UNIVERSITÀ DEGLI STUDI DI MILANO

DOCTORAL PROGRAMME IN NUTRITIONAL SCIENCES
XXXIII cycle

Role of (poly)phenols in the modulation of metabolic and functional markers: in vivo and in vitro approaches

Scientific field: MED/49

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A.A. 2019/2020

Abstract

ABSTRACT

The increase of chronic diseases, such as cardiovascular diseases, is leading to a high burden on the healthcare system. Moreover, the rise in life expectancy determined a demographic change accompanied by a boost of noncommunicable diseases and a corresponding demand for healthy aging.

Diet is a well-established modifiable lifestyle factor able to reduce the risk of chronic diseases, but also the complications associated with an overall beneficial impact on health status. In the last years, evidence has been increasing on the protective role of dietary bioactives, non-nutritive substances mainly found in plants. In particular, (poly)phenols are the most studied compounds over the last 20 years. Several clinical trials showed a protective effect of these bioactives through the modulation of different health outcomes. However, it is less clear the mechanisms through which (poly)phenols may exert their beneficial effect. This is mainly due to their wide diversity, their extensive and complex metabolism, but also to the paucity of reliable, sensitive and validated biomarkers able to bridge the gap between the intake of (poly)phenols and the different endpoints under investigation. Thus, there is a need of more evidence based nutrition able to fill this gap. Within this context, the aim of the present PhD thesis was to evaluate the role of different (poly)phenolic compounds in the modulation of several functional and metabolic biomarkers, through in vivo and in vitro approaches.

The first part of the thesis was dedicated to assessing the effect of different (poly)phenols and their metabolites on cardiovascular risk biomarkers by using two different in vitro models of atherosclerosis. Specifically, the first model was a co-culture model of human vascular endothelial cells (HUVECs) and THP-1 used to study the capacity of different (poly)phenols in counteracting THP-1 adhesion process and resolve a TNF-alpha mediated pro-inflammatory process through the modulation of different adhesion molecules. The second in vitro model consisted in a monoculture of monocytes (THP-1) derived macrophages used to study the capacity of (poly)phenolic compounds to reduce lipid accumulation (pivotal step of atherogenesis) and modulate the expression of different genes involved in lipid metabolism. The results obtained have shown the capacity of different (poly)phenols and their metabolites to reduce the THP-1 adhesion to HUVEC, the inflammatory process, including the production of adhesion molecules. In addition, (poly)phenols have shown to reduce lipid accumulation in macrophages and this reduction was accompanied by a modulation of lipid metabolism related genes.

The second part of the thesis was carried out at the Departments of Nutrition and Environmental Toxicology, University of California (Davis, USA) and devoted to investigate the role of certain phenolic compounds in the modulation of metabolic features of the adipose tissue, critically involved in the reduction of cardiovascular risk through both in vivo and in vitro models. Specifically, by using 3T3-L1 pre-adipocytes cell line, it has been studied the effect of anthocyanins and their metabolites in the promotion of mitochondrial function and differentiation from white to brown adipocytes. Furtherly, the effect of anthocyanins and metabolites was also investigated in vivo by using an animal model of C57BL/6J mice fed with a high-fat diet. The results obtained documented the capacity of anthocyanins and their metabolites to promote mitochondrial biogenesis and beiging of white adipose tissue via regulation of mitochondrial dynamics both in 3T3-L1 adipocytes cell line and in mice fed with a high fat diet.

In the third part of the thesis, the study was devoted to the evaluation of markers able to provide more insight on a novel hypothesis about the effect of (poly)phenols on intestinal permeability and related markers. Specifically, the effect of a polyphenol-rich diet (PR-diet) was evaluated on markers of oxidative stress and vascular function in a group of older

subjects with increased intestinal permeability. This last part was carried out in the context of the MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) European project, in which a positive modulation of intestinal permeability was documented. The analysis performed failed to show an effect of the intervention on markers of oxidative stress (e.g. cell resistance to DNA damage and endogenous DNA damage) and vascular function (e.g. serum levels of sVCAM-1 and sICAM-1) in this target vulnerable subjects.

In conclusion, this PhD thesis contributed to increase evidence about the beneficial effects of (poly)phenolic compounds through the exploitation of different biomarkers in in vitro and in vivo models. In particular, the in vitro studies contributed to elucidate several biological mechanisms (i.e. related to inflammation, oxidative stress and lipid accumulation/metabolism) of single metabolites and parent compounds also at physiological relevant concentrations. Finally, the studies performed on the animal model and in humans, contributed to demonstrate the potential impact of dietary polyphenols in vivo where more factors can affect and/or interfere on the final effect. The overall results seem to further support the potential benefit of these dietary bioactives even if depending on marker/outcome under study.

Future in vitro, but above all, in vivo studies should be designed to better clarify the effectiveness of (poly)phenols by considering the actual dose, their complex pharmacokinetics and extensive metabolism. However, the selection, analysis, exploitation and combination of traditional and candidate biomarkers is highlighted as a critical aspect to address.

RIASSUNTO

L'aumento delle malattie cronico, come le malattie cardiovascolari, sta portando ad un maggiore carico sul sistema sanitario. Inoltre, l'incremento dell'aspettativa di vita ha determinato un cambiamento demografico accompagnato da un'amplificazione delle malattie non trasmissibili ed una domanda sempre più crescente di linee-guida atte a promuovere un invecchiamento in salute.

La dieta rappresenta un fattore modificabile dello stile di vita capace di ridurre il rischio di malattie croniche, ma anche le complicazioni ad esse associate. Negli ultimi anni, c'è stata una crescente evidenza del ruolo protettivo dei composti bioattivi della dieta; tra questi, i polifenoli rappresentano i composti più studiati degli ultimi 20 anni. Studi clinici hanno mostrato un effetto protettivo su diversi marcatori dello stato di salute anche se tuttavia, non risultano ancora del tutto chiaro i meccanismi alla base della modulazione osservata. Questo è principalmente dovuto alla loro ampia diversità ed estensivo e complesso metabolismo, ma anche alla carenza di biomarcatori affidabili, sensibili e validati capaci di colmare il gap tra l'assunzione di polifenoli ed i diversi endpoint oggetto di studio. In questo contesto, l'obiettivo della presente tesi di dottorato è stato quello di valutare il ruolo di diversi composti fenolici su molteplici biomarcatori funzionali e metabolici, attraverso approcci in vitro e in vivo.

La prima parte della tesi ha riguardato lo studio dell'effetto in vitro di diversi polifenoli, e prodotti metabolici, su marcatori del rischio cardiovascolare, attraverso l'utilizzo di due differenti modelli di aterosclerosi. Nel dettaglio, è stato utilizzato un modello in co-coltura di monociti THP-1 e cellule endoteliali di vena ombelicale umana (HUVEC) per valutare la capacità dei composti fenolici di ridurre il processo di adesione monocitaria a seguito di uno stimolo pro-infiammatorio, come processo chiave nell'aterogenesi. Il secondo modello ha previsto il differenziamento dei monociti THP-1 in macrofagi, al fine di valutare la capacità dei composti fenolici di ridurre l'accumulo lipidico (fase cruciale della formazione della placca aterosclerotica) e modulare l'espressione di diversi geni coinvolti nel metabolismo lipidico. I risultati ottenuti hanno mostrato la capacità di diversi composti fenolici e loro metaboliti di ridurre l'adesione dei monociti THP-1 alle HUVEC, il processo infiammatorio, inclusa la produzione di molecole di adesione. In aggiunta, i polifenoli hanno mostrato di ridurre l'accumulo lipidico nei macrofagi e la riduzione è stata accompagnata da una modulazione dei geni legati al metabolismo dei lipidi.

La seconda parte della tesi è stata svolta presso il "Department of Nutrition and Environmental Toxicology, University of California, Davis" ed è stata dedicata allo studio del ruolo di alcuni composti fenolici nella modulazione metabolica e funzionale del tessuto adiposo, criticamente coinvolto nel rischio cardiovascolare, mediante l'utilizzo di modelli in vivo e in vitro. Nel dettaglio, utilizzando una linea cellulare di preadipociti 3T3-L1, è stato studiato l'effetto di alcuni antociani e loro metaboliti nella promozione della funzionalità mitocondriale e differenziamento del tessuto adiposo bianco a tessuto adiposo bruno (browning). Parallelamente, l'effetto di alcuni antociani e loro metaboliti è stato anche studiato in vivo utilizzando un modello animale di topi C57BL/6J nutriti con una dieta ricca in grassi. I risultati ottenuti hanno documentato la capacità di alcuni antociani e loro metaboliti di promuovere la biogenesi mitocondriale e il differenziamento da adipocita bianco ad adipocita bruno, attraverso la regolazione della dinamica mitocondriale, sia in un modello *in vitro* di 3T3-L1 e sia nei topi nutriti con una dieta ricca in grassi.

L'ultima parte della tesi è stata condotta nel contesto del progetto europeo MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly), nell'ambito del quale mi sono occupato di valutare l'effetto di una dieta ricca in polifenoli

su marcatori di stress ossidativo e funzione vascolare in un gruppo di soggetti anziani con aumentata permeabilità intestinale. I risultati ottenuti, hanno dimostrato un effetto di modulazione a carico della permeabilità intestinale mentre nessun effetto è stato osservato a carico dei marcatori di stress ossidativo (come la resistenza cellulare al danno al DNA indotto, e il danno endogeno) e funzione vascolare (come i livelli sierici di sVCAM-1 e sICAM-1) in questo target di soggetti fragili.

In conclusione, la presente tesi di dottorato ha contribuito ad aumentare l'evidenza riguardo l'effetto benefico di differenti composti fenolici. In particolare, gli studi in vitro hanno aiutato a chiarire alcuni meccanismi biologici legati ad infiammazione, stress ossidativo e metabolismo/accumulo lipidico. Tali effetti sono stati osservati anche a contrazioni fisiologiche sia a carico dei polifenoli che dei loro prodotti metabolici. In fine, gli studi sul modello animale e nell'uomo, hanno contribuito a dimostrare il potenziale impatto in vivo dei polifenoli della dieta anche se l'effetto era dipendente dal marker/outcome oggetto di studio.

Futuri studi in vitro, ma soprattutto, in vivo dovrebbero essere disegnati per chiarire maggiormente l'efficacia dei polifenoli, considerando la dose reale, la loro complessa farmacocinetica e l'estensivo metabolismo. Tuttavia, la selezione, l'utilizzo, l'analisi e la combinazione di biomarcatori tradizionali e candidati rimane un aspetto critico da affrontare.

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INTRODUCTION

INTRODUCTION

Food and nutritional sciences have witnessed a substantial evolution in their direction. During the last decades, the concept of conventional nutrition, focused on the achievement of nutrients requirements in order to avoid deficiencies and to allow a regular growth, has been substituted with that of optimal nutrition, oriented to the enhancement of the general health status minimizing the risk of the most common diseases. In this context, the exploitation of biomarkers is essential to study and better understand the complex relationship between diet and health (**Pande et al 2017).**

1.1 DEFINITIONS OF BIOMARKER

Despite the importance of biomarkers, univocal definition still does not exist since it depends on the specific application of the different biomarkers (e.g. *in vitro* or *in vivo* models). Consequently, this critical aspect generates confusion when these terms are used in research and clinical practice **(Califf et al 2018)**. The term "biomarker" was coined for the first time in 1989; however, only in the 1998 the National Institutes of Health Biomarkers Definitions Working Group standardized the definition as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological process, or pharmacologic responses to a therapeutic intervention*"

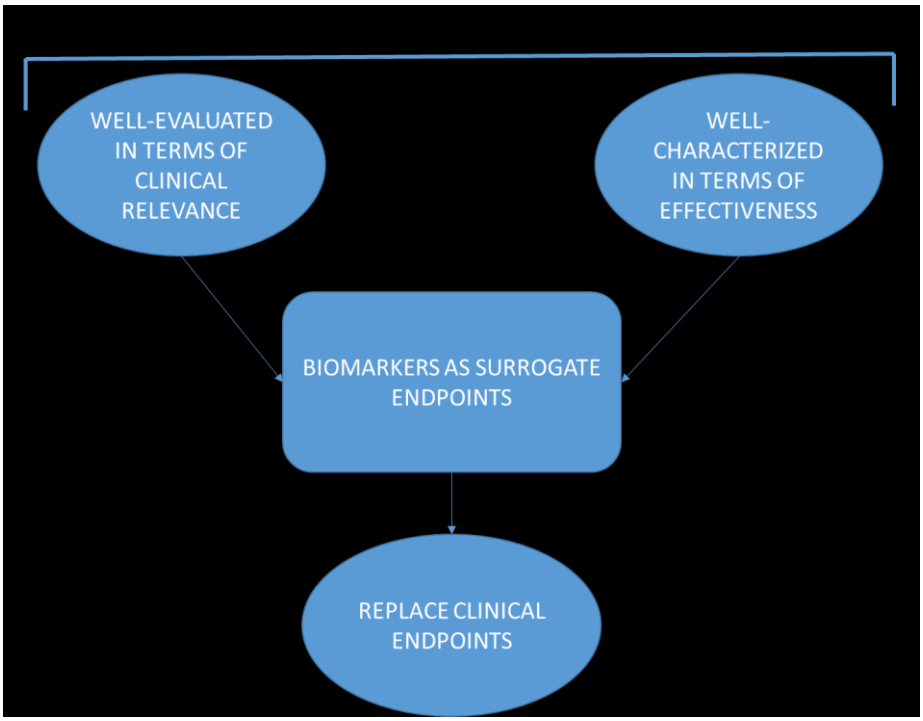
(**Biomarkers Definitions Working Group**). A further definition of biomarker was provided by Potischman and colleagues who defined a biomarker as *"any biological specimen that is an indicator of nutritional status with respect to intake or metabolism of dietary constituents. It can be a biochemical, functional or clinical index of status of an essential nutrient or another dietary constituent"* (**Potischman et al 2003**).

However, in nutrition research, biomarkers should have a wider definition since they can be identified and used to cover several aspects of relevance for nutritional science. For example, markers can be used not only to evaluate the effect of dietary interventions on physiopathological outcomes, but also to provide information on dietary intake, nutrient exposure and nutritional status. Moreover, a single biomarker could provide insights on more than one of these aspects **(Corella et al 2015)**.

Recently, in 2016, the Food and Drug Administration (FDA) and the National Institute of Health (NIH) issued the first version of the BEST (Biomarkers, EndpointS and other Tools) glossary, a continuously updated online resource to determine common definitions and to clarify terms used in translational science and medical product development **(FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource. Silver Spring (MD): Food and Drug Administration (US); Bethesda (MD): National Institutes of Health (US), www.ncbi.nlm.nih.gov/books/NBK326791/ (Last Updated: August 4, 2020).** In particular, the glossary defines a biomarker as "*a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives*." In fact, the variable that reflects how an individual feels, functions, or survives during a clinical trial is defined as clinical endpoint. Clinical endpoints represent from the study subject's perspective their health and wellbeing **(Strimbu et al 2010)**. The exploitation of biomarkers in clinical trials is relevant in order to obtain and quantify significant information providing a "proof of concept" of a treatment effect. For instance, biomarkers aimed to determine the mode of action serve as proof of mechanism. Also, biomarkers can be exploited as proof of efficacy if

they are used as clinical endpoints (**Ferber 2002**). In some cases, biomarkers can be also considered as surrogate endpoints since they can stand-in for the clinical endpoint, due to their capacity to predict the clinical outcome **(Strimbu et al 2010)**. Surrogate endpoints are biomarkers well validated from a biological and methodological point of view (**Figure 1**). Surrogate endpoints are biologically valid since they are related to the biological process that leads to the health outcome and they change with a changing of circumstances. In other words, biomarkers considered surrogate endpoints should be sensitive and specific to the health effect, thus a causal relationship should be assured. Moreover, these types of biomarkers are methodologically validated in the laboratory assessing their accuracy, precision, repeatability, reproducibility, and linear and dynamic range **(Aggett et al 2005).** However, a continuous reevaluation of the surrogate marker is necessary since not always its modification induced by a treatment predicts a true clinical endpoint. In fact, the identification of biomarkers able to be adopted as surrogate endpoints is very difficult and requires meta-analysis and large multicenter randomized intervention studies in order to have a robust statistical validation **(Buyse et al 2010)**.

Fig. 1 Identification of surrogate endpoints and their exploitation



1.2 A CHECKLIST OF BIOMARKER SELECTION AND EVALUATION

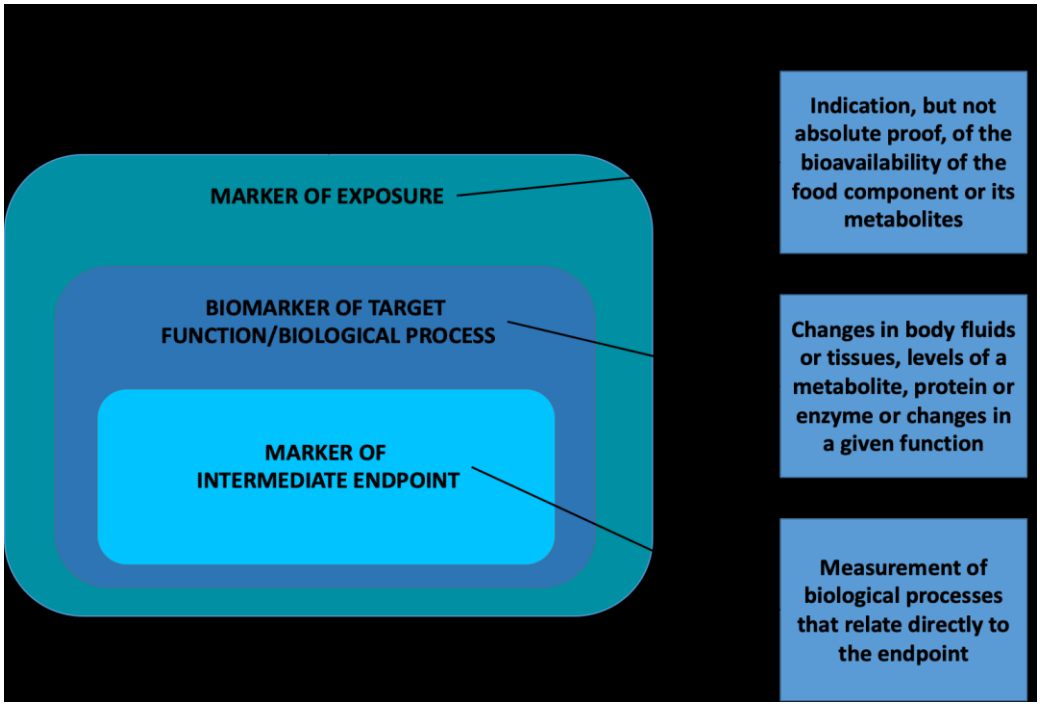
Despite the progress made, the ideal biomarker is still not defined and a general agreement for its requirement does not exist. However, De Vries and colleagues **(De Vries et al 2013)** proposed some criteria that biomarkers should satisfy:

- biomarkers should be assessed by sensitive, strong and reproducible methods. These latter should be specific and cost-effective;
- biomarkers should have a concentration in the biological sample that is sufficiently sensitive to detect minimum changes after a dietary intervention, but also that reflect the health status;
- biomarkers should enable to evaluate the responses to interventions through early detection;
- biomarkers must be specific to the intent for which they are used;
- biomarkers should be measured from biological sample that are easy to obtain;
- biomarkers should have a standard of reference, a known value in the target population that allows a correct interpretation

1.3 CLASSIFICATION OF MARKERS

In 2005, it has been published a classification of markers provided by The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) to take into consideration the relationship between markers and functional outcomes **(Figure 2)**. In particular, they were classified into: markers of exposure to the food components, markers related to the target functions or biological responses, and markers related to intermediate endpoints describing an improved health status/well-being or reduction of disease risk (**Aggett et al 2005)**.

