the assessed levels of adherence to Mediterranean diet, according to the MEDI-LITE questionnaire. Overall, mean, median and mode indicate a medium level of adherence to the Mediterranean diet (11.2 ± 2.1, 11, and 13, respectively). When analysing the single scores, apparently the dietary habits of subjects seemed to be in line with the EAT-IT dietary pattern for some aspects, since subjects declared a frequent use of olive oil (median score of 2), a limited consumption of dairy (median score of 1), and fish (median score of 1). Conversely, subjects reported an intermediate/high consumption of meat and cured meat (median score of 2, mean score of 1.6 ± 0.5) and an intermediate legumes consumption (median score of 1). The consumption of legumes is therefore confirmed as one of the main critical elements in the potential acceptability and adoption of the EAT-IT dietary pattern.

Table 2. Score given for each individual food category and final score.

	mean	SD	median	mode
Fruits	0.9	0.3	1	1
Vegetables	1.4	0.7	2	2
Legumes	0.9	0.8	1	1
Cereals	1.6	0.5	2	2
Fish	0.8	0.4	1	1
Meat and cured meat	1.6	0.5	2	2
Dairy	1.2	0.4	1	1
Alcolic beverages	1.0	0.0	1	1
Olive oil	1.9	0.3	2	2
Final score	**11.2**	**2.1**	**11**	**13**

Score can range from 0 to 2 for each food category. For fruits, vegetables, legumes, cereals, fish, and olive oil the score 2 correspond with higher declared consumption; while for meat and cured meat, and dairy the score 2 correspond to lower declared consumption. For alcoholic beverages, the score 2 correspond to intermediate consumption, while 1 correspond to lower consumption and 0 to the higher. Final score can range from 0 to 18, and the cut off score between non-adherent or adherent to Mediterranean diet is 8.5.

3.3 Metabolic and functional outcomes

The effects of the dietary interventions on the general physiological and metabolic markers are reported in Table 3. A time-dependent decrease in total cholesterol was observed within the control group (from 178 ± 45 mg/dL to 157 ± 36, p = 0.03), but considering treatment or time x treatment interactions lipid profile was not significantly different between the two groups, according to ANOVA. Fasting glucose, C-reactive protein, azotaemia, and liver function markers were not changed. Uric acid resulted significantly increased at the end of the intervention in the control group, compared to the EAT-IT group (from 5.3 ± 1.5 mg/dL to 5.9 ± 1.4 vs 4.1 ± 2.3 to 4.2 ± 2.2 respectively¸ p = 0.03). Considering blood count, the treatment did not affect the levels of haemoglobin, haematocrit, or Mean Corpuscular Volume of erythrocytes. However, a significant decrease in both haemoglobin and haematocrit levels was found within the Control group for males (from 14.8 ± 1.6 to 13.8 ± 1.5, p for time = 0.03, and from 43.9 ± 4.4% to 41.1% ± 4.5, p for time = 0.02, respectively).

Table 3. Health related markers variations.

	EAT-IT (t0)	EAT-IT (t1)	Control (t0)	Control (t1)	P value for time	P value for treatment	P value for interaction
Cholesterol (mg/dL)							
Total	181 ± 25	172 ± 32	178 ± 45	157 ± 36	***0.03**	0.69	0.36
LDL	103 ± 24	98 ± 29	89 ± 41	83 ± 33	0.10	0.51	0.92
HDL	65 ± 14	60 ± 12	64 ± 22	61 ± 17	0.22	0.98	0.73
Total/HDL ratio	2.9 ± 0.8	3.0 ± 0.8	3.1 ± 1.5	2.7 ± 0.7	0.39	0.93	0.25
Triglycerides (mg/dL)	115 ± 40	115 ± 31	94 ± 9	87 ± 38	0.74	0.25	0.78
Fasting glucose	93 ± 5	89 ± 7	94 ± 9	92 ± 3	0.14	0.63	0.62
Haemoglobin - Hb (g/dL)							
Female	*12.5 ± 0.6*	*12.2 ± 1.1*	*12.8 ± 1.2*	*12.3 ± 1.6*	0.48	0.74	0.48
Male	*15.5 ± 0.7*	*14.7 ± 0.3*	*14.8 ± 1.6*	*13.8 ± 1.5*	***0.03**	0.54	0.50
Haematocrit							
Female	37.4% ± 1.8	37.4% ± 1.2	39.1% ± 3.0	37.3% ± 3.9	0.26	0.51	0.26
Male	45.5% ± 1.5	43.5% ± 0.4	43.9 ± 4.4%	41.1% ± 4.5	***0.02**	0.60	0.35
Mean corpuscular volume (MCV)							
Female	83.5 ± 3.3	81.5 ± 1.7	78.1 ± 15.9	78.0 ± 16.0	0.22	0.78	0.44
Male	87.9 ± 2.3	88.8 ± 2.9	84.9 ± 0.6	84.4 ± 1.3	0.65	0.20	0.18
C-Reactive Protein (PCR) (mg/dL)	2.9 ± 3.4	0.9 ± 1.3	0.6 ± 0.4	0.9 ± 1.1	0.19	0.31	0.08
ALT (U/L)	15.0 ± 5.1	15.5 ± 6.1	15.7 ± 4.9	13.9 ± 3.8	0.60	0.88	0.39
GGT (U/L)	21.8 ± 11.4	24.9 ± 16.4	16.2 ± 4.3	16.7 ± 4.4	0.30	0.31	0.45
GOT (U/L)	19.1 ± 3.9	18.3 ± 5.3	15.8 ± 3.2	14.5 ± 4.2	0.51	0.14	0.89

Azotaemia (mg/dL)	30.6 ± 6.5	27.2 ± 7.3	32.4 ± 6.5	38.2 ± 7.0	0.60	0.12	0.07
Uric acid (mg/dL)	4.1 ± 2.3	4.2 ± 2.2	5.3 ± 1.5	5.9 ± 1.4	**0.004	0.25	*0.03

Values are presented as mean ± SD. Legend: ALT= Alanine transaminase; GGT= Gamma-glutamyl transferase; GOT= glutamic oxaloacetic transaminase; HDL= high-density lipoprotein; LDL= low-density lipoprotein; MCV= Mean Corpuscular Volume.

3.4 Food intake and nutrient composition of the diets

Subjects in the control diet received the general IDG instructions, while subjects in the EAT-IT dietary plan had to follow more specific dietary guidelines.

Data obtained from weighed food records are reported in Table 3. At baseline subjects had similar nutrient and energy intake that resulted about 1900 kcal on average. A time-dependent decrease in total energy was observed for the EAT-IT group (from 1923 ± 404 to 1612 ± 192 kcal, $p = 0.03$), but this difference did not result significant considering treatment and time x treatment interaction. As regards fat intake, a high energy from fat was found at baseline for both groups (34.7 ± 2.2 Vs 35.9 ± 3.8 % fat for EAT-IT and Control group, respectively). A significant increase in saturated fat intake was found in the Control group, considering time effect (from 11.2% total energy ± 0.7 to 15.1 ± 5.3, $p = 0.002$). A significant increase in ω-6 fatty acids intake was found for EAT-IT group, compared to Control group (from 2.4% of total energy ± 0.8 to 4.7% ± 1 Vs 2.3% of total energy ± 0.8 to 2.4 ± 1.1, $p = 0.02$). No statistical differences were found for other macronutrients, sugars, or fibres intake. Also, we did not find any significant differences in vitamin A, D, E, C and B12 intake. Considering minerals intake, calcium and iron were found to be very low at all timepoints for both groups, especially for calcium, but their intake was not significantly changed for time, treatment and time x treatment interaction, according to ANOVA. Calcium intake resulted equal to 370 mg at the beginning of the intervention for the EAT-IT group and ended at 459 mg at t1; while for control group initial calcium intake 554 and arrived at 492 at the end of the intervention.

Table 4. Daily mean energy and nutrients intake at the beginning and the end of the intervention in both EAT-IT and Control group.

	EAT-IT t0 (n= 5)	EAT-IT t0/t1 (n= 5)	EAT-IT t1 (n= 5)	Controls t0 (n= 5)	Controls t0/t1 (n= 5)	Controls t1 (n= 5)	P value for time	P value for treatment	P value for interaction
Energy (kcal)	1923 ± 404	1774 ± 462	1612 ± 192	1893 ± 334	1864 ± 259	1713 ± 253	***0.03**	0.69	0.73
Protein (g)	77.5 ± 9.7	67.5 ± 14.1	69.3 ± 9.7	72.0 ± 5.2	75.9 ± 11.7	67.8 ± 10.6	0.23	0.58	***0.03**
Energy protein/total energy (%)	14.1 ± 8.1	14.9 ± 0.6	17.7 ± 1.2	16.3 ± 2	16.6 ± 2.1	16.2 ± 1.1	0.50	0.89	0.10
Lipids (g)	70.5 ± 16.8	68.0 ± 18.1	65.4 ± 11.5	71.7 ± 14.2	69.7 ± 16.6	71.2 ± 23.4	0.65	0.64	0.71
Energy lipids/total energy (%)	34.7 ± 2.2	33.6 ± 4.2	37.5 ± 3.8	35.9 ± 3.8	33.9 ± 3.7	37.9 ± 10.3	0.06	0.84	0.99
SFA (g)	19.6 ± 6.1	15.9 ± 4.2	17.2 ± 5.7	23.7 ± 4.9	23.1 ± 4.4	29.6 ± 13.5	0.39	***0.03**	0.49
Energy SFA/total energy (%)	9.1 ± 1.8	7.6 ± 0.8	9.5 ± 2.4	11.2 ± 0.7	11.2 ± 1.4	15.1 ± 5.3	0.07	****0.002**	0.39
MUFA (g)	25.3 ± 10.3	30.5 ± 7.4	27.5 ± 9.3	20.8 ± 8	20.4 ± 7	25.6 ± 13.8	0.47	0.50	0.71
Energy MUFA/total energy (%)	11.5 ± 2.5	14.9 ± 3.2	15.5 ± 5.4	10.2 ± 4.9	9.7 ± 2.4	13.2 ± 7.4	0.13	0.32	0.56
PUFA (g)	7.7 ± 2.9	8.2 ± 3.3	10.0 ± 2.4	6.0 ± 1.7	6.1 ± 2	6.0 ± 2.8	0.28	0.18	0.20
Energy PUFA/total energy (%)	3.5 ± 0.7	3.9 ± 0.9	5.6 ± 1	2.9 ± 1	2.9 ± 0.8	3.0 ± 1.3	***0.03**	***0.02**	0.06
Energy ω-6/total energy (%)	2.4 ± 0.8	3.1 ± 1.0	4.7 ± 1	2.3 ± 0.8	2.3 ± 0.6	2.4 ± 1.1	****0.009**	0.07	***0.02**
Energy ω-3/total energy (%)	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.3	0.3 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.31	0.15	0.63
Cholesterol (mg)	144.3 ± 64.6	104.7 ± 25.1	110.9 ± 44.1	139.8 16.3	186.5 ± 66.2	203.9 ± 110.1	0.78	***0.048**	0.08
Carbohydrates (g)	226.3 ± 58.8	218.2 ± 78.9	184.2 ± 19.2	226.1 ± 48.7	227.0 ± 27	200.0 ± 47.1	0.09	0.65	0.94
Energy carbohydrates/total energy (%)	46.9 ± 3.7	45.6 ± 5.7	44.4 ± 3.3	46.9 ± 4.1	47.8 ± 2.6	45.4 ± 11.5	0.60	0.87	0.98
Sugars	61.6 ± 5.9	58.5 ± 19.3	58.6 ± 14.6	62.9 ± 10.7	75.4 ± 12.3	63.7 ± 13.3	0.57	0.22	0.24
Energy sugars/total energy (%)	13.2 ± 2.5	12.5 ± 2.3	14.6 ± 3.9	13.3 ± 1.2	16.2 ± 2.0	15.1 ± 4.3	0.18	0.49	0.21
Total fibres	19.3 ± 5.3	23.5 ± 2.1	20.7 ± 6	21.6 ± 6	19.4 ± 7.6	18.8 ± 6.8	0.31	0.78	0.64
Total fibres/1000 kcal	10.3 ± 3.8	13.0 ± 2.6	13.0 ± 3.9	11.8 ± 4.2	10.4 ± 4.1	10.8 ± 3.1	0.35	0.53	0.44
Vit. A - retinol eq. (µg)	709 ± 197	767 ± 487	641 ± 513	725 ± 311	998 ± 589	1195 ± 781	0.35	0.19	0.45

Vit. D (µg)	2.0 ± 1.3	2.7 ± 3.4	1.4 ± 1.5	0.6 ± 0.1	2.0 ± 1.4	2.2 ± 2.2	0.68	0.67	0.45
Vit. E (mg)	7.4 ± 1.6	11.3 ± 2.7	8.3 ± 2.6	6.8 ± 3.8	7.3 ± 3.8	7.6 ± 5.3	0.16	0.63	0.85
Vit. C (mg)	101.7 ± 78.6	94.6 ± 45.4	71.3 ± 27.2	60.8 ± 25.7	74.3 ± 56.1	79.4 ± 47.5	0.82	0.67	0.19
Vit. B12 (µg)	2.1 ± 1.2	1.9 ± 1.0	3.3 ± 2.2	1.4 ± 0.5	2.9 ± 0.8	3.1 ± 3.8	0.58	0.73	0.33
Calcium (mg)	370 ± 147	354 ± 165	459 ± 114	554 ± 129	513 ± 157	492 ± 299	0.77	0.21	0.53
Iron (mg)	9.2 ± 1.9	9.2 ± 2.3	8.9 ± 2.1	8.0 ± 2.1	7.6 ± 3	7.5 ± 2.9	0.81	0.48	0.76

Legend: SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; * p-value < 0.05; ** p-value < 0.01 (fixed effects two-way ANOVA, considering treatment as independent variable and time as dependent variables). Legend: MUFA= monosaturated fatty acids; PUFA= polyunsaturated fatty acids; SFA= saturated fatty acids.

3.5 Diet acceptability

Data regarding the global acceptability of the EAT-IT dietary plan in the five subjects of the EAT-IT group are reported in Table 5. Overall, the EAT-IT dietary pattern was well tolerated by participants, as indicated by the first question of the questionnaire (almost the best score). This pattern was also considered easy to prepare (question 2, median score of 3, mode of 2), and associated with a limited effort (question 3 and 4, median and mode score of 3 and 2 respectively). However, despite these answers, participants declared to find easier to follow their habitual diet (question 5, median score of 5 and mode of 6).

Table 5. Acceptability of EAT-IT dietary pattern and Control one, according to the questionnaire of Barnard and co-workers.

Questions (n= 5)	Median	Mode
1. How well do you like current diet? (1-7 scale, 1 = "extremely good," 7 = "extremely unappealing")	2	2
2. How easy to prepare? (1-7 scale, 1 = "extremely easy," 7 = "extremely difficult")	3	2
3. Effort required by diet? (1-7 scale, 1 = "more than is possible," 7 = "no effort at all")	3	3
4. How easy to continue this diet? (1-7 scale, 1 = "extremely easy," 7 = "extremely difficult")	2	2
5. Which diet is easier to follow? (1-7 scale, 1 = "EAT-IT much easier," 7 = "Control much easier")	5	6

6. Acceptability of the EAT-IT dietary pattern? (1-7 scale, 1 = "completely unacceptable," 7 = "extremely acceptable")	6	6
7. In future, I could stick with the EAT-IT dietary pattern (1-7 scale, 1 = "all the time," 7 = "never")	3	3
8. Adapted to the EAT-IT dietary pattern? (1-4 scale, 1 = "completely," 4 = "not at all")	2	2

4. Discussion

This study aimed at evaluating the feasibility and effect of the EAT-IT dietary pattern through a pilot randomized controlled trial in a group of young healthy subjects recruited among the university students and employees. Despite the existence of evidence that associate the adoption of the planetary health diet to positive outcomes (Springmann *et al.*, 2020; Cacau *et al.*, 2021; Laine *et al.*, 2021), to the best of our knowledge, this is the first study evaluating the effect of an optimized healthy and sustainable diet in the Italian setting, based on the EAT-Lancet indications. A dietary pilot study has been initially conducted to explore feasibility, acceptability, food choices, nutritional impact, and the effect on general health-related markers also to identify criticalities and help improving approaches. Volunteers were all healthy, young subjects with intermediate adherence to Mediterranean diet: according to MEDI-LITE questionnaire, this group of subjects declared to have generally a moderate consumption of legumes (in relation to Mediterranean diet in which an intermediate consumption is defined as 1-2 portions/week) and were already prone to consume medium/low amount of fish (1-2,5 portions/week), meat (1-1,5 portions/die) and dairy (1/1.5 portions/die). In addition, fruits consumption resulted as intermediate (1-2 portions/die).

Considering data from food records, a significant reduction in total energy intake was found for the EAT-IT group along the time (-16.2%). This reduction could be dependent on an increased difficulty in sticking to the diet in the final weeks of the intervention. We found an overall high levels of acceptability of the dietary pattern, according to the questionnaire used (Barnard *et al.*, 2000). However, at the same time it is noteworthy that the question 7, regarding the willingness to continue with this pattern in the future, obtained a medium score (3 on a 1-7 scale in which 7 correspond to "never"). Considering macronutrients intake, the main differences were found for lipids. Control groups significantly increased saturated fat intake (from 11.2% of total energy to 15.1%). Differently, the EAT-IT group increased PUFA intake, and a significant time x treatment interaction was found for ω-6 fatty acids intake, according to ANOVA (from 2.4% of total energy to 4.7%). Since nuts were present in high amount in the diet and they are rich sources of PUFA, the increased intake of this nutrient is likely to reflect compliance with the EAT-IT dietary pattern (Kendall *et al.*, 2010; Willett, 2012). However, fibre consumption resulted lower than the suggestion of introducing at least 25 g per day and it was not different between the two intervention groups. The amount was also lower compared to the theoretical expected values (around 17.5 g of fibre/1000 kcal that was slightly higher than the range of 12.6–16.7 g/1000 kcal defined by Italian recommendation for 1000 kcal) as calculated in the published model. This result, therefore, can reflect a sub-optimal adherence to the pattern. As regards cholesterol intake, that depends on animal sources and thus can be considered a proxy of animal food consumption, it resulted lower in the EAT-IT group, compared to the Control one (111 ± 44 mg/day at the end of EAT-IT intervention, vs 204 ± 110 mg/day in the Control group, $p = 0.048$ for treatment). This reduction seems to indicate that subjects were compliant with the EAT-IT plan decreasing the intake of such foods. Overall, these data suggest that reducing meat and dairy consumption resulted in a more easily implementable dietary behaviour compared to increasing legumes and wholemeal grains, at least for this group of subjects, as indicated by actual food consumption, cholesterol, and fibre intake. In this regards, it is well known that legume consumption is overall scarce on average in the Italian population, and thus a large increase of these foods can result as a dietary change hard to be implemented (Leclercq *et al.*, 2009). These products should substitute animal sources that are considered pivotal for the intake of important micronutrients such as iron, and vitamin B12 (Fanzo *et al.*, 2017; Dave *et al.*, 2021; Leroy *et al.*, 2022). Recently, some authors critically discussed the recommendation to lower meat consumption in food-based dietary guidelines (Leroy and Cofnas, 2020) , as advocated by the EAT-Lancet Commission, suggesting that it could produce important negative effects. In our pilot study, despite the initial low intake of iron with respect to recommendations in both groups (9.2 ± 1.9 mg/day in the EAT-IT group vs 8.0 ± 2.1 mg/day in the Control one), the intervention did not change these levels of intake, nor it affected blood count, considering haemoglobin, haematocrit and MCV. Conversely, we found a significant reduction in haemoglobin (-6.7%) and haematocrit (-6.4%) in male subjects within the Control group. It is difficult to define whether these fluctuation, although significant, can be attributed to the diet (actual/declared) and/or other factors, including individual (e.g., genetic polymorphisms) or environmental (e.g. climate and hydration status) (Barrera-Reyes and Tejero, 2019). Furthermore, we cannot exclude that these variations can be explained considering small sample size, since the number of male subjects is rather limited within this exploratory study. We found little effects on the other health-related markers, excluding a time-dependent effects within control group for total cholesterol, which reduced of 11.8%, and uric acid, that increased of 11.3%. Considering cholesterol reduction, it should be considered that also Control group received a list of personalized dietary suggestions to improve their diet according to Italian Dietary Guidelines (IDG) (CREA, 2018), and this could have improved their behavior. Uric acid instead in humans derive from purines degradation and can be found high at plasma level due to genetic factors, elevated cell turnover or renal issues (Saito *et al.*, 2021). However, since the cut-off values are settled to 6 mg/dL in women and 7 mg/dL in men to distinguish between normal values and hyperuricemia, and thus all subjects were within normal range, increased dietary intake of animal products such as red meat, fatty poultry, high-fat dairy, seafood products and alcohol (particularly beer from the yeast) is more likely (Puddu *et al.*, 2012; Ndrepepa, 2018).

Overall, this pilot study presents some strength as well as limitations. This is one of the first randomized controlled trials assessing the health and behavioural effects of a healthy and sustainable diet. We considered several dimensions of the diet sustainability by evaluating nutritional adequacy through weighted food records, metabolic, and health-related outcomes as well as the level of acceptability. We found that this pattern was considered overall acceptable, demonstrating the adequacy of the adaptation of the Planetary health diet to the Mediterranean food context. However, the enrolled subjects were already prone to consume low amount of diary and other animal foods, thus acceptability could be found as lower in a different group of population presenting lower adherence to Mediterranean diet. We also found that, despite possible nutritional issues, this pattern did not worsen the variables analysed in both females and males. However, independently from the intervention, calcium intake resulted markedly lower with respect to current recommendations, in all enrolled subjects. These results corroborate the epidemiological data that still some issues regarding specific micronutrients intake (iron, calcium, and vitamin D) are diffused across Italian/south European population and thus the eventual exploitation of enriched or fortified foods, or supplements, should be carefully evaluated (Balk *et al.*, 2017; Milman *et al.*, 2017; Manios *et al.*, 2018; Milman, 2019). The main limitation of the explorative pilot study reported in this thesis is that the number of subjects is very limited, and the analysis performed as parallel design at this stage, did not allow to reduce the effects of interindividual variability. Thus, a larger study on a larger sample of subjects and with the cross-over design, and longer treatment time. This is currently ongoing and will be used to better understand how healthy and sustainable diets can be tailored to maximise health and environmental outcomes.

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3.4 Chapter 4

- **Plant-Based Foods and Vascular Function: A Systematic Review of Dietary Intervention Trials in Older Subjects and Hypothesized Mechanisms of Action.**
  (Post print – published paper: doi: 10.3390/nu14132615)

- **Modulation of Adhesion Process, E-Selectin and VEGF Production by Anthocyanins and Their Metabolites in an In Vitro Model of Atherosclerosis.**
  (Post print – published paper: doi: 10.3390/nu12030655)

- **Acute effect of blueberry intake on vascular function in older subjects: study protocol for a randomized, controlled, crossover trial.**
  (Pre-print of an accepted article: doi: 10.1371/journal.pone.0275132)

- **A single portion of an anthocyanin-rich blueberry product improves endothelial function, but not arterial stiffness, in free-living older subjects: results from a randomized, controlled, crossover trial.**
  (Unpublished results)

Premise

Healthy and sustainable diets are generally plant-based patterns, with reduced consumption of animal source foods, for the reasons described in detail in the introduction. The previous chapters (3.1 – 3.3) reported the results of the research carried out within this PhD project considering healthy diets mainly optimized for macro- and micronutrients content. However, research in the last years also emphasized the great relevance of bioactive compounds such as polyphenols, carotenoids and glucosinolates, for their health-promoting effects, although it is not yet possible to define evidence-based reference intakes as previously discussed for polyphenols (Del Bo' *et al.* 2019).

These compounds, potentially able to exert positive effects on health, are not homogeneously diffused within foods and their content can be influenced by several factors including cultivar, production methods, climatic conditions, and storage (Skrovankova, 2015). This topic is generating significant scientific discussion, and recently it has also been suggested that among possible strategies that could be considered to exploit the effects of bioactives on human health there is also the selection of new crops varieties, through breeding techniques, with increased amount of bioactives compounds or the promotion of specific cultivar already cultivated (Bassolino *et al.*, 2022). This approach may be included into future research on diet sustainability if it can: i) assure additional health benefits when choosing vegetable foods or varieties characterized by interesting composition, ii) improve crop management (e.g. higher levels of flavonoids and other antioxidant are observed when reducing nitrogen output) (Bassolino *et al.*, 2022).

To achieve a comprehensive approach for the optimization of sustainable diets, the present PhD thesis also aimed at increasing the availability of evidence on the protective role of food products in relation to their bioactive components, abundantly present in plant-based diets.

In addition, since the growth of global older population poses problems and significant challenges in terms of public health (e.g. for the increased cardiovascular risk often associated to arterial dysfunction), the present thesis aimed to find evidence of the cardiovascular protective effect of vegetable food intake in older subjects. The results obtained from the analysis of the literature have been used as the basis to further demonstrate, through an acute randomized controlled trial, the effect of a specifically selected cultivar of blueberry on the modulation of markers of arterial function, in the older subjects.

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Review

3.4.1 Plant-based Foods and Vascular Function: A Systematic Review of Dietary Intervention Trials in Older Subjects and Hypothesized Mechanisms of Action

Massimiliano Tucci, Mirko Marino*, Daniela Martini*, Marisa Porrini, Patrizia Riso and Cristian Del Bo'

Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano,

Via Celoria 2, 20133 Milan, Italy; massimiliano.tucci@unimi.it (M.T.); marisa.porrini@unimi.it (M.P.); patrizia.riso@unimi.it (P.R.); cristian.delbo@unimi.it (C.D.B.)

* Correspondence: mirko.marino@unimi.it (M.M.); daniela.martini@unimi.it (D.M.)

Abstract: Cardiovascular diseases, still the leading cause of mortality in the world, are closely related to vascular function. Older subjects are more susceptible to endothelial dysfunction and therefore it is important to define possible preventive or support strategies, such as consumption of foods with health-promoting effects. This systematic review aims to summarize the currently available evidence on acute or chronic trials testing the effect of selected plant-based foods on vascular function parameters in older subjects and consider plausible mechanisms that may support the main findings. A total of 15 trials were included and analyzed, testing the effects of beetroot, plum, blueberry, and vegetable oils. We found some interesting results regarding markers of vascular reactivity, in particular for beetroot, while no effects were found for markers of arterial stiffness. The amelioration of vascular function seems to be more related to the restoration of a condition of nitric oxide impairment, exacerbated by diseases or hypoxic condition, rather than the enhancement of a physiological situation, as indicated by the limited effects on healthy older subjects or in control groups with young subjects. However, the overall set of selected studies is, in any case, rather limited and heterogeneous in terms of characteristics of the studies, indicating the need for additional high-quality intervention trials to better clarify the role of vegetable foods in restoring and/or improving vascular function in order to better elucidate the mechanisms through which these foods may exert their vascular health benefits in older subjects.

Keywords: aging; vascular function; endothelial function; dietary intervention; intervention studies; systematic review; plant-based foods; vegetable foods; nitric oxide

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases still represent the main cause of death in both developed and developing countries (2021). Cardiovascular diseases (CVDs) are thus unanimously recognized as a global burden, since they account for approximately one third of all deaths at global level (Joseph *et al.*, 2017). The incidence of this chronic condition is still rising mainly in low-and middle-income countries, and can be attributable also to the worldwide increased life expectancy of population (Bansilal *et al.*, 2015; Leong *et al.*, 2017). In particular, aging is associated with an increased risk for all CVDs, above all over 60 years (Savji *et al.*, 2013; Costantino *et al.*, 2016; Partridge *et al.,* 2018; Campisi *et al.*, 2019). In fact, it is estimated that 40% of the deaths in people aged >65 is caused by CVDs and their complications (Heidenreich *et al.*, 2011; North *et al.*, 2012). The mechanisms that lead to the increased cardiovascular (CV) risk during the aging process are related to both structural and functional changes in vascular systems (North *et al.*, 2012; Merz *et al.,* 2016).

Vascular endothelium represents the innermost monolayer of cells of the artery wall, directly in contact with blood flow, able to contribute to the regulation of vascular tone and hemostasis (Xu *et al.*, 2021). The endothelium exerts the aforementioned functions thanks to its capacity to perceive, through mechanical stimuli, changes in the blood flow and consequently induces smooth muscle cells of artery walls to relax (Félétou *et al.*, 2006; Roux *et al.*, 2020; Souilhol *et al.*, 2020). One of the main vasodilator molecules involved in this process is nitric oxide (NO), a highly reactive soluble gas mainly synthetized by the endothelium through the enzyme nitric oxide synthase (NOS) from the amino acid arginine, which diffuses rapidly (half-life of 4–30 s) and acts on the surrounding cells in a paracrine fashion (Tousoulis *et al.*, 2012; Förstermann *et al.*, 2017; Krüger-Genge *et al.*, 2019). Thus, alterations of endothelial cell monolayer integrity represent a crucial factor in the onset of endothelial dysfunction and atherosclerosis, which is considered the underlying cause of most CVDs (Sitia *et al.*, 2010; Gimbrone *et al.*, 2016). Atherosclerosis is a complex phenomenon that can be briefly described as a chronic, immunoinflammatory disease of large and medium arteries, fueled by lipids, and involving endothelial cells, leukocytes, and intimal smooth muscle cells (Falk, 2006). The pathophysiology of atherosclerosis consists in the development of plaques formed by fibrous tissues and a lipid-rich core inside the tunica intima of artery walls (Falk, 2006; Badimon *et al.*, 2010; Herrington *et al.*, 2016). These plaques can be unstable, and the acute rupture of these atheromatous plaques causes local thrombosis, thus leading to serious clinical consequences including stroke and heart disease (Sitia *et al.*, 2010; Gimbrone *et al.,* 2016). Further metabolic dysregulations linked with endothelial dysfunction include insulin resistance, diabetes, and chronic kidney failure (Rajendran *et al.*, 2013).

Aging is a gradual, continuous process of natural change that begins in early adulthood and that is associated with numerous cellular and molecular alterations such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, and stem cell exhaustion (López-Otín *et al.*, 2013). Aging process is also characterized by vascular endothelial dysfunction (Brandes *et al.,* 2005; Paneni *et al.*, 2017). This impairment affects all the major endothelium-derived vasodilators: NO, prostacyclin (PGI_2), and the endothelium-derived hyperpolarizing factors (EDHFs); bringing to an increase of vasoconstrictor agents (e.g., endothelin-1, vasopressin, angiotensin II) (Busse *et al.*, 2003). Several mechanisms have been hypothesized; for instance, aging is recognized to promote an accumulation of reactive oxygen species at mitochondrial level such as superoxide (O_2^-) able to transform NO into peroxynitrite ($ONOO^-$) and promote cumulative DNA damage. At endothelial level, the loss of NO and the consequent accumulation of high concentrations of $ONOO^-$ induce the inactivation of mitochondrial manganese superoxide dismutase (the main enzyme involved in the hydrogen peroxide detoxification) and can switch the NO synthase via the oxidation of tetrahydrobiopterin from an NO- to an O_2^-- generating enzyme (NO synthase uncoupling) (El Assar *et al.*, 2012; El Assar *et al.,* 2013). Both reactions lead to an increase in the concentration of O_2^-, in terms of a vicious circle, further promoting DNA damage, $ONOO^-$ formation, and endothelial dysfunction. This alternation is also associated with a reduced capacity of endothelium to properly regulate vascular tone and exert antithrombotic activity, thus impacting negatively on tissue perfusion and allowing the onset of atherosclerosis (Deanfield *et al*, 2007; Paneni *et al.*, 2017; Pietri *et al.*, 2021).

According to the Global Burden of Disease Study 2019, diet-related risk factors represent the second risk factor for cardiovascular diseases mortality, after systolic hypertension, in both men and women (Murray *et al.*, 2020). Diets rich in fruits and vegetables are recommended for cardiovascular health; however, current global intakes of fruits and vegetables are well below recommendations (at least 5 portions per day) (Wallace *et al.*, 2020). Extensive dose-response meta-analyses indicate significant cardiovascular risk reduction for high intake of fruit and vegetables (Wang *et al.*, 2014; Aune *et al.*, 2017; Miller *et al.*, 2017; Wallace *et al.*, 2020; Zurbau *et al.*, 2020). For example, Wang *et al.* (2014) showed in four large prospective cohort studies that higher consumption of fruit and vegetables was associated with a reduction of risk of 4% for each additional serving per day of fruit and vegetables. The large prospective cohort study of Miller and coauthors (2017) found that the lower risk of non-cardiovascular events and total mortality was associated with high fruit, vegetable, and legume consumption (three to four servings per day, equivalent to 375–500 g/day). More recently, Zurbau and colleagues (2020) reported in a meta-analysis of 117 observational studies (involving more than 4 million individuals and 100 thousand CV events) that total fruit and vegetables, fruit, and vegetables were associated with significant decreased incidence of cardiovascular disease (−7%, −9%, and −6% respectively) and associated mortality (−11%, −12%, and −13% respectively). This protective effect was particularly evident in older subjects. In fact, a greater adherence to a Mediterranean diet and/or more consumption of fruits and vegetables has been associated with a lower risk of arterial stiffness, vascular dysfunction, CV events, and mortality in older subjects (Knoops *et al.*, 2004; Alissa *et al.*, 2015; Gómez-Sánchez *et al.*, 2022) among other important functions (Del Bo' *et al.*, 2019; Martínez-González *et al.*, 2019; Martini *et al.*, 2019; Ventriglio *et al.*, 2020). The protective effects observed could be attributed to the large number of bioactive molecules contained in fruits and vegetables such as fibers, vitamins, minerals, phytochemicals (i.e., polyphenols, carotenoids), and essential fatty acids (Slavin *et al.*, 2012; Liu, 2013; Torres *et al.*, 2015; Asgary *et al.,* 2018; Martini, 2019; Grosso *et al.*, 2022). In fact, these compounds have shown several biological activities including favorable effects on endothelial function against $ONOO^-$ formation and accumulation, but also through an inhibition of angiogenesis and cell migration and proliferation in blood vessels (Marino *et al.*, 2020). Given these premises and the urgent need to adopt preventive strategies in the context of healthy aging, the aim of this review is to systematically collect and summarize the main findings deriving from acute and chronic dietary intervention trials testing the health-promoting effects of plant-based foods (e.g., fruit, vegetables, nuts, legumes, cereal-based products, and vegetable oils) on markers of vascular function in older subjects. In addition, plausible mechanistic insights have been considered to support and explain the main positive findings.

2. Materials and Methods

2.1. Search Strategy

A systematic literature search was conducted using the three different academic digital databases PubMed®, Web of Science, and Scopus. The search was conducted in July 2021 and then updated at the end of January 2022. The following Boolean search strings were used, with proper adaptation for each database: “endothelial function OR vascular function OR reactive hyperemia OR EndoPAT) AND (aged OR elderly OR frail OR older) AND (vegetable* OR vegetable food* OR fruit* OR nut* OR legume* OR cereal*) AND (intervention OR trial OR clinical study)”. The search was limited to the last 10 years, from 2011 to 2021. To ensure completeness, the search was augmented by consulting the bibliographies of the eligible articles. The literature identification process, based on the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Moher *et al.,* 2009) is illustrated in Figure 1. The following systematic review was not recorded in any register and no review protocol was prepared.

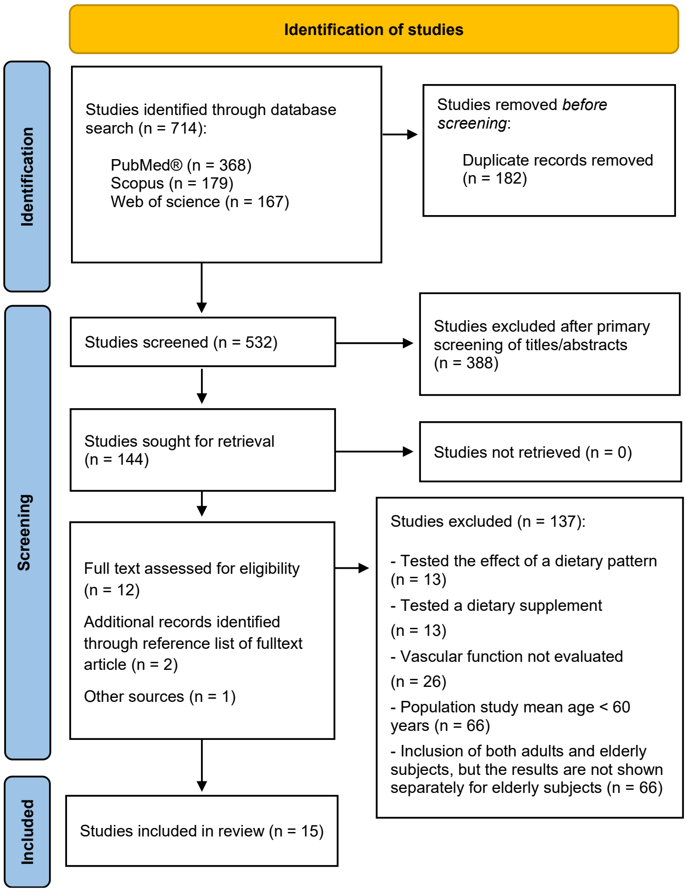

Figure 1 – PRISMA flow chart of the systematic review literature search.

2.2. Study Selection

Studies were considered eligible if they: (i) were published in the last ten years (from 2011 to 2021), (ii) were written in English, (iii) reported the results of a dietary intervention trial, (iv) tested the effect of a vegetable product, (v) analyzed markers of vascular/endothelial function, (vi) involved older subjects (population mean age >60 years; both sexes included).

Given these criteria, intervention studies were excluded when the effects of single compounds and/or supplements or specific dietary patterns (e.g., Mediterranean diet) were tested. Dietary intervention trials that involved subjects with different ages (from young to older subjects) were included only when data for older subjects were extractable.

2.3. Data Extraction and Presentation

Two authors (M.T. and M.M.) independently abstracted and tabulated data from eligible studies. Disagreement between reviewers was resolved through consultation with a third independent reviewer (D.M.) to reach a consensus. Details recorded included the year of publication, study design, location, sample size and characteristics of enrolled subjects, type and characteristics of tested plant food, characteristics of control, type of dietary assessment, primary and secondary outcomes, and results.

2.4. Risk of Bias

Risk of bias in individual studies and across the studies was carried out by two independent authors (D.M and C.D.B.). The evaluation was carried out following the criteria of the Cochrane Handbook for Systematic Reviews of Interventions. The analysis was structured into the following seven domains: (1) random sequence generation, (2) allocation concealment, (3) blinding of participants and personnel, (4) blinding of outcome assessment, (5) incomplete outcome data, (6) selective reporting, and (7) other bias. Each domain was judged as high risk, unclear risk, or low risk. Disagreements were resolved by consensus or seeking consultation with another author (P.R.).

3. Results

3.1. Study Selection

The study selection process is shown in Figure 1. A total of 531 records were identified from the database search (PubMed®, Scopus, and Web of science). After removing 120 duplicate articles, 411 studies were screened and 273 were excluded based on the title and abstract. The remaining 138 eligible papers were analyzed. At the of the process, 126 records were excluded for the following reasons: (i) mean population studied under the age of 60 years or data on older subjects not extractable, (ii) markers of vascular function not present, (iii) tested the effect of a dietary pattern (e.g., Mediterranean diet) or a single compounds supplement (e.g., grape polyphenols). At the end of the selection process, 12 trials were assessed for eligibility, and three additional trials were added following careful consultation of bibliography (two trials) and independent hand searching (one trial). Finally, 15 trials were included in this review; six studies evaluated the acute effect of vegetable foods while nine evaluated the chronic effects.

3.2. Study Characteristics

The main characteristic of the 15 studies included in the review are summarized in Table 1 (Casey *et al.*, 2015; de Oliveira *et al.*, 2016; Hughes *et al.*, 2016, 2020; Dodd *et al.*, 2019; Pekas *et al.*, 2021) and Table 2 (Gilchrist *et al.*, 2013; de Oliveira *et al.*, 2017; Shaltout *et al.*, 2017; Oggioni *et al.*, 2018; Woessner *et al.*, 2018; Jones *et al.*, 2019; Casey *et al.* 2021; do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b). Specifically, Table 1 reports the results of acute interventions (n = 6), and Table 2 the results related to the cronic dietary interventions (n = 9). The majority of the studies (n=7) were performed in the U.S.A. (Casey *et al.*, 2015; Hughes *et al.*, 2016, 2020; Shaltout *et al.*, 2017; Woessner *et al.*, 2018; Casey *et al.*, 2021; Pekas *et al.*, 2021). Of the remaining studies, 4 were conducted in the UK (Gilchrist *et al.*, 2013; Oggioni *et al.*, 2018; Dodd *et al.*, 2019; Jones *et al.*, 2019), 2 studies were performed in Australia (do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b), and 2 in Brazil (de Oliveira *et al.*, 2016, 2017). The characteristics of the study population studied are heterogenous between the studies included in the review. 7 studies involved healthy subjects (Casey *et al.*, 2015; Hughes *et al.*, 2016, 2020; Oggioni *et al.*, 2018; Dodd *et al.*, 2019; Jones *et al.*, 2019; Casey *et al.*, 2021), 4 studies involved older subjects with cardiovascular risk factors (Gilchrist *et al.*, 2013; de Oliveira *et al.*, 2016, 2017; Shaltout *et al.*, 2017), 2 studies involved subjects with overt peripheral artery disease (Woessner *et* *al.*, 2018; Pekas *et al.*, 2021), and 1 study involved subjects with mild cognitive impairment (do Rosario *et al.*, 2021b). None of this study involved only normoweight subjects. 8 studies included overweight subjects (Casey *et al.*, 2015; Hughes *et al.*, 2016, 2020; Oggioni *et al.*, 2018; Woessner *et al.*, 2018; Jones *et al.*, 2019; Casey *et al.*, 2021; do Rosario *et al.*, 2021b), while 6 studies included obese or both overweight and obese subjects (Gilchrist *et al.*, 2013; de Oliveira *et al.*, 2016, 2017; Shaltout *et al.*, 2017; do Rosario *et al.*, 2021a; Pekas *et al.*, 2021). 1 study included normoweight, overweight and obese subjects (Dodd *et al.*, 2019). Different types of plant-based foods have been tested in relation to their possible effect on vascular function. The most studied food was the beetroot, mainly administered in the form of juice (from 70 mL up to 500 mL) (Gilchrist *et al.*, 2013; Casey *et al.*, 2015; Hughes *et al.*, 2016; Shaltout *et al.*, 2017; Oggioni *et al.*, 2018; Woessner *et al.*, 2018; Jones *et al.*, 2019; Pekas *et al.*, 2021), but also as powder to be resuspended in water (amount of powder *per se* not reported) (Hughes *et al.*, 2020; Casey *et al.*, 2021) or gelified (100 g) (de Oliveira *et al.*, 2016). Beetroot products were all analytically characterized for their content in nitrates. Other food sources included: foods rich in anthocyanins (i.e., blueberry powder – 30 g to be resuspended in 300 mL of semi-skimmed milk (Dodd *et al.*, 2019), and plum juice – 250 mL/day (do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b) and polyunsaturated fatty acids (i.e., different vegetable oils – 30 mL/day) (de Oliveira *et al.*, 2017). For the chronic studies, the length of the intervention ranged from 5 days (do Rosario *et al.*, 2021a) up to 90 days (de Oliveira *et al.*, 2017), while the acute study measured the investigated outcomes after at least 1 h (Pekas *et al.*, 2021), up to 3 h (Casey *et al.*, 2015). Regarding the assessment of vascular function, the main outcome considered was vascular reactivity (n=8) (Gilchrist *et al.*, 2013; de Oliveira *et al.*, 2016, 2017; Woessner *et al.*, 2018; Jones *et al.*, 2019; do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b; Pekas *et al.*, 2021), measured with different methods, but mainly through flow mediated dilation (FMD – n=6) (Gilchrist *et al.*, 2013; de Oliveira *et al.*, 2016, 2017; Woessner *et al.*, 2018; Jones *et al.*, 2019; do Rosario *et* *al.*, 2021a; Pekas *et al.*, 2021). Also arterial stiffness was frequently measured (n=6) (de Oliveira *et al.*, 2016, 2017; Oggioni *et al.*, 2018; Woessner *et al.*, 2018; Hughes *et al.*, 2020; Pekas *et al.*, 2021) and mainly as an augmentation index (AIx – n=4) (de Oliveira *et al.*, 2016; Oggioni *et al.*, 2018; Hughes *et al.*, 2020; Pekas *et al.*, 2021).

Considering dietary and behavioral prescription, 6 acute studies included an overnight fast period before the evaluations. Four studies (Casey *et al.*, 2015; Hughes *et al.*, 2016, 2020; Dodd *et al.*, 2019) requested the participants to refrain from exercise and alcohol and caffeine intake 24 h prior assessment. In the study of de Oliveira G. *et al.* (2016), participants were also instructed to avoid the consumption of nitrates rich- food (e.g. beetroots, spinach, celery, kale, lettuce, red meats, sausages, and beans). In the study of Pekas *et al.* (2021) volunteers were asked to maintain their habitual diet, but to track what they consumed the day before the first visit. In the study performed by using blueberries, participants were asked to avoid the consumption of foods and beverages rich in polyphenols such as fruit, vegetables, tea and cocoa the day before the assessment (Dodd *et al.*, 2019).

Table 1. Characteristics of the acute intervention studies investigating the effects of plant-food consumption on markers of vascular function in older subjects.

Reference, Country	Study Design	Study Population	Test Product	Control Product	Outcome Variables	Main Findings
Casey et al., 2015, USA	Randomized, parallel, placebo-controlled	*n* = 12 healthy older subjects (9 M + 3 F), of which 7 completed the placebo trial Mean age = 64 ± 2 years Mean BMI = 25.5 ± 0.7 kg/m^2	500 mL of beetroot juice providing 9.4 mmol of nitrate	140 mL of nitrate-deprived beetroot juice + 360 mL of water	FBF, FVC, and CV tested in both condition of normoxia and hypoxia, at rest and after forearm exercise Tested prior and after 3 h from consumption of test product or placebo	↑ FBF, and FVC and in hypoxia compared to control in older subjects, but not in young subjects ←→ FBF and FVC observed in the placebo group under both normoxic and hypoxic condition
de Oliveira et al., 2016, Brazil	Randomized, crossover, placebo-controlled, double-blind Washout period of at least 1 week	*n* = 20 older subjects with cardiovascular risk factors (7 M + 13 F). Mean age = 70.5 ± 5.6 years Mean BMI = 30.2 ± 5.3 kg/m^2	100 g of beetroot gel providing 12.2 mmol of nitrate and 367.9 mg of phenolic acids (expressed as GAE)	100 g of nitrate-deprived beetroot gel (placebo)	FMD, RH, BFV, PWV, AIx, Ep, and AC. Tested 2 h after consumption of the test or control product	↑ FMD, RH, and BFV in test products vs. placebo ←→ arterial stiffness parameters
Dodd et al., 2019, UK	Randomized, crossover, controlled, double blind Washout period not indicated	*n* = 18 older subjects not taking anti-hypertensive medications (8 M + 13 F) Mean age = 68.7 ± 3.3 years Mean BMI = 25.9 ± 4.5 kg/m^2	30 g of blueberry powder providing about 500 mg of anthocyanidins and 70 mg of procyanidins, homogenized with 300 mL of semi-skimmed milk and consumed after a standardized breakfast	30 g of a powder providing the same amount of sugar and vit. C of blueberry powder.	DVP Tested 1 h after consumption of the test or control product	←→ arterial stiffness parameters

Hughes et al., 2016, USA	Comparative parallel study evaluating the differences in response to acute ingestion of dietary nitrate between young and older subjects. Uncontrolled for older subject group.	*n* = 12 healthy non-obese, non-hypertensive older subjects (9 M + 3 F). Mean age = 64 ± 5 years Mean BMI = 25.5 ± 2 kg/m^2	500 mL of beetroot juice providing 9.4 mmol of nitrate	-	AIx, AIx@75, and other hemodynamic measures Tested at six different timepoints (baseline, 1 h, 1,5 h, 2 h, 2,5 h, and 3 h).	←→ arterial stiffness and other parameters
Hughes et al., 2020, USA	Randomized, crossover, placebo-controlled, double-blind	*n* = 10 healthy non-obese non-smokers older subjects (7 M + 3 F). Mean age = 68 ± 1 years Mean BMI = 25.8 ± 1 kg/m^2	Beetroot powder in 240 mL of water, providing 4 mmol of nitrates and 0.3 mmol of nitrites.	Nitrate-deprived beetroot powder in 240 mL of water.	Leg BF and VC, tested during exercise Tested prior and 2 h after consumption of test product or placebo	←→ vascular reactivity parameters

Pekas et al., 2021 USA	Randomized, crossover, placebo-controlled, double-blind Washout period of 14 days	n = 11 older subjects with PAD (5 M + 6 F). Mean age = 70 ± 7 years Mean BMI = 29 ± 6 kg/m^2	Body mass-normalized beetroot juice, providing 0.11 mmol of nitrates/kg	Nitrate deprived beetroot beverage	Brachial and popliteal FMD, AIx, AI@75, AP, PP, PWV Tested prior and 1 h after consumption of test product or placebo	↑ FMD in test products vs. placebo

Legend: AC, arterial compliance; AIx, augmentation index; AIx@75, AIx normalized by considering a heart rate of 75 bpm; AP, augmented pressure; BMI, body mass index; BF, blood flow; BFV, blood flow velocity; CV, compensatory vasodilation; DVP, digital volume pulse; Ep, pressure-strain elasticity modulus; FBF, forearm blood flow; FMD, flow-mediated dilation; FVC, forearm vascular conductance; GAE, gallic acid equivalents; pulse wave velocity; PAD, Peripheral Artery Disease; PP, pulse pressure; PWV, carotid-to-femoral pulse-wave velocity; RH, reactive hyperemia; VC, vascular conductance.

Table 2. Characteristics of the chronic intervention studies investigating the effects of plant-food consumption on markers of vascular function in older subjects.

Reference, country	Study Design	Study Population	Test Product	Control product	Duration	Outcome variables	Main findings
Casey and Bock. 2021, USA	Randomized, cross-over, placebo-controlled, double-blind Washout period of at least 4 weeks	*n* = 10 healthy, non-obese, non-smokers older subjects (6 M + 4 F) Mean age = 67 ± 3 years Mean BMI >25.8 ± 3.3 kg/m^2.	Beetroot powder mixed with approximately 200 mL of water, providing about 4 mmol of nitrate and 0.3 mmol of nitrate	Nitrate- and nitrite-depleted Beetroot powder	4 weeks	Measures of shear profile at rest, and measure of exercise hyperemia during handgrip exercise (FBF and FVC).	Improvement in shear profile in test products group but not in placebo group
de Oliveira et al. 2017, Brazil	Randomized, parallel, double-blind	*n* = 76 obese or overweight non-diabetic older subjects (23 M + 53 F) Mean age = 67.4 ± 5.16 years Mean BMI >28 kg/m^2	30 mL/day of three different types of vegetable oil: olive, flaxseed or sunflower oil. This quantity of oil should not have been additional to the normal diet of the subjects	Absent	90 days	FMD and CIMT	↑ FMD in the group that consumed sunflower oil ↓ CIMT in all the intervention groups
do Rosario et al. 2020, Australia	Randomized, crossover, placebo-controlled, double-blind Washout period of 14 days	*n* = 16 overweight or obese, but otherwise clinically healthy elderly subjects (non-hypertensive, non-diabetic, non-drug-treated, non-smoker subjects). Mean age = 65.9 ± 6 years Mean BMI = 30.6 ± 3.9 kg/m^2	250 mL/day of high ACN queen garnet plum juice (about 200 mg of ACN)/day	250 mL colored apricot juice (placebo).	5 days	FMD and microvascular reactivity (peak shear rate, PV, PORH max, and IONT max) to evaluate postprandial response 2 h after challenging with a high energy/high fat test meal	↑ FMD, and PORH max in test products group versus control

do Rosario et al. 2020, Australia	Randomized, parallel, placebo-controlled, double-blind	*n* = 31 older subjects with mild cognitive impairment (12 M + 19 F). Mean age = 75.3 ± 6.9 years Mean BMI = 26.1 ± 3.3 kg/m^2	250 mL/day of two different types of fruit juice: low ACN queen garnet plum (about 45 mg of ACN) or high ACN queen garnet plum (about 200 mg of ACN).	Colored apricot juice (placebo). Blinding strategies included advertising and consenting participants to a “fruit juice study”, without providing information on which fruit was being investigated.	8 weeks	Microvascular reactivity evaluated POHR	↔ microvascular reactivity
Gilchrist et al. 2013, UK	Randomized, crossover, placebo-controlled, double-blind Washout period of 4 weeks	*n* = 27 non-smokers older subjects with T2DM (of at least 5 years duration) and BP 4125/85 mm Hg or on one or more antihypertensive agents (18 M + 9 F). Mean age = 67.2 ± 4.9 years. Mean BMI = 30.8 ± 3.2 kg/m^2	250 mL/day of beetroot juice, providing 7.5 mmol of nitrate	250 mL of nitrate-depleted beetroot juice (placebo).	2 weeks	FMD and microvascular endothelial function (perfusion response after skin iontophoresis of ACh and SNP)	↔ macro- and microvascular reactivity
Jones et al. 2019, UK	Randomized, parallel, placebo-controlled, double-blind	*n* = 20 non-obese older subjects. Mean age = 63 ± 6 years. Mean BMI = 26,5 kg/m^2	70 mL of beetroot juice providing 4.7 mmol of nitrate every day	70 mL of prune juice	4 weeks	FMD and markers of microvascular endothelial function (perfusion response after skin iontophoresis of ACh and SNP)	↔ compared to placebo

Oggioni et al. 2017, UK	Randomized, crossover, placebo-controlled, double-blind Washout period of at least 7 days	*n* = 20 non-smokers healthy older subjects (10 M + 10 F). Mean age = 64.7 ± 3 years. Mean BMI = 25.6 ± 3.4 kg/m^2.	70 mL of beetroot juice twice a day (for a total of 12 mmol additional nitrate/day)	70 mL of nitrate-depleted beetroot juice twice a day (placebo)	1 week	AIx	↔ arterial stiffness parameter
Shaltout et al. 2017, USA	Randomized, parallel, placebo-controlled, double-blind	26 older hypertensive patients (13 M + 13 F). Mean age = 69 ± 7 years. Mean BMI = 33.7 kg/m^2.	70 mL of beetroot juice providing 6.1 mmol of nitrate every day + aerobic exercise training 3 times/week	70 mL of nitrate-depleted beetroot juice twice a day (placebo) + aerobic exercise training	6 weeks	Hemodynamic measures (SVR, TAC, VI, AI, and LCWI)	↔ hemodynamic measures
Woessner et al. 2018, USA	Randomized, parallel, placebo-controlled, double-blind	*n* = 24 older subjects with PAD + IC (10 M + 10 F). Mean age = 64.7 ± 3 years. Mean BMI = 25.6 ± 3.4 kg/m2.	70 mL of beetroot juice providing 4.2 mmol 3 h prior training session (30 min walking sessions 3 times/week)	70 mL of nitrate-depleted beet-root juice twice a day (placebo) prior training session	12 weeks	ABI and RHBF	↑ ABI, and RHBF in the test group vs. placebo

Legend: ABI, resting ankle-brachial index; Ach and SNP, acetylcholine and sodium nitroprusside; ACN, anthocyanin; AI, acceleration index; AIx, augmentation index; BMI, body mass index; CIMT, carotid intima-media thickness; D, brachial artery diameter; FBF, forearm blood flow; FMD, flow-mediated dilation; FVC, forearm vascular conductance; IONT max, maximum perfusion following iontophoresis of acetylcholine; LCWI, left cardiac work index; LSCI combined with POHR, laser speckle contrast imaging combined with a post-occlusive reactive hyperemia test; PAD + IC, peripheral artery disease associated with intermittent claudication; PORH max, post-occlusive reactive hyperemia maximum perfusion; PV, peak value; RHBF, reactive hyperemic blood flow; SVR, systemic vascular resistance; TAC, total arterial compliance; T2DM, type 2 diabetes mellitus; VI, velocity index; Vmean, blood velocity; n.r., not reported.

3.3. Risk of Bias

In Supplementary Figures S1 and S2 are reported the risks of bias within individual studies and across the studies, respectively. Overall, the results have documented the selective reporting (reporting bias) and the blinding of outcome assessment (detection bias) to represent the highest risks of bias.

3.4. Results from Acute Intervention Studies

The results of short-term interventions are summarized in Table 1. Five studies were randomized and placebo-controlled (Casey *et al.*, 2015; de Oliveira *et al.*, 2016; Dodd *et al.*, 2019; Hughes *et al.*, 2020; Pekas *et al.*, 2021) while one study (Hughes *et al.*, 2016) followed a parallel design comparing differences in vascular response between young and older subjects, without including a control group of older subjects. All studies were performed by using beetroot, while only 1 study was carried out by testing the effect of blueberry (Dodd *et al.*, 2019). Three out of 5 studies administered beetroot in the form of juice (Casey *et al.*, 2015; Hughes *et al.*, 2016; Pekas *et al.*, 2021), in dose ranging from 70 mL up to 500 mL, while 1 study used 100 g of beetroot gel (de Oliveira *et al.*, 2016), and 1 study a drink obtained by suspending beetroot powder (powder amount not reported) in 240 mL of water (Hughes *et al.*, 2020). Beetroot administration provided from a minimum of 4 mmol (Hughes *et al.*, 2020) up to a maximum of 12.2 mmol of nitrates (de Oliveira *et al.*, 2016). Apart from the comparative study of Hughes *et al*. (2016), all studies that tested beetroot included a nitrate-deprived placebo in the same formulation as the test product. Regarding blueberry, the tested product was a flavonoid-rich blueberry beverage prepared by suspending 30 g of blueberry powder in 300 mL of semi-skimmed milk and by using a placebo drink containing the same amount and type of sugars and vitamin C of the test product (Dodd *et al.*, 2019).

Considering the outcomes, 2 studies evaluated vascular reactivity and arterial stiffness at rest (de Oliveira *et al.*, 2016; Pekas *et al.*, 2021), 2 study evaluated only arterial stiffness at rest (Hughes *et al.*, 2016; Dodd *et al.*, 2019), and the remaining 2 studies performed hemodynamics measures under during exercise (Casey *et al.*, 2015; Hughes *et al.*, 2020). In detail, de Oliveira G. *et al*. (2016) found that administration of 100 g of beetroot gel resulted in a significant increase in FMD, reactive hyperaemia index (RHI) and blood flow velocity (BFV) compared to placebo, after 2 h from the administration. Also Pekas *et al*. (2021) found that administration of beetroot in the form of juice induced a significant increase in brachial and popliteal FMD, compared to placebo. Considering the arterial stiffness, none of the 4 acute studies found a significant effect on AIx and other related markers. In particular, Hughes *et al.* (2016) compared the different response in AIx and AI@75 between healthy young and older adults after administration of 500 ml of a commercially available nitrate-rich beetroot juice beverage (providing 9.4 mmol/L of nitrates). The effect was evaluated at six different time points (baseline, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h) post consumption of beetroot or a control product (no nitrate-depleted control product in older subjects). The results show that young adults presented significantly lower values of AIx at baseline compared to the older subjects. In addition, while AIx and AI@75 levels remained nearly constant along the time in the group of older subjects, a significant decrease in AIx and AI@75 was observed in the group of young adults. This effect was particularly greater at 2 h and 2.5 h.

Two out of 6 studies considered differences in vascular response during exercise after administration of beetroot. Hughes and colleagues (2020) did not find any significant variation in hemodynamics parameters (i.e. blood flow – BF and vascular conductance – VC) measured at leg level at different intensity knee-extension exercise, after 2 h from beetroot juice ingestion in healthy older adult. Casey *et al*. (2015) measured the difference in response between healthy young and older adults on hemodynamic measure (i.e., forearm blood flow - FBF, forearm vascular conductance - FVC) during exercise in both condition of normoxia and hypoxia. The authors found a significant increase after 3 h from consumption of beetroot juice, but not after placebo, of these parameters under hypoxia condition, compared to baseline values.

All studies performed on beetroot included the evaluation of nitrate and nitrite bioavailability. Four studies (Casey *et al.*, 2015; Hughes *et al.*, 2016, 2020; Pekas *et al.*, 2021) evaluated the bioavailability at plasma or serum levels, while one study (de Oliveira *et al.*, 2016) considered nitrate and nitrate at urinary level. All studies reported a substantial increase of these compounds after beetroot ingestion while no significant effect was documented following placebo.

3.5. Results from Chronic Intervention Studies

Results from chronic intervention trials are summarized in Table 2. Six studies (Gilchrist *et al.*, 2013; Shaltout *et al.*, 2017; Oggioni *et al.*, 2018; Woessner *et al.*, 2018; Jones *et al.*, 2019; Casey *et al.,* 2021) tested the effect of beetroot, 2 studies were run evaluating the effects of plum (do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b), while 1 study used vegetable oils (de Oliveira *et al.*, 2017). All studies were randomized, placebo-controlled. Half of them showed a parallel design, while the remaining half a crossover design. Considering the intervention, 5 out of 6 studies administered concentrated beetroot juice, ranging from 70 to 250 mL, and providing a minimum content of 4.2 mmol (Woessner *et al.*, 2018; Casey *et al.*, 2021) up to 12 mmol of nitrates (Oggioni *et al.*, 2018). Only one stud did not use beetroot juice, but supplied the participants with a drink obtained by suspending a concentrated beetroot powder (amount of powder not reported) providing 4 mmol of nitrates, to be resuspended in 200 mL of water (Casey *et al.*, 2021). In 5 out of 6 studies beetroot products had to be consumed daily, while in 1 study beetroot was provided three times/week (3 hours prior a training session) (Woessner *et al.*, 2018).

Considering the main outcomes investigated, most of the studies measured macro- and micro-vascular reactivity. Macrovascular reactivity was mainly evaluated through FMD (de Oliveira *et al*., 2016; Pekas *et al*., 2021) or different indexes such as ABI, RHBF (Woessner *et al.*, 2018). Microvascular reactivity was assessed through numerous methods, but mainly measuring perfusion response after skin iontophoresis of ACh and SNP (Gilchrist *et al.*, 2013; Jones *et al.*, 2019). Only two studies evaluated the effect on arterial stiffness (Shaltout *et al.*, 2017; Oggioni *et al.*, 2018).

In detail, Gilchrist *et al*. (2013) measured FMD and endothelium-independent dilation (response to 0.4 mg of sublingual nitroglycerine), as well as microvascular endothelial function evaluated through laser Doppler perfusion imaging in response to acetylcholine and sodium nitroprusside. Authors did not find any significant effect after two weeks of daily administration of 250 mL of beetroot juice (providing 7.5 mmol of nitrates) in a group of overweight/obese older subjects with T2DM. The same outcomes were evaluated by Jones and colleagues (2019), that tested a lower portion of beetroot juice (70 mL, providing 4.7 mmol of nitrates) but for a longer period (4 weeks) in a group of non-obese older subjects. This study found an improvement in FMD after 2 and at 4 weeks compared to baseline, but not compared to placebo. Casey *et al.* (2021) evaluated the effect of 4 weeks consumption of 200 mL beetroot beverage (amount of powder beetroot not reported and providing about 4 mmol of nitrate) on different hemodynamic measures of shear profile at rest (derived from brachial artery diameter and mean blood velocity, both measured via Doppler ultrasound) and measure of exercise hyperemia during handgrip exercise (FBF – forearm blood flow, and FVC forearm vascular conductance). The authors found a significant improvement of pro-atherogenic shear stress measures and a lower pro-atherogenic oscillatory shear stress index in the beetroot group compared to the control group. Conversely, no effect on mean shear stress or anti-atherogenic antegrade velocity or antegrade shear stress was documented.