Figure 2: Classification of biomarkers and outcomes



In the US, the FDA and the NIH **(FDA-NIH Biomarker Working Group. BEST)** classified biomarkers in different categories:

- ❖ diagnostic biomarker
  - ➢ A biomarker used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease.
- ❖ monitoring biomarker
  - ➢ A biomarker measured serially for assessing status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environmental agent.
- ❖ pharmacodynamic/response biomarker
  - ➢ A biomarker used to show that a biological response has occurred in an individual who has been exposed to a medical product or an environmental agent.
- ❖ predictive biomarker

- ➢ A biomarker used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent.

❖ prognostic biomarker

- ➢ A biomarker used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest.

❖ safety biomarker

- ➢ A biomarker measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect.

❖ susceptibility/risk biomarker

- ➢ A biomarker that indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition.

Another classification of biomarkers was provided by Potischman **(Potischman et al 2003)**, who focused on diet-related markers dividing them into two groups: biomarkers of dietary exposure and biomarkers of nutritional status. However, since in nutritional studies one of the major goal is to determine the effect of the diet on physiopathological processes, Corella and colleagues **(Corella et al 2015)** furnished an extended definition of biomarkers in nutritional studies including biomarkers of health/disease:

❖ biomarkers of dietary exposure

- ➢ Different types of biomarkers aimed at assessing dietary intake of different foods,

nutrients, non-nutritive components or dietary patterns (recovery biomarkers, concentration biomarkers, recovery biomarkers and predictive biomarkers).

- ❖ biomarkers of nutritional status
    - ➢ Biomarkers which reflect not only intake but also metabolism of the nutrient (s) and possibly effects from disease processes. It is important to note that a single biomarker may not reflect the nutritional status of a single nutrient, but may indicate the interactions of several nutrients.
- ❖ biomarkers of health/disease
    - ➢ Biomarkers related to different intermediate phenotypes of a disease or even to the severity of the disease.

In this context, it is relevant to consider also the temporal relation with dietary intake that can significantly affect a final outcome. In this regard, an additional classification of markers has been made based on this consideration **(Potischman 2003)**:

- ➢ short-term markers (markers that respond to dietary intake within hours)
- ➢ medium-term markers (markers that respond to dietary intake over weeks or months)
- ➢ long-term markers (markers that respond to dietary intake over months or years)

This classification is also of utmost importance when considered in studies evaluating the association between diet (e.g. short vs. long term interventions) and the most commonly used surrogate markers of chronic diseases such as cardiovascular diseases (i.e. oxidative stress, inflammation and vascular function markers). However, due to the complexity of the phenomenon to be studied, there is no single biomarkers available to estimate the absolute risk but it is important to define the most reliable markers depending on experimental conditions. This has been also the focus of the European Food Safety Authority (EFSA) which promoted the analysis of criteria for scientific

substantiation of health claims for food products and critically discussed the exploitation of biomarkers generally used in intervention studies.

1.4 RELEVANT BIOMARKERS USED WITHIN DIETARY INTERVENTION STUDIES

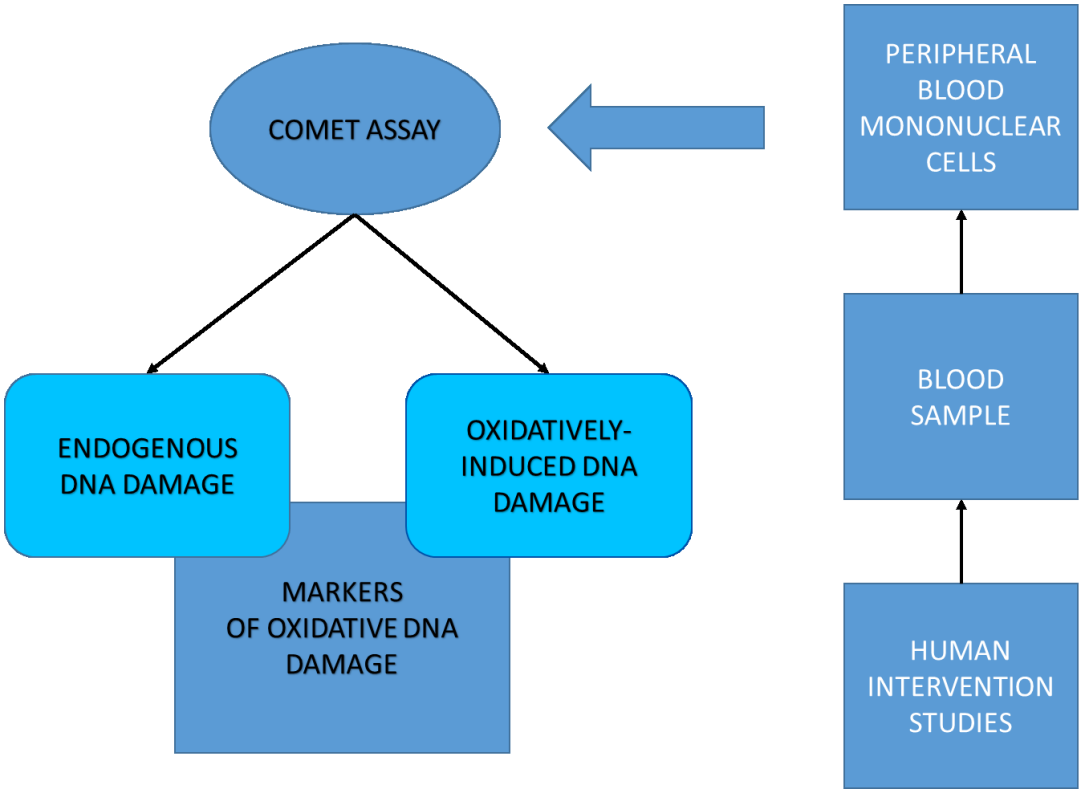
1.4.1 MARKERS OF OXIDATIVE STRESS

In 2011 the Panel on Dietetic Products, Nutrition and Allergies (NDA) commissioned by EFSA published a guidance document on the scientific requirements for health claims including *the protection against oxidative damage and cardiovascular health* **(EFSA Journal 2011;9(12):2474).** According to the panel, a marker of oxidative stress should reflect modification of body cells and molecules such as DNA, lipids and proteins. In the report, the panel considered biomarkers of oxidative damage to proteins in vivo which can be a direct measure of oxidative changes of proteins (such as oxidized –SH groups and protein tyrosine nitration products) or a measure of protein oxidation by-product (such as protein carbonyls). Concerning the oxidative damage to lipids, F2α –isoprostanes concentration is considered a direct measure in vivo since it reflects lipid peroxidation. Other biomarkers of oxidative damage to lipids in vivo are oxidized LDL particles and phosphatidylcholine hydroperoxides measured in blood. While, thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), lipid peroxides, HDL-associated paraoxonases, conjugated dienes, breath hydrocarbons, auto-antibodies against LDL particles, and ex-vivo LDL resistance to oxidation can be used as supportive evidence, but they have not been considered reliable biomarkers in vivo.

As regard oxidation of cell DNA, it has been thoroughly suggested as a good marker of a variety of stress associated factors including dietary or individual factors. The detection of oxidative damage to DNA can be measured through different techniques. For example, it can be measured *in vivo* through the modification of the comet assay, commonly using circulating lymphocytes isolated from blood as biological sample (**Figure 2**). The latter method permits the detection of oxidized DNA basis (e.g. use of endonuclease III to detect oxidized pyrimidines). The comet assay does not provide

absolute values, but it is possible to compare DNA oxidative damage with an appropriate control. On the contrary, detection of DNA strand breaks by using the traditional comet assay is considered not specific as a biomarker of oxidative stress. Another marker of oxidative damage to DNA discussed by the panel is the 8-hydroxy-2-deoxy-guanosin (8-OHdG) evaluated through HPLC – GC/MS or LC/MS - ELISA. The 8-OHdG can be measured in blood (e.g. lymphocytes), tissue (e.g. skin) and urine, and results from oxidative damage, excision repair and also oxidation of free bases or nucleotides. However, the level of free 8-OHdG in urine does not directly reflect DNA damage, but it is a biomarker of oxidative stress at the 'whole-body' level because it probably origins from removal of oxidized bases from the nucleotide pool. The most sensitive and specific methods presently available to evaluate the DNA damage are the measurement of 8-OHdG within DNA (through the formamidopyrimidine glycosylase (Fpg) enzyme-assisted comet assay) and the comet assay for single-strand breaks (**Han et al 2010**). According to the panel, markers of oxidative stress alone cannot substantiatiate such claims since they can be affected not only from the oxidative damage, but also from repairing processes. Moreover, several technical limitations can occur and impact on these markers **(EFSA Journal 2011;9(12):2474)**. However, it is emphasized that the combination of different markers of oxidative stress (and other related markers) can be considered an optimal strategy in order to get more information on the overall effect of a treatment.

Fig. 2 Biomarkers of DNA damage

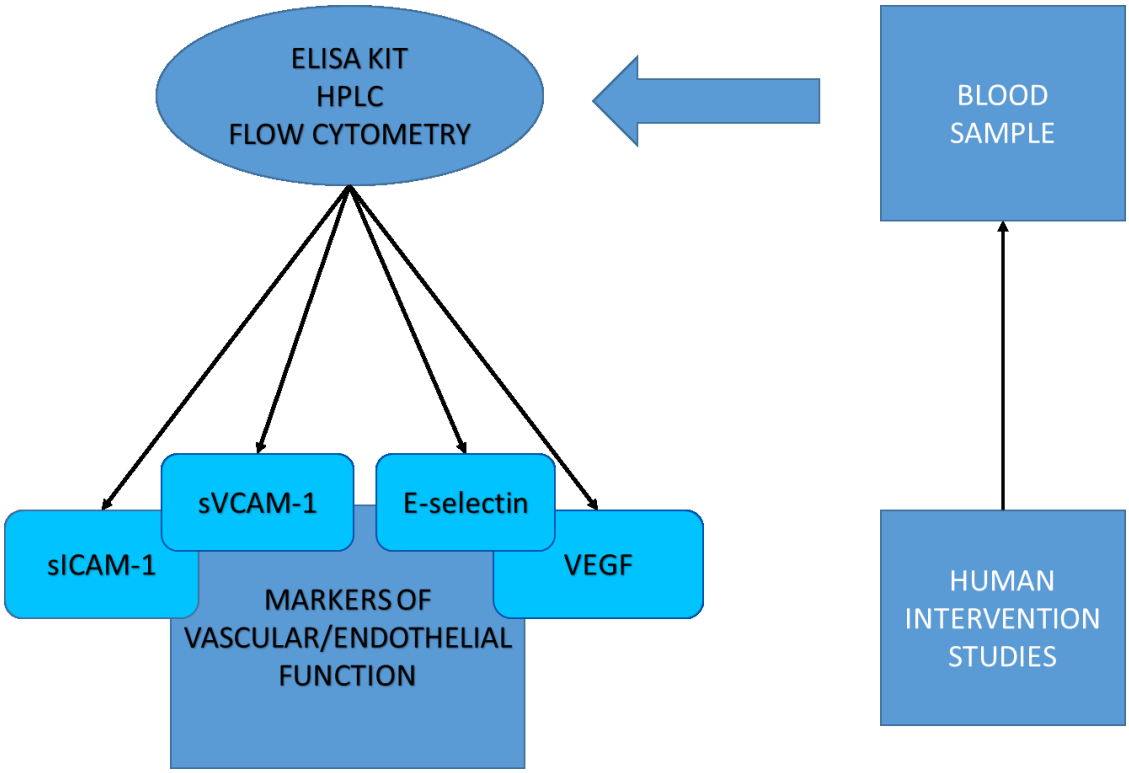


1.4.2 MARKERS OF VASCULAR/ENDOTHELIAL FUNCTION

Active factors deriving from vascular endothelium play an essential role in the modulation of several processes related to vascular system such as vasomotion, smooth muscle proliferation, thrombosis, inflammation, coagulation, fibrinolysis and oxidation **(EFSA Journal 2011;9(12):2474)**. As reported by EFSA several biomarkers of vascular/endothelial function have been used as surrogate markers to evaluate cardiovascular health. In particular, the expression of cell adhesion molecules and lipids have been reported to play a pivotal role. Among the most investigated adhesion molecules there are: intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, P-selectin and E-selectin. The concentration of cell adhesion molecules *in vivo* is commonly measured by using an enzyme-linked immunosorbent assay (ELISA) in serum samples. However, other methods can be adopted to detect levels of cell adhesion molecules, such as HPLC and flow cytometry. These molecules are involved in one of the main crucial steps of atherosclerotic plaque development

which is represented by the recruitment of circulating cells from the lumen to the interior of the vascular wall. Based on that, several studies associated the concentrations of cell adhesion molecules with the risk of cardiovascular events (**Martin-Ventura et al 2009**). However, due to the multiple functions that adhesion molecules cover in the maintenance of the vascular system, the modification in their concentration should be evaluated case by case, also based on the target population. Another biomarker whose concentrations are associated with vascular atherosclerosis is the vascular endothelial growth factor (VEGF). VEFG is a peptide involved in cell proliferation and angiogenesis that can be measured in blood sample by using commercial ELISA kits. A link between VEGF and cardiovascular diseases has been established and elevation of VEFG is associated with coronary artery disease and peripheral vascular disease (**Tsai et al 2005**).

Fig. 3 Biomarkers of vascular/endothelial function

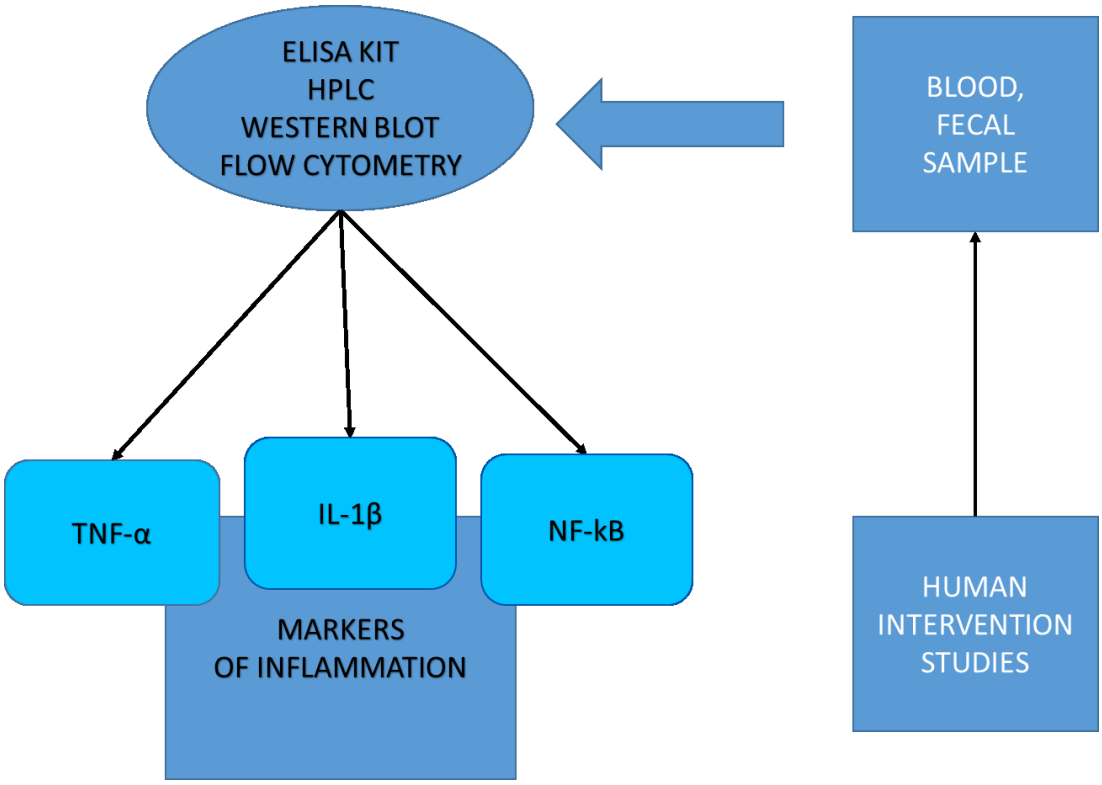


1.4.3 MARKERS OF INFLAMMATION

Among biomarkers used in dietary intervention studies there are those related to the inflammatory process. Although inflammation plays a crucial role in the protection of our organism from physical injury and infections, the chronicity of this process represents a critical step towards the development of principal chronic-degenerative diseases, such as cardiovascular disease, cancer, type 2 diabetes mellitus, neurodegenerative and autoimmune disorders (**Furman et al 2019**). A key role in the inflammatory process is covered by tumor necrosis factor alpha (TNF-α), a cytokine produced by different type of cells, including macrophages, monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts. Elevated levels of TNF-α have been found in several chronic inflammatory condition and evidence suggests this biomarker as a potential therapeutic target against the development of different chronic-degenerative diseases (**Popa et al 2007**). One of the principal molecular mechanisms of TNF-α is the induction of the nuclear factor kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) through the binding with its receptors TNFR1 and TNRF2 present on the surface of unstimulated cells (**Varfolomeev et al 2018**). One of the best characterized members of MAPK family is the extracellular signal-regulated kinase 1/2 (ERK1/2). Phosphorylation and activation of ERK1/2 promotes the inflammatory process and oxidative stress (**Sawe et al 2008**). The NF-κB pathway is a prototypical pro-inflammatory signaling pathway induced by TNF-α that in its turn leads to the expression of other pro-inflammatory genes including cytokines, chemokines, and adhesion molecules (**Lawrence et al 2009**). The phosphorylation is essential for the functioning of NF-κB and depends on the activation of IKK (IkB kinases) complex, which contains IKKα and IKKβ. Activated IKK complex determines the degradation of IkBα and phosphorylation of p65 (a NF-κB subunit), which determines a conformational change and increasing the transcriptional activity of NF-Kb (**Christian et al 2016)**. The translocation of NF-κB to the nucleus and its transcriptional activity lead to the expression of several proinflammatory mediators such as NLRP3 and IL-1β, that in their turn determine an exacerbation of the inflammatory process (**Lopez-Armada et al. 2013**). Several molecules, such as TNF-α, NF-κB, IL-1β, were used in separate dietary intervention studies as biomarkers of inflammation, assessed through different techniques (e.g. ELISA kit, western blot, flow cytometry) in different

biological samples (e.g. blood and fecal samples) (**Figure 4**) (**Jahns et al 2018**, **Vazquez-Agell et al 2011**, **Schell et al 2017**).

Fig. 4 Biomarkers of inflammation



1.5 FACTORS AFFECTING THE RELIABILITY OF BIOMARKERS

The selection of biomarkers is a critical issue in any research approach. One of the possible problems related with biomarkers regards the methodology through which they are measured. In fact, the same biomarker can be evaluated using different methods. Consequently, different laboratories could obtain different results from the same sample only due to different methods used. Moreover, even if the same method has been adopted, also the different calibration reference material could determine a different final result **(Allinson et al 2018).**

Another issue is represented by the poor specificity of many biomarkers to one disease state. It implies that very few biomarkers can be adopted as surrogate endpoints since they are not able to replace the clinical endpoint. Thus, in order to overcome this

limitation, a biomarker panel is adopted, i.e. multiple biomarkers associated with a particular condition are measured simultaneously in order to better target the actual condition. Moreover, specific statistical analysis, such as the multivariate analysis, contribute to increase confidence in the significance of change compared to the utilization of a single biomarker. Finally, different biomarkers can have different physiological variation. Therefore, also the magnitude of the change should be taken into account during the interpretation of the results **(Allinson et al 2018)**.