The studies of Shaltout *et al.* (2017) and Woessner *et al.* (2018) involved an exercise intervention together with the consumption of beetroot juice. Shaltout *et al*. (2017) used an impedance cardiograph to measure mean arterial pressure and obtained by derivation several hemodynamic measure (e.g., systemic vascular resistance, total arterial compliance, velocity index) after 6 weeks of aerobic training (3 times/week) associated with daily consumption of beetroot juice (70 mL, providing about 6 mmol of nitrates) or placebo juice (nitrate-deprived juice), in a group of 26 older subjects with controlled hypertension. However, the authors did not find any significant improvement in hemodynamic parameters, apart from the final value of total arterial compliance which was significantly increased compared to baseline, but in both the test and control groups compared to their baseline. Conversely, Woessner *et al*. (2018) measured index of vascular function (i.e. resting ankle-brachial index – ABI, and reactive hyperemic blood flow - RHBF) after 12-week of 3 times/week mild training session associated with the consumption of 70 mL of beetroot juice (4,2 mmol of nitrates, to be consumed 3 hours before each exercise training visit) or placebo in a group of 24 non-obese older subjects with a condition of peripheral artery disease associated with intermittent claudication. The study found a significant improvement in ABI and RHBF parameters compared to baseline in the group of subjects consuming beetroot and performing exercise, but not in those consuming the placebo. Finally, the study of Oggioni *et al.* (2018) focused on arterial stiffness, but did not found any significant improvement in AIx after one week of daily consumption of 2 portions of 70 mL of beetroot juice (providing a total amount of 12 mmol of nitrates) in 20 healthy, non-obese older subjects.

Two studies tested the effect of a medium-long term intervention with a daily consumption of 250 mL anthocyanins-rich plum juice (Queen Garnett plum cultivar; providing about 200 mg of total anthocyanins). Both studies were conducted in Australia by the same research group (do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b). In one study, 16 overweight healthy older subjects consumed 250 mL of plum juice per 4 days at the end of which an acute evaluation was performed. Within this test, a high energy/high fat meal (providing approximately 850 kcal and 65 g of total fats) was accompanied by the intake of 250 ml of a high anthocyanins plum juice or placebo (do Rosario *et al.*, 2021a). The intervention documented a significant improvement in FMD, as well as post-occlusive reactive hyperemia (PORH), in the group consuming plum juice compared to control. In the second study the same authors tested the effect of 250 mL of two different plum juices (presenting low and high anthocyanins content, equal to 45 and 200 mg respectively) or placebo (apricot juice) on the vascular reactivity of 31 obese older subjects with mild cognitive impairment (do Rosario *et al.*, 2021b). The study, a randomized, parallel design, involved a period of 8-week intervention in which participants consumed 250 mL/day of plum or a placebo juice. At the end of the intervention period, no significant differences in vascular reactivity were detected.

Finally, one study tested the effects of a daily consumption of 30 mL of three different vegetable oils (i.e., olive, flaxseed, and sunflower oil) on FMD and carotid intima-media thickness – CIMT in a group of overweight elderly subjects (de Oliveira *et al.*, 2017). This study followed a randomized, uncontrolled, parallel design and it was 90-day long. At the end of the trial, a significant improvement in CIMT was observed after vegetable oils supplementation. In addition, a significant improvement in FMD was also documented but only in the group of subjects consuming sunflower oil.

3.6. Other Markers Analyzed and Main Findings Obtained from Acute and Chronic Intervention Studies

Other marker, linked to cardiovascular risk, but not specifically related to vascular function, were considered within the studies included. Here, we have reported a brief report of the results obtained for vegetable products.

Regarding beetroot, several studies involved the assessment of blood pressure markers (e.g., systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure), measured at brachial or aortic level, but most of them did not find any significant variation (Gilchrist *et al.*, 2013; Casey *et al.*, 2015; de Oliveira *et al.*, 2016; Oggioni *et al.*, 2018; Hughes *et al.*, 2020; Casey *et al.*, 2021). However, 3 studies (Shaltout *et al.*, 2017; Jones *et al.*, 2019; Pekas *et al.*, 2021) found a reduction in systolic blood pressure (Shaltout *et al.*, 2017; Pekas *et al.*, 2021), while one study (Jones *et al.*, 2019) reported a decrease in both systolic and diastolic blood pressure. Furthermore, Oggioni *et al*. (2018) also considered the effect of beetroot in cardiac output at rest and during cardiopulmonary exercise testing, but without finding a significant effect. Shaltout *et al.* (2017) tested the effect of beetroot on peak oxygen consumption and time to exhaustion during aerobic exercise in their study on hypertensive subjects, without showing a significant improvement. Pekas *et al.* (2021) found a beneficial effect of beetroot in maximal walking time and levels of deoxygenated hemoglobin in older subjects with peripheral artery disease, while Woessner *et al.* (2018) reported a significant effect on claudication onset time and tissue deoxygenation characteristics in subjects with peripheral artery disease with intermittent claudication.

Considering biochemical parameters, the effects were tested on biomarkers of inflammation (i.e., IL-6), as well as endothelial integrity (e.g. E-Selectin, P-Selectin), and glucose metabolism markers (including insulin sensitivity), but without finding any significant effect (Gilchrist *et al.*, 2013; Oggioni *et al.*, 2018).

Regarding ACN-rich foods (plum and blueberry), the 2 chronic studies also evaluated blood pressure and several metabolic and functional parameters associated to vascular health such as lipid profile and inflammation showing different results. One study (do Rosario *et al.*, 2021a) reported a significant effect of plum juice in the reduction of C-reactive protein levels and a trend for the reduction of IL-6, but no effect on blood pressure, triacylglycerol, and total cholesterol, while the second study (do Rosario *et al.*, 2021b) showed a significant reduction in the levels of TNF-α, but not on IL-6, Il-1β, C-reactive protein and blood pressure. Regarding blueberries (Dodd *et al.*, 2019), no significant effect was found for cognitive function or levels of brain-derived neurotrophic factor, but there was a trend for systolic blood pressure, which increased less in the blueberry group compared to controls.

Finally, the study performed by using vegetable oils (de Oliveira *et al.*, 2017) evaluated also the impact on biochemical parameters such as CRP, APO B, Apo A, and ApoB/Apo/A ratio, but unfortunately no significant effect was reported.

4. Discussion

The aim of this review was to assess the evidence deriving from intervention trials testing the vasoactivity of bioactive-rich vegetable foods in older subjects. Overall, the number of trials is limited and involve mainly the beetroot. The discussion has been organized in different sections, discussing the main results in relation to each plant-based food and by providing the potential mechanisms identified from preclinical models and, when available, theorized by the authors of the papers included in the present revision.

4.1 Beetroot

Considering the effects of acute beetroot intake on vascular reactivity, two studies indicate an increase in FMD and related markers. The effects were observed in subjects with CV factors or peripheral artery disease (PAD) (de Oliveira *et al.*, 2016; Pekas *et al.*, 2021), suggesting a potential short-term contribution of beetroot in the modulation of FMD, RHI and BFV in subjects at risk. Regarding chronic studies, the majority of dietary interventions reported no effect following beetroot consumption. For example, Gilchrist *et al.* (2013) did not find any significant effect on FMD and microvascular endothelial function after 2 weeks of daily administration of 250 mL of beetroot juice (providing 7.5 mmol of nitrates) in a group of overweight/obese older subjects with T2DM. Oggioni *et al.,* (2018) reported no variation on arterial stiffness parameters (e.g., AIx) after 1-week intervention with 70 mL of beetroot juice, provided twice a day (12 mmol nitrate), in healthy older subjects. Similar findings were also found by Jones and colleagues (2019) showing a lack of significance on hemodynamic measures after 4-week intervention with a comparable dose of beetroot juice (70 mL providing 4.7 mmol of nitrate) in a group of healthy older subjects. This discrepancy could be attributed to the different population considered; in fact, while acute studies were conducted in subjects with CV factors, chronic interventions were carried out mainly on healthy subjects. Another plausible reason could be related to the rapid metabolization of nitrites that are converted into NO and quickly disappear from blood (Tousoulis *et al.*, 2012). Finally, it is considered quite unlikely an increase in NO levels above a certain threshold in healthy subjects through NOS-independent mechanisms, given that in healthy subjects the impairment in NO production is less pronounced than in subjects who already show an endothelial dysfunction. However, some chronic studies have reported a beneficial effect on vascular function following the consumption of beetroot but in non-older subjects. For example, Asgary and co-workers (2016) found an increase in FMD after 2 weeks in a group of 24 subjects presenting mild hypertension (systolic blood pressure between 130-140 mmHg), while Velmurugan *et al.*, (2016) documented an improvement after 6-week beetroot consumption in subjects with CV factors.

The rest of the studies were carried out in older subjects wherethe effect of beetroot was combined with physical activity. Casey *et al.* (2015) indicated a significant effect of acute beetroot juice intake (500 mL providing 9.4 mmol of nitrate) on FBF and FVC in subjects performing physical exercise in a hypoxia condition (80% arterial O_2 saturation, induced by a tight-fitting oronasal facemask), while no improvement was found when tested in normal conditions (normoxia) or in young subjects. Hughes *et al.*, (2016, 2020) documented a lack of significant effect on several vascular and hemodynamic markers (e.g., leg blood flow, vascular conductance AI, AI@75) following the acute intake of beetroot beverage (240 and 500 ml) measured during physical exercise and at different times in normoxia (Hughes *et al.*, 2016, 2020). When considering chronic intervention, Casey *et al.* (2021) found that 4-week intervention with beetroot (providing at about 0.4 mmol of nitrate and 0.3 mmol of nitrite) improved conduit artery shear profiles, thus suggesting a reduction in the resistance of the arteries and a restoration of normal laminar flow in older adults. This result was not observed in a condition of handgrip exercise hyperemia. Woessner and coworkers (2018) documented a significant increase in vascular parameters (ABI and RHBF) following 12-week intervention with 70 mL beetroot in subjects with PAD and intermittent claudication. The analysis of vascular parameters was carried out before and after a rehabilitation process that included 30 minutes of walking 3-time per week. Conversely, Shaltout and co-workers (2017) showed no significant effect on hemodynamic markers in a small group of hypertensive older subjects after 6 weeks intervention (70 mL of beetroot per day, providing 7.5 mmol nitrate) associated with an aerobic exercise training (3 times per week). The lack of univocal findings could be attributed to numerous factors. First, the different characteristics of the study population considered within the studies (e.g., healthy subjects, subjects with risk factors); second, the different exercise training program attended by the subjects during the experimental period that cannot be comparable; third, the difference in terms of experimental design including the amount of beetroot administered, nitrate content, duration of intervention, presence/absence of the control group and its characteristics; fourth, differences in terms of biomarkers analyzed that make the comparison difficult.

In respect to the effects on physical exercise, some premises are required. During the activity in normoxia, local vasodilation responds to the increased needs of skeletal muscles of oxygen for the oxidative phosphorylation of mitochondria, nutrients, as well as for the need to remove metabolites and dissipate the excess heat produced (Hammond, 2014). In order to respond adequately to these requests, the mechanisms of local vasodilation need to be multiple and involve not only vasoactive endothelial molecules (e.g., NO and PGI2), but also other factors (e.g., K + and adenosine produced directly by the muscles under stress, as well as mechanical effect of the "muscle pump") (Joyner *et al.,* 2015). In fact, exercise hyperaemia can also occur when NO and PGI2 are inhibited and it is estimated that NO contributes to exercise hyperaemia only about 20% (Dinenno, 2016). Thus, NO impairment during physical exercise is less easy to evaluate. However, exercise during a hypoxic condition depends much more on NO, probably since hypoxia induces further vascular adaptations to cope with the reduction of oxygen supply (Casey *et al.*, 2010). In fact, trials that evaluated exercise hyperemia during hypoxic conditions show that the consumption of beetroot could compensate for the lack of NO production in case of impairment by providing nitrate and nitrite to use for its manufacture through alternative ways than NOS, as reported above.

These data support the idea that when NO levels are not impaired (as occurs in healthy subjects) there is no beet effect, but also that the deoxygenation of red blood cells can induce an increase in their ability to release nitric oxide from nitrate and nitrites, as suggested in the Dinenno review (2016). These results seem also in line with the observations reported in the review of Jones (2014), which indicates that the ergogenic effect of beetroot seems more easily detectable for activities shorter than 30 min, that generally are more intense than longer activities and thus more likely to recruit the contribution of the endothelial mechanism of vasodilation.

In Figure 2 are summarized the main potential mechanisms through which nitrates (NO3-), nitrite (NO2-) could exert a vasodilatory effect, perhaps in synergy with other beetroot compounds such as vitamins, minerals and betalains (e.g., betanin, isobetanin, vulgaxanthin I); these latter responsible of the red color of the root (Fu *et al.*, 2020). Several studies have reported a health-promoting effect of betalains (Hadipour *et al.*, 2020), thus their potential contribution in the modulation of vascular function should not be excluded. Accumulating evidence indicates that NO3- and NO2- can be reduced to NO, that can thus be obtained independently from arginine transformation catalyzed by NOS. The conversion of NO3- into NO is dependent on the activity of the oral microbiota that is able to reduce NO3- into NO2-. The latter are then subsequently reduced into NO thanks to the activity of the enzyme xanthine oxidoreductase (XOR) and deoxyhemoglobin (Deoxy-Hb) at the level of red blood cells (Lundberg *et al.* 2005; Bryan, 2006). In this regard, Ghosh *et al.* (2013) documented that beneficial effect on blood pressure after beetroot consumption was due to the conversion of NO3- into NO by XOR, and that this conversion occurred significantly more in hypertensive subjects compared to normotensive controls.

Regarding safety issue, the European Food Safety Authority (EFSA) has set the Acceptable Daily Intake (ADI) for nitrate at 3.7 mg/kg (approximately 260 mg for a 70 kg adult, equivalent to 4,2 mmol). A similar limit is also suggested by the World Health Organization (WHO) (Hord *et al.,* 2009). Safety limits exist since the intake of nitrites can brings to the formation of carcinogenic compounds (i.e. N-nitrous compounds) or to the formation of methaemoglobin, which is particularly dangerous for infants (Benjamin, 2000). It should be taken into account that the majority of the studies testing beetroot products included in this review used a dosage higher than EFSA's ADI (Gilchrist *et al.*, 2013; Casey *et al.*, 2015; de Oliveira *et al.*, 2016; Hughes *et al.*, 2016; Shaltout *et al.*, 2017; Oggioni *et al.*, 2018; Pekas *et al.*, 2021)

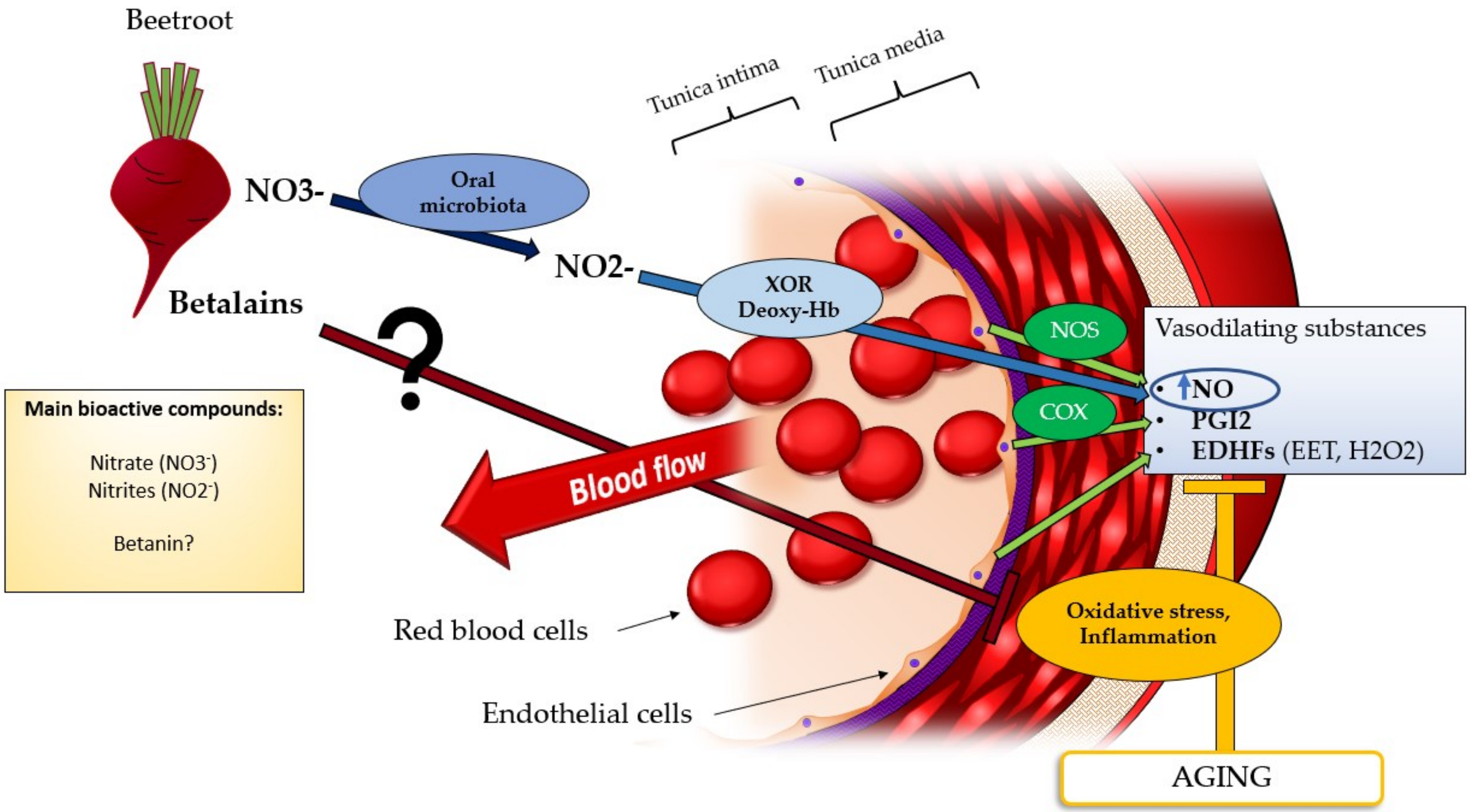

Figure 2 – Schematic representation of the mechanism of action of beetroot in the increase of vascular function in older subjects. Endothelial cells produce vasodilating substances through different enzymes in response to physiological stimuli. Aging can be

associated with high levels of oxidative stress and inflammation that cause an impairment of those vasodilating substances. Dietary $NO3^-$ from nitrates rich vegetables, such as beetroot, can acutely contribute to replenish the pool of NO in case of impairment thanks to NOS independent pathway, such as XOR and Deoxy-Hb of red blood cells. Other beet bioactives (e.g., betanin, the main betalains contained in beetroot) can also be involved in these processes, through synergistic or independent effects. Legend: COX, cyclooxygenase Deoxy-Hb, deoxyhemoglobin; EDHFs, endothelial derived hyperpolarizing factors; EET, epoxyeicosatrienoic acid; H_2O_2, hydrogen peroxide; $NO2^-$, nitrites; $NO3^-$, nitrate; NO, nitric oxide; NOS, nitric oxide synthase; PGI2, prostacyclin2; XOR, xanthine oxidoreductase.

4.2. Effect of Fruit on Vascular Function

The role of fruits in the modulation of vascular function has been debated for a long period. Fruit is a rich source of numerous bioactive compounds such as vitamins, minerals, and phytochemicals including (poly)phenols and carotenoids. Three studies investigated the effects of fruit on vascular health in older subjects. Two were chronic studies testing the effects of plum (do Rosario *et al.*, 2021a; do Rosario *et al.*, 2021b), while one was an acute study carried out with blueberries (Dodd *et al.*, 2019).

Regarding the effect of plums, a study has shown to counterbalance the detrimental effect a high-calorie and high-fat meal over a period of four days in a group of overweight/obese older subjects (do Rosario *et al.*, 2021a). Conversely, another study showed no effect on micro-vascular reactivity at the end of an 8-week intervention with plum in older subjects with mild cognitive impairment (do Rosario *et al.*, 2021b). However, the authors were able to document a reduction in TNF-α serum levels, suggesting a contribution of the intervention on the inflammatory response. These two studies are difficult to compare due to their heterogeneity in terms of study population, duration and experimental design adopted. Another consideration is related to the dietary prescription followed by the subjects within the intervention periods. In the first study (do Rosario *et al.*, 2021a), participants were requested to avoid ACN-rich foods and a standardized, low-flavonoid, frozen meal was provided at dinner prior to the experiment. In the second study (do Rosario *et al.*, 2021b), subjects maintained their habitual diet. It cannot be excluded that this lack of standardization could have affected the results on vascular function.

To the best of our knowledge, only one study investigated the effects of blueberry in older subjects. Specifically, Dodd *et al.* (2019) evaluated the acute effect of a highbush blueberry beverage (30 g of blueberry powder providing at about 580 mg of ACNs) on arterial stiffness in a group of 18 healthy older subjects. The results have documented a lack of significant effect. These findings seem in line with the observations deriving from acute blueberry studies, in which a significant improvement on vascular function, but not arterial stiffness, was documented in young/adult healthy subjects (Del Bo′ *et al.*, 2013; Rodriguez-Mateos *et al.*, 2013, 2014; Del Bo' *et al.*, 2014, 2017). Also the results from chronic intervention studies failed to observe an effect on arterial stiffness from the consumption of blueberry (Riso *et al.*, 2013). Conversely, Johnson and colleagues (2015) found an amelioration in arterial stiffness in post-menopausal women following 8 weeks intervention with a drink containing 22 g of a highbush blueberry powder. Specifically, the authors documented a significant decrease in blood pressure and brachial-ankle pulse wave velocity (marker of arterial stiffness), but not in carotid-femoral pulse wave velocity. The authors attributed the beneficial effects to an increase in NO levels than to a ROS reduction, since the levels of SOD did not appear to be increased.

The putative mechanism of actions through which ACNs could have a potential health benefit on vascular function is depicted in Figure 3. ACNs are the most studied bioactives in the CV field in relation to numerous experimental evidence that indicate a health-promoting effect, deriving from both *in vivo* studies (observational and RCT) and *in vitro* studies (Reis *et al.*, 2016; Krga *et al.*, 2019). Experimental evidence indicates that ACNs are capable of positively affecting vascular function, inflammation, and counteract pro-atherogenic processes (Kalt *et al.*, 2019). Consistent literature indicates that these beneficial effects are mainly mediated by a regulatory effect on numerous genes involved in endothelial function especially by inducing an increased NO production (Speciale *et al.*, 2014; Krga *et al.*, 2016; Martini *et al.*, 2019; Ma *et al.*, 2021). This effect seems to be mediated by an increased activity of eNOS and/or by its preservation from the oxidation. Moreover, ACNs are considered as antioxidant compounds thanks to their capacity to counteract the detrimental effect of reactive oxygen species (ROS) by preventing the formation of peroxynitrite and consequently the reduction of NO availability (Alappat *et al.*, 2020). Several reviews have tried to explain the antioxidant activity of ACNs. One putative mechanism is related to their direct antioxidant activity against ROS, although this effect is influenced by the actual bioavailability (Garcia *et al.*, 2021). The second more plausible mechanism consists in the regulation of genes involved with oxidative stress, such as antioxidant defense enzymes (e.g., SOD, CAT, and GPx), through the activation of Nrf2. In this regard, Rodriguez-Mateos *et al*. (2013) found that the increase in vascular function observed in their study corresponded to a significant reduction in NADPH oxidase activity, an enzyme that catalyzes the formation of ROS (Johnson *et al.*, 2015). ACNs are also able to inhibit the activation of NF-kB, thus interfering with inflammation response also at endothelial level by reducing the production of several interleukins and cytokines with a direct effect on vascular function (Speciale *et al.*, 2014; Reis *et al.*, 2016).

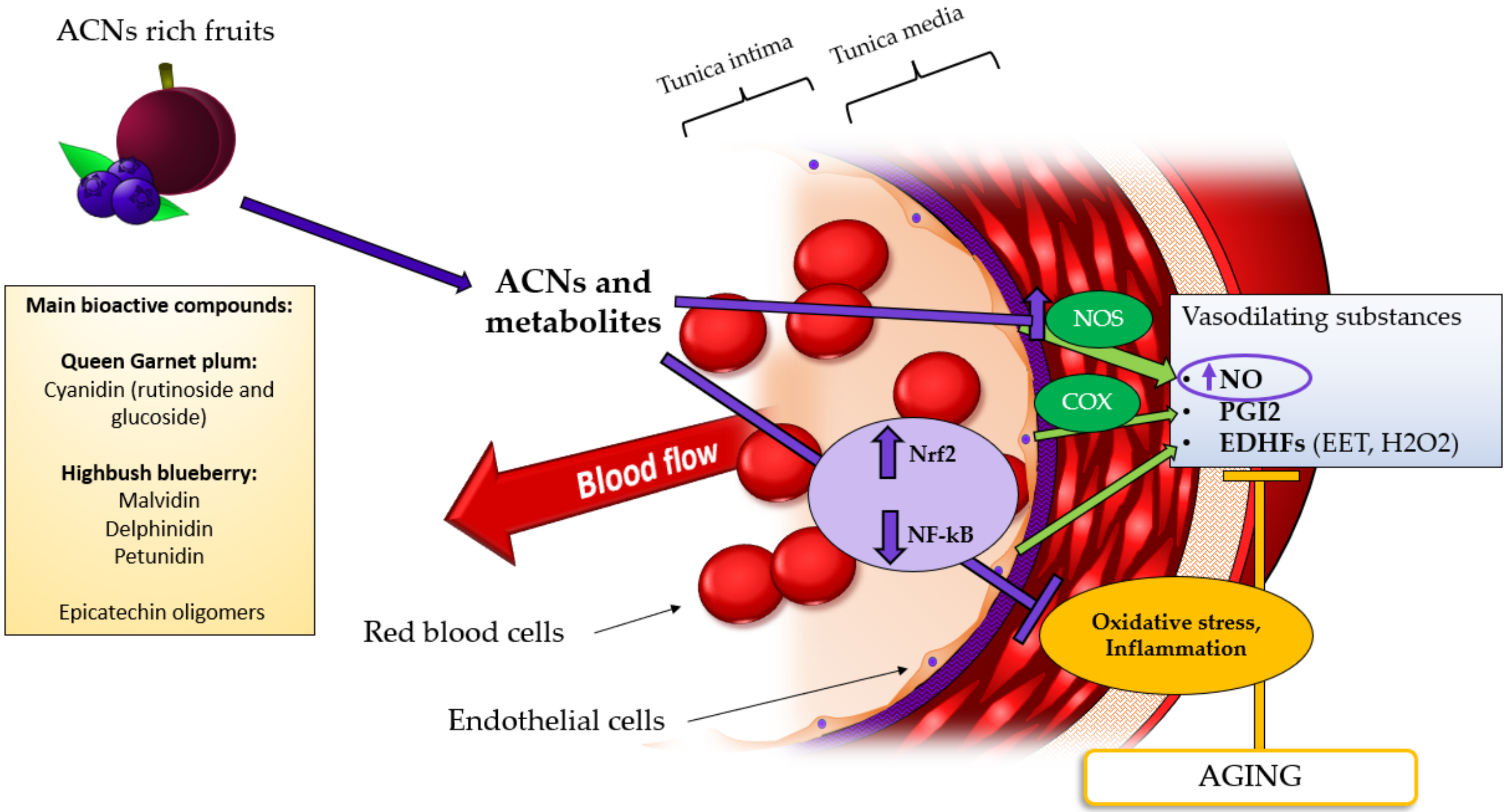

Figure 3 – Schematic representation of the mechanism of action supposed for ACNs rich fruits in the increase of vascular function in the elderly. In contrast to nitrates that act by increasing NO levels through NOS-independent pathways, ACNs increase the expression

of NOS and counteract the detrimental effects of oxidative stress (due to activation of Nrf2, that lead to increased defense from ROS) and inflammation (inhibiting the activation of NF-kB), thus increasing NO production and contribute to the restoration of its availability. Legend: ACNs, anthocyanins; COX, cyclooxygenase; EDHFs, endothelial derived hyperpolarizing factors EET, epoxyeicosatrienoic acid; H_2O_2, hydrogen peroxide; NF-kB, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; Nrf2, nuclear receptor factor 2; PGI2, prostacyclin 2.

4.3. Effect of Vegetable Oils on Vascular Function

The impact of vegetable oils on vascular function in older subjects has been evaluated only in one study (de Oliveira *et al.*, 2017). de Oliveira P. *et al.* (2017) performed a 12-week intervention testing the effect of 30 g/day of three different vegetable oils (flaxseed, olive and sunflower oil) on markers of vascular function (FMD) and stiffness (carotid intima-media thickness, CIMT). The results have shown a different effect that was dependent on the type of oil. Specifically, a significant amelioration of vascular response (evaluated by FMD) was reported only in the group that consumed sunflower oil, while a significant reduction in CIMT was documented in all the three intervention groups, even if the effect seemed to be more pronounced in the group of subjects consuming flaxseed and olive oils (de Oliveira *et al.*, 2017). These results could be explained by the different composition in fatty acids (FAs); sunflower is rich in ω-6 PUFA (linoleic acid – LA), flaxseed oil in ω-3 PUFA (α-linolenic acid) while olive oil in MUFA (oleic acid). Although the role of FA on vascular function has been poorly investigated and results from *in vivo* studies are controversial (Hall, 2009), growing evidence from preclinical models seem to suggest a relevant influence of FA in this context in both positive and negative ways. In particular, FA can be associated with vascular function through the modulation of inflammatory processes, oxidative stress, apoptosis of endothelial cells, and NO production (Ghosh *et al.*, 2017; Mallick *et al.*, 2022), as depicted in Figure 4. In fact, ω-3, -6 and -9 FA differently modulate processes associated with vascular function and CV risk. Results from both *in vivo* and *in vitro* studies indicate that ω-3 could have a beneficial effect on vascular endothelium (Colussi *et al.*, 2017). Among them, it has been observed that ω-3 are capable of increasing the availability of NO in the aorta of rats through the activation of eNOS and the enhancement of NOS activity (López *et al.*, 2004). Moreover, NO increase, induced by ω-3, can prevent the formation of oxLDL due to the antioxidant effect of NO via the termination of radical chain propagation reactions (Donnell *et al.*, 2001). In addition, evidence from a apoE-knockout mice model suggests that ω-3 play an important role on oxidative stress by increasing the activity of antioxidant enzymes (i.e. SOD, CAT, and GPx), determining lower levels of ROS (Wang, 2004). Since oxidative stress induces endothelial cell damages, inflammation, cell permeability, increased apoptosis and vascular remodeling (Weseler *et al.,* 2010), the capacity of ω-3 to counteract the excess of free radicals, could represent a relevant mechanism in preventing vascular dysfunction. Beside antioxidant properties, ω-3 are also involved in the reduction of endothelin-1, in particular EPA supplementation in human endothelial cells determined lower levels of this potent vasoconstrictor, independently of the production of NO (Chisaki *et al.*, 2003). Evidence from a human study demonstrated that a 12-week intervention with EPA and DHA in older subjects was able to decrease carotid-femoral PWV, an important measure of arterial stiffness, but not arterial blood pressure and arterial wave reflections (central augmentation index) (Monahan *et al.*, 2015).

On the other hand, the role of ω-6 FA on vascular function is still controversial and dependent on the individual FA considered. Despite the possible rationale to the effect of LA in the amelioration of vascular function, it should be underlined that evidence on the effects of specific FA in vascular function, even if not conclusive and overall limited, currently leans more towards an effect of ω-3 PUFA on vascular function, rather than ω-6 PUFA (Colussi *et al.*, 2017). Dietary EFA are modified by specific enzymes to produce the eicosanoids, i.e. second messengers highly produced by endothelial cells and particularly relevant for CV health (Horrobin, 1993). For instance, from arachidonic acid (AA), a well-known metabolites of LA, derives prostacyclin (PGI_2), an important contributor to vasodilation together with NO (Horrobin, 1993), but also thromboxane A2 (TXA2), a well-known vasoconstrictor factor (Nava *et al.*, 2019). Furthermore, Hennig *et al*. also reported that linoleic acid (LA) hydroperoxide may be associated with endothelial dysfunction and atherogenesis through inflammation-promoting effects (2001). Moreover, several mechanisms through which LA exerts its negative role on vascular functions are supposed. Among them, LA mainly consumed from refined vegetable oils is incorporated in LDL and seems to be responsible for triggering LDL oxidation due its high susceptibility to oxidative processes. Once oxidized, apoB LDL is not recognized by liver receptor (due to its binding with oxidation-derived products such as malondialdehyde and 4-hydroxynonenal), while macrophages, through their scavenger receptor, are able to internalize ox-LDL and promote atherosclerotic plaque development (DiNicolantonio *et al.*, 2018). Additionally, lipoprotein lipase at the level of the endothelium acts on VLDL determining the release of oxidized lipids from linoleic acid such as 13-hydroxyoctadecadienoic acid. These metabolites represent a harmful stimulus directly to the endothelium, by enhancing the release of ROS (mainly by inducing p47 and NADPH oxidase enzyme complex) and cell adhesion molecules (CAMs) and exacerbating the inflammatory process and vascular permeability (DiNicolantonio *et al.*, 2018). However, a systematic review that specifically addressed this hypothesis found that no evidence from randomized, controlled intervention studies on healthy subjects indicates a negative effect of LA on inflammation markers (Johnson *et al.*, 2012). Furthermore, a systematic review highlighted that changes in LA intake are not associated with significant variation in tissue AA content in an adult population consuming a Western-type diet (Rett *et al.*, 2011). Thus, another possible interpretation of the results indicated by de Oliveira P. *et al.* (2017) that should be considered is a matrix effect in which other bioactives (e.g. sunflower oil polyphenols) contributed to the amelioration of vascular response (Visioli *et al.*, 2020).

Differently to ω-6 PUFA, ω-9 MUFA are less susceptible to oxidation, indeed, in samples of human atheroma the amount of oxidized LA was higher than oxidized oleic acid (OA) (Carpenter *et al.*, 1993). In Sprague Dawley rat cardiomyocytes, OA reduced oxidative stress and inflammation induced by SFA, exerting an antiatherogenic effect (Al-Shudiefat *et al.*, 2013). Also, in healthy individuals, the enrichment of milk with oleic acid was able to lower total cholesterol (TC), low-density lipoproteins cholesterol (LDL-C) and triglycerides (TG) levels (Lopez-Huertas, 2010). Different clinical studies suggest a beneficial effect of OA, the main constituent of olive oil, on markers of endothelial function such as FMD (Ghosh *et al.*, 2017). At the same time, some studies using *in vitro* models reported a controversial role of OA on vascular function. For instance, it has been documented that OA induced the disruption of calcium signaling and the following NO production and release in aortic endothelial cells (Kuroda *et al.*, 2001).

Besides unsaturated FA, the detrimental role on vascular function attributed to SFA is due to their effect on increasing TC and LDL-C, both markers of CV risk. However, results are still debating and, again, depending on the individual SFA and also the triacylglycerol structure that could determine a potential different effect (Fattore *et al.*, 2014). In a RCT conducted on 195 subjects with moderate CVD risk, the replacement of SFA with MUFA or ω-6 PUFA did not observe effect on vascular function measured as FMD and other measures of arterial stiffness such as AI and PWV. While, the same intervention determined a reduction of night systolic blood pressure, E-selectin, TC and LDL-C (Vafeiadou *et al.*, 2015). Although a short-term intervention, healthy subjects supplemented with a diet rich in SFA observed a reduction in FMD and increased P-selectin levels compared to MUFA and PUFA groups (Keogh *et al.*, 2005). Conversely, another RCT did not observe differences between MUFA- or SFA-based oils in endothelium-dependent and independent vascular responses (Khan *et al.*, 2003). Results deriving from *in vitro* studies indicate a detrimental effect of SFA on endothelial function through MAPK pathway that leads to ET-1 induction and the following vasoconstriction, but also an increase in CAMs and plasminogen activator inhibitor-1 (PAI-1) (Newens *et al.*, 2011).

Taken together, the existing evidence suggest a key role of vegetable oils and their relative main FAs on vascular function, and also several potential mechanisms of action have been assumed. However, the scarcity of well-designed interventional studies does not allow a clear interpretation of the role of individual vegetable oils/fatty acids.

5. Strengths and Limitations

Overall, this review shows several strengths and limitations. The main strength regards the systematic approach used for the searching and the selection of the studies. In fact, the use of different databases allowed us to be more inclusive in our research. In addition, the choice to focus only on older subjects (aged more than 60 years), by excluding studies including mixed populations, but not only older individuals, has allowed us to have a clear overview of the current studies available on this target population. Another important strength is the assessment of the study quality by using validated tools able to provide detailed information on the methodological quality (risk of bias) of the studies included and to make a weight of the evidence. Regarding the limitations, the first one is related to the few dietary intervention trials available in older subjects. The second is related to the high heterogeneity among studies in terms of the type and dose of products tested, way of administration (i.e., fruit/product, drink), nutritional composition/characterization, and also in terms of bioactives. Third, differences in terms of experimental protocols adopted (i.e., acute versus medium-long term, with and/or without physical activity) and characteristics of the study population (i.e., healthy and/or with risk factors) make the comparison among studies difficult to perform. Lastly, differences in terms of outcomes (i.e., FMD, PWV, AI) and their measurements (i.e., gold standard techniques versus surrogate methodologies). All these limitations made it impossible to perform a quantitative analysis (meta-analysis) of the effects of the different plant-based products tested.

6. Conclusions and Future Directions

In conclusion, the results of the studies included in the present review, although few and with several limitations, seem to corroborate the possible contribution of selected plant-based foods in the improvement of vascular function in older subjects. In particular, the main effects seem to derive from beetroots and their bioactives (such as nitrate/nitrite and betalains) on markers of vascular reactivity including FMD, FBF, and BFV, while no changes were found for markers of arterial stiffness (i.e., AI, PWV). The effects on vascular function seem to be more related to the restoration of a condition of nitric oxide impairment (exacerbated by diseases or hypoxic condition), which in turn ameliorates vascular function, rather than the enhancement of a normal physiological condition. Interestingly, the positive observation seems also mediated by a contribution of oral microbiota able to reduce NO_3^- into NO_2^- and in turn exert a physiological effect. However, the limitations of the studies described above, mainly in terms of heterogeneity among trials, reduce the significance of the findings on beetroots. This aspect also regards the results of the studies performed by using plums, blueberries, and vegetable oils that are too limited. Thus, future well-designed intervention studies focused on this target group are highly recommended to corroborate the current findings and to better elucidate the mechanisms through which plant-based foods may help in improving vascular health in aging. In addition, dose and time-dependent studies are needed to better identify the products, and related bioactives which are able to have a vasodilator effect. The results of such studies could be useful for the development of new products able to maintain and/or improve vascular health and reduce the incidence of vascular disorders in older subjects.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14132615/s1, Figure S1: Risk of bias for each item assessed in each of the included studies; Figure S2: Risk of bias for each item assessed, presented as a percentage across all included studies combined.

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Article

3.4.2 Modulation of Adhesion Process, E-Selectin and VEGF Production by Anthocyanins and Their Metabolites in an In Vitro Model of Atherosclerosis

Mirko Marino [1], Cristian Del Bo'[1,*], Massimiliano Tucci [1], Dorothy Klimis-Zacas [2], Patrizia Riso[1,†] and Marisa Porrini[1,†]

[1]Division of Human Nutrition, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, 20133 Milan, Italy;

mirko.marino@unimi.it (M.M.); massimiliano.tucci@unimi.it (M.T.); patrizia.riso@unimi.it (P.R.); marisa.porrini@unimi.it (M.P.)

[2] School of Food and Agriculture, University of Maine, Orono, ME 04469, USA; dorothea@maine.edu

*Correspondence: cristian.delbo@unimi.it; Tel.: +39-0250-316-730

[†]These authors contributed equally to this work.

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Abstract: The present study aims to evaluate the ability of peonidin and petunidin-3-glucoside (Peo-3-glc and Pet-3-glc) and their metabolites (vanillic acid; VA and methyl-gallic acid; MetGA), to prevent monocyte (THP-1) adhesion to endothelial cells (HUVECs), and to reduce the production of vascular cell adhesion molecule (VCAM)-1, E-selectin and vascular endothelial growth factor (VEGF) in a stimulated pro-inflammatory environment, a pivotal step of atherogenesis. Tumor necrosis factor-α (TNF-α; 100 ng mL−1) was used to stimulate the adhesion of labelled monocytes (THP-1) to endothelial cells (HUVECs). Successively, different concentrations of Peo-3-glc and Pet-3-glc (0.02 μM, 0.2 μM, 2 μM and 20 μM), VA and MetGA

(0.05 μM, 0.5 μM, 5 μM and 50 μM) were tested. After 24 h, VCAM-1, E-selectin and VEGF were quantified by ELISA, while the adhesion process was measured spectrophotometrically. Peo-3-glc and Pet-3-glc (from 0.02 μM to 20 μM) significantly ($p < 0.0001$) decreased THP-1 adhesion to HUVECs at all concentrations (−37%, −24%, −30% and −47% for Peo-3-glc; −37%, −33%, −33% and −45% for Pet-3-glc). VA, but not MetGA, reduced the adhesion process at 50 μM (−21%; $p < 0.001$). At the same concentrations, a significant ($p < 0.0001$) reduction of E-selectin, but not VCAM-1, was documented. In addition, anthocyanins and their metabolites significantly decreased ($p < 0.001$) VEGF production. The present findings suggest that while Peo-3-glc and Pet-3-glc (but not their metabolites) reduced monocyte adhesion to endothelial cells through suppression of E-selectin production, VEGF production was reduced by both anthocyanins and their metabolites, suggesting a role in the regulation of angiogenesis.

Keywords: anthocyanins and metabolites; inflammation; adhesion molecules; vascular endothelial growth factor; monocytes; endothelial cells

1. Introduction

Inflammation represents the initial response of the body to harmful stimuli (i.e., pathogens, injury) and involves the release of numerous substances known as inflammatory mediators. Normally, inflammatory stimuli may activate intracellular signaling pathways that promote the production of inflammatory mediators including microbial products (i.e., lipopolysaccharide) and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). However, the inflammatory response also involves the activation of cells such as macrophages and monocytes that are able to mediate local responses resulting from tissue damage and infection (Libby, 2002). In particular, activated endothelial cells release numerous cell surface adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intra-cellular adhesion molecule (ICAM)-1, P-selectin and E-selectin (also known as the endothelial leucocyte adhesion molecule—ELAM), which attract neutrophils and monocytes at the endothelial level, permit their adhesion and transmigration into the tissue and increase microvascular permeability (Liao, 2013). Generally, inflammation is of relatively short duration. When uncontrolled, inflammation becomes chronic and can contribute to the pathogenesis of many diseases, including chronic inflammatory diseases and degenerative diseases such as atherosclerosis. Inflammation may also promote angiogenesis, a process that involves the formation of new blood vessels from preexisting ones. Angiogenesis is associated with the activation and proliferation of endothelial cells, and structural changes of the vasculature. Vascular endothelial growth factor (VEGF) is important for endothelial integrity, vascular function and angiogenesis. In fact, VEGF can stimulate endothelial cell survival, invasion and migration into surrounding tissues and increase proliferation and vascular permeability. On the other hand, during atherosclerosis, VEGF may enhance the pathophysiologic mechanism of plaque formation and destabilization by increasing the risk of plaque rupture (Michel *et al.*, 2014; Camaré *et al.*, 2017). Polyphenols are a heterogeneous class of bioactive compounds found abundantly in the plant kingdom. They are generally classified into phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (flavanols, flavonols, flavons, flavanones, isoflavons and anthocyanidins), stilbens and lignans. Polyphenols are responsible for the color, bitterness, astringency, flavor and smell of numerous plants including fruits, vegetables, coffee, chocolate and tea (Donovan *et al.*, 2007). In foods, most of them are present as glycosides. After ingestion, polyphenols move intact through the gastrointestinal tract to the small intestine where they are absorbed through passive (i.e., aglycones) and/or active transport (i.e., glycosides). Polyphenols that enter intestinal epithelial cells are metabolized in the intestine and liver through methylation, glucuronidation and sulfation reactions (Fang, 2014; Fernandes *et al.*, 2014). Unabsorbed polyphenols reach the colon where they are extensively metabolized by gut microbiota (Tomás-Barberán, Selma and Espín, 2016). Additionally, the microbial derivatives after absorption undergo conjugation and are metabolized in the liver. Polyphenols reach maximal plasma concentration within 1.5 h after absorption and disappear from the bloodstream by 6 h post-consumption, while their metabolites may display a biphasic phase (depending on microbiota and endogenous metabolism) and appear in the blood 8–10 h or 16–24 h after consumption (de Ferrars *et al.*, 2014; Krga and Milenkovic, 2019). It is estimated that plasma concentrations range between nanomolar for anthocyanins and other polyphenols (native forms), up to low micromolar for their derivates (de Ferrars *et al.*, 2014; Krga and Milenkovic, 2019). In recent years, polyphenols have received extensive interest for their health benefits in the prevention of numerous cardiovascular diseases (Grassi *et al.*, 2016; Del Bo' *et al.*, 2017; Bondonno *et al.*, 2018; Leyva-Soto *et al.*, 2018; Martini *et al.*, 2019). The mechanisms through which polyphenols may exert their bioactivity are not completely understood since it is not clear whether their activity is linked to the native forms, their derivatives or both. Some of the most proposed protective mechanisms of action include the increase of antioxidant/detoxification enzymes activity (i.e., glutathione S-transferase, superoxide dismutase, glutathione peroxidase) (Guglielmi *et al.*, 2003; Martín *et al.*, 2010; Iskender *et al.*, 2016), and the decrease of pro-inflammatory cytokines (i.e., tumor necrosis factor alpha (TNF-α), interleukin-1, interleukin-6) (Ferrari *et al.*, 2017; Le Phuong Nguyen *et al.*, 2018; Li *et al.*, 2018). Furthermore, polyphenols have been documented to have the capability to modulate some factors involved in atherosclerosis, such as the release of numerous vasoconstrictor and vasodilator agents at the endothelial level including nitric oxide, endothelin-1 and soluble vascular cell adhesion molecules-1 (sVCAM-1) (Oak *et al.*, 2018). In this regard, we have previously reported the ability of different anthocyanins and metabolites to counteract and/or resolve an inflammation-driven adhesion of monocytes on endothelial cells (HUVECs). In the present study, we focused on the effects of peonidin (peo) and petunidin (pet)-3-glucoside, two anthocyanins mainly found in berries and grapes (Zamora-Ros *et al.*, 2011), and their respective metabolites (vanillic and methyl-gallic acids; VA and MetGA) on their capacity to resolve a TNF-α-mediated inflammatory process responsible for the adhesion of monocytes to HUVECs through the production of the mediators VCAM-1 and E-selectin. In addition, since TNF-α and monocytes play a crucial role in angiogenesis (Jaipersad *et al.*, 2014), we evaluated whether polyphenolic compounds were also able to reduce VEGF production, one of the main angiogenic factors. To the best of our knowledge, very few studies have explored this topic, as the majority of them focus on oncology.

2. Materials and Methods

2.1. Chemicals and Reagents

Lyophilized standards of peonidin-3-glc (Peo-3-glc) and petunidin-3-glucoside (Pt-3-glc) were purchased from Polyphenols Laboratory (Sandes, Norway). Lyophilized standards of vanillic acid (VA) and methil-gallic acid (MetGA), Hanks balanced salt solution, fetal bovine serum (FBS), tumor necrosis factor-alpha (TNF-α), MTT kit, Trypan blue and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium pyruvate, RPMI-1640, HEPES, gentamicin and trypsin–EDTA (0.05%) and gelatine (0.1%) were from Life Technologies (Monza Brianza, MB, Italy). Human endothelial cell basal medium and the growth supplement were obtained from Tebu-Bio (Magenta, MI, Italy), while 5-chloromethylfluorescein diacetate (CellTrackerTM Green; CMFDA) was obtained from Invitrogen (Carlsbad, CA, USA). Methanol and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany), while water from a Milli-Q apparatus (Millipore, Milford, MA, USA) was used. VCAM-1 and VEGF ELISA kits were purchased from Vinci-Biochem Srl (Vinci, FI, Italy) and the E-Selectin ELISA kit was purchased from Aurogene Srl (Roma, RM, Italy).

2.2. Preparation of Anthocyanin and Metabolite Standards

The stock solutions of Peo-3-glc, Pet-3-glc, VA and MetGA (Figure 1) were prepared by dissolving the powder of each standard in acidified methanol (0.05% HCl). Successively, standards were quantified spectrophotometrically and stored in dark vials at −80 ◦C until use.

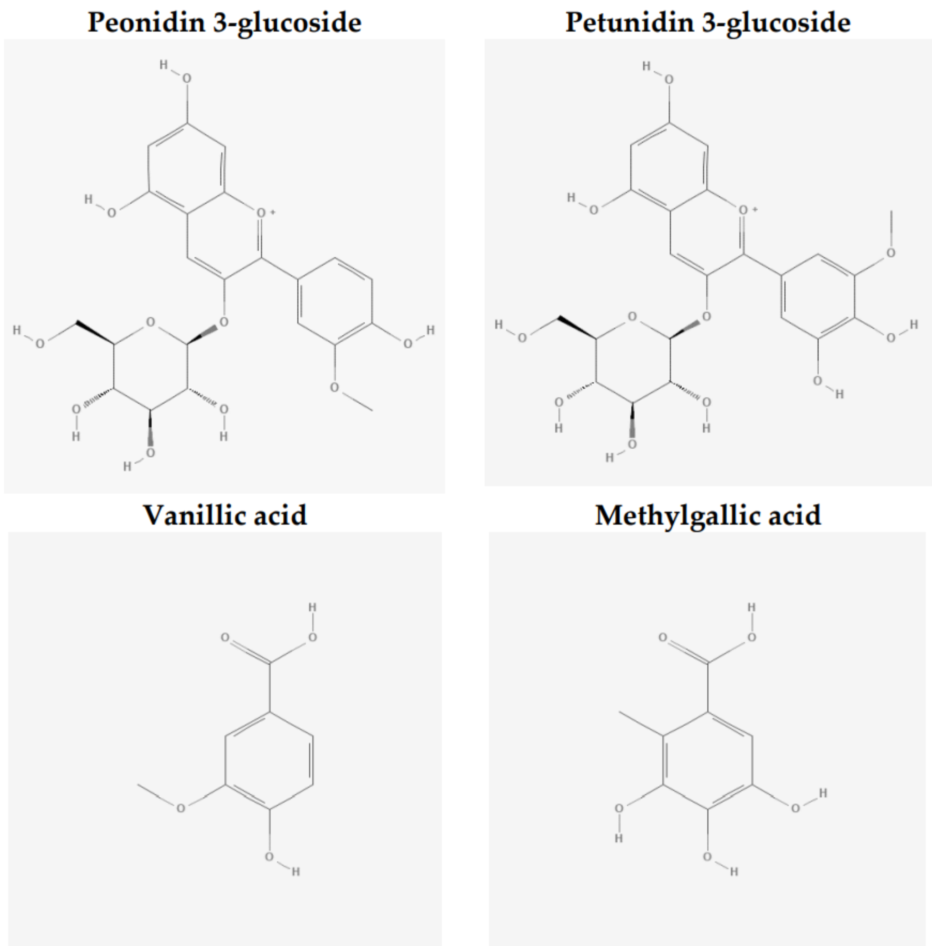


Figure 1 – Chemical structure of peondin and petunidin-3-glucoside, vanillic and methylgallic acids.

2.3. Cell Culture

Monocytic cells (THP-1; Sigma-Aldrich, St. Louis, MO, USA) were cultured in complete RPMI cell medium (RPMI-1640 medium supplemented with 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin and 10% FBS). For the experiment, 1 × 105 cells were grown in a flask until the concentration of 1 × 106 cells/mL was reached. Human umbilical vein endothelial cells (HUVECs; Tebu-Bio Srl, Magenta, MI, Italy) were seeded at the concentration of 1 × 105 cells on a pre-coated flask with 0.1% gelatine and grown in a cell medium kit containing 2% serum until they reached confluence.

2.4. Cytotoxicity Assay

The cytotoxicity of the compounds was tested by Trypan blue and (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on HUVECs, according to the manufacturer's instructions. Triton X-100 was used as positive control. Two independent experiments were performed in which each compound and concentration was tested in quadruplicate.

2.5. Evaluation of Monocytes Adhesion on Activated Human Umbilical Vein Endothelial Cells

When the confluency reached about 80%, HUVECs were removed using trypsin (0.05 mM) and seeded on a 0.1% gelatin pre-coated 96-well black plate at the concentration of 2 × 104 cells/well at 37 ◦C and 5% CO2. After 24 h incubation, THP-1 (2 × 106) cells were labelled with CellTrackerTM Green CMFDA (1 μM) in 1 mL serum-free RPMI medium (containing 1% HEPES, 1% sodium pyruvate and 0.1% gentamicin) for 30 min. Successively, cells were washed twice, re-suspended in HUVEC medium at a final concentration of 2 × 105 cells mL−1 and added to HUVECs. The adhesion process was induced for 24 h with 100 ng mL−1 of TNF-α. Then, 200 μL of new medium containing the single compounds (0.02 μM, 0.2 μM, 2 μM and 20 μM for Peo and Pet-3-glc and 0.05 μM, 0.5 μM, 5 μM and 50 μM for VA and MetGA) was added and the cells were further incubated for 24 h. Medium from each well was collected and stored at −80 ◦C until ELISA analysis. Cells were rinsed twice with 200 µL of Hanks balanced salt solution and the fluorescence (excitation: 485 nm, emission: 538 nm) associated with the number of labeled THP-1 cells attached to the HUVECs was measured by a spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy). Each compound and concentration were tested in quintuplicate in three independent experiments.

2.6. ELISA Quantification of Soluble VCAM-1, E-Selectin and VEGF

At the end of the experiment, the recovered cell culture supernatants were used to quantify the concentrations of soluble VCAM-1 (Cat# EK0537, BosterBio), E-selectin (Cat# MBS355367, MyBioSource) and VEGF (Cat# V3-200-820-VEF, Vinci-Biochem). The analysis was performed using ELISA kits according to the manufacturer’s instruction. The analyses were conducted in quadruplicate and the results derived from three independent experiments.

2.7. Data Analysis

STATISTICA software (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. All the results are expressed as means ± standard error of the mean (SEM). One-way ANOVA was applied to verify the effect of Peo-3-glc, Pet-3-glc, VA and MetGA supplementation on cell cytotoxicity, adhesion process and production of soluble VCAM-1, E-selectin and VEGF. The least significant difference (LSD) test was used to assess differences between treatments by setting the level of statistical significance at $p < 0.05$.

3. Results

3.1. Effect of Peo-3-glc, Pet-3-glc, VA and MetGA on Cell Cytotoxicity

Table 1 presents the effects of the compounds tested on cell cytotoxicity measured by Trypan blue assay at all concentrations tested. Peo-3-glc and Pet-3-glc (from 0.02 µM to 20 µM), VA and MetGA (from 0.05 µM to 50 µM) did not have any cytotoxic effect, maintaining cell viability above 90%. The results were also in line with those obtained following the MTT assay tested only at the maximum concentration (20 μM for anthocyanins (ACNs) and 50 μM for metabolites). Conversely, incubation of HUVEC cells with Triton X-100, as a positive control (data not shown), significantly reduced ($p < 0.0001$) cell viability up to 20% compared to the cells treated with and without TNF-α (cell viability at 99%).

Table 1. Percentage of cell viability following supplementation with peonidin-3-glucoside (Peo-3-glc), petunidin-3-glucoside (Pet-3-glc), vanillic acid (VA) and methyl-gallic acid (MetGA) evaluated by Trypan blue and MTT assays

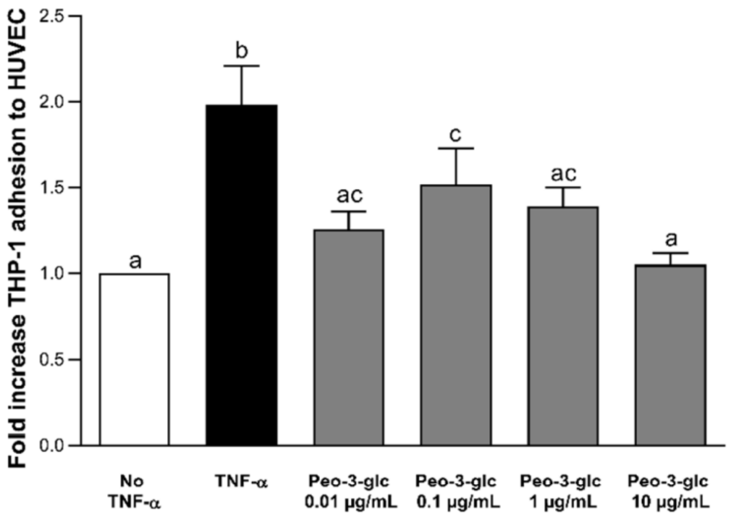
Trypan Blue Assay	**Anthocyanins**			**Gut Metabolites**	
Concentrations	Peo-3-glc	Pet-3-glc	Concentrations	VA	MetGA
0.02 μM	99.7 ± 0.33	110 ± 0	0.05 μM	100 ± 0	99.7 ± 0.33
0.2 μM	100 ± 0	97.0 ± 1.0	0.5 μM	99.7 ± 0.33	99.3 ± 0.67
2 μM	99.3 ± 0.67	97.7 ± 0.33	5 μM	99.7 ± 0.66	98.7 ± 1.33
20 μM	99.3 ± 0.33	100 ± 0	50 μM	99.3 ± 0.67	97.3 ± 1.77
MTT assay	**Anthocyanins**			**Gut metabolites**	
Concentration	Peo-3-glc	Pet-3-glc	Concentration	VA	MetGA
20 μM	98.5 ± 0.12	94.4 ± 0.45	50 μM	99.7 ± 0.32	96.7 ± 0.43

Results derived from three independent experiments. Peo-3-glc, Pet-3-glc, VA and MetGA were tested in the presence of tumor necrosis factor-α (TNF-α) stimulus. Each concentration was tested in triplicate. Data are reported as mean ± standard error of the mean.

3.2. Effect of Peo-3-glc, Pet-3-glc, VA and MetGA on THP-1 Adhesion to HUVECs

The results of THP-1 adhesion to HUVECs after incubation with Peo-3-glc and Pet-3-glc are shown in Figure 2. Data on the adhesion process are reported as fold increase compared to the control cells without TNF-α or (poly)phenolic compounds. Stimulation with 100 ng mL^{-1} of TNF-α significantly increased ($p < 0.0001$) the adhesion process of THP-1 cells to HUVECs compared to the negative control (no TNF-α). The treatment with Peo-3-glc and Pet-3-glc significantly decreased the ($p < 0.0001$) adhesion of monocytes to HUVECs compared to the TNF-α treatment. The size of the effect was similar between Peo-3-glc (−37%, −24%, −30% and −47%; Figure 2a) and Pet-3-glc (−37%, −33%, −33% and −45%; Figure 2b) at all the concentrations tested (0.02 µM, 0.2 µM, 2 µM and 20 µM, respectively). Figure 3 shows the results of THP-1 adhesion to HUVECs after incubation with VA and MetGA (metabolites of Peo-3-glc and Pet-3-glc, respectively). Only VA (Figure 3a) significantly reduced the adhesion process at the concentration of 50 µM (−21%; $p < 0.001$), while no effect was observed for MetGA (Figure 3b).

a)



b)

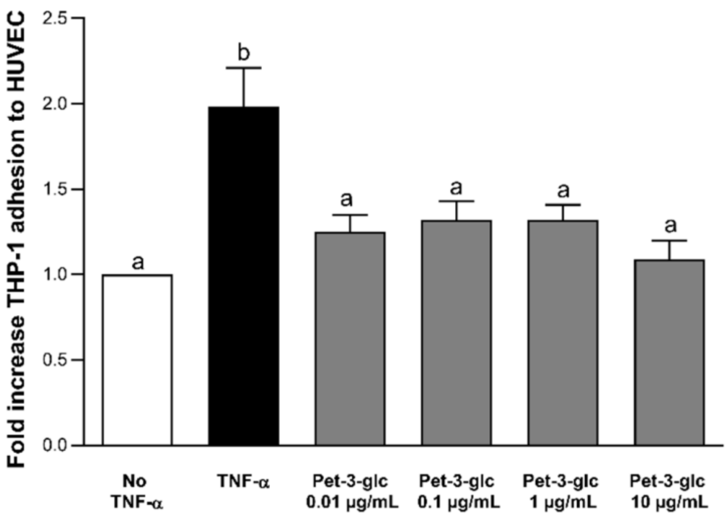


Figure 2 – Effect of different concentrations (0.02–20 μM) of Peo-3-glc (a) and Pet-3-glc (b) on THP-1 (monocytes) adhesion to HUVECs (vascular endothelial cells). Results are expressed as mean ± standard error of mean. a,b,c Nutrients 2020 Bar graphs reporting different letters are significantly different ($p \leq 0.05$).

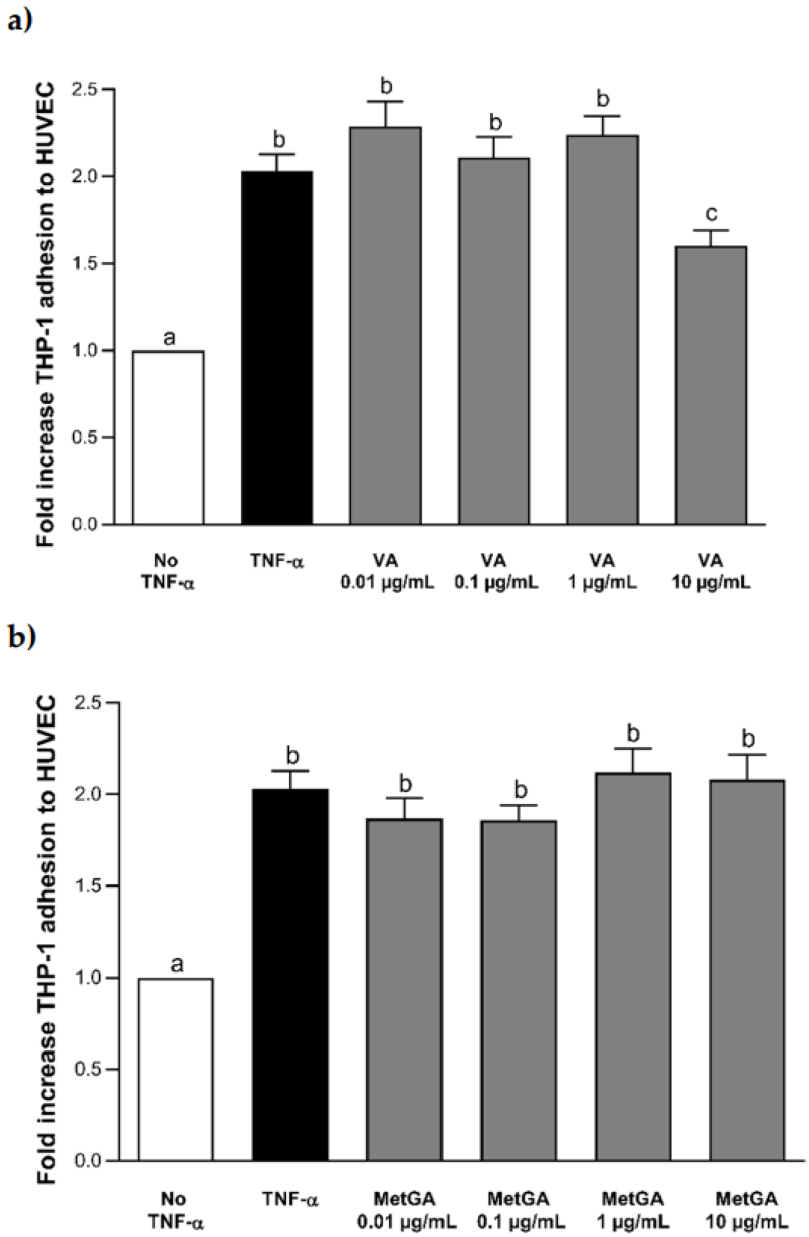


Figure 3 – Effect of different concentrations (0.05–50 µM) of VA (a) and MetGA (b) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. a,b,c Bar graphs reporting different letters are significantly different ($p \leq 0.05$).