Another aspect regards the impact of inter-individual factors which can contribute to heterogeneous data collection and misinterpretation of results. Such factors include: sex, age, cigarette smoking, alcohol consumption, drug assumption, physical activity and other lifestyle factors. Diet is another important factor that, for instance, can affect the measurement of biomarkers, e.g. through nutrient-nutrient interactions. Also genetic polymorphisms can determine different behavior in response to nutritional intervention. Further aspects not to be overlooked are accounted for by the type of sample and its management. Evaluating the same biomarkers in different biological samples, rather than using different conditions and time of storage could lead to inaccurate values **(Rubio-Aliaga et al 2012)**.

Overall, it is noteworthy to underline how the availability of appropriate biomarkers validated in the specific context (e.g. similar or different conditions and experimental models) and adequately elaborated to obtain results on a given outcome are fundamental to elucidate the effect of diet or dietary compounds on physiopathological processes.

1.6 EXPLOITATION OF BIOMAKERS FOR THE EVALUATION OF DIETARY POLYPHENOLS ON HUMAN HEALTH

A class of food bioactives that received a great and growing interest during the last 20 years, for their potential beneficial role on human health, is certainly that of polyphenols. Phenolic compounds are abundant in several fruits and vegetables present in our diet and the most widespread classes are represented by anthocyanins (mainly content in berries), flavanols (cocoa, tea, apples and bean), phenolic acids (coffee and

several fruits), flavanones (like hesperidin in citrus fruits) and flavonol (mainly quercetin in onion, apple and tea) **(Williamson et al 2017)**.

The low oral bioavailability of phenolic compounds is one of the most important limitations that affect considerably the variability of their effect observed in clinical trials between different individuals. This latter, in its turn determines an ambiguous therapeutic effect and the incapacity to establish a daily intake recommended value. To this aim research on absorption, metabolism and bioavailability of these compounds obtained a great interest during the last two decades **(Teng et al 2019)**.

- **The role of bioavailability in the modulation of bioactivity**

Polyphenols are generally present in foods as esters, glycosides and polymeric forms. When they are linked to other molecules they are not absorbed directly, but need to be hydrolyzed through enzymatic reactions **(Chen et al 2018)**. At the level of oral cavity and gastrointestinal tract, before entering into circulation, polyphenols undergo an extensive metabolism. The initial part of absorption already occurs in the oral cavity, but to a limited extent. Most of the absorption takes place in the gastrointestinal tract **(Metere et al 2017)**. Differences in lactase-phlorizin hydrolase can determine extensive inter-individual variations (**Hostetler et al 2017**), so a different metabolism is supposed in intolerant subjects to this sugar. While different phenolic compounds, such as quercetin glucosides, naringenin, catechin and epicatechin, are transported by SGLT1 (Na^+-glucose cotransporter). Therefore, there is the inhibition of this transporter due to a competitive nature, which affects sugar absorption (glucose and fructose) determining a lower postprandial glycaemia and potential protective effect against type 2 diabetes and obesity **(Loureiro et al 2019)**. Moreover, quercetin also showed *in vivo* to inhibit GLUT2-mediated glucose transport, determining a decrease in hyperglycemia **(Song et al 2002)**. The uptake at the level of enterocyte can happen through passive, active or facilitated transportation. Once in the portal vein, phenolic compounds arrive to the liver, where they undergo a further transformation thanks to cytochrome P 450 enzymes **(Chen et al 2018)**. However, one of the main enzymes involved in polyphenols' metabolism is already present in the gut and it is the CYP3A4. Thus, polyphenols are recognized by these enzymes as xenobiotics and transformed into more hydrophilic compounds in order to be eliminated from the body via urine and

bile. In particular, through phase I enzymes a hydroxyl group is introduced to the molecule, making it available for phase II enzymes (e.g. UDP-glucuronosyltransferases, sulfotransferases), which transform the xenobiotic in the methylated, sulfated and glucuronidated form **(Basheer et al 2015)**. The mechanisms of conjugation are efficient, indeed very low concentrations of free aglycones are present in the circulation after the consumption of food. Moreover, the proportion of different conjugated forms can vary based on species and sex. Since the position and the type of conjugation affect the biological properties and activity, it is important to characterize and to study circulating metabolites **(D'archivio et al 2007)**. Once in the blood, metabolites bind to proteins, in particular to the albumin. The affinity of phenolics to this protein, that depends by the chemical structure, determines their rate of clearance and their transport to target cells and tissues. Thus, the cellular uptake of circulating conjugated could be proportional to the concentration unbound to albumin **(Dangles et al 2001; Dufour et al 2005)**. Plasmatic concentration of intact polyphenols rarely exceeds 1μM and it is affected by different factors such as the nature of polyphenol, the food source and the quantity ingested. Also, it is necessary to ingest polyphenol-rich food recurrently in order to maintain elevated circulating level of the compound if interest, since the maximum concentration is achieved after 1-2 hours after the ingestion, except for polyphenols that need to be metabolized before to be absorbed **(D'Archivio et al 2007)**.

In addition to their already complex absorption and metabolism, several factors of different nature can affect the bioavailability of dietary antioxidants such as polyphenols. Porrini and colleague **(Porrini et al 2008)** outlined these factors:

- factors related to the antioxidant
  - chemical structure, species/form, molecular linkage, concentration in foods, amount introduced, interaction with other compounds
- factors related to the food/preparation
  - matrix characteristics, technological processing, presence of positive effectors of absorption (fat, protein, lecithin), presence of negative
  - effectors of absorption (fiber, chelating agents), duration of storage
- factors related to the host

- ➢ disorders and/or pathologies, enzyme activity, gender and age, genetics, hormonal status, intestinal transit time, microflora, nutritional and antioxidant status, physiological conditions, secretion of HCl

❖ external factors

- ➢ exposure to different environments, food availability

Among all these factors, in the last period, the intestinal microbiota obtained a relevant interest. Considering that a large extent of the polyphenols ingested are not absorbed in the small intestine and reach the colon, the gut microbiota plays a pivotal role in phenolic compounds metabolism and bioavailability. Firstly, glycosides are hydrolyzed into aglycones and furtherly transformed into aromatic acids. In detail, the enzymes of the microorganisms are able to cut the heterocycle of the aglycones at different points based on their chemical structure, leading to the formation of acids which are metabolized to derivatives of benzoic acid **(D'Archivio et al 2007)**. Afterwards, these polyphenols' metabolites deriving from gut microbiome are excreted through feces, but in part are absorbed via epithelial cells of the colon and could be responsible for the protective role of dietary polyphenols. Moreover, already at the level of the colon, gut metabolites exert a beneficial activity on intestinal microbiota through the stimulation of the immune system and the activation of short chain fatty acids excretion **(Kawabata et al 2019)**. Furthermore, maintaining an appropriate gut microbiota in terms of richness and diversity will contribute to producing bioactive metabolites from dietary polyphenols, which in their turn demonstrated to have health benefits **(Marìn at al 2014)**.

- **Polyphenols and derived metabolites can share molecular mechanisms or act through peculiar effects**

According to a recent review **(Rasouli et al 2017)** polyphenols and secondary metabolites share molecular mechanisms of action and exert different activities such as anti-proliferative, anti-inflammatory and antioxidant activity. They are capable of affecting signaling pathways through the modulation of different transcriptional factors. The same review **(Rasouli et al 2017)** summarized potential biomarkers that have been used to provide evidence on their functional effects:

- ❖ Molecular target of anti-cancer activity
  - ➢ cJNK, Her2/neu, ERK1/2; AP-1, MAPK, STAT3; PKA, PKC, PTK; EGFR, VEGF, VEGFR; Bcl-XL, Ras/MAPK, JAK/STAT; NF-kB, cAMP/PKA, PEPCK; p38/MAPK, IL-6, IL-8
- ❖ Molecular target of antioxidant activity
  - ➢ ROS, RNS, NO, OH, H_2O_2, O_2.
- ❖ Molecular target of anti-obesity, anti-inflammatory and anti-hypertension activity and associated to improved cardiovascular diseases and gastrointestinal pain
  - ➢ carbohydrate digestion, glucose uptake; insulin secretion; insulin sensitivity; adipocyte differentiation, adipocyte proliferation and body weight; IRS2, PEPCK, G6Pase; CaMKII, ERK1/2, NK-kB; GLUT2/4, JAK/STAT; PPARg; MCP-1, TNF-a, adiponectin; LDL, HDL, VLDL, cholesterol; adipogenesis, lipolysis, adipokine

However, it is always more clear that there can be a compound specific response differentiating not only polyphenol parent compounds but also their endogenous and microbial derived metabolites (**Luca et al 2020**)

1.7 ROLE OF POLYPHENOLS ON PROTECTION AGAINST DNA OXIDATIVE DAMAGE IN HUMANS

Several human intervention studies observed a significant genoprotective effect exerted by polyphenol rich food and beverages. Del Bo' and colleagues reported a reduction in H_2O_2-induced DNA damage after blueberry intake in young volunteers. However, no significant effect was demonstrated for endogenous DNA after blueberries intake **(Del Bo' et al 2013)**. Another placebo-controlled, cross-over supplementation study conducted on healthy volunteers showed a reduction in DNA damage, assessed through Fpg-assisted comet assay, induced by green tea rich in polyphenolic antioxidants **(Han et al 2010)**. Bakuradze and coworkers observed a significant reduction in background and total DNA strand breaks in healthy male volunteers after 8 weeks of intervention with anthocyanin-rich fruit juice consumption, demonstrating a protective effect on DNA (**Bakuradze et al 2019**). Healthy non-smokers subjects after consumption of one

glass of dealcoholized red wine showed a reduction of H2O2-induced (300 μM, 20 min) strand breaks. However, the same intervention did not exert any effect in single cell gel electrophoresis (Comet Assay) in untreated cells. On the other hand, red wine (not dealcoholized) exerted opposite effects, demonstrating conflicting results **(Arendt et al 2005)**. Weisel et al in their intervention study provided 700 mL of anthocyanin-rich juice to 18 healthy non-smoking male for a 4-week period. After the analysis of DNA damage through comet assay, they observed a protective effect of anthocyanin-rich juice in reducing oxidative cell damage **(Weisel et al 2006)**. On the whole, the endogenous DNA damage and the DNA damage induced *ex vivo* in blood cells seem to be positively affected by intervention with polyphenol-rich products, although well-designed human intervention studies are still necessary in this direction.

Tab. 1 Effect of polyphenol-rich products on DNA damage in humans

Author	**Study Subjects**	**Intervention**	**Result**
Del Bo' et al. 2013	10 healthy male subjects (aged 20.8 ± 1.6)	300 g of blueberries Single portion	↓ H_2O_2-induced DNA damage ↔ endogenous DNA damage
Han et al. 2010	18 healthy subjects (aged 42.6 ± 3.6)	2 x 150 ml/d of 1 % (w/v) green tea 4 weeks	↓ H_2O_2-induced DNA damage ↓ endogenous DNA damage
Bakuradze et al. 2019	62 healthy male subjects (aged 24 ± 3)	750 ml/d anthocyanin-rich juice 8 weeks	↓ endogenous DNA damage
Arendt et al. 2005	78 healthy subjects (aged 27.1 ± 9)	200 ml red wine or 175 ml dealcoholized red wine 6 weeks	↓ H_2O_2-induced DNA damage

Weisel et al. 2006	18 healthy subjects (aged 27.7 ± 4.9)	700 ml/d anthocyanin-rich juice 9 weeks	↓ endogenous DNA damage

1.8 ROLE OF POLYPHENOLS ON ENDOTHELIAL FUNCTION MARKERS IN HUMANS

Epidemiological studies show an association between high polyphenol intake and protection against atherosclerosis. Also, different clinical trials demonstrated a positive modulation of endothelial function exerted by polyphenol-rich products, although data are not always consistent. Vazquez-Agell and colleagues supplementing twenty healthy males with red wine or gin for 28 days, showed a decrease of VCAM-1 and E-selectin exerted by both the beverages. However, only the red wine also reduced the levels of ICAM-1, probably due to their content in phenolic compounds compared to the gin (**Vazquez-Agell et al 2007**). Still Vazquez-Agell and coworkers, in a subsequent intervention study demonstrated the capacity of cocoa to reduce the concentrations of sICAM-1 and E-selectin, but not sVCAM-1. In particular, the intervention provided for an acute consumption of 40 g of cocoa powder in 18 healthy male volunteers (**Vazquez-Agell et al 2013**). A randomized controlled study, consisting on 4-weeks intervention with apple juice (2 glasses x 250 ml/ day), did not show any effect on endothelial function markers such as sVCAM-1 and sICAM-1 in twenty healthy volunteers (**Soriano-Maldonado et al 2014**). Also, Hodgson et al were not able to demonstrate a positive modulation of adhesion molecules concentrations after providing 5 cups/day of black tea for 4 weeks to twenty-two subjects compared to hot water (**Hodgson et al 2001**). While, another intervention study on thirtyfive reported a reduction in adhesion molecules after consumption of red wine. In particular, after 4-week intervention the levels of VCAM-1 and E-selectin were reduced, whereas the levels of ICAM-1 did not observe any significant effect compared to the control group (**Sacanella et al 2007**).

Tab. 2 Effect of polyphenol-rich products on adhesion molecules levels in humans

Author	Study Subjects	Intervention	Result
Vazquez-Agell et al. 2007	20 healthy males (aged 27.7 ± 4.9)	300 ml/d of red wine 2 weeks	↓ sVCAM-1 ↓ sICAM-1 ↓ E-SELECTIN
Vazquez-Agell et al. 2013	18 healthy subjects (aged 19-49)	40 g/d cocoa powder Acute	↔ sVCAM-1 ↓ sICAM-1 ↓ E-SELECTIN
Soriano-Maldonado et al. 2014	20 healthy subjects (aged 21-29)	2 x 250 ml/d apple juice 4 weeks	↔ sVCAM-1 ↔ sICAM-1
Hodgson et al. 2001	22 healthy subjects (aged 59.1 ± 1.6)	5 x 250 ml cups/d black tea 4 weeks	↔ sVCAM-1 ↔ sICAM-1
Sacanella et al. 2007	35 healthy women (aged 38 ± 8.5)	200 ml/d red wine 4 weeks	↓ sVCAM-1 ↔ sICAM-1 ↓ E-SELECTIN

1.9 ROLE OF POLYPHENOLS ON INFLAMMATORY BIOMARKERS IN HUMANS

The protective effect exerted by polyphenol rich foods and beverages on inflammation has been reported by several human intervention studies. A recent review regarding the effect of anthocyanins on inflammation, via the modulation of NF-kB and MAPK, concluded about the capacity of these polyphenols to reduce the levels of proinflammatory mediators and to increase the levels of anti-inflammatory molecules (**Vendrame et al 2015**). Schell et al observed a reduction of IL-1β and IL-6 levels in 17 subjects with radiographic evidence of knee osteoarthritis after 12-week intervention with 50 g of freeze-dried strawberry powder (equivalent to approximately 500 g of fresh strawberries). However, no significant effect was reported for hs-CRP after strawberry powder intake (**Schell et al 2017**). On the other hand, Ederisinghe and

coworkers did not report any significant effect on IL-1β and TNF-α levels after an acute intervention with strawberry drink (10 g strawberry powder containing 81.65 mg of anthocyanins) in twenty-six healthy subjects. However, the same intervention was able to reduce the levels of hs-CRP (**Ederisinghe et al 2011**). Similarly, in another clinical trial, the consumption of a portion (150 g) of blueberries failed to affect hs-CRP levels in subjects with metabolic syndrome, while was able to reduce the levels of IL-6 mRNA (**Sobolev et al. 2019**). **Basu and colleagues (2011)** did not show any modifications in the levels of IL-1β and TNF-α following 8-week supplementation with green tea (4 cups/day) or green tea extract (2 capsules and 4 cups water/day) in obese subjects with metabolic syndrome. Finally, **Vàzquez-Agell and colleagues (2013)** reported that administration for 3 weeks of 40g of cocoa powder (containing 28.2 mg of (-)-epicatechin) dissolved in 250 mL of whole milk or water were able to decrease NF-kB activation in healthy volunteers.

Tab. 3 Effect of polyphenol-rich products on inflammation in humans

Author	Study Subjects	Intervention	Result
Schell et al. 2017	17 subjects with radiographic evidence of knee osteoarthritis (aged 57 ± 7)	50 g of freeze-dried strawberry powder (equivalent to approximately 500 g of fresh strawberries) reconstituted in water (twice a day). 12 weeks	↓ IL-1β ↓ IL-6 ↔ hs-CRP
Vazquez-Agell et al 2013	18 healthy subjects (aged 19-49)	40 g of cocoa powder 3 weeks	↓ NF-kB
Ederisinghe et al. 2011	26 healthy subjects (aged 50.9 ± 15)	Strawberry drink (10g strawberry powder) Acute	↔ IL-1β ↔ TNF-α ↓ IL-6 ↓ hs-CRP
Sobolev et al. 2019	5 subjects with	150g blueberry	↔ IL-1β

	metabolic syndrome (aged 26-61)	Acute	↔ TNF-α ↔ IL-10 ↔ IL-4 ↓IL-6 mRNAs
Basu et al 2011	35 obese subjects with metabolic syndrome (aged 42.5 ± 1.7)	green tea (4 cups/day) or green tea extract (2 capsules and 4 cups water/day) 8 weeks	↔ IL-1β ↔ IL-6 ↔ CRP

However, multiple aspects make it difficult to accurately determine the magnitude of the effect played by polyphenols in counteracting the onset or the progression of the condition on which they are tested **(Spencer et al 2007)**. Among the limitations that complicate the interpretation of results deriving from studies on polyphenols there is the great diversity of content between different foods. Moreover, for some foods there is not an adequate characterization in terms of polyphenols' content reported in main databases. Additionally, the common tools used to estimate the food intake suffer from several limitations that furtherly reduce the capacity to establish the amount of polyphenols assumed. To make it even more complex, there is the fact that phenolic compounds have a challenging metabolism and absorption, that up to date it is not completely elucidated.

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AIM OF THE STUDY

Aim of the study

The general aim of the present PhD thesis was the study of biomarkers helpful to investigate the role of the diet on health status, elucidating the molecular mechanisms of action underlying their effect. In particular, it was carried out using a specific class of dietary bioactive compounds widely diffused in plant-derived foods, which is represented by polyphenols. In order to achieve the objective, phenolic compounds were tested as individual compounds, extracts and whole food on several biomarkers, moreover on different models, both *in vitro* and in *vivo* (Table 1).

The objective of the individual chapters is briefly reported in the following list:

- Chapter 1
  - to assess the effect of different polyphenols and their metabolites on cardiovascular risk biomarkers via two separate *in vitro* models of atherosclerosis.
- Chapter 2
  - to evaluate how a high fat diet can modulate the browning of white adipose tissue and mitochondria dynamics, using an anthocyanins-rich extract in mice model and individual anthocyanins and their metabolites in 3T3-L1 adipocytes.

- Chapter 3
  - to investigate the effect of a polyphenol-rich diet on markers of oxidative stress and inflammation in older subjects with increased intestinal permeability: the MaPLE project.