3.3. Effect of Peo-3-glc, Pet-3-glc, VA and MetGA on the Levels of E-Selectin

Table 2 reports the levels of E-selectin quantified in the cell supernatant following incubation with ACNs and metabolites. Cell stimulation with TNF-α significantly increased ($p < 0.001$) the levels of E-selectin compared to the negative control (without TNF-α). The incubation with Peo-3-glc and Pet-3-glc significantly attenuated ($p < 0.001$) the production of E-selectin. The size of the effect was similar between Peo-3-glc (−55%, −66%, −65% and −76%) and Pet-3-glc (−64%, −60%, −67% and −72%) at all the concentrations tested (0.02 μM, 0.2 μM, 2 μM and 20 μM, respectively). In addition, Peo-3-glc at the high doses (0.2 μM, 2 μM and 20 μM) significantly reduced ($p < 0.05$) the levels of E-selectin (−32%, −31% and −53%, respectively) compared to the negative control (without TNF-α). A similar effect was documented for Pet-3-glc which showed a reduction ($p < 0.05$) at low (0.02 μM; −28%) and high doses (2 μM and 20 μM; −36% and −45%, respectively). Vanillic acid decreased E-selectin levels at the high dose (50 μM) with respect to the positive TNF-α control (−70%; $p < 0.001$) and the negative control without TNF-α (−46%; $p < 0.05$). Conversely, no effect was observed following MetGA exposure.

Table 2. Effect of peonidin-3-glucoside, petunidin-3-glucoside, vanillic acid and methyl-gallic acid on the levels of E-selectin.

	Anthocyanins			**Gut Metabolites**	
Concentrations	**Peo-3-glc**	**Pet-3-glc**	**Concentrations**	**VA**	**MetGA**
TNF-α 0 ng mL^{-1}	160 ± 7.9 [a]	164 ± 5.8 [a]	TNF-α 0 ng mL^{-1}	160 ± 7.9 [a]	164 ± 5.8 [a]
TNF-α 100 ng mL^{-1}	316 ± 8.1 [b]	317 ± 6.3 [b]	TNF-α 100 ng mL^{-1}	316 ± 8.1 [b]	317 ± 6.3 [b]
0.02 μM	143 ± 4.3 [a]	115 ± 7.5 [c]	0.05 μM	312 ± 14.1 [b]	299 ± 7.5 [b]
0.2 μM	108 ± 5.3 [c]	123 ± 11.8 [a,c]	0.5 μM	312 ± 11.2 [b]	297 ± 7.5 [b]
2 μM	109 ± 7.2 [c]	104 ± 6.3 [c]	5 μM	305 ± 7.4 [b]	297 ± 8.0 [b]
20 μM	76 ± 8.4 [c]	88 ± 12.1 [c]	50 μM	95 ± 13.2 [c]	295 ± 7.3 [b]

Results derived from three independent experiments. Peo-3-glc, Pet-3-glc, VA and MetGA were tested in the presence of TNF-α stimulus. Each concentration was tested in triplicate. Data are reported as mean ± standard error of the mean. a,b,c Data with different letters are significantly different ($p < 0.05$).

3.4. Effect of Peo-3-glc, Pet-3-glc, VA and MetGA on the Levels of Soluble VCAM-1

Table 3 presents the levels of VCAM-1 quantified in the cell supernatant following incubation with ACNs and metabolites. Cell stimulation with TNF-α significantly increased ($p < 0.001$) the levels of VCAM-1 compared to the negative control (without TNF-α). Incubation with Peo-3-glc significantly reduced ($p < 0.0001$) the levels of soluble VCAM-1 (−195%, −203%, −69%

and −112%) at all concentrations tested (0.02 μM, 0.2 μM, 2 μM and 20 μM, respectively) with maximum reduction at the low doses. Pet-3-glc attenuated soluble VCAM-1 production only at the maximum dose (−270%; 20 μM, $p < 0.0001$) while VA and MetGA had no effect.

Table 3. Effect of peonidin-3-glucoside, petunidin-3-glucoside, vanillic acid and methyl-gallic acid on the levels of sVCAM-1.

Anthocyanins			Gut Metabolites		
Concentrations	**Peo-3-glc**	**Pet-3-glc**	**Concentrations**	**VA**	**MetGA**
TNF-α 0 ng mL^{-1}	59 ± 9.0 [a]	64 ± 10 [a]	TNF-α 0 ng mL^{-1}	59 ± 9.0 [a]	64 ± 10 [a]
TNF-α 100 ng mL^{-1}	316 ± 16 [b]	307 ± 11 [b]	TNF-α 100 ng mL^{-1}	316 ± 16 [b]	307 ± 11 [b]
0.02 μM	107 ± 15 [c]	311 ± 13 [b]	0.05 μM	308 ± 11 [b]	299 ± 15 [b]
0.2 μM	104 ± 16 [c]	297 ± 15 [b]	0.5 μM	299 ± 22 [b]	297 ± 15 [b]
2 μM	186 ± 12 [c]	300 ± 14 [b]	5 μM	295 ± 12 [b]	297 ± 16 [b]
20 μM	149 ± 24 [c]	83 ± 10 [c]	50 μM	315 ± 16 [c]	295 ± 14 [b]

Results derived from three independent experiments. Peo-3-glc, Pet-3-glc, VA and MetGA were tested in the presence of TNF-α stimulus. Each concentration was tested in triplicate. Data are reported as mean ± standard error of the mean (SEM). a,b,c Data with different letters are significantly different ($p < 0.05$).

3.5. Effect of Peo-3-glc, Pet-3-glc, VA and MetGA on the Levels of VEGF

In Table 4, the levels of VEGF quantified in the cell supernatant following incubation with ACNs and metabolites are reported. Cell stimulation with TNF-α induced a small but significant increase ($p< 0.01$) in VEGF levels compared to the negative control (without TNF-α). Incubation with Peo-3-glc and Pet-3-glc significantly reduced ($p < 0.001$) VEGF concentrations. The size of the effect was similar between Peo-3-glc (−27%, −28%, −30% and −30%) and Pet-3-glc (−24%, −27%, −28% and −30%) at all concentrations tested (0.02 μM, 0.2 μM, 2 μM and 20 μM, respectively) and comparable to the negative control ($p > 0.05$). A reduction was also reported for VA (−12%; −17%, −13% and −21%) and MetGA (−9%; −17%, −17% and −19%) at all concentrations tested (0.05 μM, 0.5 μM, 5 μM and 50 μM, respectively). However, the size effect was smaller compared to their native compounds and significantly different ($p < 0.05$) compared to the negative control.

Table 4. Effect of peonidin-3-glucoside, petunidin-3-glucoside, vanillic acid and methyl-gallic acid on the levels of VEGF.

	Anthocyanins			**Gut Metabolites**	
Concentrations	**Peo-3-glc**	**Pet-3-glc**	**Concentrations**	**VA**	**MetGA**
TNF-α 0 ng mL^{-1}	120 ± 6.9 [a]	121 ± 6.1 [a]	TNF-α 0 ng mL^{-1}	120 ± 6.9 [a]	121 ± 6.1 [a]
TNF-α 100 ng mL^{-1}	170 ± 8.5 [b]	172 ± 7.9 [b]	TNF-α 100 ng mL^{-1}	170 ± 8.5 [b]	172 ± 7.9 [b]
0.02 μM	120 ± 6.9 [a]	129 ± 10 [a]	0.05 μM	149 ± 3.0 [c]	153 ± 2.5 [c]
0.2 μM	123 ± 1.7 [a]	123 ± 7.4 [a]	0.5 μM	141 ± 8.3 [c]	142 ± 3.0 [c]
2 μM	123 ± 6.0 [a]	123 ± 2.9 [a]	5 μM	147 ± 4.9 [c]	141 ± 4.9 [c]
20 μM	119 ± 2.6 [a]	117 ± 9.9 [a]	50 μM	135 ± 5.7 [c]	137 ± 6.0 [c]

Results derived from three independent experiments. Peo-3-glc, Pet-3-glc, VA and MetGA were tested in the presence of TNF-α stimulus. Each concentration was tested in triplicate. Data are reported as mean ± standard error of the mean (SEM). a,b,c Data with different letters are significantly different ($p < 0.05$).

4. Discussion

In the present study, we documented the capacity of anthocyanins (Peo-3-glc and Pet-3-glc) to reduce the adhesion of monocytes to vascular endothelial cells, either when tested at physiological or supra-physiological concentrations. Conversely, the effect of their metabolites to counteract the adhesion of THP-1 to HUVECs was controversial. In particular, MetGA did not show any significant effect at each concentration tested, while VA was effective only at the maximum concentration. The present findings agree with our previous studies that reported the ability of an anthocyanin-rich fraction, single anthocyanins (cyanidin, delphinidin and malvidin-3-glucoside) and their relative metabolites (protocatechuic, gallic and syringic acid) to differentially prevent and/or resolve (depending on the compound and dose tested) an inflammatory response and mitigate the adhesion of monocytes to endothelial cells—an important initial step of the atherogenic process (Del Bo' *et al.*, 2016, 2019). The ability of anthocyanins and metabolites to reduce/prevent the adhesion of monocytes/macrophages to endothelial cells has been reported in several studies, even if the results are not always in agreement with each other. This could be due to the different compounds and concentrations tested. Most of the studies reported in the literature used supra-physiological concentrations as it is well recognized that anthocyanins are scarcely absorbed (Krga and Milenkovic, 2019). Generally, their blood concentrations range from 0.06 nM up to 0.4 µM, while those of their metabolites range between 0.2 µM and 2 µM (Krga and Milenkovic, 2019). In our experimental conditions, we tested both plasma relevant concentrations (0.02 µM and 0.2 µM for anthocyanins and 0.05 µM, 0.5 µM and 5 µM for their metabolites) and supra-physiological (2 μM and 20 μM for anthocyanins and 50 μM for metabolites), supporting (at least in part) the role of physiological doses in the modulation of the adhesion process. However, the results obtained were dependent on the molecules used. The effect of anthocyanins and metabolites at plasma concentrations has been evaluated in few studies. For example, Krga and colleagues (Krga *et al.*, 2016) tested the effects of 10 different phenolic compounds (five anthocyanins and five degradation products/gut metabolites) on the capacity to counteract the adhesion of monocytes to endothelial cells. The authors reported a significant reduction in the adhesion process following delphinidin-3-glucoside treatment at all the concentrations tested, cyanidin-3-glucoside, galactoside and arabinoside in the range between 0.1–2 μM, while Peo-3-glc was effective only at the lowest concentration. Considering anthocyanin metabolites, protocatechuic acid reduced monocyte adhesion at all concentration tested, VA at 0.2 μM and 2 μM only, while ferulic and hippuric acids were only effective at 1 μM and 2 μM [28]. The results obtained on Peo-3-glc and VA are only partially in line with our observations, since the effects of VA were detected only at the maximum dose (50 μM). In addition, we cannot exclude that other factors could have affected the findings obtained; for example, an important factor of variability may depend on the different experimental design adopted between the two studies. We tested Peo-3-glc and VA after an overnight stimulation with 100 ng mL−1 of TNF-α and a co-incubation with monocytes, while Krga and coworkers (Krga *et al.*, 2016) incubated them for different times (3 h for Peo-3-glc e and 18 h for VA) and the stimulation with TNF-α was performed for 4 h while monocyte co-incubation was limited to 15 min. The mechanisms of action through which polyphenols can reduce/prevent the adhesion process and consequently exert their anti-atherosclerotic effect are still not completely understood. It is widely recognized that atherosclerosis is a multifactorial process involving several pathways. It is also well-known that chronic inflammation may activate this process, starting with the over-expression and production of different cytokines, interleukins and adhesion molecules such E-selectin, VCAM-1 and ICAM-1. E-selectins are Ca2+-dependent transmembrane lectins, produced following different stimuli such as TNF-α, IL-1β and LPS, that permit the rolling of monocytes to endothelial cells. Moreover, this process enhances the expression of β2-integrin which allows the strong adhesion and the transmembrane migration of the monocytes at the endothelial level (McEver, 2015). For this reason, E-selectin plays a major role and represents an important molecular target in the study of atherosclerosis. Together with E-selectin, VCAM-1 is also an important protein involved in the initiation of the atherosclerotic process. In fact, the activation of endothelial cells stimulates the expression of VCAM-1 which is able to bind α4β1 integrin located on monocyte membranes by determining the rolling-type adhesion and later the firm adhesion phase (Ley and Huo, 2001). It has been observed that administration of monoclonal antibodies against VCAM-1 can reduce monocyte adhesion to endothelial cells and decrease plaque formation in apolipoprotein E-deficient (ApoE−/−) mice (Park *et al.*, 2013). Few studies that examined the role of polyphenols on the modulation of E-selectin and VCAM-1 expression/production have documented different results depending on the type of compound tested. For example, Warner *et al.* reported that phenolic metabolites of different flavonoids, but not their unmetabolized precursors, were able to reduce the secretion of VCAM-1 at a range of concentrations between 1 µM and 100 µM (Warner *et al.*, 2016). Similar results were reported by Kunts and colleagues, showing that microbial fermentation of an anthocyanin-rich grape/berry extract (50 µM) reduced the expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1. However, this effect was dependent on bacterial species and is most likely due to their capacity to biotransform anthocyanins (Kuntz *et al.*, 2016). Amin *et al.* showed that the incubation of cyanidin-3-glucoside and different metabolites, in particular ferulic acid, at different concentrations (0.1 µM, 1 µM, and 10 µM) were able to alter the expression of VCAM-1 at physiologically relevant concentrations (Amin *et al.*, 2015). More recently, Calabriso *et al.* reported the capacity of a biofortified bread polyphenol extract (containing mainly ferulic, sinapic and p-coumaric acids) to inhibit in a concentration-dependent manner (1 μg mL−1 , 5 μg mL−1 , 10 μg mL−1) the adhesion of monocytes to LPS-stimulated endothelial cells through a reduction in the expression of different adhesion molecules, with a significant effect on VCAM-1 (Calabriso *et al.*, 2020). In our in vitro model, Peo-3-glc and Pet-3-glc significantly inhibited the production of E-selectin at all tested concentrations while VA was effective only at supra-physiological concentrations according to the results regarding the adhesion process. Differently, Peo-3-glc was the only compound able to decrease the levels of VCAM-1 at physiologically-relevant concentrations while no effect was observed for Pet-3-glc, VA and MetGA, confirming the results of our previous publication (Del Bo' *et al.*, 2016) and in line with results found by others researchers (Krga *et al.*, 2016; Tang *et al.*, 2019), suggesting that high concentrations are needed in order to exert an effect. The different effects obtained with anthocyanins compared to their metabolites may be explained by their variable structures, chemical properties, and thus their heterogeneous capacity to interact with biological systems and to modulate target molecules. The presence of several functional groups, but also the size of the molecule or different conformations could be all factors affecting the binding of these compounds to specific membrane receptors, the interaction with transcriptional factors, or the capacity to act as a free-radical scavenger. Moreover, the potential synergistic role of phenolic compounds on the regulation of the main processes in which they are involved should also be taken into account. The role of angiogenesis in atherosclerotic plaque progression is still not completely understood. Despite several in vitro studies showing that VEGF-induced angiogenetic processes increase plaque instability, the administration of anti-angiogenic drugs (mainly anti-VEGF) for cancer therapy causes adverse cardiovascular effects in human studies. A recent review asserts that the long-term treatment of oncological patients with anti-VEGF drugs could promote adverse cardiovascular effects through hypertension, suggesting a different mechanism of action of VEGF inhibitors compared to in vitro studies that aim to evaluate the role of angiogenesis within the plaque (Katsi *et al.*, 2015). Neocapillaries inside the atherosclerotic plaque are more fragile and can easily undergo damage due to the high level of oxidative stress that mainly occurs during the later stage of atherosclerosis. This latter condition could lead to plaque rupture, one of the main factors responsible for cardiovascular events (Camaré *et al.*, 2017). Arterial injuries are followed by arterio-intimal angiogenesis that induces intimal hyperplasia and a subsequent intimal hemorrhage (Sueishi *et al.*, 1997). Repeated intraplaque hemorrhages play an essential and promoting role in plaque progression and rupture. Intraplaque hemorrhages are mainly induced by angiogenesis from the adventitia to the intima, where the atheroma starts to develop (Michel *et al.*, 2014). To support the hypothesis of the involvement of angiogenesis in atherosclerosis, Qiu et al. showed that arterial regions with higher shear stress also exhibit an elevated number of intraplaque microvessels, characterized by abnormal endothelial cells, in particular with intracytoplasmatic vacuoles and leukocyte infiltration that could lead to rupture-prone plaque formation (Qiu *et al.*, 2017). In cancer research, multiple in vitro studies demonstrated the anti-angiogenic effect of anthocyanins, in particular concerning delphinidin, as a potential chemopreventive agent (Lamy *et al.*, 2006; Keravis *et al.*, 2015; Kim *et al.*, 2017). We found that Peo-3-glc, Pet-3-glc and their metabolites (VA and MetGA) reduced the levels of VEGF, corroborating the hypothesis of a protective mechanism of action through which these compounds inhibit angiogenesis within the atheroma, therefore reducing atherosclerotic disease progression. Tanaka *et al.*, using a purple rice extract and its constituents cyanidin and peonidin tested at 10 μg mL−1 and 30 μg mL−1 on HUVECs and HRMECs, showed a reduction of migration and proliferation. In detail, these polyphenols seem to act through the inhibition of extracellular signal-regulated kinase (ERK) 1/2 and p38 pathways in reducing VEGF-induced angiogenesis (Tanaka *et al.*, 2012). Similar results were observed by Negrao *et al.*, who reported that 1 μM of catechin was able to reduce migration and invasion capacity in smooth muscle cells. This latter effect seems to depend on the presence or absence of angiogenesis stimuli, such as VEGF, emphasizing a potential use of some phenolic compounds against pathological situations where angiogenesis is stimulated (Negrão *et al.*, 2013). Also, Calabriso *et al.* demonstrated that 0.1 μg mL−1 to 10 μg mL−1 of olive oil polyphenol extract suppressed endothelial cell migration induced by VEGF. The inhibition was dose-dependent, and the lowest concentration reduced the migration by about 35% (Calabriso *et al.*, 2016). For the first time, Tsakiroglou *et al.* reported a different modulation of endothelial cell migration through the regulation of ras homolog family member A (RHOA) and ras-related C3 botulinum toxin substrate (RAC)-1 (two proteins involved in cell motility), induced by anthocyanin and the phenolic fraction from wild blueberries (dependent on dose and compound). In detail, time-lapse videos showed that the anthocyanin fraction at 60 μg mL−1 decreased the migration rate of endothelial cells, while treatment with the phenolic acid fraction at 0.002 μg mL−1 , 60 μg mL−1 and 120 μg mL−1 significantly increased the endothelial cell migration rate [46]. Cerezo *et al.* tested a wide range of polyphenols on VEGF-dependent vascular endothelial growth factor receptor (VEGFR)-2 activation. In particular, 11 of these phenolic compounds showed an half-maximal inhibitory concentration (IC50) < 1 μM, demonstrating efficacy at physiologically relevant concentrations. These compounds act by binding to a specific site of VEGF while avoiding the interaction with its receptor VEGFR2. The inhibitory potency is strongly correlated to the binding affinity that, in turn, is related to structural features such as the galloyl group at the 3-position of flavan-3-ols, the degree of polymerization of procyanidin oligomers, the total number of hydroxyl groups on the B-ring and hydroxylation of position 3 on C-ring (Cerezo *et al.*, 2015). In a subsequent study, Perez-Moral *et al.* reported that polyphenols with a strong inhibitory effect toward VEGF also have a lower IC50, demonstrating the increased formation of complexes between VEGF and polyphenols (and vice versa for those with a higher IC50), highlighting that the level of VEGF inhibition is strongly correlated to VEGF–polyphenol complex formation. To strengthen these results, polyphenols with lower IC50 values also demonstrated lower dissociation rate constants and equilibrium dissociation constants, indicating a stronger interaction and higher affinity (Perez-Moral *et al.*, 2019). A recent review reported that the anti-angiogenic role of anthocyanins is more consistent compared to phenolic acids, for which results are still mixed. According to Tsakiroglou *et al.*, this heterogeneity is mainly due to the use of different types, combinations and concentrations of the compounds tested, but also to different cell lines, co-cultures and types of stimulation (2019). Therefore, enhanced scientific cooperation, using common extracts and experimental protocols, could lead to a consensus among different studies, thereby formulating robust conclusions (Tsakiroglou *et al.*, 2019).

5. Conclusions

Taken together, our results have shown that Peo-3-glc and Pet-3-glc, but not VA and MetGA, decrease the attachment of monocytes to endothelial cells via E-selectin reduction. These results were documented both at physiological and supra-physiological concentrations, providing further evidence of the capacity of polyphenols to blunt inflammation and to counteract the processes involved in the onset of atherosclerosis. Moreover, we documented (for the first time) the important role of Peo-3-glc and Pet-3-glc and their metabolites to reduce VEGF and thus exert an important role on the modulation of angiogenesis. Studies are ongoing in order to corroborate the findings obtained and to elucidate the contribution of these and other polyphenols (alone or in combination) in the modulation of further important molecules potentially involved in the adhesion process, such as intercellular adhesion molecules 1, L- and P-selectin, and endothelin-1.

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Article

3.4.3 Acute effect of blueberry intake on vascular function in older subjects: study protocol for a randomized, controlled, crossover trial

Cristian Del Bo'[#], Massimiliano Tucci[#], Daniela Martini, Mirko Marino, Simona Bertoli, Alberto Battezzati, Marisa Porrini and Patrizia Riso*

Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), Division of Human Nutrition, Via Celoria, 2, 20133, Milano, Italy

[#]Cristian Del Bo' and Massimiliano Tucci contributed equally to this work

*Corresponding author

E-mail: patrizia.riso@unimi.it

Abstract: Aging is associated with an increased risk of developing cardiovascular disease which is often accompanied by a decline in vascular health and function. Current evidence suggests that berries may have a potential role in the modulation of vascular function, but dietary interventions are still needed to confirm findings, especially in older subjects. In the context of the MIND FoodS HUB project, this study aims to investigate the effect of a single serving of blueberry (250 g of blueberry versus a control product) in a group of older subjects (≥ 60 y) through a randomized, controlled, cross-over dietary intervention trial. Specifically, the study evaluates the absorption kinetics of bioactives following the blueberries intake and the effects on markers related to oxidative stress, inflammation, and vascular function analyzed at different time points. By considering a drop-out rate estimate of 25%, at least 20 subjects will be recruited in the study. The study will provide evidence to support the potential beneficial effects of blueberry and its bioactive compounds on vascular function in a group of population more

susceptible to vascular dysfunction and to the development of cardiovascular diseases. Moreover, the study will contribute the analysis of several metabolic and functional markers that can support the biological plausibility of the results obtained. Finally, the trial will provide data on the absorption and metabolism of blueberry bioactives which will be used to study their association with the different markers under study. The trial is registered at ISRCTN (http://isrctn.com/ISRCTN18262533); May 7, 2021.

Introduction

Aging is a complex and progressive phenomenon characterized by a decrease in functionality of numerous physiological processes at both molecular, cellular, tissue, and organ level, leading to an increased risk of oxidative stress, inflammation and, in turn, development of certain chronic degenerative diseases (Richter *et al.*, 2003; Niccoli and Partridge, 2012; Minciullo *et al.*, 2016; Schmeer *et al.*, 2019; Di Micco *et al.*, 2021). According to the Global Burden of Disease Study 2019, cardiovascular diseases (CVDs) still represent the leading cause of death in the world, particularly in older subjects (GBD Diseases Injuries Collaborators, 2020). The increased risk of developing CVDs during aging is mainly due to modifications to arteries and the establishment of vascular endothelial dysfunction (Donato, Machin and Lesniewski, 2018). The senescent process in endothelial cells leads to an alteration of arterial structure and functionality by reducing the production of vasodilators, such as nitric oxide (NO), and shifting toward the production of vasoconstrictors, procoagulants, proliferative and pro-inflammatory intermediates (North and Sinclair, 2012). These factors are considered as critical for the development of endothelial dysfunction, arterial stiffness and vascular diseases (North and Sinclair, 2012; Moriya and Minamino, 2017; Jia *et al.*, 2019).

Diet and dietary factors play a crucial role in maintaining normal physiological functions and promoting health. Among dietary factors, (poly)phenols, including flavonoids and phenolic acids, have been identified as potential bioactives able to improve CV health (Habauzit and Morand, 2012). Recent observational studies report an overall inverse association between (poly)phenol intake and CV risk events and mortality (Wang *et al.*, 2014; Rienks, Barbaresko and Nöthlings, 2017; Del Bo' *et al.*, 2019; Giacco *et al.*, 2020). In addition, mechanistic and human intervention studies seem to support the potential role of polyphenols in the modulation of several biomarkers related to vascular/endothelial function and CVDs (Reis *et al.*, 2016; Tresserra-Rimbau et al., 2018; Martini *et al.*, 2019; Di Pietro *et al.*, 2020). Blueberries are one of the richest foods in (poly)phenols, particularly anthocyanins (ACNs), which are, among the (poly)phenols sub-classes, those mostly associated with CV health (Reis *et al.*, 2016; Krga and Milenkovic, 2019). In addition, blueberries are a source of minerals, vitamins, fiber, and salicylates (Wood *et al.*, 2011, 2019) that could contribute, alone or synergistically, to the overall beneficial effect on vascular function (Martini *et al.*, 2019; Wood *et al.*, 2019). Short and long-term human intervention studies have documented that the consumption of blueberries may improve vascular function, reduce blood pressure and arterial stiffness in healthy subjects but also in those with risks for CVDs, metabolic syndrome, and type 2 diabetes (Johnson *et al.*, 2015; Stull *et al.*, 2015; Stote *et al.*, 2017; Curtis *et al.*, 2019). To the best of our knowledge, few and contrasting studies have investigated the contribution of plant-based foods in the promotion of vascular function in older subjects (Tucci *et al.*, 2022) and only one trial tested the effect of blueberries, reporting no effect on markers of arterial stiffness (Dodd *et al.*, 2019). Based on these premises, providing evidence of the potential health benefits of polyphenol-rich foods on vascular health is relevant in the current demographic context also due to the almost world-wide increase in the number of older people (GBD 2019 Demographics Collaborators, 2020). The goal of this study is to evaluate the effect of a single portion of blueberries on markers of vascular function, oxidative stress, and inflammation in older subjects. In addition, the absorption kinetics of blueberries' bioactive compounds will be assessed, and the data obtained correlated with the other biological markers under study.

Methods/design

Protocols and study design

This study follows a randomized, controlled, cross-over design (single portion of blueberry versus a control product) in a sample of free-living older subjects (≥60 y). The study involves two phases (Phase 1 and 2), both including two different appointments, each one spaced by a 1-week wash-out period, as illustrated in the SPIRIT schedule of enrollment, interventions, and assessments (Fig 1). Phase 1 is aimed at evaluating the absorption of blueberry polyphenols and the effect of blueberries consumption on markers of vascular function (i.e., endothelin-1, NO, ICAM-1, VCAM-1 and VEGF), inflammation (i.e., IL-6, IL-8, and TNF-α), oxidative stress (i.e., levels of DNA damage), blood glucose and insulin response (Fig 2). Phase 2 is devoted to the evaluation of vascular functionality, using a non-invasive biosensor and the assessment of reactive hyperemia index (RHI), Framingham reactive hyperemia index (fRHI) and arterial stiffness (i.e., augmentation index (AI) and AI@75); in addition, blood pressure is recorded, as depicted in Fig. 3. The approach, divided into two phases, is necessary to facilitate blood drawing (venous cannula) and the evaluation of vascular function (at the level of the brachial artery) avoiding potential sources of interferences between the two measurements. The day before the experiment, participants receive a list of instructions to follow. Following these instructions, subjects are asked to maintain their habitual lifestyle habits (also in terms of physical activities) and to refrain from consuming foods rich in (poly)phenols (e.g., berries, tea, coffee, chocolate, fruit juices). In addition, to limit the possible noise deriving from a long-fasting period the day of the experiment, subjects participating at the

Phase 1 are invited to consume a standardized light breakfast. Specifically, volunteers can consume 200 mL of partially skimmed milk or 125 g of yogurt and 3 biscuits (i.e., shortbread) or 3 rusks. The meal has to be consumed at least 90 min before blood collection and at the same hour each test day. The adherence to instructions is evaluated through a food diary and a face-to-face interview that will be scheduled the day of the experiment. In addition, subjects are motivated to complete all the intervention by providing them with free meals the test days and with the access to all laboratory analysis at the end of the trial.

STUDY PERIOD									
	Enrolment	Allocation	Post-allocation						Close-out
TIMEPOINT	*-t1*	0	*Day before*	*t0 (baseline)*	*t1 (1 h)*	*t2 (1 h 30')*	*t3 (2 h)*	*t4 (4 h)*	
ENROLMENT									
Eligibility screen	X								
Informed consent	X								
General anamnesis	X								
Allocation		X							
PHASE I	ASSESSMENT OF BIOACTIVE BIOAVAILABILITY AND OTHER MARKERS								
Consumption of blueberry product				⟵	—	—	—	⟶	
Consumption of control product				⟵	—	—	—	⟶	
Weighted food and drug record			X						
Bioactives absorption assessment				⟵	—	—	—	⟶	
Oxidative stress markers				⟵	—	—	—	⟶	
Inflammation markers				⟵	—	—	—	⟶	
Vascular function markers				⟵	—	—	—	⟶	
Glycemic and insulinemic response				⟵	—	—	—	⟶	
PHASE II	ASSESSMENT OF NON-INVASIVE VASCULAR FUNCTION MARKERS								
Consumption of blueberry product				⟵	—	—	—	⟶	
Consumption of control product				⟵	—	—	—	⟶	
Weighted food and drug record			X						
Reactive hyperemia index				X			X		X
Arterial stiffness				X			X		
Blood pressure				X			X		
Heart rate				X			X		

Figure 1 - Schedule of enrollment, interventions, and assessment: recommendations for interventional trials (SPIRIT).

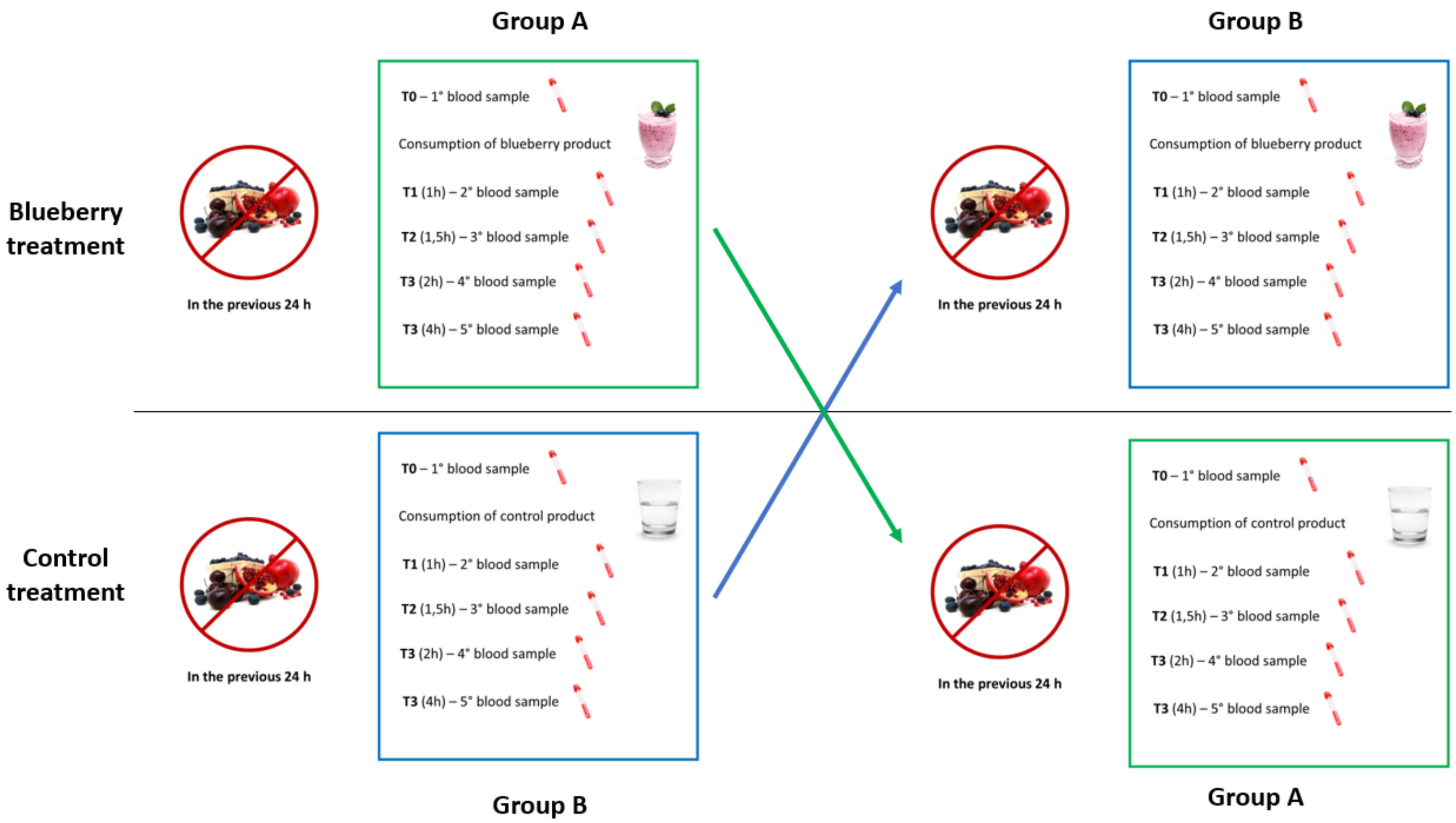

Figure 2 - Schematic representation of the study design for the evaluation of blueberry bioactive absorption and of different metabolic/functional markers (phase 1).