Tab. 1 Summary of the main polyphenols and biomarkers under study

EXPERIMENTAL MODEL	POLYPHENOL	MECHANISM	BIOMARKER
IN VITRO			
THP-1/HUVECs co-culture	Anthocyanin-rich fraction, single anthocyanins (Cy, Dp, Peo, Pet and Mv-3-glc) and related metabolites (PrA, GA, VA, SA and MetGA)	Monocytes adhesion to vascular endothelial cells	E-selectin; VCAM-1; ICAM-1; VEGF
THP-1 derived macrophages	Chlorogenic acid and caffeic acid as main dietary polyphenols	Lipid accumulation into macrophages	PPAR-γ1; C/EBPβ
3T3-L1 derived adipocyte-like cells	Single anthocyanin (Cy and Del-3-glc) and related metabolites (PCA, HB, GA)	Browning (differentiation of white adipocytes into brown adipocyte) and mitochondrial function NLRP3 inflammasome and inflammatory cascade activation	PPAR-γ; PRDM-16; PGC-1α; UCP-1 NLRP-3; Caspase-1; IL-1β; pIKK; pp65; pERK
ANIMAL MODEL			
C57BL/6J	Anthocyanin-rich extract (mainly Cy and Del)	Browning and mitochondrial function	pDRP-1; Fis1; MFF; OPA1; MFN-2; PINK-1; Parkin; Nix; citrate synthase; ATP5B; COX IV; PRX1; PPAR-γ, PRDM-16; PGC-1α; UCP-1
HUMAN INTERVENTION STUDY			
Older subjects	Polyphenol-rich diet (mainly rich in flavonoids and phenolic acids)	Oxidative stress Vascular function	DNA damage (8-oxo-7,8-dihydroguanine) VCAM-1; ICAM-1

Cy, cyanidin; Del, delphinidin; Peo, peonidin; Pet, petunidin, Mv-3glc, malvidin-3-glucoside; PCA, protocatechuic acid; HB, 4-hydroxybenzaldehyde; GA, gallic acid; VA, vanillic acid; MetGA, methyl-gallic acid; SA, syringic acid

CHAPTER 1

3. Effect of berries' polyphenols and their metabolites on atherosclerosis biomarkers: an *in vitro* approach

In this chapter, the effect of several polyphenols and their metabolites on markers of inflammation and vascular function has been investigated through the exploitation of different cell culture models. In particular, two studies describe the capacity of anthocyanins and their metabolites to reduce the inflammatory response and the production of cellular adhesion molecules by using a co-culture (monocytes and umbilical vein endothelial cells) model. The third study, provides evaluates the ability capacity of select phenolic acids (i.e. chlorogenic and caffeic acid) to decrease lipid accumulation in THP-1-derived macrophages and to regulate the expression of the main genes involved in such modulation.

Original article
3.1 Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin
and adhesion of monocytes to endothelial cells
(Post-print of published paper; DOI: 10.1016/j.cbi.2019.01.002)

Original article
3.2 Modulation of Adhesion Process, E-selectin and VEGF Production by Anthocyanins and Their Metabolites in an *in vitro* Model of Atherosclerosis
(Post-print of published paper; DOI: 10.3390/nu12030655)

Original article
3.3 A Mix of Chlorogenic and Caffeic Acid Counteracts Lipid Accumulation and Downregulates C/EBPß and PPAR-γ1 Gene Expression in Macrophages
(Post-print of submitted paper)

3.1 Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin and adhesion of monocytes to endothelial cells

Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin and adhesion of monocytes to endothelial cells

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RUNNING TITLE: Anthocyanins and metabolites reduce inflammation

ABBREVIATIONS: ACN-RF, anthocyanin-rich fraction; Cy-3-glc, cyanidin-3-glucoside; Dp-3-glc, delphinidin-3-glucoside; GA, gallic acid; HUVEC, humbelical vein endothelial cells; Mv-3-glc, malvidin-3-glucoside; PrA, protocatechuic acid; SA, syringic acid; THP-1, human monocytic cells; TNF-α, tumor necrosis factor-alpha;VCAM-1, vascular cell adhesion molecule-1.

KEYWORDS: anthocyanins; metabolites; E-selectin; VCAM-1; cell culture; atherogenesis

FINALCIAL SUPPORT: This work was supported by a contribution of the "Piano di sostegno alla ricerca- Linea 2, azione A- grant number PSR2017-CDELB"

ABSTRACT

This study investigated the capacity of an anthocyanin-rich fraction (ACN-RF) from blueberry, single anthocyanins (cyanidin, delphinidin and malvidin-3-glucoside; Cy, Dp and Mv-3-glc) and related metabolites (protocatechuic, gallic and syringic acid; PrA, GA and SA) to resolve an inflammation-driven adhesion of monocytes (THP-1) on endothelial cell (HUVECs) and secretion of cell adhesion molecules E-selectin and vascular cell adhesion molecule 1 (VCAM-1). The adhesion of THP-1 to HUVECs was induced by tumour necrosis factor α (TNF-α, 100 ng mL^{-1}). Subsequently, ACN-RF, single ACNs and metabolites (from 0.01 to 10 μg mL^{-1}) were incubated for 24 h. The adhesion was measured in a fluorescence spectrophotometer. E-selectin and VCAM-1 were quantified by ELISA. No toxicological effects were observed for the compounds and the doses tested. ACN-RF and Mv-3-glc reduced THP-1 adhesion at all the concentrations with the maximum effect at 10 μg/ml (-60.2% for ACNs and-33.9% for Mv-3-glc). Cy-3-glc decreased the adhesion by about 41.8% at 10 μg mL^{-1}, while PrA and GA reduced the adhesion of THP-1 to HUVECs both at 1 and at 10 μg mL^{-1} (-29.5% and -44.3% for PrA, respectively, and -18.0%and -59.3% for GA, respectively). At the same concentrations a significant reduction of E-selectin, but notVCAM-1 levels, was documented. No effect was observed following Dp-3-glc and SA supplementation. Overall, ACNs and metabolites seem to resolve, in a dose-dependent manner, the inflammation-driven adhesion of THP-1 to HUVECs by decreasing E-selectin concentrations. Interestingly, Mv-3-glc was active at physiologically relevant concentrations.

INTRODUCTION

Anthocyanins (ACNs) are a group of abundant and widely consumed flavonoids providing the red, blue, and violet colours in fruit- and vegetable-based food products. The dietary intake of ACNs is up to 9-fold higher than that of other dietary flavonoids. Epidemiological studies have found an inverse association between the consumption of ACNs and risk of cardiovascular diseases [1-6]. Their role in prevention of cardiovascular disease is strongly linked to the protection against oxidative stress and inflammation [7-10]. Atherosclerosis is the main underlying cause of cardiovascular disease in humans. The early stage, i.e. atherogenesis, is characterized by activation of endothelial cells to express cell adhesion molecules and recruit monocytes. This process is identical to the vascular responses to tissue inflammation, which resolves when the underlying cause of inflammation (e.g. an invading infectious agent) has been removed. However, the prolonged inflammatory milieu in early atherosclerotic foci stimulates the transformation of monocytes foam cell [11].

It has been shown that ACNs prevent endothelial cell dysfunction by modulating the expression and activity of several enzymes involved in nitric oxide production [12-13]. Furthermore, recent evidence suggests that ACNs can down-regulate the expression of adhesion molecules and prevent the adhesion of monocytes to endothelial cells challenged by pro-inflammatory cytokines [12;14]. The absorption of ACNs is low (<1%), but most of them are rapidly transformed by human gut to metabolic products, reaching a plasmatic concentration much higher than that of parental ACNs, indicating their contribution in the biological activity observed should be considered [15]. We have reported that ACNs and phenolic acid-rich fractions from a wild blueberry powder counteracted the adhesion of monocyte to endothelial cells in a pro-inflammatory milieu [16]. In the same study, single ACNs and certain gut metabolites (delphinidin-3-glc and gallic acid) prevented the attachment of monocytes to endothelial cells, while malvidin-3-glc and syringic acid exacerbated the adhesion process [16].

In the present study, we investigated the capacity of the same ACNs to resolve an inflammatory process by reducing the adhesion of monocytes to activated endothelial cells and the production of vascular adhesion molecules as potential mechanisms in the atherogenesis. To this end, monocytic (THP-1) cells were cultured with human

umbilical endothelial cells (HUVECs) in the presence of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) to promote the expression of cell adhesion molecules and interaction between the cells. TNF-α is produced by immune cells and it stimulates endothelial cells to express adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) as well as chemokines (i.e. interleukin-8 and monocyte chemoattractant protein-1) that promote the recruitment of monocytes to inflamed luminal endothelium and induce their adhesion to endothelial cells at the site of activation [17]. The expression of E-selectin occurs early following stimulation of pro-inflammatory cytokines such as TNF-□ in endothelial cells (4 and 6 h after stimulation and remains elevated up to 24 h) [18]. E-selectin mediates the initial attachment of free-flowing leukocytes to the arterial wall, while the expression of VCAM-1 provides a stronger interaction between leukocytes and endothelial cells and mediates the transmigration of the cells into the tissue [18-19].Cytokine-induced expression, and subsequent down-regulation after cessation of exposure, in endothelial cells occurs later for VCAM-1 than E-selectin [20]. We assessed the production of E-selectin and VCAM-1 to cover this "early" and "late" phase of the endothelial production of cell adhesion proteins.

MATERIALS AND METHODS

Reagents

Standard of cyanidin, delphinidin and malvidin-3-glucoside (Cy, Dp and Mv-3-*O*-glc) were obtained from Polyphenols Laboratory (Sandes, Norway), while those of gallic, protocatechuic, and syringic acid (GA, PrA and SA) from Sigma-Aldrich (St. Louis, MO, USA). Human Endothelial Cells Basal Medium and Human Endothelial Cells Growth Supplement were purchased from Tebu-Bio (Magenta, MI, Italy). Hanks balanced salt solution, foetal bovine serum (FBS), TNF-α were from Sigma-Aldrich (St. Louis, MO, USA). Gentamin, RPMI-1640, HEPES, Sodium Pyruvate, trypsin-EDTA were from Life Technologies (Monza Brianza, MB, Italy)while the 5-Chloromethylfluorescein Diacetate (CellTrackerTM Green CMFDA) from Invitrogen (Carlsbad, CA, USA).Hydrochloric acid and methanol were purchased from Merck (Darmstadt, Germany), while water was obtained from a Milli-Q apparatus (Millipore, Milford, MA).

Preparation and characterization of the ACN-rich fraction, single anthocyanins and metabolites

The extraction of the ACN-rich fraction from a wild blueberry powder (Future Ceuticals, Momence, IL, USA)was performed as reported by Del Bo' et al. [16]. The fraction was characterized for the content of ACNs, phenolic acids as well as other bioactives as previously published [16]. The total ACN content was 45.11 ± 0.35 mg mL^{-1} and constituted predominantly of Mv-3-glc (about 26%), Mv-3-gal (15%) followed by Dp-3-glc (9%) and Petunidin-3-glc (8%). No phenolic acids or other bioactives were detectable.

Lyophilized standards of Mv, Cy, Dp-3-*O*-glc (native compounds) and SA, PrA and GA (corresponding metabolites) are shown in Figure 1. The standards were prepared as previously reported [16]. These single compounds were tested since found in the blood stream of volunteers after consumption of a blueberry portion [21].

Cell culture and viability

Human umbilical vein endothelial cells (HUVECs; Tebu-Bio SrL, Magenta, MI, Italy) were cultured in endothelial cell growth medium kit containing 2% serum at 37°C and 5% CO_2 until reaching confluence (generally after 1 week). THP-1 cells were grown in a complete RPMI cell media (RPMI-1640 medium supplemented with 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin, and 10% FBS at 37 °C and 5% CO_2 and maintained in culture for up to 3 months.

Cell viability was performed for each compound and concentration by Trypan blue and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, showing cells viability above 90% as previously published [16].

THP-1 adhesion to HUVECs

An aliquot of $2x10^4$ HUVECs was seeded on 0.1% gelatine pre-coated 96-well black plate and maintained at 37°C and 5% CO_2 for 24h.Subsequently, monocytic ($2x10^6$) THP-1 cells (American Type Culture Collection, Manassas, VA, USA) were re-suspended in 1 mL serum free RPMI cell medium (containing 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin) and labelled with CellTrackerTM Green CMFDA (1 μM, 30 min at 37°C and 5% CO_2). THP-1 were washed twice, re-suspended in HUVEC

medium ($2x10^5$ cells mL^{-1}density) and added to HUVECs with TNF-α (100 ng mL^{-1}). After 24 h incubation (37°C, 5% CO_2) medium was removed and 200 µL of new medium, containing the single ACNs (Mv, Cy and Dp-3-glucoside) and their corresponding metabolites (SA, PrA and GA, respectively) was added at the concentrations of 0.01, 0.1, 1 and 10µg mL^{-1} for 24 h at 37°C and 5% CO_2. Then, media was collected and stored at -80°C until analysis. Cells were rinsed twice before the measure of the fluorescence (excitation: 485 nm, emission: 538 nm; mod. F200 Infinite, TECAN Milan, Italy). The level of fluorescence is associated with the number of labeled-THP-1 cells attached to the HUVECs. The results derive from three independent experiments in which each concentration was tested in quintuplicate. Data are reported as fold increase compared to the control cells without stimulation with TNF-α or bioactive compounds.

Visualization at the microscope

The adhesion of THP-1 to HUVECs was visualized at the microscope. HUVEC ($4x10^4$/well) were seeded onto 0.1 % gelatin pre-coated 12-well plate for 24 h. THP-1 ($8x10^4$/well) were stained with CellTrackerTM Green CMFDA and added with TNF-α to HUVECs as previously reported. After treatment, cells were rinsed with Hank solution in order to remove the non adherent cells and inspected with an inverted wide-field microscope with 10 × magnifications.

Determination of soluble VCAM-1 and E-selectin concentration in cell supernatant

The concentrations of soluble VCAM-1 and E-selectin, in recovered cell culture supernatants, were quantified by ELISA kits according to the manufacture's instruction. The analyses were conducted in quadruplicate and the results derived from three independent experiments.

Statistical analysis

One-way ANOVA was applied to verify the effect of the different concentrations of ACNs and metabolites on fold increase THP-1 adhesion to HUVECs and on percentage changes in soluble VCAM-1 and E-selectin concentration. Differences between treatments was assessed by the Least Significant Difference (LSD) test with $p<0.05$ as level of statistical significance. Results are reported as mean ± standard error of mean. The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Effect of ACN-rich fraction on monocytes adhesion process

In **Figure 2** are reported **t**he effects of ACN-RF on THP-1 adhesion to HUVECs. There was a significant increase in THP-1 cell adhesion to HUVECs following stimulation with TNF-α ($p<0.0001$), while the incubation with ACN-RF significant reduced the process ($p<0.0001$) at all the concentrations tested (from 0.01 to 10 μg mL^{-1}).The maximum effect of reduction was observed at 10 μg mL^{-1} (-60.2%) with respect to the control with TNF-α.

Effect of anthocyanins and metabolic products on monocytes adhesion process

Figure 3 (A-C) shows the results on THP-1 adhesion to HUVECs after incubation with the single ACNs. The incubation with Mv-3-glc significantly decreased ($p<0.0001$) the adhesion of monocytes to HUVECs at all the concentrations tested (from 0.01 to 10 μg mL^{-1}) compared to TNF-□ (**Fig. 3A**). The maximum reduction was observed for the concentration at 10 μg mL^{-1} (-33.9%; $p<0.0001$) as also reported in **Figure 4** that shows the adhesion of labelled THP-1 to endothelial cells following 24 h stimulation with TNF-α (A), TNF-α + 10 μg/mL Mv-3-glc (B) and control (C). Regarding Cy-3-glc, a significant reduction in the adhesion of THP-1 to HUVEC was observed only at 10 μg mL^{-1} (-41.8%; $p<0.01$) (**Fig. 3B**), while no significant effect was found for Dp-3-glc (**Fig. 3C**).

Figure 5 (A-C) reports the results on THP-1 adhesion to HUVECs after incubation with SA, PrA and GA (metabolites of Mv-3-glc, Cy-3-glc, and Dp-3-glc, respectively). No significant effect was observed following SA supplementation (**Fig. 5A**) in line with the results reported in **Fig. 4** that shows the adhesion of labelled THP-1 to endothelial cells following stimulation with TNF-α + 10 μg/mL SA (D). The supplementation with PrA (**Fig. 5B**) and GA (**Fig. 5C**) significantly decreased the adhesion of monocytes to endothelial cellsat 1 μg mL^{-1} (-18.0%; $p<0.05$ for GA, -29.5%; $p<0.05$ for PrA) and 10 μg mL^{-1} (-59.3%; $p<0.001$ for GA, -44.3%; $p<0.01$ for PrA) compared to TNF-□.

Effect of anthocyanins and metabolic products on soluble E-selectin and VCAM-1 levels in cell supernatant

Table 1 shows the levels of E-selectin quantified in the cell supernatant following incubation with ACNs and metabolites. There was a significant increase in E-selectin following stimulation with TNF-□ compared to negative control (without TNF-□). The incubation of cells with Mv-3-glc significantly reduced ($p<0.001$) the levels of E-selectin at all concentrations tested. This reduction was not concentration dependent and the maximum effect was observed at 0.01 and 0.1 μg mL^{-1} (-66% and -67%, respectively). Cy-3-glc reduced the E-selectin concentration at 10μg mL^{-1} (-72%; $p<0.01$), PrA at 1 and 10 μg mL^{-1} (-74 and -76%; $p<0.001$, respectively), and GA at 1μg mL^{-1} (-34%; $p<0.01$) and 10 μg mL^{-1} (-40%; $p<0.01$). No effect was found after Dp-3-glc and SA incubation in line with the lack of the positive effect on the adhesion of THP-1 to HUVECs.

The levels of VCAM-1 quantified in the cell supernatant following incubation with ACNs and metabolites are reported in **Table 2**. There was a significant increase ($p<0.05$) following stimulation with TNF-□ compared to negative control (without TNF-□). However, no significant effect was observed following incubation with ACNs and gut metabolites.

DISCUSSION

Chronic inflammation is a common factor in endothelial dysfunction and atherosclerosis [11;22]. Different cell models have been used to assess the interaction between endothelial cells and monocytic cell lines (e.g. THP-1, U937, MonoMAC) or freshly isolated leukocytes as early event in atherosclerosis. We obtained a two-fold increase in attachment of THP-1 cells to HUVECs which is in line with earlier observations with the same co-culture [23-24]). The TNF-induced attachment of monocytic U937 cells to endothelial cells seems to be in the range of a 2-3-fold increase [25-26], whereas MonoMAC cells may have higher sensitivity and response to TNF-mediated adhesion to HUVECs (i.e. 6-fold increase at 10 μg/mL TNF-□) [27] Poussin 2014).

In the last years, several studies have focused on the mechanisms through which polyphenols modulate the adhesion process and the vascular inflammation [28-29]. Here we evaluated the capacity of Mv, Cy, and Dp-3-glc, and corresponding metabolites, to resolve an inflammation-driven adhesion of THP-1 to HUVECs and the production of vascular adhesion molecules. The results obtained documented that ACN-RF and Mv-3-glc had an effect at all the concentrations tested, while Cy-3-glc, GA and PrA resolved the adhesion process only at the high concentrations (1 and 10 μg mL^{-1}). These findings are in contrast with those documented in a previous experiment, in which Mv-3-glc led to an exacerbation of the adhesion process, while Cy and PrA failed to affect the interaction between monocytes and endothelial cells [16]. In light of our results, we hypothesize that these compounds are more active in resolving than preventing the adhesion process. *In vitro* studies reported a beneficial effect on the prevention of atherogenesis only at supra-physiological concentrations in according with our findings [25-33]. However, recent *in vitro* studies showed a positive effect of ACNs, phenolic acids and gut metabolites also at physiological relevant concentrations [34-35]. For example, Kraga et al., [35] reported that Cy-3-glucoside, galattoside and arabinoside, as well as Dp and Peondin-3-glucoside and phenolic acids/gut metabolites (vanillic acid, ferulic acid, hippuric acid, 4-hydroxybenzaldehyde and PrA) decreased the adhesion of monocytes to HUVECs from 0.1 to 2 μM. The effect was also confirmed when ACNs and phenolic acids were used as a mix, suggesting an additive effect of the compounds.

In our experimental conditions, the reduction of adhesion of THP-1 to TNF-α-activated HUVECs after supplementation with ACNs and metabolites can be attributed to different non-specific and/or specific complex mechanisms of action. Further insight into the mechanisms can be gained by high content screening and transcriptomics of inflammatory and oxidative stress pathways as used in co-culture studies of monocytes and HUVECs [36]. Inhibition of NF-κB activity could have reduced the synthesis of numerous cytokines by decreasing the levels of inflammation at endothelial level. In this regard, the inhibition of pro-inflammatory cytokines such as TNF-□ and the reduction of leukocyte adhesion to endothelial cells are key mechanisms in the control of atherogenesis and atherosclerosis. Moreover, ACNs have a pivotal role in the modulation of mitogen-activated protein kinase pathways implicated in several cellular processes including proliferation, differentiation, apoptosis, cell survival, cell motility, metabolism, stress response and inflammation [8]. Alternatively, the use of ACNs and phenolic acids may repress the secretion of chemokine (C-C motif) ligand 2 (MCP-1), which pilots the migration of monocytes toward the intracellular cleft between adjacent endothelial cells, or reduce the production of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin that regulate the recruitment of monocytes into atherosclerosis-prone area. In our experimental conditions, we found that the alleviating effects on cell adhesion, induced by the single compounds, were associated with changes in the levels of E-selectin, but not VCAM-1 levels. We found that Mv-3-glc was more effective in reducing the production of E-selectin compared to the other compounds tested. In fact, the decrease was observed both at low and high concentrations, while for Cy-3-glc, PrA and GA the effects were detected only at the high doses. The increased E-selectin production at high concentration may be due to a stimulation of the cells as also shown in a previous study where Mv-glc led to an exacerbation of the adhesion process [16]. Dp-3-glc and SA supplementation did not show any reduction in line with the lack of an effect on THP-1 adhesion to HUVECs. Conversely, different studies report changes in the expression/levels of VCAM-1, ICAM-1, other than E-selectin, following ACNs and metabolites supplementation; most of them showed a beneficial effect only at supra-physiological concentrations. For example, Ferrari et al., [38] demonstrated that Cy-3-glc (20 μM) counteracted the acute pro-inflammatory effects of TNF-α in HUVECs, reduced leukocyte recruitment from microcirculation, and decreased the

gene expression levels of E-selectin and VCAM-1. Huang et al., [39] reported that the supplementation with different concentrations of Mv-3-glc (1-100μM) inhibited the TNF-α-induced inflammatory response in a concentration-dependent manner and reduced the production of MCP-1, ICAM-1 and VCAM-1 in endothelial cells. Nizamutdinova and colleagues [40] found that ACNs from black soybean seed coats (rich in Cy, Dp and Petunidin-3-glucoside) reduced TNF-□-mediated VCAM-1 induction in a concentration-dependent manner (10, 50, and 100 μg/mL), but not ICAM-1 in HUVEC. Amin et al., [41] showed that simulated human vascular endothelial cells with oxidized-LDL and co-treated with Cy-3-glc (0.1, 1, and 10 μM concentrations) significantly reduced VCAM-1 protein production. In addition, phenolic acids affected the expression and the levels of adhesion molecules. Warner et al., [42] tested the capacity of 20 different phenolic acids to reduce the secretion of VCAM-1 in activated TNF-α endothelial cells showing a significant effect for PrA in a concentration-dependent manner (1-100 μM). Similar results were also found following vanillic, isovanillic, ferulic, hyppuric acids and derivates supplementation [37;41-42].

CONCLUSIONS

In conclusion, this study documented the capacity of Mv-3-glc, Cy-3-glc, PrA and GA to reverse an atherogenic condition. This reduction can be explained by a significant decrease in the adhesion of monocytes to endothelial cells and in the production of E-selectin, but not VCAM-1 in the present short-term incubation period. Mv-3-glc seems the most potent anti-atherogenic compound since it actives both at supraphysiological and physiological concentrations.

ACKNOWLEGMENTS

Cristian Del Bo' designed the study, performed the experiments and wrote the first draft of the manuscript. Mirko Marino performed the analysis and reviewed the manuscript. Peter Moller and Patrizia Riso critically revised the manuscript. Marisa

Porrini supported the research, supervised the analysis and critically revised the manuscript.

We would like to thank Mr. Lorenzo Battisti, Alessandro Moreletti and Miss Maria Tsoumis for their help and support in cell growth. The authors are grateful for support granted by Ministero delle Politiche Agricole, Alimentari, Forestali e del Turismo (Mipaaft) and the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL) MaPLE.

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FIGURE CAPTIONS

Figure 1- Chemical structure of anthocyanins and their metabolites used in this study

Legend: *Mv-3-glc,* malvidin-3-glucoside; *Cy-3-glc,* cyanidin-3-glucoside; *Dp-3-glc,* delphinidin-3-glc; *SA,* syringic acid; *PrA,* protocatechuic acid; *GA, g*allic acid;

Figure 2- Effect of *ACN-RF* (0.02 and 18.9 μM, expressed as Mv-3-glc as the main compound) 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 ($p \leq 0.05$).

Legend: *TNF-α* tumor necrosis factor alpha, *ACN-RF* anthocyanin-rich fraction, *NO TNF-α* (control).

Figure 3- Effect of **A)** *Mv-3-glc* (0.02-18.9 μM), **B)** *Cy-3-glc* (0.03–25.9 μM) and **C)** *Dp-3-glc* (0.02–19.9 μM) 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 ($p \leq 0.05$).

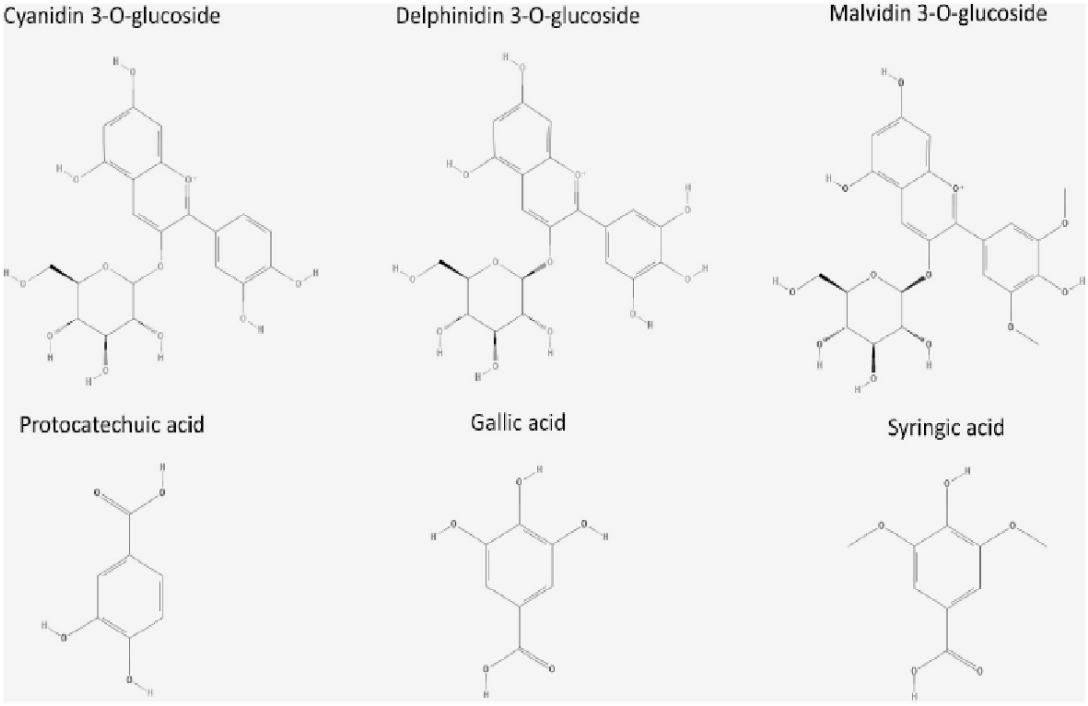
Legend: *TNF-α,* tumor necrosis factor alpha; *Mv-3-glc,* malvidin-3-glucoside; *Cy-3-glc,* cyanidin-3-glucoside; *Dp-3-glc,* delphinidin-3-glc; *NO TNF-α* (control).

Figure 4 Visualization of THP-1 adhesion to HUVEC following 100 ng mL^{-1} of TNF-α (**a**), TNF-α +10 μg mL^{-1} of Mv-3-glc (**b**), TNF-α + 10 μg mL^{-1} of SA (**c**), and NO TNF-α (**d**).

Legend: *TNF-α,* tumor necrosis factor alpha; *Mv-3-glc,* malvidin-3-glucoside; *SA,* syringic acid; *NO TNF-α* (control). Round yellow cells represent THP-1 cells adhered to HUVECs. The black arrows indicate an example of adhered THP-1, while the red arrows indicate HUVECs.

Figure 5- Effect of **A)** *SA* (0.05-50.5 μM), **B)** *PrA*(0.03–64.9 μM) and **C)** *GA* (0.03–58.8 μM) 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 ($p \leq 0.05$).

Legend: *TNF-α,* tumor necrosis factor alpha; *SA,* syringic acid; *PrA,* protocatechuic acid; *GA, g*allic acid; *NO TNF-α* (control).



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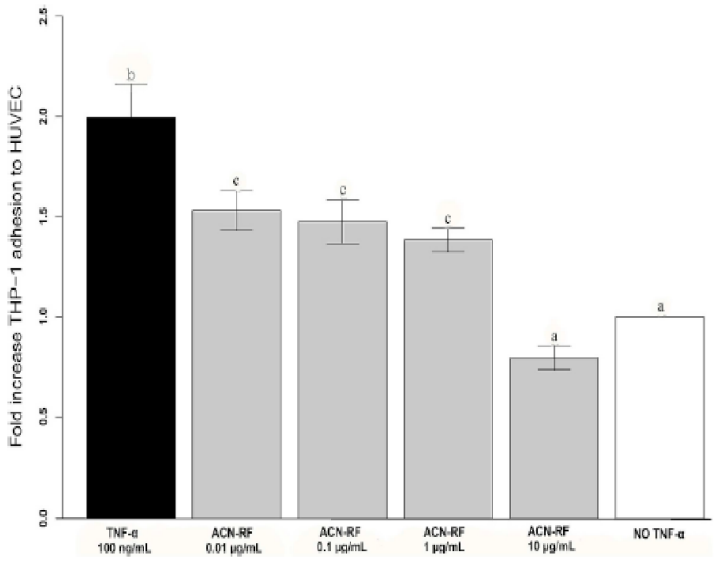

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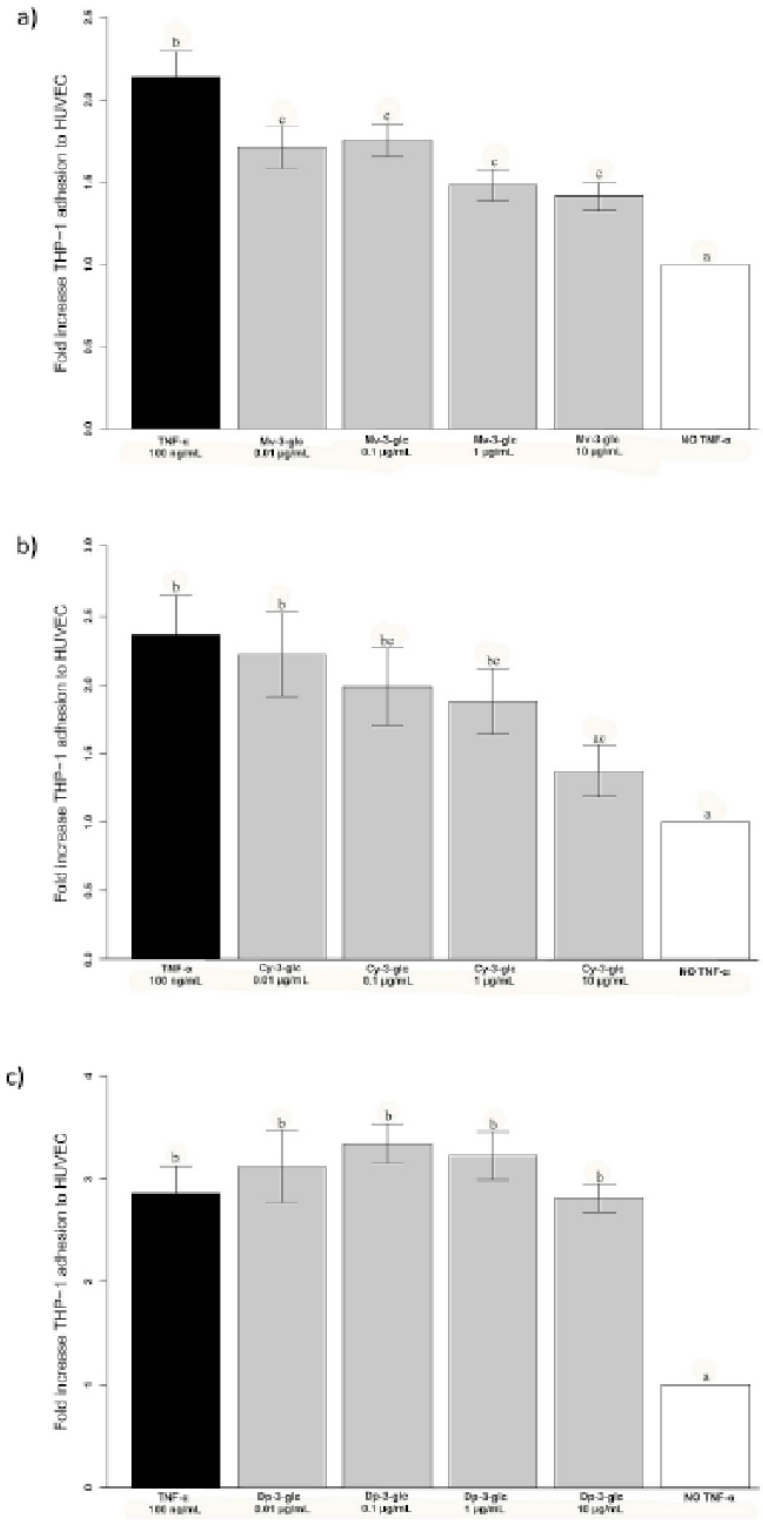

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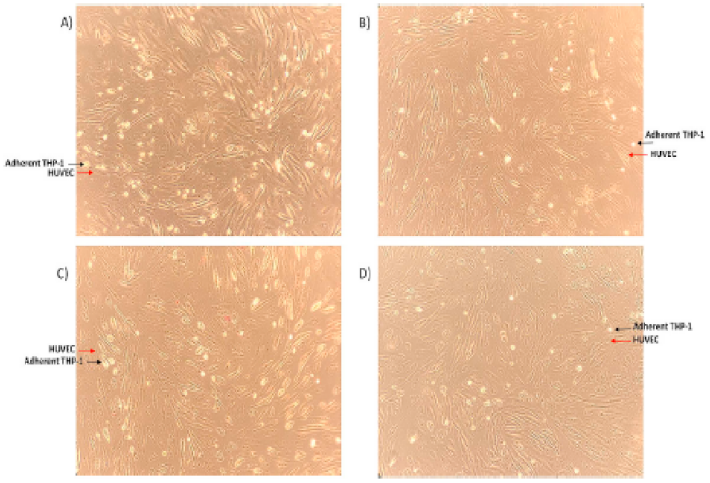

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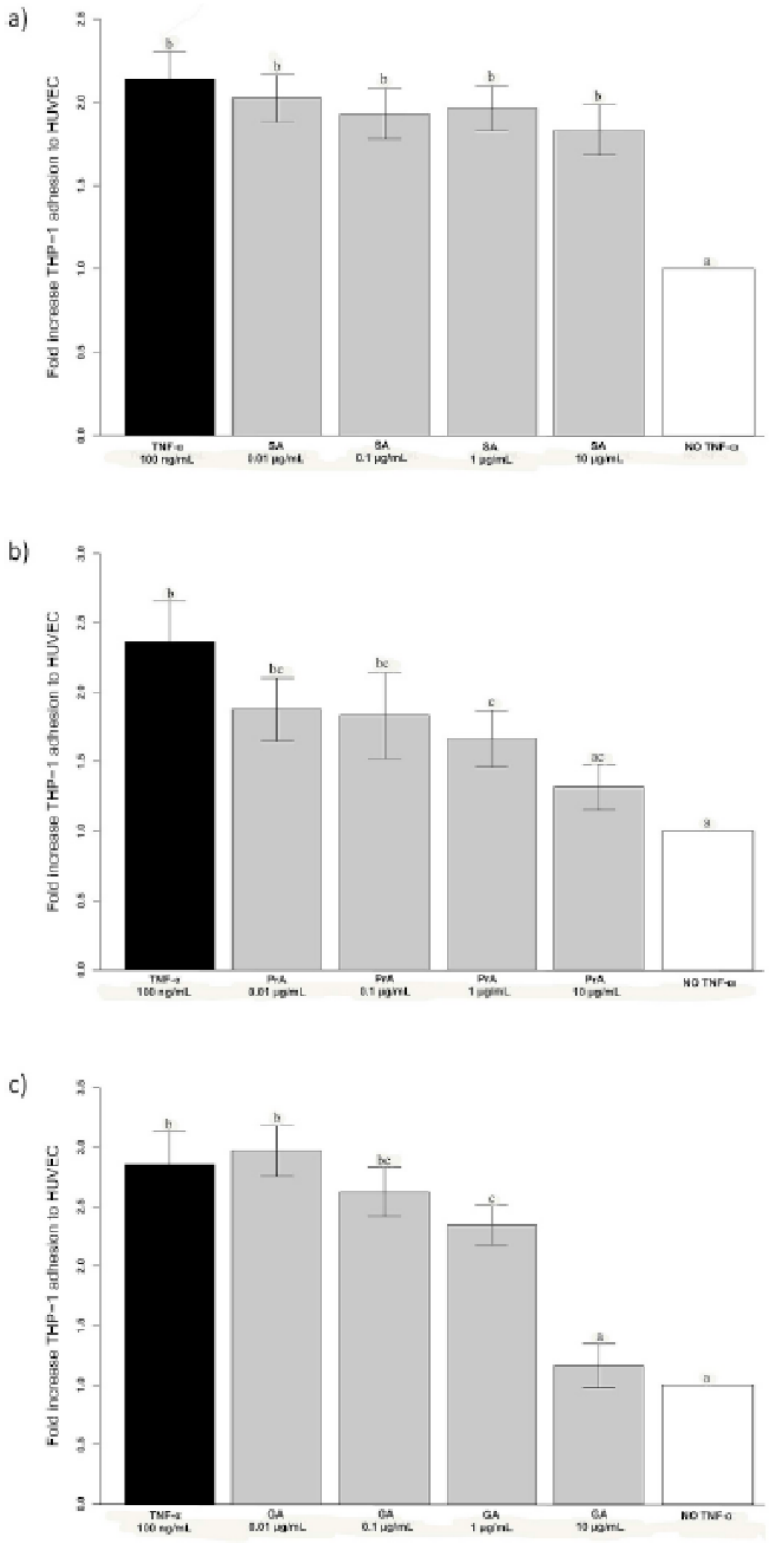

Fig. 5. Effect of **A)** *SA* (0.05–50.5 µM), **B)** *PrA*(0.03–64.9 µM) and **C)** *GA* (0.03–58.8 µM) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. [a,b,c]Bar graphs reporting different letters are significantly different ($p \leq 0.05$).