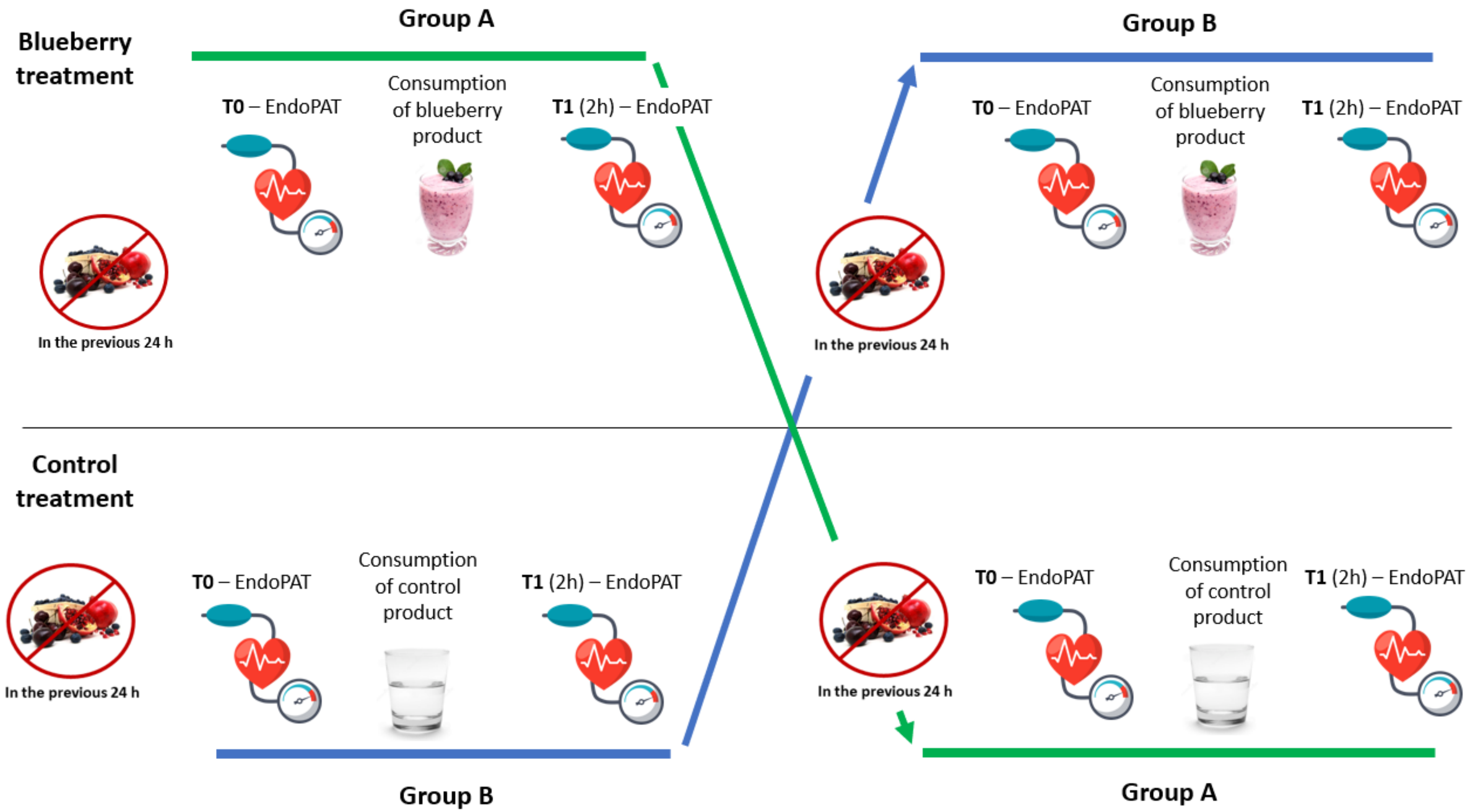

Figure 3 - Schematic representation of the study design for the evaluation of the effects of intervention on vascular function markers (phase 2).

Trial status

The trial has been prospectively registered (May 7, 2021; ISRCTN18262533) and is currently ongoing.

Location

The study is carried out at the Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano (Milan, Italy) through its International Center for the Assessment of Nutritional Status (ICANS).

Participant enrollment

For the study, a group of 20 subjects (age ≥ 60 y; 10 women and 10 men) are enrolled. The enrollment is carried out through advertisements by using emails, social networks, bulletin boards, and by word of mouth. The subjects who express their willingness to participate undergo a first general medical anamnesis through questions about their health status and their lifestyle (e.g., diseases, use of drugs and supplements, dietary habits, alcohol consumption, and physical activity) followed by an accurate clinical evaluation to confirm their eligibility to participate. Subjects interested in participating in the study sign an informed consent in which they agree with all the information on the dietary intervention, the analysis, and protocols that they are asked to follow. Volunteers are selected according to the inclusion and exclusion criteria reported below:

Inclusion criteria

- Age ≥ 60 years

Exclusion criteria

- Diabetes
- Major condition related to cardiovascular system (e.g., a medical history of thrombosis or myocardial infarction).

- Presence of allergies (or other adverse reaction) to the ingestion of blueberry

Changes in the volunteer status during the experimentation (e.g. occurrence of an exclusion criteria such as drug prescription, not evidenced at the enrolment) will be assessed to define the need of final exclusion from the study.

Blueberry and control product

Blueberry product consists of a mousse obtained by shredding a serving of blueberries (organic highbush blueberries, cultivar Legacy) able to provide at least 300 mg of anthocyanins (ACNs). This amount of ACNs is considered sufficient to induce an improvement of vascular function as previously reported (Del Bo' *et al.*, 2014, 2017). The control product consists of a drink prepared by suspending the same amount of fructose, glucose, and saccharose of blueberry (matching the blueberry product for energy) in water, as already published (Del Bo' et al., 2014, 2017).

Information on potential adverse effects

Even though no reports of adverse effects due to a blueberry consumption have been registered or reported in the literature, subjects are advised to annotate and communicate any adverse symptom perceived during the intervention period.

Biological sampling

Blood samples (7 mL) are collected at baseline (t0) and after 1 h (t1), 1 h 30 '(t2), 2 h (t3) and 4 h (t4) from the consumption of blueberry or control product, as depicted in Figure 2. A cannula needle is used to facilitate blood collection procedures and minimize the discomfort of the volunteers. Tubes containing silicon for serum and tubes containing heparin as anticoagulants for peripheral blood mononuclear cells (PBMCs) are used. An aliquot of blood (500 µL) is immediately processed to obtain PBMCs, while the rest of the blood is maintained at room temperature (22 °C) for 30 min before being processed by centrifugation at 1088 g for 15 min at 4 °C. The serum obtained is collected, divided in aliquots, and stored at -80 °C until analysis.

Outcome measurements

A complete list of the included markers of vascular function, inflammation, and oxidative stress is reported in Table 1. The primary selected outcome of the study is RHI as a vascular function marker, while other markers including vascular function (i.e., fRHI, AI, AI@75, Endothelin-1, NO, ICAM-1, VCAM-1 and VEGF), inflammation (i.e., IL-6, IL-8, and TNF-α) and oxidative stress markers (DNA damage), together with circulating levels of (poly)phenols, salicylates, glycaemia and insulin, are included as secondary outcomes to support and validate the study hypothesis.

Table 1. Brief description of the markers analyzed within this study

Vascular function markers	**Brief description**
RHI	The Reactive Hyperemia Index represents a measure of endothelial function, obtained through peripheral artery tonometry techniques. This method assesses reactive hyperemia through the changes in the amplitude of pulsations in finger pressure after inducing a hyperemic response in the brachial artery (Rosenberry and Nelson, 2020).
fRHI	The Framingham Reactive Hyperemia Index is an alternative reactive hyperemia score, proposed by Framingham Heart Study researchers, which differs from RHI since uses the natural logarithmic transformation of the RHI ratio, does not include the baseline correction factor, and utilizes only the readings from 90 to 120 seconds of post occlusion recordings (McCrea *et al.*, 2012) .
AI	The augmentation index is an indirect measure of arterial stiffness. It represents the measure of contribution that the wave reflection makes to the systolic arterial pressure, and it is calculated as the augmentation pressure divided by pulse pressure and expressed as a percentage (Fantin *et al.*, 2006).
AI@75	AI@75 is an alternative measure of arterial stiffness normalized for a heart rate of 75 bpm, since an inverse, linear relationship between augmentation index and heart rate is well established (Wilkinson *et al.*, 2000; Durmus *et al.*, 2014).

ET-1	Endothelin-1 is a potent endogenous vasoconstrictor, mainly secreted by endothelial cells. It also stimulates the production of ROS and contributes to the development of inflammatory processes.
ICAM-1	Intercellular Adhesion Molecule 1 is a molecule which promotes the trans-endothelial migration of leukocytes and the activation of T cells during inflammatory processes.
VCAM-1	Vascular Cell Adhesion Molecule 1 is a molecule induced on endothelial cells during inflammatory processes and acts mediating the adhesion of monocytes and other immune system cells to vascular endothelium.
VEGF	Vascular Endothelial Growth Factor is an angiogenic factor that regulates angiogenesis by inducing proliferation, migration, and permeability of endothelial cells.
NO	Nitric oxide is a soluble gas produced continuously by endothelial cells in response to homeostatic stimuli. It regulates several functions including the modulation of vascular tone.
Inflammation markers	
IL-6	Interleukin-6 is a cytokine connected to immune system activation, through several mechanisms. It is primarily pro-inflammatory but can also exert anti-inflammatory effects.
IL-8	IL-8 is an important protein related to inflammation, where it plays a key role in the recruitment of neutrophils and other immune cells to the site of infection.
TNF- α	Tumor necrosis factor alpha is an inflammatory cytokine produced by macrophages and monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to inflammation, necrosis or apoptosis.
Oxidative stress markers	
% DNA damage	The percentage of DNA damage in PBMCs is evaluated by the comet assay, a.k.a. single cell gel electrophoresis (SCGE). The comet assay is a versatile method used for measuring DNA damage and repair at individual cell level (Costa and Teixeira, 2014). The comet assay can be used to analyze endogenous DNA damage and DNA resistance to oxidatively-induced (H_2O_2) DNA damage in PBMCs (peripheral blood mononuclear cell).

Anthropometric measurements

Body weight and height are assessed at the beginning of the study by trained personnel following the international guidelines reported by Lohman et al. (1988). BMI calculation is obtained by dividing a person's weight by the height to the power of 2 (BMI = weight (kg) / height (m)^2).

Blood pressure

Volunteers are monitored during each intervention period measuring both systolic and diastolic blood pressure (SBP and DBP) obtained in a resting, seated position based on the validated JNC 7 guidelines (Chobanian *et al.*, 2003).

Metabolic and functional markers

At enrollment, metabolic and functional parameters (i.e., glucose, insulin, lipid profile, liver, and renal function) are analyzed by a standardized validated protocol, using an automatic biochemical analyzer (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer, Marshall Scientific, Hampton, VA, USA)). In details, non-high density lipoprotein cholesterol (non-HDL-C) is calculated by subtracting high density lipoprotein cholesterol (HDL-C) from total cholesterol (TC) while low density lipoprotein cholesterol (LDL-C) concentration is estimated using the Friedewald formula (Knopfholz *et al.*, 2014). The levels of insulin sensitivity are assessed using the widely used homeostasis model assessment for insulin resistance (HOMA-IR), that is calculated considering fasting plasma concentrations of glucose and insulin, as indicated by Wallace et al. (Wallace, Levy and Matthews, 2004). The blood samples are used for the evaluation of: i) kinetics of the absorption of the anthocyanins by gas chromatography–mass spectrometry analysis (GC–MS); ii) kinetics of the absorption of salicylates by means of ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS); iii) serum markers of vascular function (i.e. ET-1, NO, ICAM-1, VCAM-1 and VEGF) and inflammation (i.e. IL-6, IL-8, and TNF-α) evaluated through ELISA kit, while serum markers of oxidative stress (i.e. levels of DNA damage) evaluated through comet assay iv) blood glucose concentrations evaluated by oxidation with YSI 2300 STAT Plus™ Glucose & Lactate Analyzer and insulin levels determined by ELISA kit.

Vascular function and arterial stiffness

Endothelial-dependent vasodilation in the small finger arteries is assessed by a non-invasive plethysmographic method (Endo-PAT 2000, Itamar Medical Ltd, Caesarea, Israel) as described elsewhere (36-37). Briefly, the Endo-PAT equipment consists of two finger-mounted probes, which include a system of inflatable latex aircushions. The pulsatile volume changes of the fingertip are sensed by a pressure transducer, located at the end of each probe, and transferred to a personal computer where the signal is transduced, amplified, displayed, and stored. For the evaluation, subjects are requested to lie down in a supine position, with both hands on the same level, in a comfortable and thermoneutral environment. Then, a blood pressure cuff is placed on one upper arm (study arm), while the contralateral arm serves as a control arm. After a 10 min equilibration period, the blood pressure cuff on the study arm is inflated to 200-220 mmHg for 5 min. The cuff is then deflated to induce reactive hyperemia while the signals from both PAT channels (Probe 1 and Probe 2) are recorded by a computer. The RHI, an index of the endothelial-dependent flow-mediated dilation, is derived automatically in an operator independent manner. In fact, RHI is calculated as the ratio of the average pulse wave amplitude during hyperemia (60-120 s of the post-occlusion period) to the average pulse wave amplitude during baseline in the occluded hand, divided by the same values in the control hand and then multiplied by a baseline correction factor. According to manufacturing indications, an RHI value of 1.67 provides a sensitivity of 82% and a specificity of 77% for diagnosing endothelial dysfunction (38).

The Endo-PAT tool also provides the digital augmentation index (AI), a measure of pulse wave reflection and a surrogate marker for arterial stiffness. AI derives from digital pulse volume waveforms and it has been reported to be strongly correlated with aortic AI. Peripheral AI is calculated from the shape of the pulse wave recorded during baseline (39-40). Since digital AI is affected in an inverse and linear manner by heart rate (HR) (41), the AI was automatically normalized by considering a HR of 75 bpm (AI@75).

Vascular and inflammatory markers

The concentrations of several markers related to vascular and inflammatory processes are quantified at least in duplicate using specific ELISA or colorimetric assay kits according to the instructions of each producer (Boster Bio, Valley Ave, Pleasanton, CA; Cayman Chemical Company, Ann Arbor, MI, USA). The following kits are used on serum samples obtained at each time points during the intervention periods: IL-6: BSR-EK0410 - Human IL-6 PicoKine ELISA Kit, IL-8: BSR-EK0413 - Human IL-8 PicoKine ELISA Kit, TNF-α: BSR-EK0525 - Human TNF alpha PicoKine ELISA Kit, Endothelin-1: BSR-EK0945 - Human Endothelin PicoKine ELISA Kit, ICAM-1: BSR-EK0370 - Human ICAM-1 PicoKine ELISA Kit, VCAM-1: BSR-EK0537 - Human VCAM-1 PicoKine ELISA Kit, VEGF: BSR-EK0539 - Human VEGF PicoKine ELISA Kit, NO: CAY-780001-2x96 - Nitrate/Nitrite Colorimetric Assay Kit.

Oxidative stress markers

The levels of oxidatively-induced DNA damage, as markers of oxidative stress, are assessed in PBMCs by the comet assay on an aliquot of fresh blood, immediately after its withdrawal. Oxidatively-induced DNA damage is measured by treating the cells with hydrogen peroxide and by evaluating the capacity of cells to counteract this oxidative insult. This procedure is performed according to Del Bo' et al., (Del Bo' *et al.*, 2015). Briefly, cell resistance against oxidatively induced DNA damage is measured by treating the isolated PBMCs with hydrogen peroxide (H_2O_2). Two slides with two gels each are prepared for each sample. One slide is treated with H_2O_2 (500 µmol/L in PBS) for 5 min in the dark, while the other slide is treated with PBS without H_2O_2, as it is used as a control. Following oxidative treatment, the slides are placed in lysis buffer (2.5M NaCl, 0.1M Na2 EDTA, 10mM Tris, 1% Triton X-100, 1% DMSO, and 1% N-lauroylsarcosine sodium salt, pH

10) for 1h at 4°C in the dark. After lysis, the gels are washed in buffer (40nM HEPES, 0.1M KCl, 0.5mM EDTA and 0.2mg/ml bovine serum albumin, pH 8) and then transferred to electrophoresis buffer (0.3M NaOH and 1mM Na2 EDTA) and incubated for 40min at 4°C in the dark. Electrophoresis is performed at 1.1V/cm2 for 20min. Slides are successively neutralized (0.4M Tris–HCl, pH 7.5) for 15min at 4°C in the dark, stained with ethidium bromide (2 µg/ml), washed in PBS, drained and coverslipped to permit the subsequent quantification of DNA damage through an epifluorescence microscope attached to a high sensitivity CCD video camera and to a computer equipped with an image analysis system (QCapture 2.9.13, Irvine, CA, USA; DNA COMET1.50.00, University of Milan, Italy). The levels of DNA damage are calculated as the percentage of DNA in the tail.

Serum anthocyanins

Serum samples are used to determine the kinetics of absorption of blueberry ACNs and salicylates. The extraction and analysis of ACNs from serum are performed through a microelution solid phase extraction (SPE) and ultra-HPLC methods, according to Martí et al., (2010). Briefly, prior to injection in the UPLC-MS/MS, ACNs are extracted using µElution Plates 30 mm conditioned with 250 mL of methanol and 250 mL/mL of 0.2% acetic acid. Then 350 µL of plasma sample is mixed with 350 µL of phosphoric acid 4%, loaded into the plate. The loaded plates are washed with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained ACNs are eluted with 2 x 50 µL of acetone/Milli-Q water/acetic acid solution (70:29.5:0.5, v/v/v) and injected. Injection volume is 2.5 µL. UPLC-ESI-MS/MS is constituted by a stationary phase of 100% silica particles while the mobile phase by 10% acetic acid (eluent A) and acetonitrile (eluent B). The flow rate is set at 0.4 mL/min.

Blood salicylates

The analysis of salicylates in serum samples is performed using stable isotope dilution and gas chromatography–mass spectrometry analysis (GC–MS), as described previously (Battezzati *et al.*, 2006). Briefly, following adjustment for the pH to about 1.0 with hydrochloric acid, the organic material is extracted twice with ethyl ether and ethyl acetate (2 mL), and then is collected for derivatization prior to GC–MS analysis. Analysis is then carried out using a gas chromatograph interfaced with a single-quadrupole mass spectrometer equipped with a chamber provided with an electron ionization source and using helium as carrier gas. Finally, the identification of derivative salicylic acid is achieved by comparing the gas chromatographic retention times and the mass spectra of the serum samples with those of the standards.

Data management

Data management includes a strict control of data quality and check of accuracy of data entry. To ensure data protection, data will be saved in a password-protected Excel file on an electronic device equipped with an antivirus system locked up in a secure location when not in use. Once completed final data will be stored in an electronic database (Dataverse). Identifiable data are not recorded in the database or other documents, and participants are identified by a unique trial ID only. Hard copies of data sheets linking the participant identification number to the person's contact details are kept securely within the University premises that are accessible only to dedicated research team members. Participant files and other source data (including copies of protocols, questionnaires, and original reports of test results, correspondence, records of informed consent and other documents pertaining to the conduct of the study) are kept for the maximum period of time permitted by the institution.

Sample size, randomization, and statistical analysis

The sample size is determined based on previous studies conducted in our laboratories (Del Bo' *et al.*, 2014, 2017) and other research groups (Rodriguez-Mateos *et al.*, 2013; Stull *et al.*, 2015; Dodd *et al.*, 2019) to test the efficacy of a blueberry consumption on markers of vascular function. Twenty volunteers are enrolled for the study. This number is considered sufficient (alpha = 0.05, 80% power) to determine an increase of 0.30 in the RHI parameter (endothelial function marker) after blueberry intake. This calculation also considers a possible drop out of 25% of the volunteers. Subjects are randomly divided using a computer random number generator. The randomisation and allocation is performed by a person not involved in the trial and blinded to the investigators and researchers involved in samples analysis.

Statistical analysis

The statistical analysis is performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Only complete data obtained will be analysed. The Shapiro–Wilk test is applied to verify the normal distribution of the variables under study. In particular, the following statistical elaborations are 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. Furthermore, regression and correlation analyses (Spearman and Kendall test) are carried out to highlight potential associations between vascular activity, and markers of inflammation, vascular function, oxidative stress, other than biochemical and physiological markers. Corrections for potential confounding factors (i.e., age, gender, drugs/medications) are performed. When appropriate, a post-hoc p-value adjustment is performed using the Hochberg-Benjamin correction. Significance is set at $p < 0.05$; significance in the range $0.05 < p < 0.10$ is indicated as trend.

Monitoring

Given the limited objectives and the short-term nature of the intervention, this trial is monitored by the dedicated research team without the use of a formal data monitoring committee and will be performed independently from the sponsor. Data access is restricted to trained staff with unique password protected accounts. Eventual adverse events such as unfavorable and unintended signs, abnormal laboratory findings, and symptoms temporarily associated with the intervention are collected from the time of intervention until the end, whether or not considered related to the intervention study. All adverse events are considered until they are resolved, but adverse effects are unlikely to occur.

Ethics and dissemination

The study is conducted in accordance with the Declaration of Helsinki and the Data Protection Act. Trial personnel obtain informed consent from all participants prior to inclusion to the study, including consent for the use of data and biological specimens in other ancillary studies. The principal investigator stores informed consent. All subjects must agree to participate voluntarily and are free to withdraw from the study at any time. The study received ethical approval by the Ethics Committee of the University of Milan on the 12 December 2020. The Research Ethics Committee (REC) approval includes the trial protocol, information sheet and consent form. The trial is registered at the International Standard Randomized Controlled Trial Number (ISRCTN18262533, date of registration: 07 May 2021). Any amendment to the protocol and information provided to participants are submitted to the REC for approval prior to implementation. Substantial amendments may only be implemented after written Ethics Committees approval has been obtained, whereas non-substantial amendments can be implemented without written approval from the Ethics Committee. The Principal Investigator (PI) has to ensure that the participant's privacy is maintained. Data and source documents are stored in such a way that they can be accessed later for the purposes of monitoring or inspection by the Ethics Committee. Public access to data and other materials could be requested to the PI at the end of the study after completion of elaboration and publication of data. At the end of the study, participants can receive a copy of the results of the study from the PI. The results from the trial are submitted for publication in a peer-reviewed journal irrespective of the outcome. Authorship of presentations and reports related to the study are in the name of the collaborative group and the list will be based on the extent of contribution. Results will be also communicated through other dissemination means including relevant scientific conferences.

Discussion

The prevention of the decline of endothelial function, especially in older subjects, represents an important task in view of CV events prevention. In this context, diet and dietary factors can contribute not only to optimal aging, but they can also play an important role in the preservation of the vascular system. As reported above, diet is a well-known pillar of optimal aging; however, little evidence is available about the effect of bioactive rich-foods (such as polyphenol-rich foods) on vascular function in older subjects. Thus, there is a need for research that pushes towards acquiring solid evidence. The exploitation of well-controlled and specifically targeted dietary intervention trials represents the correct scientific approach for the evaluation of the health promoting properties of food bioactives, overcoming and completing the observations deriving from epidemiological studies and pre-clinical models. On the other hand, human interventions are difficult to implement in practice, as they can be affected by several confounding factors (e.g., environment, diet, lifestyle) and require considerable efforts in terms of organizational skills, in particular when they address specific target populations such as older subjects.

Previous human intervention studies conducted in our laboratories have documented that the intake of a single portion of blueberries (300 g, providing at about 300 mg of ACNs) counteracted (2 hours post consumption) an impairment in vascular function and blood pressure in a group of healthy young smokers with normal endothelial function (Del Bo' *et al.*, 2014). In another study, we found that the same blueberry portion increased vascular function in both young smokers and non-smokers with endothelial dysfunction (2 hours from intake) (Del Bo' *et al.*, 2017). Conversely, we have shown that a portion of blueberry purée (300 g) did not affect vascular reactivity (measured 1 hour after intake), but reduced oxidative stress, in a group of young healthy volunteers with normal vascular function (Del Bo′ *et al.*, 2013). Similarly, we could not demonstrate an effect on vascular function and inflammation, while there was a reduction in oxidative stress markers, after a 6-week intervention with a wild blueberry-based drink (250 mL/d, providing 475 mg of ACNs) in adults with CVD risk factors (Riso *et al.*, 2013).

Within this study, we want to test the hypothesis that the consumption of blueberries can positively affect markers of vascular function in older subjects. Considering that aging is associated with an increased risk of oxidative stress and inflammation, and that these two conditions are crucial in the onset and progression of vascular dysfunction, we want also to ascertain whether the intake of a single blueberry portion can exert a positive modulation on a plethora of direct and indirect markers of oxidative stress and inflammation. Furthermore, since the potential health benefits should be attributed to the absorption of the bioactive compounds, the kinetic of absorption of (poly)phenols and salicylates from blueberries is evaluated. To this aim, a randomized, controlled, crossover post-prandial study is performed in which the absorption of bioactives from the intake of a single portion of blueberries and the effects on metabolic and functional markers (e.g., vascular function, oxidative stress, and inflammation) is evaluated. The exploitation of a crossover design allows us to reduce the interindividual variability of the subjects, with advantages in terms of sample size and control of the experimental plan for potential confounding factors. The intervention is planned at the facilities of the University in a controlled environment in which subjects can rest, comply with the instructions provided and consume the products. The products consist of a serving of blueberries and a control product. The blueberry portion is defined by considering the amount of ACNs present and potentially capable of exerting a beneficial effect, but also that the portion should be easily consumed as part of a balanced diet. Based on our previous studies, the portion should be in the range 250-300 g (raw product) able to provide at least 300 mg of ACNs (Del Bo' *et al.*, 2014, 2017). The control product consists in a sugar drink (250-300 mL) containing the same sugars and amount present in the blueberries, matching for macronutrients and energy intake. The inclusion of a control drink with these characteristics allows us to maintain a controlled experimental setting. Subjects also have to follow, 3-day before and during the experimentation, specific dietary instructions consisting of a list of foods allowed and not allowed. Furthermore, weighed food diaries are assessed by each volunteer both before and during the intervention to permit an accurate estimation of nutrient intake and to check the compliance with the dietary instructions by maintaining a high degree of control.

The study is performed in two different phases. Phase 1 consists in the evaluation of the absorption of blueberry bioactives, metabolic and functional markers analyzed in the blood samples collected at different time points (time zero (0 h; baseline), 1 h, 1 h 30 min., 2 h and 4 h from the consumption of blueberry or control products) selected by taking into consideration the "regular" pharmacokinetic of ACNs in terms of time of maximum plasma concentration (t_{max}; at 2 h), peak of maximum plasma concentration (C_{max}) and clearance from blood. These times have been selected based on the results of previous studies showing the absorption of ACNs and a modulation of metabolic and functional markers, including oxidative stress and inflammation, in young healthy subjects or subjects with risk factors following the consumption of blueberries and/or blueberry-bioactives (Bo *et al.*, 2012; Del Bo′ *et al.*, 2013; Riso *et al.*, 2013; Rodriguez-Mateos *et al.*, 2013, 2014). Regarding phase 2, the analysis of vascular function is evaluated at baseline and after 2 h from the intake of blueberry and control products. Also in this case, the choice to measure vascular function at 2 h derives from previous observations, performed both in our laboratories and in others, in which a significant increase in RHI was documented at that specific time from the intake of blueberries both in healthy subjects and in those with an endothelial dysfunction or other risk factors (Rodriguez-Mateos *et al.*, 2013, 2014; Del Bo' *et al.*, 2014, 2017). The decision to split the blood samples collection and the analysis of vascular function into two different days was chosen to overcome the overlapping of the two procedures by reducing possible stress conditions to the volunteers, by facilitating the operating procedures and by limiting the occurrence of potential noises that could affect the different evaluations (vascular function and related markers).

As regards the primary outcome, RHI has been identified as a marker of vascular function that reflects endothelial function of the microvasculature (i.e., resistance vessels) (Hamburg *et al.*, 2011). RHI represents an alternative method to flow-mediated dilatation (FMD), the reference method, since it has the advantage of being simple, operator independent, and therefore less prone to operator bias and variability (Kuvin *et al.*, 2003; McCrea *et al.*, 2012). In addition, RHI has shown a significant correlation ($r = 0.55$, $p < 0.0001$) with FMD (Onkelinx *et al.*, 2012; Rosenberry and Nelson, 2020) and it is widely used, alone or in combination with FMD and/or other markers of vascular function, not only for clinical practice but also to study the effect of drugs, supplements, foods and exercise in numerous human interventional trials (Hong *et al.*, 2013; Gerstgrasser *et al.*, 2015; Giallauria *et al.*, 2016; Garu *et al.*, 2021; Krishnan *et al.*, 2022). Regarding the secondary outcomes, a wide range of metabolic and functional markers related to the aging process, and directly and/or indirectly associated to vascular function, has been identified. In particular, glycaemia and insulin are analyzed in order to ascertain the impact of blueberries, as an important source of single sugars, on glucose metabolism and whether the eventual sugar metabolic response is able to influence vascular function. Finally, the analysis of markers of oxidative stress (e.g., DNA damage at cellular levels), inflammation and angiogenesis (e.g., interleukin-6, IL-6; tumor necrosis factor alpha, TNF-α; vascular endothelial growth factors; VEGF), adhesion molecules (vascular cell adhesion molecules 1, VCAM-1; intercellular adhesion molecules, ICAM-1), vasoconstrictors and vasodilators (ET-1 and NO, respectively) is useful to verify the contribution of blueberry in their modulation.

This study has some limitations. First, the difficulty of obtaining a real placebo, thus subjects are not blind to the treatment. Second, the relatively small sample size that, however, was defined based on previous studies obtained in different target groups (Del Bo′ *et al.*, 2013; Rodriguez-Mateos *et al.*, 2013; Del Bo' *et al.*, 2017) including older subjects (Dodd *et al.*, 2019). It is noteworthy that the translation to the general older population could be limited since this target is characterized by wide heterogeneity, thus the demonstration in larger groups of volunteers could be recommended. Third, to limit exclusion criteria in order to enroll subjects with phenotypes that are typical of older age, including those presenting hypertension or mild pharmacological treatments even because in the cross over design adopted each subject will serve as their own control (i.e. also maintaining the same pharmacological treatments). In this regard, characteristics of subjects will be duly considered in data interpretation.