Legend: *TNF-α*, tumor necrosis factor alpha; *SA*, syringic acid; *PrA*, protocatechuic acid; *GA*, gallic acid; *NO TNF-α* (control).

Table 1
Effect of ACNs and metabolites on the levels of E-selectin.

	Compounds					
Concentrations	Mv-3-glc	Cy-3-glc	Dp-3-glc	SA	PrA	GA
0.01 μg mL^{-1}	107 ± 15^{a}	311 ± 13^{a}	308 ± 11^{a}	299 ± 15^{a}	290 ± 13^{a}	304 ± 15^{a}
0.1 μg mL^{-1}	104 ± 16^{a}	297 ± 15^{a}	299 ± 22^{a}	297 ± 15^{a}	257 ± 12^{a}	321 ± 11^{a}
1 μg mL^{-1}	186 ± 12^{a}	300 ± 14^{a}	295 ± 12^{a}	297 ± 16^{a}	83 ± 15^{b}	206 ± 10^{b}
10 μg mL^{-1}	149 ± 24^{a}	83 ± 10^{b}	315 ± 16^{a}	295 ± 14^{a}	74 ± 18^{b}	188 ± 17^{b}
(TNF-α) 100 ng mL^{-1}	316 ± 16^{b}	307 ± 11^{a}	318 ± 12^{a}	316 ± 16^{a}	307 ± 11^{a}	318 ± 12^{a}
(TNF-α) 0 ng mL^{-1}	59 ± 9.0^{c}	64 ± 10^{c}	65 ± 4.6^{b}	59 ± 9.0^{b}	64 ± 10^{c}	65 ± 4.6^{c}

Data derived from three different experiments and each concentration tested in triplicate. Each ACN and metabolite was tested in presence of TNF-α stimulus. Results are expressed as mean ± SEM. *Mv-3-glc*, malvidin-3-glucoside; Cy-3-glc, cyanidin-3- glucoside; *Dp-3-glc*, delphinidin-3-glc; *SA*, syringic acid; *PrA*, protocatechuic acid; *GA*, gallic acid; *TNF-α*, tumor necrosis factor alpha. ^{a,b,c}Data with different letters are significantly different ($p < 0.05$). Concentration range: 0.02–18.9 μM for Mv-3-glc, 0.02–19.9 μM for Dp-3-glc, 0.02–20.6 μM for Cy-3-glc, 0.25 and 50.5 μM for SA, 0.32–64.9 μM for PrA and 0.29–58.8 μM for GA.

Table 2
Effect of ACNs and metabolites on the levels of VCAM-1.

	Compounds					
Concentrations	Mv-3-glc	Cy-3-glc	Dp-3-glc	SA	PrA	GA
0.01 μg mL^{-1}	13.16 ± 0.78	15.10 ± 0.35	15.98 ± 0.76	15.43 ± 0.41	14.38 ± 0.17	16.98 ± 1.76
0.1 μg mL^{-1}	13.64 ± 0.04	14.56 ± 0.23	15.80 ± 1.10	16.59 ± 0.28	14.83 ± 0.53	14.99 ± 1.90
1 μg mL^{-1}	14.15 ± 0.33	14.65 ± 0.20	16.94 ± 0.51	18.85 ± 0.23	15.28 ± 0.42	16.64 ± 0.71
10 μg mL^{-1}	14.38 ± 0.11	15.10 ± 0.24	16.30 ± 0.40	17.45 ± 0.29	16.19 ± 0.37	16.26 ± 0.80
(TNF-α) 100 ng mL^{-1}	15.74 ± 1.14	15.17 ± 1.08	16.97 ± 1.81	15.74 ± 1.14	15.17 ± 1.08	16.97 ± 1.81
(TNF-α) 0 ng mL^{-1}	11.04 ± 0.37*	10.99 ± 0.35*	11.27 ± 0.28*	11.04 ± 0.37*	10.99 ± 0.35*	11.27 ± 0.28*

Data derived from three different experiments and each concentration tested in triplicate. Each ACN and metabolite was tested in presence of TNF-α stimulus. Results are expressed as mean ± SEM. *Mv-3-glc*, malvidin-3-glucoside; Cy-3-glc, cyanidin-3- glucoside; *Dp-3-glc*, delphinidin-3-glc; *SA*, syringic acid; *PrA*, protocatechuic acid; *GA*, gallic acid; *TNF-α*, tumor necrosis factor alpha. *Significantly different ($p < 0.05$). Concentration range: 0.02–18.9 μM for Mv-3-glc, 0.02–19.9 μM for Dp-3-glc, 0.02–20.6 μM for Cy-3-glc, 0.25 and 50.5 μM for SA, 0.32–64.9 μM for PrA and 0.29–58.8 μM for GA.

3.2 Modulation of Adhesion Process, E-selectin and VEGF Production by Anthocyanins and Their Metabolites in an *in vitro* Model of Atherosclerosis

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Received: 10 January 2020; Accepted: 25 February 2020; Published: 27 February 2020

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

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 [1]. 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 [2,3]. 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 [4,5].

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 [6]. 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 [7,8]. Unabsorbed polyphenols reach the colon where they are extensively metabolized by gut microbiota [9]. 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 [10,11]. It is estimated that plasma concentrations range between nanomolar for anthocyanins and other polyphenols (native forms), up to low micromolar for their derivates [10,11].

In recent years, polyphenols have received extensive interest for their health benefits in the prevention of numerous cardiovascular diseases [12–16]. 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) [17–19], and the decrease of pro-inflammatory cytokines (i.e., tumor necrosis factor alpha (TNF-α), interleukin-1, interleukin-6) [20–22]. 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) [23]. 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 [24], 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 [25], 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.

MATERIALS AND METHODS

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 (CellTracker^{TM} 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) 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).

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.

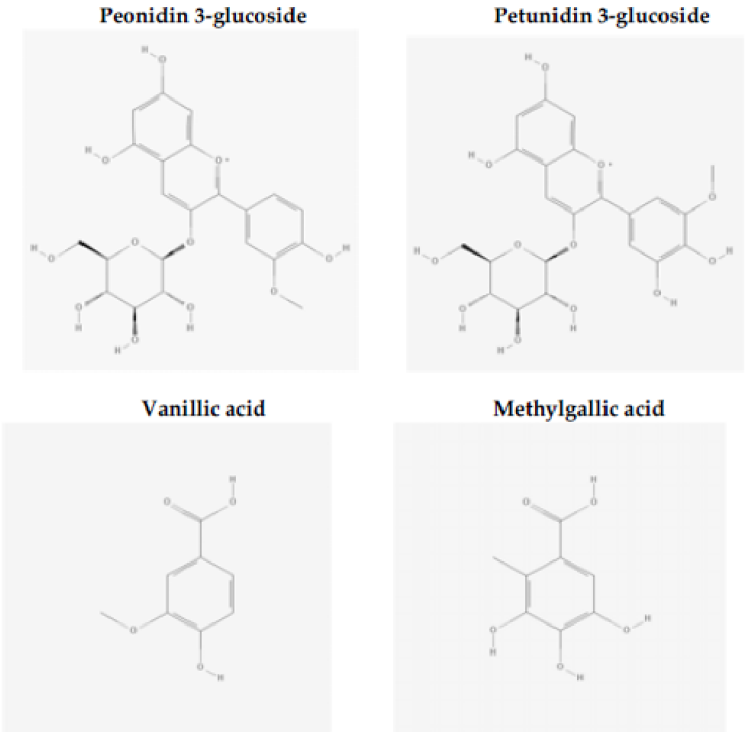

Figure 1. Chemical structure of peondin and petunidin-3-glucoside, vanillic and methylgallic acids.

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, 1×10^5 cells were grown in a flask until the concentration of 1×10^6 cells/mL was reached. Human

umbilical vein endothelial cells (HUVECs; Tebu-Bio Srl, Magenta, MI, Italy) were seeded at the concentration of 1×10^5 cells on a pre-coated flask with 0.1% gelatine and grown in a cell medium kit containing 2% serum until they reached confluence.

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.

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 2×10^4 cells/well at 37 °C and 5% CO_2. After 24 h incubation, THP-1 (2×10^6) cells were labelled with CellTracker^{TM} 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 2×10^5 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.

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.

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 $p < 0.05$.

RESULTS

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%).

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.

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.

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).

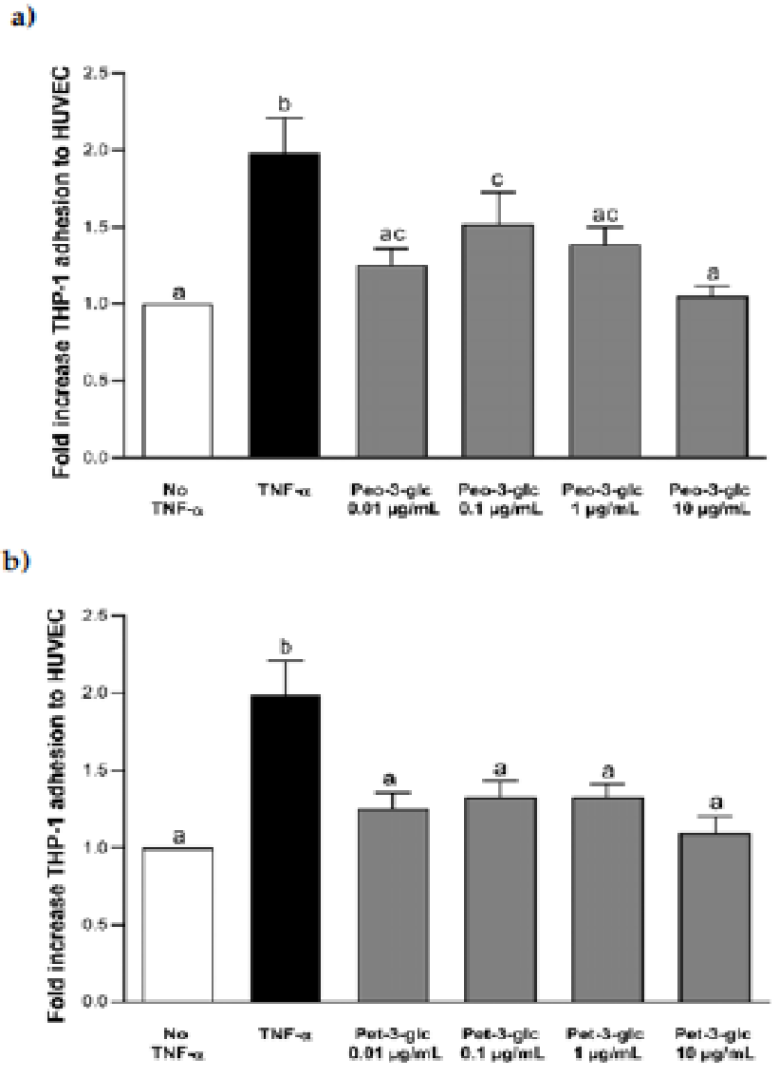

Figure 2. Effect of different concentrations (0.02–20 µM) of Peo-3-glc (**a**) and Pet-3-glc (**b**) on THP-1 (monocytes) adhesion to HUVECs (vascular endothelial cells). Results are expressed as mean ± standard error of mean. [a,b,c] Bar graphs reporting different letters are significantly different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

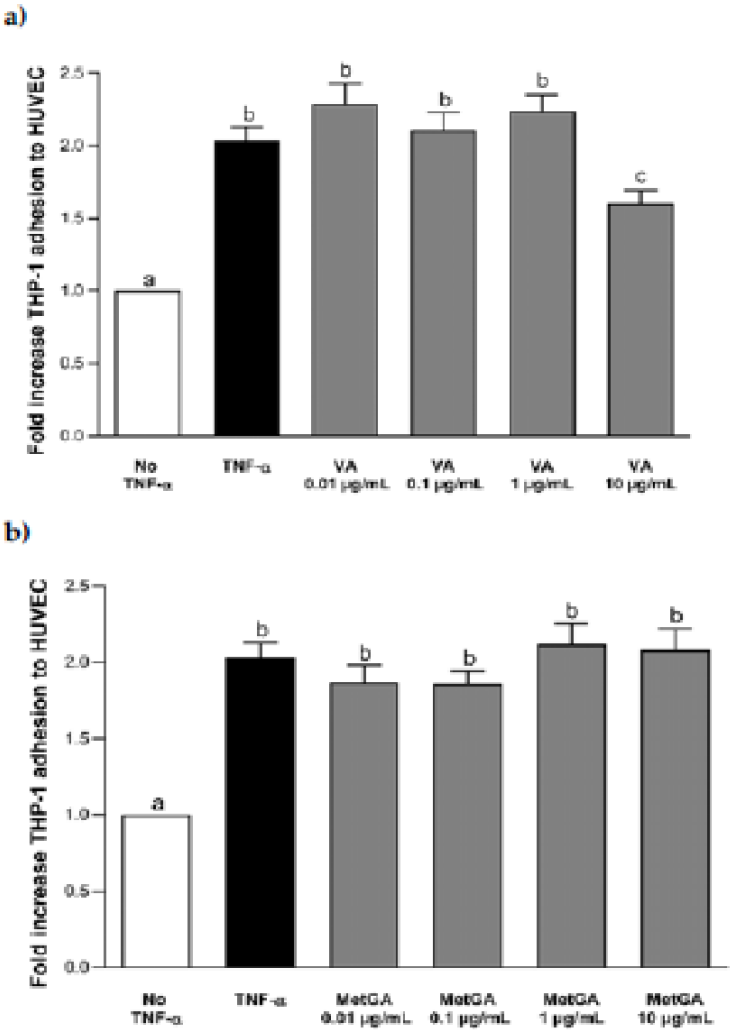

Figure 3. Effect of different concentrations (0.05–50 μM) of VA (**a**) and MetGA (**b**) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. [a,b,c] Bar graphs reporting different letters are significantly different ($p \leq 0.05$).

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-□). 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-□ (−46%; $p < 0.05$). Conversely, no effect was observed following MetGA exposure.

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-□ significantly increased ($p < 0.001$) the levels

of VCAM-1 compared to the negative control (without TNF-□). 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.

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-□ induced a small but significant increase ($p < 0.01$) in VEGF levels compared to the negative control (without TNF-□). 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 significanlty different ($p < 0.05$) compared to the negative control.

	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^{b,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 ($p < 0.05$).

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

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 ($p < 0.05$).

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}	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^{b}	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 ($p < 0.05$).

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

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 [26,27]. 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 [10]. 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 [10]. 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 [28] 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 [28] 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 Ca^{2+}-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 [29]. 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 [30]. 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 [31]. 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 [32]. 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 [33]. 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 [34]. 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 [35]. 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 [26] and in line with results found by others researchers [28,36], 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 [37]. 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 [4]. Arterial injuries are followed by arterio-intimal angiogenesis that induces intimal hyperplasia and a subsequent intimal hemorrhage [38]. 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 [5]. 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 [39]. In cancer research, multiple in vitro studies demonstrated the anti-angiogenic effect of anthocyanins, in particular concerning delphinidin, as a potential chemopreventive agent [40–42]. 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 [43]. 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 [44]. 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% [45]. 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 [47]. 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 [48]. 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. Therefore, enhanced scientific cooperation, using common

extracts and experimental protocols, could lead to a consensus among different studies, thereby formulating robust conclusions [49].

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.

Author Contributions: M.M. performed the experiments and wrote the first draft of the manuscript, C.D.B. designed the study, performed the experiments, the statistical analysis and wrote/improved the first draft of the manuscript. M.T. contributed by performing the experiments and conducting the analysis of the ELISA kits. P.R. and M.P. critically revised the manuscript and partially supported the research. D.K.-Z. critically revised the manuscript and edited the paper for language.

Funding: This work was supported by a contribution of the "Piano di sostegno alla ricerca- Linea 2, azione A-grant number PSR2019 and 2018-CDELB".

Acknowledgments: The authors are grateful for support granted by Ministero delle Politiche Agricole, Alimentari, Forestali e del Turismo (Mipaaft) and the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL) MaPLE. P.R. and C.D.B. acknowledge the European Cooperation for Science and Technology (COST Action) CA16112 "NutRedOx: Personalized Nutrition in Aging Society: Redox Control of Major Age-related Diseases".

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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A Mix of Chlorogenic and Caffeic Acid Counteracts Lipid Accumulation and Downregulates C/EBPß and PPAR-γ1 gene expression in Macrophages

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ABSTRACT

Purpose: Chlorogenic acid (CGA) and caffeic acid (CA) are bioactive compounds in whole grains, berries, apples, some citrus fruits and coffee, which are hypothesized to promote health-beneficial effects on the cardiovascular system. The present study aimed to evaluate the capacity of CGA and CA to reduce lipid accumulation in macrophages, recognized as critical stage in the onset of atherosclerosis. In addition, the modulation of CCAAT/enhancer-binding protein β (C/EBPβ) and peroxisome proliferator-activated receptor gamma (PPAR-γ1), as transcription factors involved in lipid metabolism, was evaluated.

Methods: THP-1 derived macrophages were treated for 24 h with 0.03, 0.3, 3 and 30 μM of CGA and CA, tested alone or in combination, and a solution of oleic/palmitic acid (500 μM, 2:1 ratio). Lipid storage was assessed spectrophotometrically through Nile red. The kinetic of the expression of C/EBPβ and PPAR-γ1 was evaluated by RT-PCR.

Results: The mix of CGA + CA (1:1 ratio), but not individual compounds, was able to counteract lipid accumulation at all concentrations tested, except for the highest one. The greatest effect (-65%; $p<0.01$) was observed at the concentration of 0.3 μM for each compound. In addition, the same concentration was able to exert a significant ($p<0.01$) downregulation of C/EBPβ and PPAR-γ1 gene expression at 2 h and 24 h, respectively.

Conclusion: The results obtained support the capacity of the mix of CGA + CA, but not the single compounds, to reduce lipid storage in macrophages possibly due to a modulation of gene expression of the transcription factors C/EBPβ and PPAR-γ1.