The results of the study will be able to provide several important pieces of information. First, to improve knowledge on the absorption of the bioactive compounds in older subjects, on which data are still lacking in literature. Second, to determine whether the intake of blueberries has a beneficial effect on vascular function also in relation to the absorption of its bioactives. Third, to verify the capacity of blueberries to positively modulate metabolic and functional markers, and whether their combination can provide evidence on the mechanisms by which the food/constituent could exert the protective effect. Overall, these results will be pivotal for the development of new dietary approaches in which blueberries and/or blueberry-products can be used, within a healthy dietary pattern, for improving vascular function with a view to promoting healthy aging.

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Author Contributions

Conceptualization: Cristian Del Bo', Daniela Martini, Patrizia Riso

Data curation: Mirko Marino, Massimiliano Tucci

Methodology: Alberto Battezzati, Cristian Del Bo', Mirko Marino, Massimiliano Tucci

Writing – original draft: Cristian Del Bo', Massimiliano Tucci

Writing – review & editing: Alberto Battezzati, Simona Bertoli, Daniela Martini, Marisa Porrini, Patrizia Riso

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3.4.4 A single portion of an anthocyanin-rich blueberry product improves endothelial function, but not arterial stiffness, in free-living older subjects: results from a randomized, controlled, crossover trial

Abstract: Several studies have reported the capacity of blueberries to positively affect vascular function; however, data on older subjects, that are more prone to a vascular dysfunction, are still lacking. Aim of the following crossover, single-blind, randomized-controlled trial was to assess the acute effects of a single portion of a highbush blueberry cultivar, particularly rich in anthocyanins, on reactive hyperaemia index (RHI), as primary outcome of vascular reactivity, in a group of 17 healthy older (mean age 69 ± 5 y) subjects. RHI was measured at baseline and after 2 h from blueberry or control product consumption. Secondary outcomes included the measure of arterial stiffness (augmentation index – Aix) at 2 h, as well as serum markers of vascular function and inflammation (i.e., endothelin-1 (ET-1), Intercellular cell adhesion molecule-1 (ICAM-1), Vascular cell adhesion protein 1 (VCAM-1) and total nitrates and nitrites as a measure of NO production, TNF-α, and IL-6; by ELISA kits), measured at baseline and at four different time-points, up to 4 h. ANOVA revealed a significant ($p<0.05$) increase in RHI following blueberry consumption compared to the control (+50% vs. +23%, respectively). No effect was found for the markers of arterial stiffness as well as for those of inflammation. Regarding vascular function markers, a post-hoc analysis revealed a significant reduction in serum ET-1 and sVCAM-1 (at 1 h and 4 h, respectively), while a significant increase was observed for total nitrates and nitrites at 1.5 h post blueberry consumption. In conclusion, blueberry seems to improve vascular reactivity, but not arterial stiffness, in older subjects. This effect could be potentially attributed to a modulation of some markers of vascular function such as ET-1, NO and sVCAM-1. Further research is required to corroborate these findings in older subjects.

1. Introduction

Among foods associated with low environmental impact able to contribute to health, blueberries are particularly interesting. In fact, researchers are particularly active in Europe nowadays in developing modern berry cultivar able to fulfill the demand of high-quality berries from sustainable production (Senger *et al*. 2022). The yields of blueberry production are in fact increasing rapidly worldwide, thus reducing per unit environmental impacts (Liu *et al.* 2021). This is true also in Italy which has experienced important growth (around +46% from 2010 to 2017), in relation to increased demands, and is improving the sustainability of blueberry supply chains through different agronomic and technological approaches (Peano et al. 2017).

From a health-promoting perspective, blueberries are a rich source of (poly)phenols such as anthocyanins and phenolic acids (i.e., chlorogenic acids) which bioactivity has been extensively investigated in *in vitro* and in vivo studies (Wood et al. 2019). Although the results from the pre-clinical models seem to be promising, those deriving from humans are still conflicting. This could be attributed to different factors such as the type of intervention, the duration, the type of markers analysed, the sample size, the type of population, but also the type of product tested, the way of administration, the amount/portion of product and its nutritional composition in terms of bioactives such as (poly)phenols, that can be influenced by the cultivar and the production methods. In this regard, the amount and the composition of (poly)phenols may vary greatly from product to product also in relation to the cultivar by contributing significantly to the overall biological effect. Within the MIND FoodS HUB project "*Innovative concept for the eco-intensification of agricultural production and for the promotion of dietary patterns for human health and longevity through the creation in MIND of a digital Food System hub*" have been identified and analysed several foods, including different blueberry cultivars, for their nutritional and bioactives profile. In this context, a blueberry cultivar ("Brigitta") has been identified for its high content in polyphenols, in particular anthocyanins, and tested in a dietary intervention study.

2. Materials and methods

2.2 Subject selection

Subjects were included in the present study according to specific inclusion and exclusion criteria as previously reported (see chapter 3.4.3). Older subjects under chronic drug treatment for mild hypertension, or chronic conditions not related to vascular functions (e.g. gastroesophageal reflux disease) were considered eligible (more consideration on this aspect is provided in the Discussion section). For the trial, 26 subjects aged ≥ 60 years were initially recruited through advertisement and peer-to-peer engagement within the Milan area. Subjects were interviewed to ascertain their general condition and their eligibility for the study. Four subjects were considered ineligible for participation while 4 subjects declined to participate. Thus, 18 subjects were randomized to the two treatments, and 17 subjects completed the study, as schematized in the CONSORT 2010 Flow Diagram (Figure 1).

The demographic characteristics of the 17 subjects that completed the study are reported in Table 2.

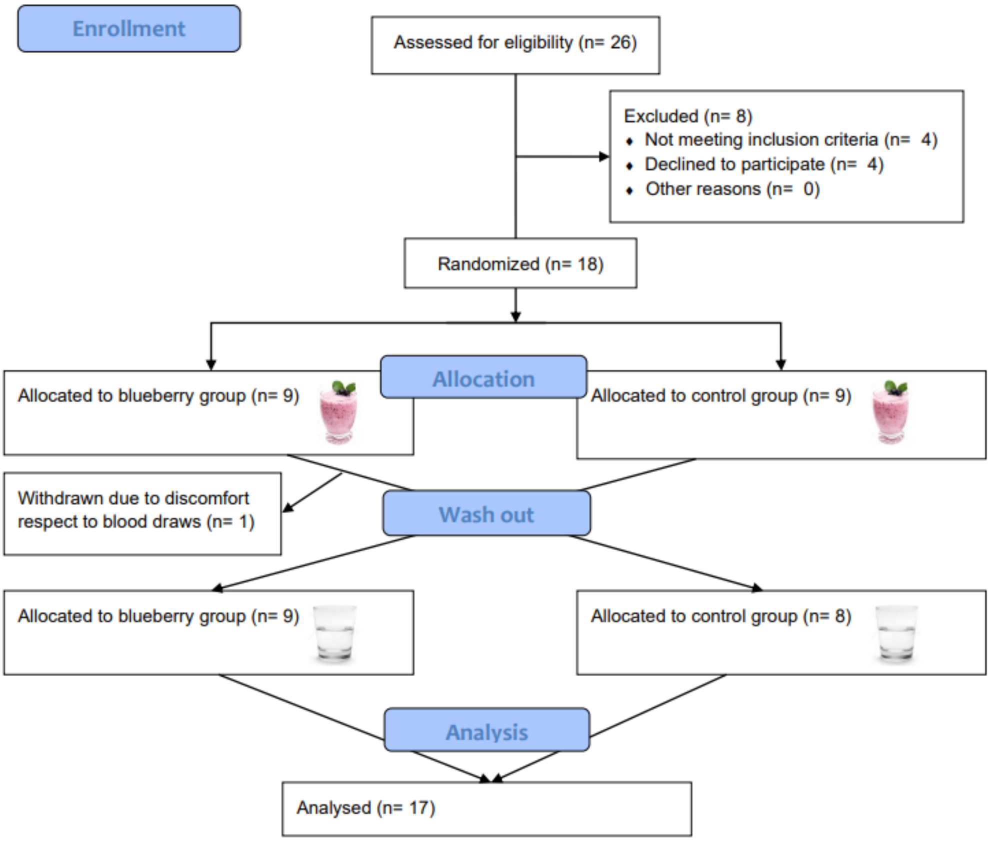


Figure 1 – CONSORT flow diagram.

2.3 Food preparation and composition

The study product consisted in a single portion of 250 g of an anthocyanin-rich cultivar (cv. Legacy) of highbush blueberry (*Vaccinium corymbosum*), that was selected after characterization of seven different cultivars grown under organic conditions in the Lombardy region. The blueberry product provided about 192 mg of anthocyanins/100g of fresh product. The anthocyanins profile is depicted in Fig. 2, and was mainly represented by delphinidin, malvidin, and petunidin glycosides. The control products consisted in 250 ml of water containing the same type and amount of sugars of the blueberry portion (i.e., 8.0 g of fructose, 7.3 g of glucose, and 1.5 g of saccharose). The blueberry product was prepared starting from previously washed and frozen blueberries and mixing them in a specific blender, obtaining a mousse.

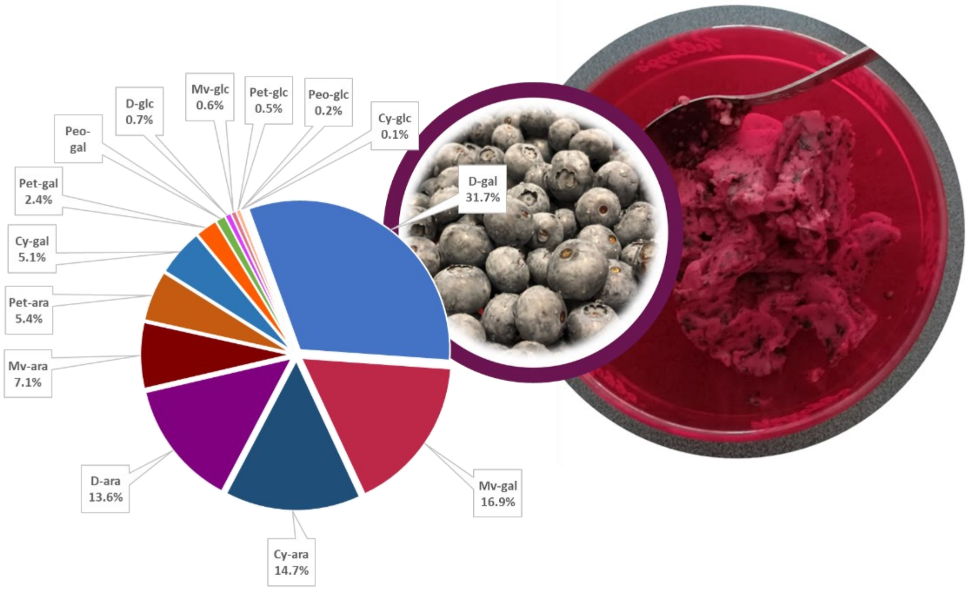


Figure 2 – Blueberries' anthocyanin profile in the test product.

Anthocyanins identified in the blueberry product are indicated as a percentage of total anthocyanins (192 mg of anthocyanins/100 g of fresh blueberry). For the study, 250 g of product was provided (i.e. 480 mg anthocyanins). This portions has been decided based on previous studies that tested fresh blueberry portions (or equivalent freeze-dried powder) of 150-300 g (Wood *et al.*, 2019).

2.4 Primary and secondary outcomes

The complete list of primary and secondary outcomes was previously reported (see chapter 3.4.3). Briefly, the primary outcome of the study was the modulation of Reactive Hyperemia Index (RHI), while the secondary outcomes included: the digital augmentation index (AI), as surrogate marker of arterial stiffness, blood pressure and heart rate. Further outcomes include serum markers of inflammation (i.e., tumor necrosis factor alfa (TNF-α), interleukin-6 – (IL-6)) and vascular function (Intercellular Adhesion Molecule 1 (ICAM-1), Vascular cell adhesion protein 1 (VACM-1), total nitrate/ nitrite as a biomarker of nitric oxide (NO) production, and endothelin-1 (ET-1)), glucose and insulin response.

2.5 Experimental design

The study followed a randomized, controlled, cross-over design (single portion of blueberry versus a control product). The trial was carried out at the facilities of the University of Milan in collaboration with the International Center for the Assessment of Nutritional Status (ICANS). The protocol was in accordance to the ethical standards established in the 1964 Declaration of Helsinki and it was approved by the Ethics Committee of the University of Milan. All participants signed the informed consent form. The study was registered at http://www.isrctn.org as ISRCTN18262533.

The study involved two phases (Phase 1 and 2), both including two different appointments, each one spaced by a 1 week wash-out period, (see the study protocol reported in the chapter 3.4.3). Phase 1 aimed at evaluating the effect of blueberries consumption on markers of vascular function (i.e., endothelin-1, NO, ICAM-1, VCAM-1 and VEGF), inflammation (i.e., IL-6, IL-8, and TNF-α), blood glucose and insulin response. Phase 2 was devoted to the evaluation of vascular functionality, using a non-invasive biosensor and the assessment of reactive hyperemia index (RHI), Framingham reactive hyperemia index (fRHI) and arterial stiffness (i.e., augmentation index (AI) and AI@75); in addition, blood pressure was recorded. The approach, divided into two phases, was necessary to facilitate blood drawing (venous cannula) and the evaluation of vascular function (at the level of the brachial artery) avoiding potential sources of interferences between the two measurements.

The day before the experiment, participants receive a list of instructions to follow. In particular, subjects were asked to maintain their habitual lifestyle habits also in terms of physical activities. Specifically, subjects followed a low-(poly)phenols diet (e.g., reducing fruit and vegetables intake, limiting them to peeled apple and pears, kiwi, potatoes, green salad, carrot, and zucchini), avoiding consumption of anthocyanins-rich foods (e.g., berries, red plum, red orange, red wine) as well as avoiding coffee or tea. Subjects were requested to eat, before 9:00 p.m., a standardized dinner consisting of a light meal of pasta or rice, meat, fish or cheese and bread.

In addition, to limit the possible noise deriving from a long-fasting period the day of the experiment, subjects participating at the Phase 1 were invited to consume a standardized light breakfast. Specifically, volunteers consumed 200 mL of partially skimmed milk or 125 g of yogurt and 3 biscuits (i.e., shortbread) or 3 rusks. The meal was consumed at least 90 min before blood collection and at the same hour each test day. Blood samples were collected at baseline (time 0) and after 1 h, 1.5 h, 2 h and 4 h from the blueberry or control products consumption.

For the Phase 2 (vascular function assessment) subjects were requested to reach the facilities of the University after a fasting night. The adherence to instructions was evaluated through a food diary and a face-to-face interview scheduled the day of the experiment. During the experiments (Phase 1 and Phase 2), the subjects remained at the university facilities. Subjects could consume water, while foods (excluding the text foods), coffee and other drinks were not allowed till the end of the text.

2.6 Blood samples

Blood samples were collected into vacutainers containing K-EDTA as anticoagulant for plasma, or silicon for serum. A detailed description about the procedures used for blood sampling was reported previously (see chapter 3.4.3).

2.7 Anthropometric, metabolic, inflammatory, and vascular function markers analysis.

A full description of the different markers was reported in the chapter 3.4.3. Briefly, body weight and height were assessed following the international guidelines reported by Lohman *et al.* XXX, while both systolic and diastolic blood pressure (SBP and DBP) were measured position based on the validated JNC 7 guidelines (Chobanian *et al.*, 2003). Metabolic parameters (i.e., glucose, insulin, lipid profile, liver, and renal function) were analyzed by a standardized validated protocol, using an automatic biochemical analyzer. LDL-C was estimated using the Friedewald formula (Knopfholz *et al.*, 2014), while insulin sensitivity was detected as indicated by Wallace *et al.* (2004). RHI, AI in the small finger arteries were assessed by a non-invasive plethysmographic method (Endo-PAT 2000, Itamar Medical Ltd, Caesarea, Israel) as described previously and elsewhere (Del Bo' *et al.*, 2014).Markers of vascular function (i.e. ET-1, NO, ICAM-1, VCAM-1 and VEGF) and inflammation (i.e. IL-6, IL-8, and TNF-α) were evaluated in duplicate in the serum by following the instructions reported in the ELISA or colorimetric assay kits according to the instructions of each producer (Boster Bio, Valley Ave, Pleasanton, CA; Cayman Chemical Company, Ann Arbor, MI, USA).

2.8 Statistical analysis

The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Data were analyzed by ANOVA (analysis of variance) for repeated measures design. ANOVA with treatment (blueberry vs. control) and time (before and after each treatment) as dependent factors was applied in order to evaluate the effect of blueberry on the variables under study. Only complete data obtained were analyzed. Significance was set at $p < 0.05$; significance in the range $0.05 < p < 0.10$ was indicated as trend. Post-hoc analysis of differences between treatments was carried out by the Least

Significant Difference (LSD) test with $p < 0.05$. Dara is reported as means ± standard deviation (SD).

3. Results

3.1 Subjects characteristics

Baseline characteristics of the enrolled subjects are reported in Table 2. Mean age was close to 70 years. The subjects were overall in good health status, even if with a non-negligible level of heterogeneity in health parameters such as fasting glycemia (from 77 to 124 mg/dL) or the drugs therapy. 8 subjects were completely free from chronic drug therapy, while most of them, 9 subjects, used drugs chronically, mainly anti-hypertensive.

Table 2. Baseline characteristics of the study population (n = 17).

Variables	Value
Age (years)	69 ± 5 (62 – 76)
Male	10 (58.8%)
Female	7 (41.2%)
Height (cm)	169 ± 8 (150 – 179)
BMI (kg/m^2)	24.8 ± 2.4 (21.1 – 29.4)
Drug treatment*	9 (52.9%)
SBP (mmHg)	120 ± 7 (108 – 138)
DBP (mmHg)	78 ± 6 (70 – 90)
RHI	1.91 ± 0.45 (1.11 – 2.81)
AIx	19% ± 17% (50 – 80)

HR (bpm)	62 ± 8 (-40% – 47%)
Glucose (mg/dl)	100 ± 13 (77 – 124)
Insulin (µUI/ml)	12.6 ± 6.9 (3.1 – 30.8)
TC (mg/dL)	210 ± 29 (154 – 260)
LDL-C (mg/dL)	124 ± 21 (86 – 158)
HDL-C (mg/dL)	51 ± 12 (33 – 73)
TC/HDL-C (ratio)	4.3 ± 1.0 (2.8 – 6.4)
LDL/HDL-C (ratio)	2.6 ± 0.7 (1.5 – 3.9)
TG (mg/dL)	124 ± 55 (71 – 242)
CRP (mg/L)	1.5 ± 1.5 (0.2 – 4.2)
ALT (U/L)	14.7 ± 4.7 (7.0 – 24.2)
GOT (U/L)	21.2 ± 8.1 (13.8 – 46.7)
GGT (U/L)	26 ± 28.9 (8.2 – 130.8)

Data are expressed as mean ± SD. Legend: AIx= augmentation index; ALT= Alanine transaminase; BMI= body mass index; CRP= C-reactive protein; DBP= diastolic blood pressure; GGT= Gamma-glutamyl transferase; GOT= glutamic oxaloacetic transaminase; HDL= high-density lipoprotein; HR= heart rate; LDL= low-density lipoprotein; MCV= Mean Corpuscular Volume; RHI= reactive hyperemia index; SBP= systolic blood pressure; TC= total cholesterol; TG= triglycerides; Minimum and maximum is reported in parenthesis. * Refers mainly to anti-hypertensive drugs (6/9).

3.2 Effect of intervention on peripheral arterial function

In Figure 2 are reported RHI values detected for all the 17 subjects that have completed the trial. Data are expressed as mean RHI variation after 2 h from the intake of blueberry or control product compared to baseline. The intake of blueberry intake increased RHI by 50% (from 1.79 ± 0.42 to 2.58 ± 0.61) compared to baseline, while the intake of the control product increased RHI by 23% (from 2.03 ± 0.46 to 2.46 ± 0.62). The variations registered in RHI following the two treatments resulted statistically different ($p < 0.05$) as evaluated with ANOVA.

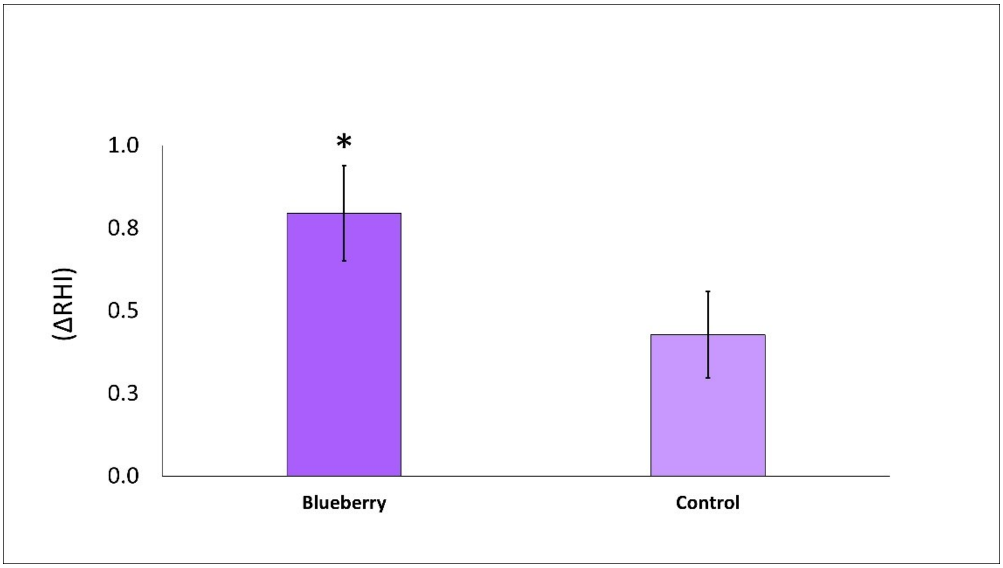


Figure 2 – Mean RHI variation after 2 h from blueberry or control product. Legend: Data are reported as mean ± SEM. *Significantly different compared to control treatment ($p < 0.05$).

3.3 Effect of intervention on arterial stiffness, blood pressure and heart rate

Arterial stiffness increased from baseline values after both blueberry and control products (from 18% to 36% and from 19% to 33%, respectively), as reported in Figure 3. However, statistical analysis did not show significant differences ($p > 0.05$).

Blood pressure did not change significantly compared to baseline after either the blueberry or control product (from 119 ± 6 to 119 ± 6 mmHg, and from 110 ± 6 to 118 ± 5 mmHg respectively, for systolic blood pressure; and from 77 ± 5 to 76 ± 3 mmHg, and from 77 ± 4 to 76 ± 4 mmHg respectively, for diastolic blood pressure). Also, heart rate did not result to be affected by blueberry or control treatment (from 61 ± 8 to 61 ± 9 beats per min, and from 58 ± 7 to 57 ± 6 beats per min, respectively).

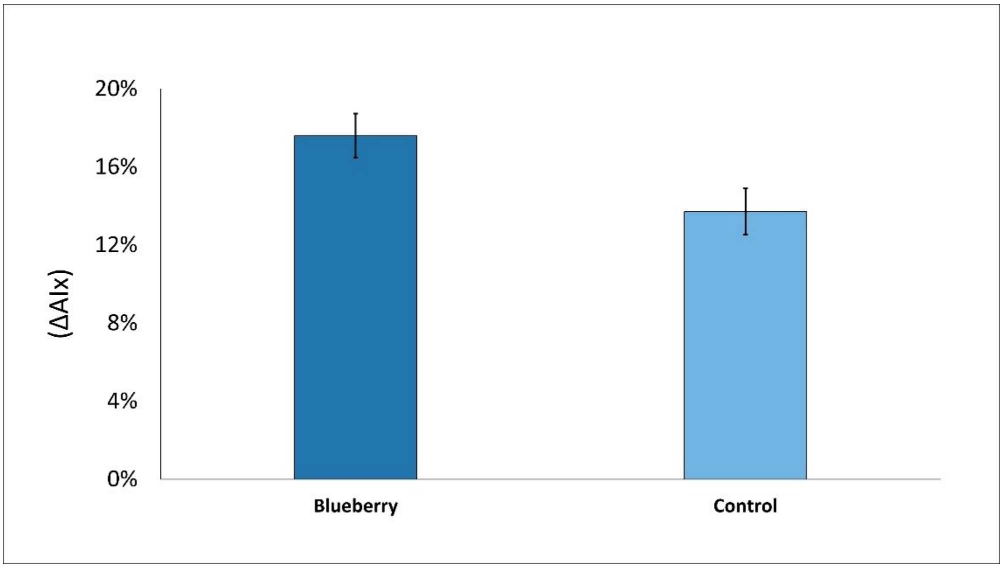
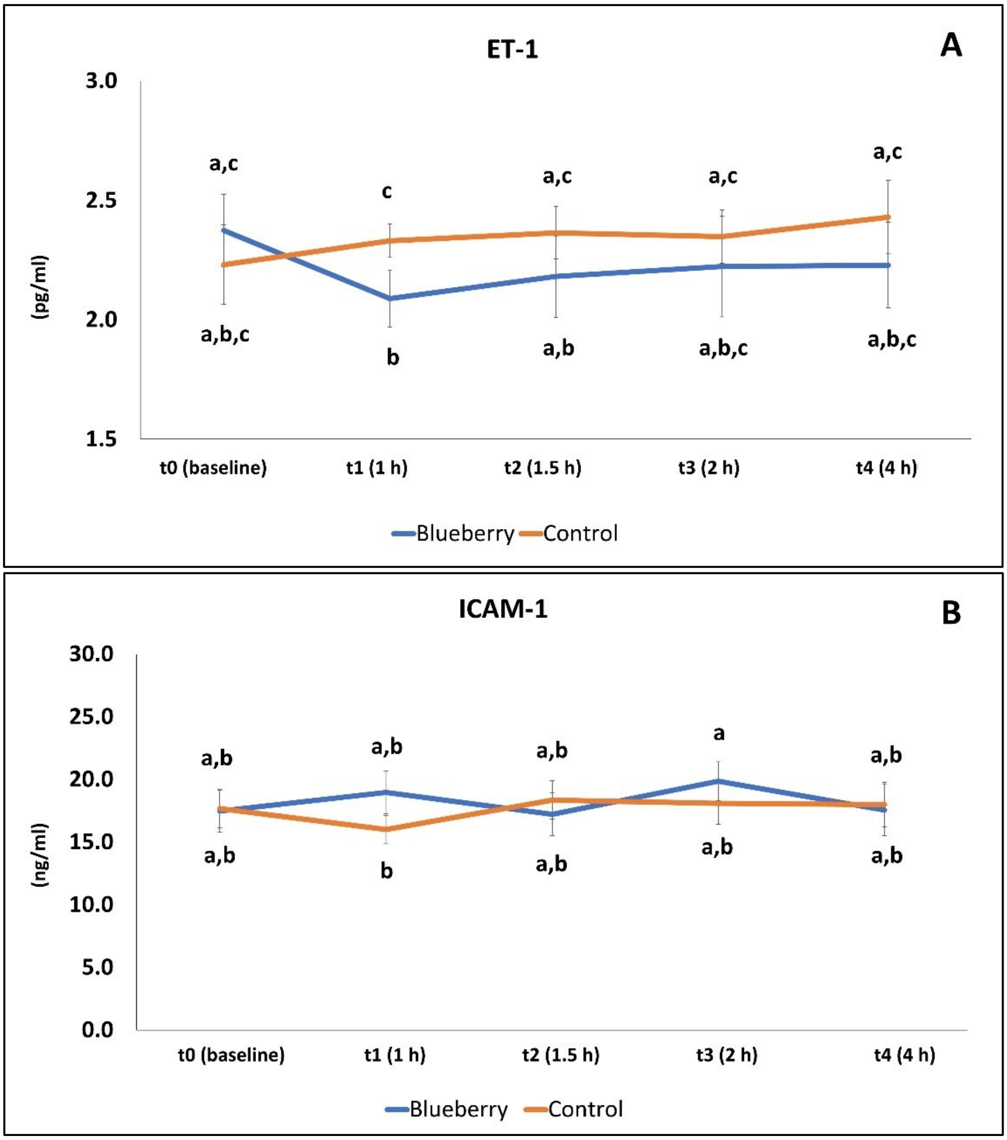


Figure 3 – Mean AIx variation after 2 h from blueberry or control product. Legend: Data are reported as mean ± SEM.

3.4 Serum markers of adhesion and vascular function

Cell adhesion molecules (i.e., ICAM-1 and VCAM-1), total nitrate/nitrite as a biomarker of nitric oxide (NO) production, and endothelin-1 were quantified as markers of vascular function. Repeated measures ANOVA did not indicate an overall effect of treatment, time, or interaction; however, the post hoc analysis showed some differences between the two treatments at specific time-points. In particular, the levels of endothelin-1 resulted significantly lower after 1 h from blueberry consumption, compared to controls (2.09 ± 0.17 pg/mL vs 2.33 ± 0.46 pg/mL, respectively. Fig 4a), Total nitrate/nitrite concentration resulted significantly higher after 1,5 h from blueberry consumption, compared to controls (50.5 ± 8.3 μM vs 42.1 ± 9.5 μM, respectively. Fig 4b), and VCAM-1 levels resulted significantly lower after 4 h from blueberry consumption, compared to controls (609.5 ± 156.6 ng/mL vs 645.2 ± 235.7 ng/mL, respectively. Fig 4c). No significant variations were found for total nitrate/nitrite levels (Fig 4d).


ET-1
A
3.0
2.5
2.0
1.5
(pg/ml)
a,c
c
a,c
a,c
a,c
a,b,c
b
a,b
a,b,c
a,b,c
t0 (baseline)
t1 (1 h)
t2 (1.5 h)
t3 (2 h)
t4 (4 h)
Blueberry
Control
ICAM-1
B
30.0
25.0
20.0
15.0
10.0
5.0
0.0
(ng/ml)
a,b
a,b
a,b
a
a,b
a,b
b
a,b
a,b
a,b
t0 (baseline)
t1 (1 h)
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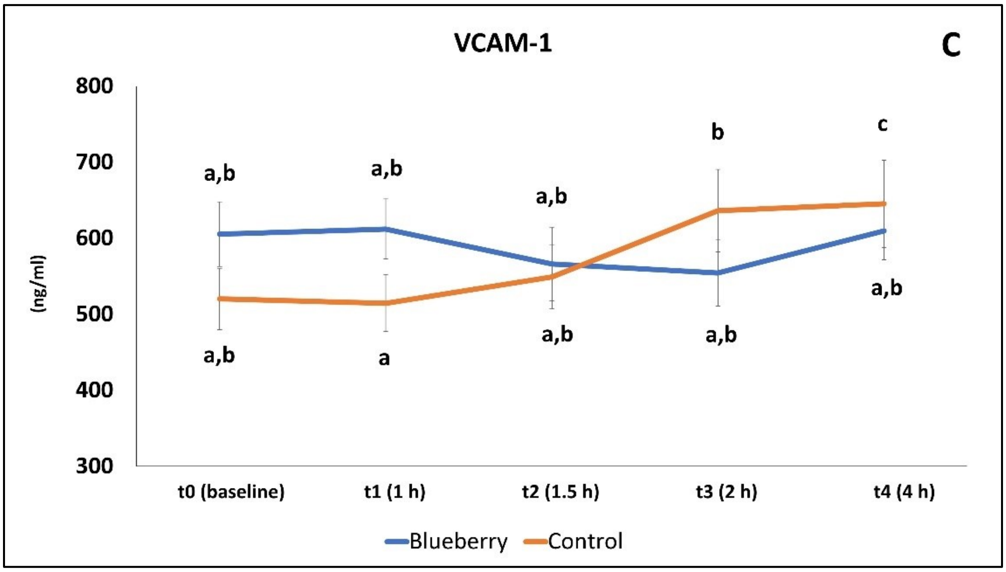
t3 (2 h)
t4 (4 h)
Blueberry
Control



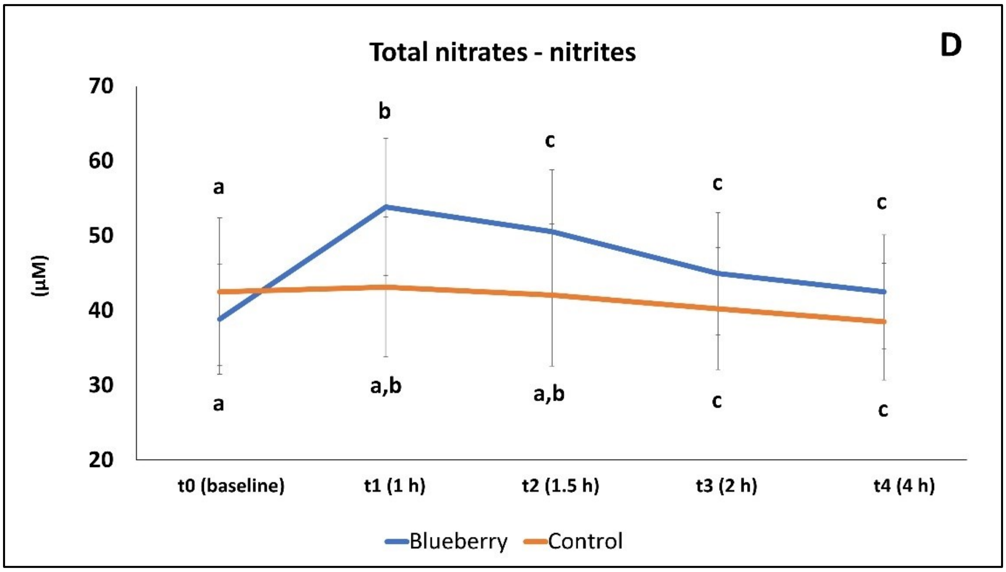


Figure 4a, 4b, 4c, and 4d – Levels of ET-1, ICAM-1, VCAM-1, and total nitrate/nitrite. Data are expressed as mean ± SEM for the two treatments over time. The ANOVA for repeated measures did not show an effect of the treatment, time, or interaction. Post-hoc analysis indicated statistical differences at specific time points.

3.4 Effect of intervention on serum markers of inflammation

In figure 5 is reported the serum levels of TNF-α (5a) and IL-6 (5b) measured in serum, for each treatment, at baseline (time 0 min) and after 1, 1,5, 2 and 4 h from the intake of blueberry or control product. Neither of these markers were significantly modulated after the intervention.

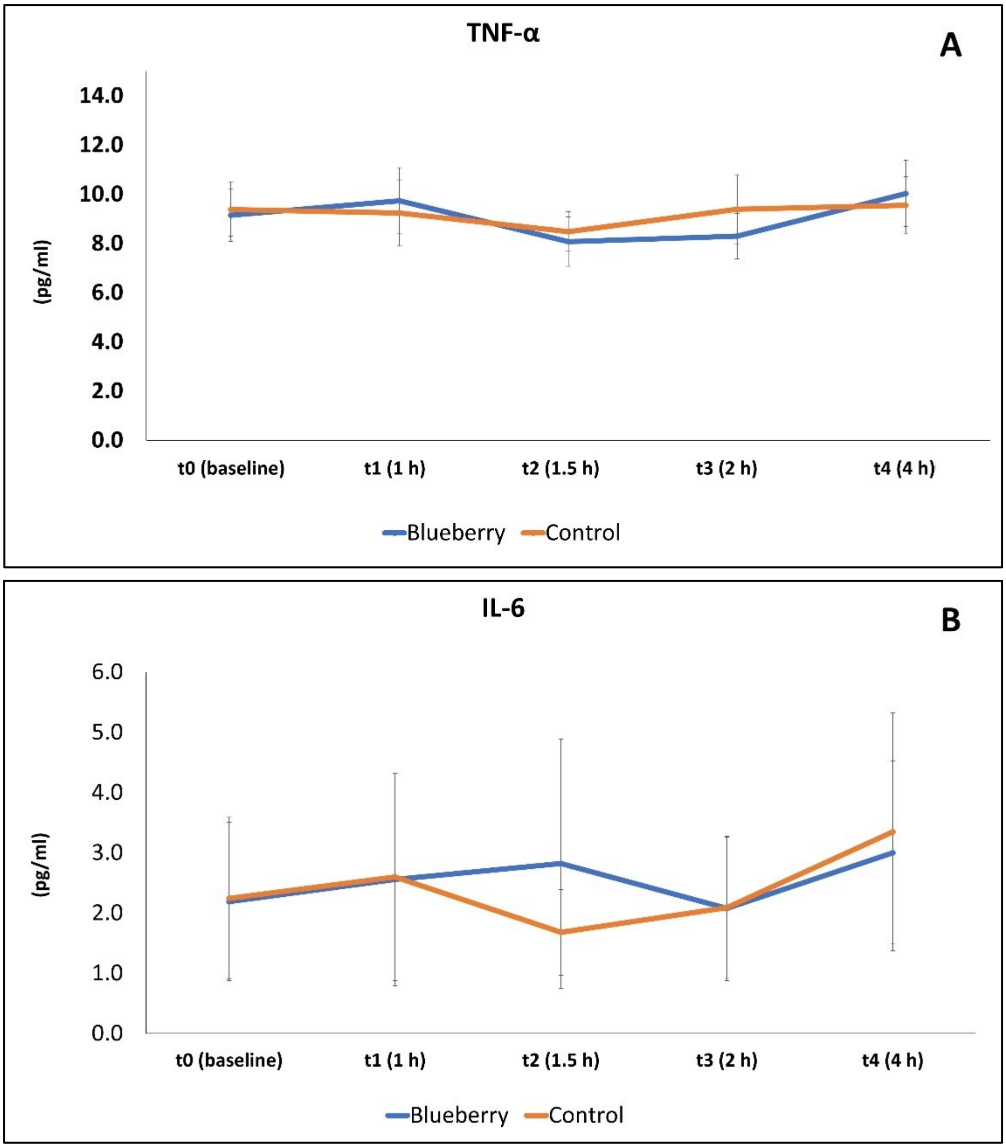
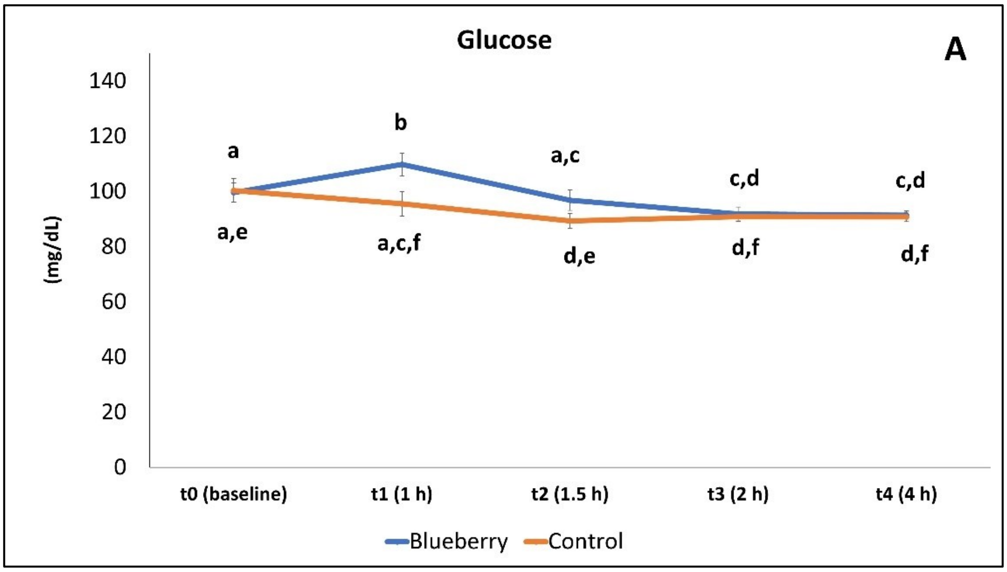


Figure 5a and 5b – Legend: Data are expressed as mean ± SEM. [a,b] Data with different letters are significantly different compared considering both time and treatments, according to post-hoc analysis.

3.6 Markers of glucose metabolism

Glucose and insulin were quantified as markers of glucose metabolism. Statistical analysis reported a significant effect of time ($p < 0.001$) and treatment *x* time interaction ($p < 0.01$) for both glucose and insulin. Levels of glucose (Fig. 6a) and insulin (Fig. 6b) temporary increased at 1 h and 1.5 h following blueberry consumption, compared to baseline and control drink, while no significant effect was observed after 2h from the consumption of the products.



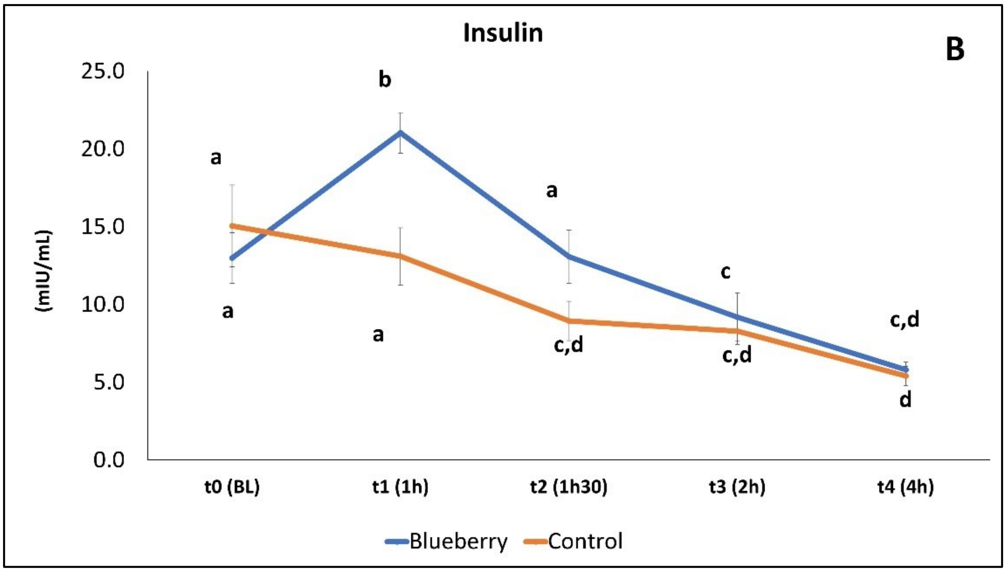


Figure 6a and 6b – Data are expressed as mean ± SEM for the two treatments over time. [a,b,c,d] Data with different letters are significantly different compared considering both time and treatments, according to post-hoc analysis.

4. Discussion

Despite consistent evidence on the effects of berries on vascular function in adult population (Martini *et al.*, 2019; Wood *et al.*, 2019), this is one of the few study that evaluated the effect of blueberry on vascular function parameters in older subjects (Tucci *et al.*, 2022). Blueberries are a rich source of different bioactive compounds, in particular anthocyanins (Krga *et al.*, 2019; Wood *et al.*, 2019) whose potential vasoactive properties have been largely investigated, and their supply chain is improving to increase yields and sustainable production (Liu *et al.*, 2021; Senger *et al.*, 2022). In this regard, our study aimed at providing data on the protective activity, in older subjects, of a specifically selected cultivar previously analyzed, Specifically, we tested the effect of an anthocyanin-rich highbush blueberry, providing around 200 mg of anthocyanins/100 g of fresh fruits, mainly constituted by delphinidin- and malvidin-3-galactoside. The cultivar used for the present study presented higher amount of anthocyanins compared to the one used for previous studies conducted by our research team (cv “Brigitta”, providing around 100 mg/100 g fresh fruits) (Del Bo′ *et al.*, 2013a; Del Bo’ *et al.*, 2014, 2017).

The role of anthocyanins in the improvement of vascular function has been widely investigated through *in vivo* and *in vitro* studies, that support their causal role through several, but still not completely elucidated, mechanisms of action (Del Rio *et al.,* 2010; Krga *et al.*, 2019; Martini *et al.*, 2019). In this regard, previous *in vitro* studies carried out by my research group found that testing five different anthocyanins (in the form of glucoside) and their metabolites (hydroxybenzoic acids) were differently active in the reduction of inflammation-induced adhesion of monocytes to endothelial cells and production of adhesion molecules (a process linked to the onset of atherosclerosis) also at physiological concentration (Del Bo’ *et al.*, 2019; Marino *et al.*, 2020). Due to the time-points considered for this study (up to 4 h for the bioavailability and 2 h for non-invasive vascular function and arterial stiffness evaluation), we focused on the assessment of the effect of anthocyanins *per se* or their precocious metabolites. In fact, anthocyanins can be absorbed intact through active or passive transportation, and reach maximum plasmatic concentration between 0,5 and 2 h (Del Rio *et al.*, 2013; de Ferrars *et al.*, 2014; Fang, 2014). Anthocyanins are also widely metabolized by both our organism and the gut microbiota after reaching large intestine, generating a wide range of metabolites, mainly represented by hydroxycinnamic acids, which peak appears in in the blood after a minimum of 2 h and up to 30 h from the consumption of an anthocyanin-rich food (Rodriguez-Mateos *et al.*, 2013; de Ferrars *et al.*, 2014; Lila *et al.*, 2016).

In the present experimental conditions, it was found an increase in post-occlusive reactive hyperemia index at 2 h after blueberry intake, compared to control product, while no significant changes were observed for arterial stiffness. These results are in line with previous acute studies performed in our laboratory after blueberry consumption in young adults. For example, in a study on a group of 16 males smokers, it was found a significant effect of blueberry at 2 h in counteracting the detrimental effects of smoking on RHI, but not arterial stiffness, compared to controls (Del Bo' *et al.*, 2014, 2017). In another study, it has been documented the capacity of blueberry to affect RHI values, but not arterial stiffness, in a group of 12 young subjects with vascular dysfunction (RHI < 1.67), while no significant effect was observed in subjects with a normal vascular function (Del Bo′ *et al.*, 2013a). Positive findings were also found by other acute studies testing the effects of freeze-dried wild blueberry (*Vaccinium angustifolium*) powder administered as drink and/or products (e.g., bakery products) on flow-mediated dilation (FMD), as marker of vascular reactivity, in a group of young healthy subjects (Rodriguez-Mateos *et al.*, 2013, 2014). For example, Rodriguez-Mateos and coworkers (2013) documented an increase in flow mediated dilation (FMD) after 1 h and 6 h from the intake of a wild BB drink (11 g of freeze-dried wild BB powder in 500 mL of water) and an increase in FMD at 1, 2, and 6 h after the intake of different BB-products (e.g., BB drink and BB bun) with a maximum effect observed at 1 and 2 h from their ingestion, respectively, in healthy subjects (Rodriguez-Mateos *et al*., 2014).

Regarding the effect on arterial stiffness, most of the studies reported not significant effect, in line with previous studies conducted by our research group (Del Bo′ *et al.*, 2013a, 2013b; Riso *et al.*, 2013) in young adults, and those reported by Dodd and coworkers (Dodd *et al.*, 2019) in healthy older adults following the consumption of a blueberry smoothie (30 g of blueberry powder providing about 500 mg of anthocyanidins). Conversely, a significant reduction in augmentation index (Aix), as marker of arterial stiffness, was reported up to 6 h from the intake of five different BB drinks (containing increased doses of freeze-dried wild BB powder). The effects were observed in healthy subjects in a time- and intake-dependent manner (Rodriguez-Mateos *et al.*, 2013).

Considering the effects on serum biomarkers of vascular function and inflammation, no differences were documented except for some biomarkers at specific time-points. For example, endothelin-1 resulted significantly lower at 1 h from blueberry consumption, giving a possible explanation for the increase in RHI, since endothelin-1 is a potent vasoconstrictor substance produced by the endothelium (Seals *et al.*, 2011; Nava *et al.*, 2019). It has also found an increase at 1.5 h of the levels of total nitrates and nitrites, an index of nitric oxide (NO) production. NO impairment is strictly connected with decreased levels of endothelial function in older subjects (Sitia *et al.*, 2010; El Assar *et al.*, 2012; Tucci *et al.*, 2022). A putative mechanism of action of anthocyanins or their metabolites at cellular level is indeed linked to NO restoration (Tucci *et al.*, 2022). Finally, it was found a significant reduction in VCAM-1 levels at 4 h from blueberry consumption, compared to controls, while no effect was found for ICAM-1. Cell adhesion molecules represent one of the link between inflammation and atherosclerosis and *in vitro* experiments found that anthocyanins and metabolites are able to lower their levels (Richter *et al.*, 2003; Asgary *et al.*, 2016; Del Bo' *et al.*, 2019).

Regarding markers of inflammation, no effect was documented for IL-6 and TNF-α. The acute effect of blueberry on vascular and inflammatory markers has been poorly investigated in vivo. Only 4 studies have analyzed the effect. Three studies have reported no significant effect (Ono-Moore *et al.*, 2016; McAnulty *et al.*, 2018; Lemay *et al.*, 2019), while one trial documented a reduction. Specifically, (Sobolev *et al.*, 2019) in a pilot trial observed an increase in TGF-β and a reduction in IL-6, but not IL-1β, TNF-α, IL-10 and IL-4, mRNAs gene expression after 2-4 h from the intake of 150 g of BB with a high fat and high glycemic load meal in subjects with Metabolic Syndrome.

When considering glucose metabolism, a significant increase in serum glucose and insulin response was documented at 1h following blueberry intake but not the control product. This temporary increase could be misleading and potentially attributed to a matrix effect. The control product was a solution of sugars in water; thus it can postulate that the peak of sugars absorption could be occurred within the first 1h of administration. Conversely the presence of fiber and other bioactive components in the blueberry, together with the physiological characteristics of the subjects (older individuals), could have induced a slow release and absorption of the sugars which result in a longer glucose and insulin response. However, at 2h from the intake (in concomitance with the analysis of vascular function), the glucose and insulin profiles were comparable between products (Curtis *et al.*, 2022). These results are in contrast with those reported in the literature. For example, Curtis *et al*. (2022) reported the capacity of 26 g freeze-dried blueberries to reduce post-prandial glucose and insulin response in metabolic syndrome subjects but after the consumption of a high energy meal. Palma and colleagues (2021) showed that 150 g of unprocessed frozen BB decreased postprandial glucose area under the curve and increased insulin levels at time 15 min in sedentary volunteers, while White *et al.* (2021) documented that the intake of 25 g freeze-dried whole wild blueberry improved glucose and insulin response within 2 h after the consumption of blueberry in middle-aged adults.

The present study shows several strengths such as the selection of a homogenous population (older subjects), the standardization of the experimental protocol, the selection of a cross-over design able to reduce inter-individual variability, the selection and analysis of a wide panel of markers of inflammation and vascular function. On the other hand, it has also some limitations related mainly to the relatively small group of individuals (n=17) and the difficulty to produce a real placebo (water and sugars). Another limitation could be the lack of data on plasma anthocyanins/metabolites and other bioactives (salicylates) to associate (possibly) with the improvement of vascular function. However, these markers are still under analysis and when available will clarify their possible contribution in the modulation observed.

In conclusion, these results support the capability of blueberry, a vegetable food associated with low environmental impacts that can potentially be exploited to optimize healthy and sustainable diets, to improve vascular reactivity in older subjects, as documented by the significant increase in RHI. This effect seems to be attributed to a modulation of endothelin-1 as marker involved in the vasoconstriction process. However, no effect was observed for arterial stiffness parameters and for most of the other serum markers of vascular function and inflammation. Further studies are required to ascertain the effects of blueberries and/or other selected plant-based foods on vascular function in older subjects and to identify the putative mechanisms of action.

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4. GENERAL CONCLUSION

In conclusion, this PhD thesis attempted to provide useful data to deepen the discussion on the multifaceted nutritional dimension that should be investigated within the context of healthy and sustainable diets developed based on the current scenario and implementing different approaches.

In the first chapter an updated overview on the actual level of inclusion of sustainability guiding principles, as defined by FAO and WHO, within global Food-Based Dietary Guidelines (FBDG), has been provided. FBDGs should condense a large body of evidence on optimal diets into appropriate actionable recommendations for the general population. Despite changes in dietary habits have been urged to contribute to the preservation of environmental resources also at consumer level, it was found that currently FBDGs are poorly aligned with FAO/WHO's sustainability principles. Given this background and considering practical approaches to address the issue of achieving a healthy and sustainable diet suitable for the whole population, an Italian/Mediterranean adaptation of the Planetary health diet of the EAT-Lancet Commission has been elaborated and its theoretical nutritional adequacy assessed. We found that this proposal, when elaborated into a more actionable scheme (the EAT-IT dietary pattern), resulted in a plant-based diet, rich in legumes and nuts, but with low amount of dairy products and meats. On principle, based on the elaborations performed on a 2500 kcal plan, the adoption of this diet should guarantee nutritional adequacy, except for vitamin D and calcium for which it is opened the discussion on the potential need of different strategies, such as the implementation of well-designed and targeted fortified foods or novel foods. Finally, starting from the data that I previously elaborated, a procedure of adaptation of the dietary plan to different energy targets has been proposed since re-elaborations can significantly affect final nutritional characteristics of the diet and its ability to cover individual needs. In addition, since these diets should be also healthy and sustainable, further investigations and approaches are required to improve knowledge about the intersection between the associated health and sustainability parameters thus minimizing the trade-offs between nourishing populations and safeguarding the environment.

In the second chapter of this thesis, the carbon footprint (CF) and water footprint (WF) associated with the EAT-IT dietary pattern was estimated using data of environmental impact for different food items, obtained from a recent well-designed database. Since among the environmental constraints the limitation of animal food products is determinant in providing the most critical nutrients, efforts were made in the evaluation of how dietary choices among different food items, within the same food category, can affect final CF and WF. We found that the EAT-IT dietary pattern, or the application of the Italian Dietary Guidelines, are associated with significantly lower CF, but not WF, compared to the average impact of the actual diet of the Italian population. Furthermore, we demonstrated that dietary choices can markedly increase these two indicators. Thus, further research is needed to identify best practice and promote the most effective messages through FBDGs. This also involves the implementation of databases with larger number of food records, and indicators of impacts, obtained through validated methodologies.

In the third chapter, I actively contributed to settle and carrying out an exploratory pilot study to assess the feasibility and efficacy of the EAT-IT dietary pattern. In this regard, evidence about healthy and sustainable diets is rapidly increasing, however, data derives almost exclusively from modelling studies, while intervention studies are lacking. Such studies could help understanding the acceptability and the actual adoption of optimized healthy and sustainable diets, as well as their effects in terms of nutritional and health benefits. Although preliminary, the results obtained have indicated a good level of acceptability and overall compliance, however, a reduction in energy intake was registered. Interestingly, a reduction of cholesterol and increase in PUFA and, specifically, ω-6 intake was revealed while for fiber intake only a trend was observed. Regarding health related-parameters they were not apparently affected following the EAT-IT intervention. Overall, further trials involving larger number of subjects are required to investigate the effects of such pattern on numerous health and behavioral outcomes.

In the fourth chapter, it has also been considered that, despite nutritional sustainability rarely focuses on bioactive compounds, nutritional research is putting a lot of efforts to integrate the importance of bioactives compounds in the biological explanation of the association of specific food/food categories intake and human health. Furthermore, the implementation of healthy and sustainable diet cannot disregard the presence of vulnerable groups having specific nutritional needs. Oder subjects are rapidly increasing worldwide, and represent a critical target in the context of dietary sustainability. Thus, an overview of the more effective plant-based foods in the prevention of the onset of cardiovascular disease in older subjects has been carried out, detailing putative mechanisms of action. Also, data corroborating the effects of anthocyanins on vascular health from *in vitro* experiments in which I actively contributed on the experimental parts have been provided. Finally, the methodology and results of a crossover randomized controlled trial, aiming to ascertain whether a selected anthocyanin-rich cultivar of blueberry can acutely improve vascular function in older subjects, has been included in this chapter. In this study we found a significant increase in vascular response, but not arterial stiffness, after blueberry consumption when compared with the results obtained following the intake of a control product. This improvement was also accompanied by a modulation of specific serum markers of vascular function (e.g., endothelin-1 reduction). These results in part reinforce the plausibility of further optimized plant-based diets with specific products, selected or processed to improve their nutritional composition including bioactives, which could provide additional benefits for specific target groups of population. Further research is needed to assess the most effective compounds, their mechanisms of action and the factors that affect individual response.

5. IMPLICATION AND FUTURE DIRECTIONS

Sustainability is nowadays considered a pivotal aspect of research, also including nutrition field. Environmental impact associated with food systems cannot be reduced at consumer level just by dietary choices, given that food production, accounting for the largest share of impact, has already occurred when it is made available to the consumers. However, to achieve a substantial transformation of food system, multiple stakeholders need to be involved, from individual consumers to all the actors in the food supply chain. In this regards, dietary habits, all together, represent the main driver that create the demand for food production, thus helping consumers to make conscious and favorable choices is mandatory. In addition, from a nutritional perspective, diet still represent one of the main modifiable risk factors for the development of chronic diseases, leading to death and disability. As also specified by the Sustainable Development Goals, the aim of human development is to improve life-quality, health, and longevity. Thus, the definition of sustainable diets cannot simply focus on lowering diet-associated environmental impact but must also aim at the maximization of health outcomes. The Planetary health diet represents an attempt to define a possible roadmap to achieve improvement in both population health and diet-associated environmental impact. In this regard, the elaboration of the EAT-IT dietary pattern allowed to assess theoretical nutritional adequacy and feasibility issues that can prevent its implementation. Furthermore, by considering the presence in the population of large share of subjects with specific nutritional needs, the evaluation of possible strategies (e.g., of adaptation) to meet these requirements seems advisable, rather than a “one size fits all” approach. The need for further research in this context is also desirable considering the expected effect of climate change on food quantity and quality potentially affecting their impact on nutritional status in the future. The data provided within this PhD thesis can be useful for defining indexes and other adaptations strategies that can contribute to the promotion of healthy and sustainable diet in the population. The work performed highlighted that methodologies to ascertain individual diet-associated environmental impacts are important as well as the improvement of current databases that are often limited to few environmental indicators. Future perspective involves the exploitation of well-designed randomized controlled trials assessing the actual feasibility of such dietary patterns and their capacity to exert positive effects on health outcomes, since scarce human studies are currently available. In this regard, data from the pilot study reported in the thesis, although preliminary, have tried to contribute to this demand. Finally, the possibility of implementing these plant-based patterns could imply the development of *ad hoc* food products, defined to address critical nutrients (e.g., in specific target population) or wisely selecting among already available foods the ones able to provide additional health benefits (e.g., considering different cultivars of crops). This goal represents a possible perspective not only for nutrition research, but also for other associated research fields, such as food technology and agronomy, whose collaboration could help defining best solutions to achieve a sustainable and healthy food environment.

6. APPENDICES

Published papers:

- Martini, D., Marino, M., Venturi, S., **Tucci, M.**, Klimis-Zacas, D., Riso, P., Porrini, M., Del Bo', C. 2022. Blueberries and their bioactives in the modulation of oxidative stress, inflammation and cardio/vascular function markers: a systematic review of human intervention studies. J Nutr Biochem. 20:109154. DOI: 10.1016/j.jnutbio.2022.109154.

- **Tucci, M.**, Marino, M., Martini, D., Porrini, M., Riso, P., Del Bo', C. (2022). Plant-Based Foods and Vascular Function: A Systematic Review of Dietary Intervention Trials in Older Subjects and Hypothesized Mechanisms of Action. Nutrients. 14 (13), 2615. DOI: 10.3390/nu14132615.

- Marino, M., Del Bo', C., **Tucci, M.**, Venturi, S., Mantegazza, G., Taverniti, V., Møller, P., Riso, P., Porrini, M. A mix of chlorogenic and caffeic acid reduces C/EBPß and PPAR-γ1 levels and counteracts lipid accumulation in macrophages. European Journal of Nutrition. 61 (2), 1003-1014. DOI: 10.1007/s00394-021-02714-w.

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Submitted papers:

- Del Bo', C., **Tucci, M.**, Martini, D., Marino, M., Bertoli, S., Battezzati, A., Porrini, M., and Riso, P. (2022). Acute effect of blueberry intake on vascular function in older subjects: study protocol for a randomized, controlled, crossover trial. Plos One (accepted: 10.1371/journal.pone.0275132)

- **Tucci, M.**, Martini, D., Marino, M., Del Bo', C., Vinelli, V., Biscotti, P., Parisi, C., De Amicis, R., Battezzati, A., Bertoli, S., Porrini, M., and Riso, P. (2022). The environmental impact of an Italian-Mediterranean dietary pattern based on the EAT-Lancet reference diet (EAT-IT). Foods (pending minor revision)

Poster presentations:

- **Tucci Massimiliano**, Cristian Del Bo', Daniela Martini, Alberto Battezzati, Simona Bertoli, Leone Alessandro, Aldini Giancarlo, and Patrizia Riso on behalf of the MIND FoodS Hub consortium. Effect of an anthocyanin-rich blueberry product on vascular function in a group of older subjects (presented at 3rd International Conference on Food Bioactives & Health 2022).

- **Tucci Massimiliano**, Daniela Martini, Cristian Del Bo', Mirko Marino, Alberto Battezzati, Simona Bertoli, Marisa Porrini, Patrizia Riso. Evaluation of potential nutritional issues associated to a 2000 kcal Mediterranean-adapted planetary diet for Italian population (EAT-IT). Nutrition, Metabolism and Cardiovascular Diseases (presented at

SINU national congress 2021). DOI: https://doi.org/10.1016/j.numecd.2021.08.023.

- Marino Mirko, **Tucci Massimiliano**, Taverniti Valentina, Riso Patrizia, Porrini Marisa, Klimis-Zacas Dorothy, Del Bo' Cristian. Role of caffeic and chlorogenic acid in the modulation of cellular fatty acid uptake. Proceedings of the Nutrition Society, 79(OCE2), E486. 2020. DOI: 10.1017/S0029665120004346.

- **Tucci Massimiliano**, Marino Mirko, Venturi Samuele, Riso Porrini, Porrini M, Del Bò Cristian. Peonidina-3-glucosie e acido vanillico riducono il processo di adesione dei monociti alle cellule endoteliali e produzione di molecole di adesione. SINU national congress 2019 (Genoa, Italy).

- Del Bo' Cristian, Marino Mirko, **Tucci Massimiliano**, Riso Patrizia, Porrini Marisa. Effect of caffeic and chlorogenic acid in the modulation of lipid accumulation in THP-1 derived macrophages. 3rd World Congress on Nutrition, Dietetics and Nutraceuticals. 2019 (Prague, Czech Republic). DOI: 10.4172/2472-1921-C1-005.

- Marino Mirko, Porrini Marisa, Tadini Jacopo, **Tucci Massimiliano**, Riso Patrizia, Del Bo' Cristian. Ruolo di acido caffeico e clorogenico nella modulazione dell'accumulo dei lipidi in un modello di aterogenesi. SINU national congress 2018 (Naples, Italy).

Participation in training schools:

- Novel approaches to the food-health relationship: from molecules to sociotypes. Lake Como School of Advanced Studies. From 19.10.2021 to 22.10.2021. Advanced school held in Como (Italy), aiming to stimulate provocative and innovative thinking and a broader view of the impact of nutritional research

- International Summer School on Food Sustainability. School of advanced studies on food and nutrition. University of Parma. First On-line Edition 1st June – 31st July 2020 (80 hours). International Summer School aiming at encompassing the environmental, socio-economical and political issues that need to be taken into account to speculate on the sustainability of agri-food systems.

- Training school Eurocaroten COST CA15136. From 10.02.2020 to 14.02.2020. Training school held in Palma de Mallorca (Spain) related to the management and proper setting of research with carotenoids.

Projects:

This PhD thesis has been developed within the context of:

i) the project “MIND FoodS HUB (Milano Innovation District Food System Hub): Innovative concept for the eco-intensification of agricultural production and for the promotion of dietary patterns for human health and longevity through the creation in MIND of a digital Food System Hub”, cofunded by POR FESR 2014- 2020_BANDO Call HUB Ricerca e Innovazione, Regione Lombardia;

ii) the project SYSTEMIC: “An integrated approach to the challenge of sustainable food systems: adaptive and mitigatory strategies to

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