Keywords: polyphenols, THP-1 derived macrophages, lipid accumulation, PPAR-γ1, C/EBPβ, atherosclerosis

INTRODUCTION

In the last years, there has been increasing attention on the potential role of dietary polyphenols in the modulation of lipid metabolism and prevention of cardiovascular events [1-7]. Polyphenols are a heterogenous class of more than 8000 bioactive compounds in plants. The main food sources are vegetables, fruits, whole grains, legumes and chocolate, but they are also abundant in beverages such as tea, coffee, and red wine [8]. Polyphenols are most commonly classified into flavonoids and nonflavonoids. Flavonoids are characterized by the basic structure of a diphenyl propane and contain several hydroxyl groups on the aromatic rings; while, nonflavonoids share a single aromatic ring as basic structure [9]. Flavanols, anthocyanins, isoflavones, flavones, flavonols and flavanones belong to the group of flavonoids, while phenolic acids, stilbenes and lignans to the group of nonflavonoids. Among the latter, the main class is covered by phenolic acids (hydroxycinnamic and hydroxybenzoic acids) such as ferulic, chlorogenic acid (CGA), and its metabolite caffeic acid (CA) widely found in whole grains, apples, some citrus fruits, coffee and berries where they are rarely found free but esterified with other molecules such as carbohydrates and organic acids [9]. The concentration in foods is variable; for example, different types of berries contain from 1 to 200 mg/100g CGA and CA [10], while the total CGA concentration in coffee ranges from 30 to 1000 mg/100 ml of product [11,12].

The absorption of CGA occurs mostly in the intestine by passive diffusion. After absorption, CGA undergoes mainly sulfation and glucuronidation reactions, while the unabsorbed portion is extensively hydrolyzed by gut bacteria [12]. The colonic metabolites can be absorbed and excreted in urine. Also due to this extensive metabolism, the *in vivo* concentrations of CGA and CA are widely variable. The maximum plasma concentration (C_{max}) of CGA and metabolites (including CA) ranges from nM to μM levels in humans [13-15], depending on the type and magnitude of food administered, individual xenobiotic metabolism response, but also the analytical method used for their detection. The role of phenolic acids in the modulation of cardiovascular risk has not been fully investigated. Some studies seem to support their cardiovascular protection by counteracting oxidative stress and inflammation, and by regulating cell adhesion process, migration and lipid accumulation [16-22].

The entrapment of monocytes in the intimal area and the subsequent transformation to macrophage-like foam cells is a pivotal step in atherosclerosis, with a significant impact on plaque inflammation, progression and stability. In particular, lipid accumulation in macrophages determines cell dysfunction, dysregulating several transcriptional factors involved in the atherosclerotic process [23]. Lipid overload at the level of arterial tunica intima triggers foam cells development, which is the first event in atherosclerosis that can be noticed [24]. Influx, esterification and efflux of lipids are critical stages involved in intracellular lipid droplets generation. Indeed, alteration of these processes involved in lipid metabolism is one of the major determinants of the transformation of macrophages into foam cells [25]. Excessive lipid deposition in foam cells leads to persistent endoplasmatic reticulum (ER) stress which in turn induces apoptosis [26]. The increasing number of dead cells, further worsened by decreased clearance via impaired autophagy, promotes an exacerbation of the inflammatory response and a subsequent establishment of the necrotic core that leads to plaque progression [27]. There are several transcription factors associated with lipid metabolism in foam cells, including peroxisome proliferator-activated family of receptors (PPARs), CCAAT enhancer binding proteins (C/EBPs), liver-X family of receptors (LXRs), and sterol regulatory element binding proteins (SREBPs). In addition, although not a transcription factor, Micro-RNA 33 (miR-33) has also been implicated in regulating lipid metabolism [28]. The peroxisome proliferator activated receptor-γ (PPAR-γ) and the proliferator-activated receptor-coactivator 1β have a crucial role in the conveyance of lipid metabolism and in the polarization of metabolic profile of macrophages. PPAR-γ in particular has been described as a sensor of fatty acids for its high responsivity to these molecules [29]. CCAAT enhancer-binding proteins (C/EBP) are a family of transcription factors involved in adipocyte differentiation with key roles during macrophage polarization [29]. Recent evidence connect C/EBPβ with atherosclerosis since its expression seems to be stimulated by oxidized low-density lipoprotein (oxLDL) in primary human macrophages, suggesting that C/EBPβ may be involved in inflammatory processes triggered by oxLDL during foam cell development [30]. The LXRs belong to the nuclear receptor superfamily of ligand activated transcription factors of which there exist two isoforms, LXRα and LXRβ. These transcription factors are strongly connected to atherosclerosis protective

effects since they promote cholesterol efflux from intimal macrophages, thus reducing atherosclerotic plaques through numerous mechanisms involving ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) [31]. Furthermore, there is evidence that LXRs regulate multiple key metabolic pathways, such as coordinating cholesterol homeostasis, repressing inflammation, promoting lipogenesis etc. [31]. The sterol regulatory element binding proteins (SREBPs) are transcription factors that regulate gene expressions of enzymes involved in lipid and glucose metabolism, known for fostering cholesterol biosynthesis in macrophages through the induction of HMG-CoA reductase [32].

We have previously reported the capacity of anthocyanin- and phenolic acid-rich fractions, from a wild blueberry powder, to reduce lipid accumulation in human THP-1 derived macrophages [21]. We documented that the effects for anthocyanin-rich fraction, the single anthocyanins and their derivates were compound dependent. Regarding the phenolic acid-rich fraction, rich mainly in CGA, we have shown that the effects were dose dependent and effective only at the low-medium concentrations. Based on these results, the aim of this study was to evaluate the effect of CGA and its metabolite (i.e. CA) on the reduction of fatty acids accumulation in monocyte-derived macrophages. The compounds were tested alone and in combination both at physiological and supraphysiological concentrations. Moreover, the impact of CGA and CA on the expression of PPAR-γ1 and C/EBPβ as pivotal transcription factors involved in lipid metabolism of macrophages and atherosclerotic plaque development, has been investigated.

MATERIALS AND METHODS

Chemicals and reagents

Standard of CGA and CA, bovine serum albumin (BSA), palmitic acid, oleic acid, fetal bovine serum (FBS), Hanks balanced salt solution, Trypan Blue, Pluronic F127, Nile Red, phorbol 12-myristate 13-acetate (PMA), DNase I amplification grade kit, hydrochloric acid, methanol and ethanol were purchased from Merck (Darmstadt, Germany). Water was obtained from Milli-Q apparatus (Millipore, Milford, MA). Hepes, sodium pyruvate, gentamicin, RPMI-1640, and trypsin–EDTA were obtained

from Life Technologies (Monza Brianza, Italy). RNeasy Mini kit was purchased from QIAGEN (Hilden, Germany). iScript cDNA Synthesis Kit and SsoFast EvaGreen Supermix were purchased from BIORAD (Segrate, Milano, Italy). Forward and reverse primers of PPAR-γ1 and C/EBPβ genes were obtained from biomers.net (Ulm, Donau, Germany). Forward and reverse primers of 18S were acquired from Primmbiotech (Cambridge, MA, USA).

Preparation of chlorogenic and caffeic acid

A stock solution of CGA and CA standards (**Figure 1**) was prepared. These phenolic acids were selected based on our previous publication in which CGA and CA were the most representative compounds found in a phenolic-rich fraction obtained from wild blueberry [21]. Lyophilized standards (50 mg) were dissolved in acidified methanol (5 ml; HCl 0.05 mM). Aliquots (1 mL) of standard were prepared, quantified spectrophotometrically, and finally stored at −20 °C until use.

Preparation of fatty acids and control solution

The stock solution of free fatty acids (FFA; 0.2 M oleic/palmitic acid; 2:1 ratio) was prepared in EtOH and stored at −20 °C. On the day of use, 5 mM of FFA water-soluble solution (FFA/BSA solution) was generated by incubating the FFA in Hanks solution containing 10 % BSA at 37 °C for about 30 minutes with occasional shaking. The FFA/BSA solution was added to the medium to obtain final 500 μM FFA concentration for the exposure. Equal volumes of the Hanks solution/EtOH/fatty acid-free BSA were applied to control cells. The final concentrations of EtOH and BSA in exposure media were 0.25 and 0.1 %, respectively.

Cell culture

The monocytic THP-1 cell line (human monocytic leukaemia) was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were maintained in culture in RPMI-1640 medium containing 10% of heat inactivated FBS and supplemented with 1% hepes, 1% sodium pyruvate and 0.1% gentamicin. According to Tsuchiya et al [33], the cells maintain their monocytic characteristics for over 14 months of continuous growth. In our study, the cells were maintained for up to 3 months and used at passage 3.

Viability assay

The toxicity of the compounds was tested on THP-1 derived macrophages by Trypan blue using a TC20^{TM} automated cell counter and dual-chamber cell counting slides (BIORAD, Segrate, Milano, Italy). THP-1 cells were differentiated to macrophages and treated with CGA, CA and the mix of CGA and CA at the maximum concentration tested (30 μM of each compound) for 24 hours and in presence of FFA/BSA solution (500 μM). Subsequently cells were trypsinized, resuspended and used for the trypan blue assay. Three independent experiments were performed in which each compound was tested in triplicate.

Lipid accumulation in THP-1 derived macrophages

Cells were cultured in complete RPMI cell medium at 37 °C and 5% CO_2. After growth, cells were differentiated into macrophages by treatment with 5 ng/mL PMA for 72 hours. Macrophages become adherent to the surface of the culture flasks; thus, cells were washed with Hanks Solution to remove all non-adherent cells. Subsequently, 3 mL trypsin (0.05 %)–EDTA (0.53 mM) was added and the cells were incubated for 2 minutes at 37 °C and 5% CO_2 in order to release the attachment to the flask. After incubation, 2 mL of complete RPMI cell medium was added to inactivate the trypsin. Cells were collected in Falcon tubes, quantified in a TC20^{TM} automated cell counter and then centrifuged (mod. Eppendorf 5804R Centrifuge, Milano, MI, Italy) for 5 minutes at 250×g. After centrifugation, cells were resuspended in new complete RPMI medium (without PMA) in order to reach a final concentration of 2.5×10^5 cells/mL. Two hundred microliters of cell suspension (5×10^4 cells) were added into each well of a black 96-well plate and incubated for 24 h at 37 °C and 5 % CO_2, in order to allow the cell adhesion to the surface of the plate. Medium was removed and 200 μL of new complete RPMI medium, containing 500 μM FFA and different concentrations of CA and CGA alone or in combination, was added. All these compounds were prepared in acidified methanol (0.05 mM HCl) and then diluted in the culture medium before use. The highest methanol content was <0.03% while the concentration of HCl in cell media was less than 0.001% without impact on pH and cell viability, as also documented in our previous papers [21]. The concentrations for each compound were 0.03, 0.3, 3 and

30 μM tested alone or in combination. These concentrations were selected in order to mimic both physiological and supraphysiological conditions in accordance with data found after oral administrations of foods or single compounds [13-15]. Cells were incubated for 24 h at 37 °C and 5 % CO_2. The lipid accumulation was measured by using the fluorescent dye Nile red [34]. Nile red is not fluorescent in aqueous solution but can be extensively fluorescent in lipid rich environments. Nile red is thus suitable for measuring lipid accumulation both in cells and in tissue. To measure the lipid accumulation in macrophages, the cells were washed with Hanks solution and then stained in Hanks solution containing 0.5 μg/mL Nile red and 0.01 % Pluronic F127 for 15 min at 37 °C and 5–7 % CO_2. Later, cells were washed twice with 200 μL of Hanks solution and then added 100 μL Hanks solution. The fluorescence (excitation: 544 nm, emission: 590 nm) was measured in a fluorescence spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy), and the fold increase compared to the control (without FFA) was calculated.

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR)

Cells were obtained using the same conditions as the lipid accumulation assay, except the plating of THP-1 derived macrophages was done in a 24 well plate (5×10^5 cells) instead of 96 well plate (5×10^4 cells) in order to have a suitable extraction of RNA. RNA from THP-1 macrophages was extracted immediately after the experiment by RNeasy Mini kit according to manufacturer's protocol. DNAase treatment, using DNase I amplification grade kit (Merck), was used to degrade DNA in the RNA preparations in a 15-minute digestion at room temperature. The reaction was then stopped adding 1 μl of Stop Solution and DNase I was heat inactivated for 10 min at 70°C. Purified RNA was quantified through spectrophotometer (Power Wave XS2, BioTek, Winooski, VT, USA) by loading each sample in duplicate; the A260/A280 ratio was verified to be above 1.8 as sufficient quality of RNA purity. An equal amount of RNA for each sample was immediately reverse transcribed using iScript cDNA Synthesis Kit and Mastercycler nexus gradient (mod. Eppendorf, Milano, MI, Italy) by using the following thermal cycle: 25°C × for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. Controls without retrotranscriptase (No-RT) and without RNA template (no RNA) were performed to be used in qPCR in order to rule out residual genomic DNA contamination in RNA samples and reagents contamination with nucleic acids.

Afterwards, the obtained cDNA was diluted with nuclease free water to a concentration of 3 ng/µl. qRT-PCR was performed by using C1000 Thermal Cycler / CFX96 Real-Time System (BIORAD, Segrate, Milano, Italy) and SsoFast EvaGreen Supermix according to manufacturer's protocol. A total amount of 15 ng of cDNA was used. Controls included No-RT, no RNA and a control sample without cDNA for each primer pair. Thermal cycling conditions for the analysis of PPAR-γ1 were 15 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C, 20 seconds at 55°C, and 10 seconds at 72°C with the primer pairs indicated in Table 1. The cycling program for the analysis of C/EBPβ was 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds with the primer pairs indicated in Table 1. Primer pairs have been used at a concentration of 0.5 µM. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product. The expression levels of all target genes were normalized against the expression of the ribosomal 18S housekeeping gene, which served as the endogenous control was used as most suitable reference gene in the cell model employed. Relative fold changes between different experimental conditions were calculated with the comparative Ct method (2−ΔΔCt Method). For detailed information refer to Supplementary file.

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 CGA and CA supplementation on cell viability, lipid accumulation process and gene expression of PPAR-γ1 and C/EBPβ. The least significant difference (LSD) test was used to assess differences between treatments by setting the level of statistical significance at $p < 0.05$.

RESULTS

Effect of CGA and CA on Cell Viability

Table 2 presents the effects of the compounds on the cellular viability measured by Trypan blue assay at all the concentrations tested for 24 h and in presence of FFA/BSA

solution (500 μM). The control condition is represented by cells in their normal growth medium without phenolic acids and fatty acids solution. CGA, CA and the mix of CGA and CA did not affect the cell viability that remained higher than 90%.

Effect of CGA and CA on lipid accumulation in THP-1 derived macrophages

The results of lipid accumulation in THP-1 derived macrophages after incubation with CGA and CA are shown in **Figure 2a** (CGA) and **2b** (CA). Stimulation with 500 μM of FFA significantly increased ($p < 0.05$) the lipid accumulation process in macrophages compared to cells without stimulation (No FFA). While, no effect was observed after treatment with different concentrations of CGA and CA, tested alone, compared to the FFA treatment.

Effect of the mix CGA + CA on lipid accumulation in THP-1 derived macrophages

The results of lipid accumulation in THP-1 derived macrophages after incubation with the mix CGA + CA are shown in **Figure 3**. Stimulation with 500 μM of FFA significantly increased ($p < 0.05$) the lipid accumulation process in macrophages compared to cells without stimulation (No FFA). The treatment with CGA and CA tested in combination significantly decreased ($p < 0.01$) the lipid accumulation in macrophages compared to the FFA treatment, except for the maximum concentration. In particular, the size of the effect was similar between the different concentrations tested, -56%, -65% and -48% respectively for the mix CGA + CA at 0,03 μM, 0,3 μM and 3 μM.

Effect of the mix CGA + CA on C/EBPβ gene expression

The results of gene expression kinetic of C/EBPβ after the administration of the mix of CGA + CA at the physiological concentration of 0.3 μM (i.e. the most effective concentration in counteracting the lipid accumulation) are shown in **Figure 4**. Gene expression has been evaluated at 5 different timepoints (1, 2, 4, 8 and 24 h) in 3

independent experiments in which every experimental condition has been tested in triplicate.

There is a statistically significant increase of C/EBPβ gene expression following 2 hours ($p < 0.01$) from the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA induced, after 2 hours, a statistically significant ($p < 0.01$) reduction in C/EBPβ gene expression compared to the positive control (only FFA administration); these results were in line and not different from those obtained in the negative control (No FFA). No difference has been also reported for the other timepoints analyzed.

Effect of the mix CGA + CA on PPAR-γ1 gene expression

The results of gene expression kinetic of PPAR-γ1 after the administration of the mix of CGA + CA at the physiological concentration of 0.3 μM are shown in **Figure 5**. Gene expression has been evaluated at 5 different timepoints (1, 2, 4, 8 and 24 h) through 3 independent experiments in which every experimental condition has been tested in triplicate.

There was a statistically significant increase ($p < 0.01$) of PPAR-γ1 gene expression at 2 and 24 hours following the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA increased PPAR-γ1 gene expression; the magnitude of increase was comparable with the positive control (only FFA administration). Conversely, no significant effect was observed after 24 h; here, the levels of expression were lower compared to the positive control and not statistically different from the negative control.

DISCUSSION

In the present manuscript we documented the ability of CGA and CA, administered also at physiological relevant concentrations, to counteract lipid accumulation in monocyte-derived macrophages and to positively modulate the expression of PPAR-γ1 and C/EBPβ as important transcription factors involved in lipid metabolism.

The role of (poly)phenols in the modulation of lipid accumulation and metabolism has been evaluated in different *in vitro* studies. Results are equivocal probably due to the different experimental conditions used including cell lines, phenolic components tested, concentrations, and duration of exposure. Here, we found that the administration of CGA and CA alone did not reduce lipid accumulation in THP-1 derived macrophages. This result could be partially explained by the relatively low concentrations of CGA and CA used in our experimental conditions. Other studies reported a beneficial effect when polyphenols were tested at supraphysiological concentrations. For example, Aranaz et al [38] observed that *p*-coumaric, ellagic and ferulic acids when tested at 100 μM reduced the content of triglycerides in 3T3-L1 preadipocytes, while no effect was observed at the concentrations of 10 and 50 μM. Analogous results were obtained by Zhao et al [39] reporting the capacity of 25 and 50 μg/mL, but not 5 μg/mL, of a pomegranate peel polyphenol-rich extract to counteract lipid accumulation in RAW264.7 macrophages. In another *in vitro* model, Yeh et al [40] showed the ability of high concentrations of a Chinese olive polyphenol-rich extract (100, 200 and 400 μg/mL) to reduce lipid content in FL83B hepatocytes. Finally, Liu et al [41] documented the reduction of lipid accumulation in RAW264.7 macrophages following incubation with 80 μg/mL of a wild blueberry extract rich in CGA. Conversely, Liu et al [42] observed an increase of lipid droplets in HepG2 cells supplemented with 0.5-2 mM of CGA and these effects were attributable to the induction of the expression of SREBP-1c and PNPLA3, two important pathways involved in adipogenesis.

Interestingly, we found that CGA and CA when tested in combination reduced the amount of intracellular lipids in our *in vitro* model, suggesting a possible synergistic effect of the two compounds. This synergistic effect has been observed also by other authors in several *in vitro* studies after the administration of different polyphenols [43-46]. We also documented that the positive effect of the mix was observed only at low-medium concentrations while no effect was observed at the maximum concentration tested. This result seems in line with the findings obtained in our previous study on whole anthocyanin- and phenolic acid-rich fractions of wild blueberry extracts [21]. Specifically, we showed that the administration of high concentrations (5 and 10 μg/mL) of a wild blueberry phenolic acid-rich fraction, rich in CGA, was not able to

reduce lipid accumulation in THP-1 derived macrophages, while the low-medium concentrations (0.05-0.3 μg/mL) were effective [21]. By excluding the toxic concentrations, the lack of effect could be attributed to other factors. For example, we postulated a possible involvement of the hormetic response in which biological systems exhibit a reaction when exposed to low concentration of a stimulus, while they are inhibited at high concentrations [47-49]. In addition, we cannot exclude interactions of polyphenols with components in the cell culture medium, as well as problems related to the possible formation of CGA-CA complex, when tested at maximum concentration, with a consequent loss of bioactivity of the molecules.

Another important aspect evaluated within this study was the identification of the potential mechanisms of action through which CGA and CA can modulate lipid accumulation. In particular, we tested the gene expression of two important transcription factors, i.e. PPAR-γ1 and C/EBPβ. Numerous studies reported a connection between the expression of C/EBPβ and PPAR-γ1; for example, the differentiation of pre-adipocytes is regulated by a specific pathway in which, after hormonal induction, C/EBPβ activates the expression of C/EBPα and PPAR-γ1 [50-53]. There is also clear evidence reporting that PPAR-γ activation upregulates CD-36-mediated lipid accumulation in macrophages [54]. The role of phenolics in the modulation of C/EBPβ and PPAR-γ1 has been poorly evaluated. The main results regard the potential beneficial effects of epigallocatechin, genistein and resveratrol in the modulation of adipogenesis [55], while the contribution of CGA and CA deserves investigation. In our experimental conditions, we reported that fatty acids induced the expression of PPAR-γ1 (at 2 and 24 h) and C/EBPβ (at 2 h) genes. Conversely, the administration of CGA + CA induced a downregulation in C/EBPβ and PPAR-γ1 gene expression respectively at 2 h and at 24 h post administration of the mix. No effect was reported on PPAR-γ1 gene expression at 2 h which was comparable to the positive control (FFA). Our results on PPAR-γ1 are different from those reported by Wu and colleagues [56], who found an increment (+50%) of PPAR-γ1 expression after 24 h stimulation with ox-LDL (50 mg/ml) in relation to the administration of two different concentrations of CGA (1 and 10 μM) in RAW264.7 macrophages. This discrepancy in the results could be related to the different CGA concentrations tested (more physiological in our study) and/or to the different cell model used, including species

differences. For instance, RAW264.7 cells are actively proliferating macrophages, which is an unusual characteristic as macrophages usually do not proliferate. THP-1 cells are monocytes that must be treated with e.g. PMA to develop a macrophage-like characteristic and cease to proliferate. The treatment of THP-1 cells with PMA is known to induce protein kinase C [57]. Protein kinase C stimulates signaling pathways involved in sensing of intracellular energy resources [58]. However, the uptake of lipids in activated THP-1 cells is not affected by the protein kinase C activation [59]. Thus, murine macrophages such as RAW264.7 and human PMA-stimulated monocytes may respond differently to polyphenols due to differences in species background and regulation of energy sources such as lipids. In addition, we cannot exclude that lower level of PPAR-γ1 gene expression observed in our experimental conditions is due to an effective reduction of intracellular lipids at 24 h, after the administration of the mix CGA + CA. Another possibility is that the primary expression of C/EBPβ at 2 h, found after administration of FFA, led to the second peak of PPAR-γ1 at 24 h after its transcription and translation. Thus, it is possible that CGA + CA mix acted on PPAR-γ1 by reducing its expression through an indirect way. This hypothesis seems in line with the observations reported by Murase and coworkers [60], who documented that a coffee phenolic extract, rich in CGA, did not stimulate the expression of PPAR-γ. This data could support the hypothesis of an involvement of other pathways not directly related to PPAR-γ.

Regarding C/EBPβ gene expression, Zhao et al [61] observed that palmitate induced lipid accumulation in HepG2 cells, also increasing mRNA expression of C/EBPβ. Knockdown of C/EBPβ inhibited lipid storage and promoted lipolysis in palmitate-treated cells, highlighting a key role of this transcription factor in lipid metabolism. Also, Ma et al [62] reported the capacity of ox-LDL (100 μg/mL) to stimulate C/EBPβ gene expression at 12, 24 and 48 hours in RAW 264.7 macrophages, that in turn led to the induction of NF-kB and p65 gene, and consequently the production of inflammatory markers such as IL-1β.

The role of polyphenols in the modulation of C/EBPβ has been poorly investigated. To the best of our knowledge, only Mosqueda-Solis et al [63] tested the effect of polyphenols (luteolin, genistein, hesperidin, kaempferol, pterostilbene, or vanillic acid at a concentration of 25 μM) in the modulation of C/EBPβ and other genes (e.g. PPAR-

γ) in pre-adipocytes treated from day 0 to day 8. The authors found that genistein, kaempferol and pterostilbene were effective in reducing the expression of C/EBPβ (about -55%) other than PPARγ (about -65%). However, this result is difficult to compare with our findings since the compounds and the concentrations tested were different.

The different effect on C/EBP-ß and PPAR-γ1 gene expression could be dependent by different factors including for example the type of cells, lipid stimulation, duration of exposure as well as different polyphenols tested. A possible hypothesis regards the association of the rapid modulation of C/EBP-ß and PPAR-γ1 gene expression and the reduced lipid accumulation observed at 24 h; this effect could be attributed to the inhibition of C/EBP-ß by CGA and CA, which in turn would result in a lower activation of PPAR-γ1, as demonstrated by other authors [50-53]. Consequently, the inhibition of PPAR-γ1 is supposed to downregulate CD-36-mediated lipid accumulation in macrophages, as reported by Poznyak and colleagues [54]. However, downstream targets have not been assessed in our experiments; thus, the results do not cover the array of pathways through which CGA and CA exert their effect in reducing lipid accumulation. Another potential explanation is represented by the inhibition of inflammatory pathway due to the suppression of C/EBP, that is strongly demonstrated to be responsible for lipid accumulation [64,65]. In this case, PPAR would not be involved in the mechanism of action of phenolics tested in our *in vitro* model and its reduction in gene expression observed at 24 h is essentially caused by the absence of lipid excess in the cells, which represents a stimulus in the expression of this transcriptional factor.

Furthermore, to explain the limited effects observed on C/EBP-ß and PPAR-γ1 *versus* lipid accumulation data it is possible to formulate alternative hypothesis relying to different mechanisms. For example, it is known that the liver X receptors, LXRA and LXRB, or rather a subfamily of the nuclear receptor superfamily, are key regulators of macrophage function, controlling transcriptional programs involved in lipid homeostasis and inflammation [65]. The inducible LXRA is highly expressed in macrophages and tissues such as lung and kidney, whereas LXRB is ubiquitously expressed. Ligand-activated LXRs form heterodimers with retinoid X receptors and regulate expression of target genes containing LXR response elements [66]. Tangirala

et al. [67] documented, in vivo, the crucial role of LXRs in atherosclerosis through the elimination of LXR activity in bone marrow-derived cells, which caused lipid accumulation in macrophages. Joseph et al [68] reported that LXRs and their ligands were negative regulators of macrophage inflammatory gene expression with consequence in lipid metabolism and the innate immune response. Finally, Zelcer et al [69] showed that LXRs can modulate lipid metabolism, thereby limiting cholesterol uptake and enhancing cholesterol efflux through ABC transporters. Based on these premises, we cannot exclude that further pathways, other than PPARγ and C/EBPβ, can have contributed to the modulation of lipid accumulation in our experimental conditions. A limitation in our findings relates to the lack of proteins quantification associated with the transcriptional regulatory elements PPARγ and C/EBPβ. This critical point should be considered in the future in order to verify, and possibly corroborate, the results of the gene expression with those obtained by proteomic analysis and other post-translational regulation of the activity. Another limitation is related to the choice of only two transcriptional factors, instead of performing a complete downstream transcriptional pathway of the different genes involved in the lipid metabolism that it would have been helpful for a better understanding of the results obtained. Finally, since CGA and CA are also rapidly metabolized *in vivo* mainly in the liver, the contribution of their metabolites in the modulation of PPARγ and C/EBPβ, or other genes, cannot be excluded.

In conclusion, the mix of CGA and CA reduced lipid accumulation in monocytes-derived macrophages probably *via* the modulation of PPAR-γ1 and C/EBPβ signaling pathways. These results are promising since the modulation of these important genes was obtained at concentrations close to physiological conditions and thus achievable *in vivo* through dietary intake of these bioactives. Moreover, lipid accumulation represents an important step of the atherogenic process and blunting its progression by dietary intervention can be considered crucial in the prevention of cardiovascular events. However, further studies are necessary in order to corroborate these findings and to verify the contribution of CGA and CA also in the regulation of other transcription factors potentially involved in lipid metabolism and atherogenesis.

Acknowledgements: This work was supported by a contribution of the "Piano di sostegno alla ricerca- Linea 2, azione A-grant number PSR2019 and 2018-CDELB". We are grateful to Samuele Venturi and Jacopo Tadini for their support in the experiments. The authors are grateful for support granted by Ministero delle Politiche Agricole, Alimentari e Forestali (Mipaaf) and the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL) MaPLE. P.R. and C.D.B. acknowledge the European Cooperation for Science and Technology (COST Action) CA16112 "NutRedOx: Personalized Nutrition in Aging Society: Redox Control of Major Age-related Diseases". The results provided in the present manuscript were obtained in the DeFENS Cell Culture Laboratory (University of Milan, Italy).

Author contributions: M.M. performed the experiments and wrote the first draft of the manuscript, C.D.B. designed the study, performed part of the experiments, the statistical analysis and wrote the first draft of the manuscript. M.T. and G. M. performed the experiments on gene expression supervised by V.T.
P.R. and M.P. critically revised the manuscript and partially supported the research. P.M. critically revised the manuscript and edited the paper for language. All authors have read and approved the final version of the manuscript.

Conflict of interest: None of the authors has a conflict of interest to declare.

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Table 1 Forward and reverse primer sequences of housekeeping and target genes

Table 2 Percentage of viability of THP-1 derived monocytes following supplementation with free fatty acids (FFA), chlorogenic acid (CGA), caffeic acid (CA), and the mix of the two compounds (CGA + CA) evaluated by Trypan blue assay

Fig. 1 Chemical structure of chlorogenic and caffeic acid

Fig. 2 Effect of different concentrations (0.03 – 30 μM) of CGA (a) and CA (b) on lipid accumulation in THP-1 derived macrophages

Legend: Results are expressed as mean ± standard error of mean. Each value represents the mean of 7 values ± SEM from 3 individual experiments. Data are reported as fold increase compared to the control cells without FFA (average fluorescence values in the control was at about 23000 a.u). ^{a,b} Bar graphs reporting different letters are significantly different ($p \leq 0.05$) between each other. No FFA: control without free fatty acids; FFA: free fatty acids (500μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 3 Effect of different concentrations (0.03 – 30 μM) of the mix CGA + CA on lipid accumulation in THP-1 derived macrophages

Legend: Results are expressed as mean ± standard error of mean. Each value represents the mean of 7 values ± SEM from 3 individual experiments. Data are reported as fold increase compared to the control cells without FFA (average fluorescence values in the control was at about 23000 a.u). ^{a,b,c,d} Bar graphs reporting different letters are significantly different ($p \leq 0.01$) between each other. No FFA: control without free fatty acids; FFA: free fatty acids (500μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 4 Effect of the mix of CGA + CA in the modulation of the levels of expression of C/EBPβ in macrophages

Legend: Results are expressed as mean of fold increase ± standard error of mean. Each value represents the mean of 3 values ± SEM from 3 individual experiments. ** Significantly different ($p < 0.01$) compared to time zero and CA+CGA at 2h. No FFA: control without free fatty acids; FFA: free fatty acids (500 μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 5 Effect of the mix of CGA + CA in the modulation of the levels of expression of PPAR-γ1 in macrophages

Legend: Results are expressed as mean of fold increase ± standard error of mean. Each value represents the mean of 3 values ± SEM from 3 individual experiments. ** Significantly different ($p < 0.01$) compared to time zero and at 24h also compared to CA+CGA at the same time. No FFA: control without free fatty acids; FFA: free fatty acids (500 μM); CGA: chlorogenic acid; CA: caffeic acid

Table 1 Forward and reverse primer sequences of housekeeping and target genes

Target Gene	Forward primer	Reverse primer	References
PPAR-γ1	5′-GAAAGACAACGGACAAATCACC-3′	5′-GGGGGTGATATGTTTGAACTTG-3′	[35]
C/EBPβ	5′-AACCTGGAGACGCAGCACAA-3′	5′-CTTGAACAAGTTCCGCAGGGTG-3′	[36]
Reference			
18S	5′-ATCCCTGAAAAGTTCCAGCA-3′	5′-CCCTCTTGGTGAGGTCAATG-3′	[37]

PPAR-γ1, peroxisome proliferator activated receptor-γ1; C/EBPβ, CCAAT-enhancer-binding proteins

Table 2 Percentage of viability of THP-1 derived monocytes following supplementation with free fatty acids (FFA), chlorogenic acid (CGA), caffeic acid (CA), and the mix of the two compounds (CGA + CA) evaluated by Trypan blue assay

Trypan blue assay	% Cell viability
No FFA	91.9 ± 1.1
FFA	93.1 ± 1.0
CGA (0.03μM)	90.3 ± 0.9
CGA (0.3μM)	90.0 ± 0.3
CGA (3μM)	90.0 ± 0.5
CGA (30μM)	92.9 ± 0.9
CA (0.03μM)	91.7 ± 0.9
CA (0.3μM)	90.7 ± 0.7
CA (3μM)	90.0 ± 0.2
CA (30μM)	91.8 ± 0.5
CGA (0.03μM) + CA (0.03μM)	91.6 ± 1.0
CGA (0.3μM) + CA (0.3μM)	91.6 ± 0.9
CGA (3μM) + CA (3μM)	90.8 ± 0.6
CGA (30μM) + CA (30μM)	92.3 ± 0.7

Results derived from three independent experiments in which each compound was tested in triplicate. Data are reported as mean ± standard error of the mean

Fig. 1

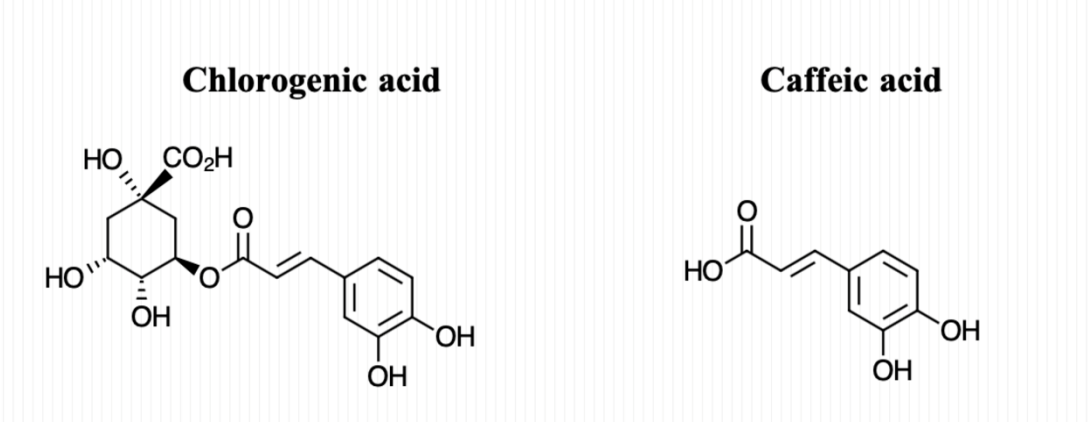


Fig. 2a

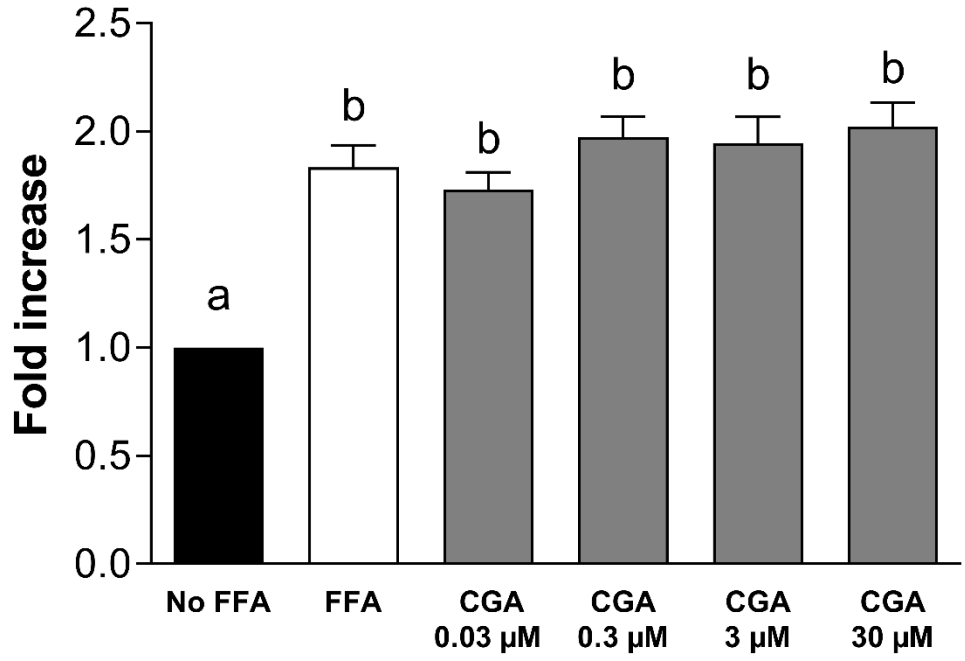


Fig. 2b

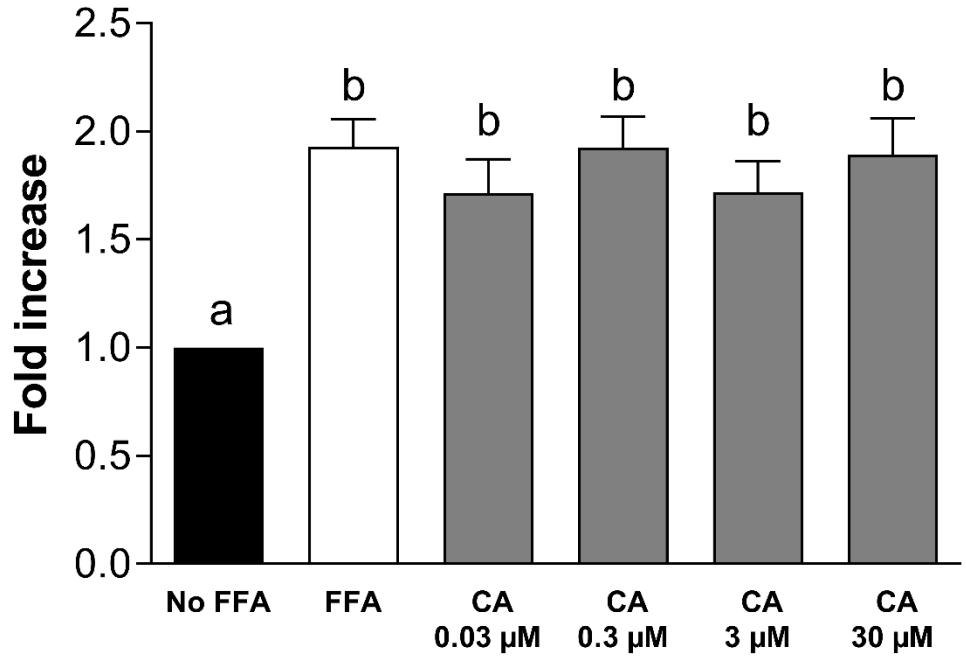

Fold increase
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
b
b
b
No FFA
FFA
CA 0.03 µM
CA 0.3 µM
CA 3 µM
CA 30 µM

Fig. 3

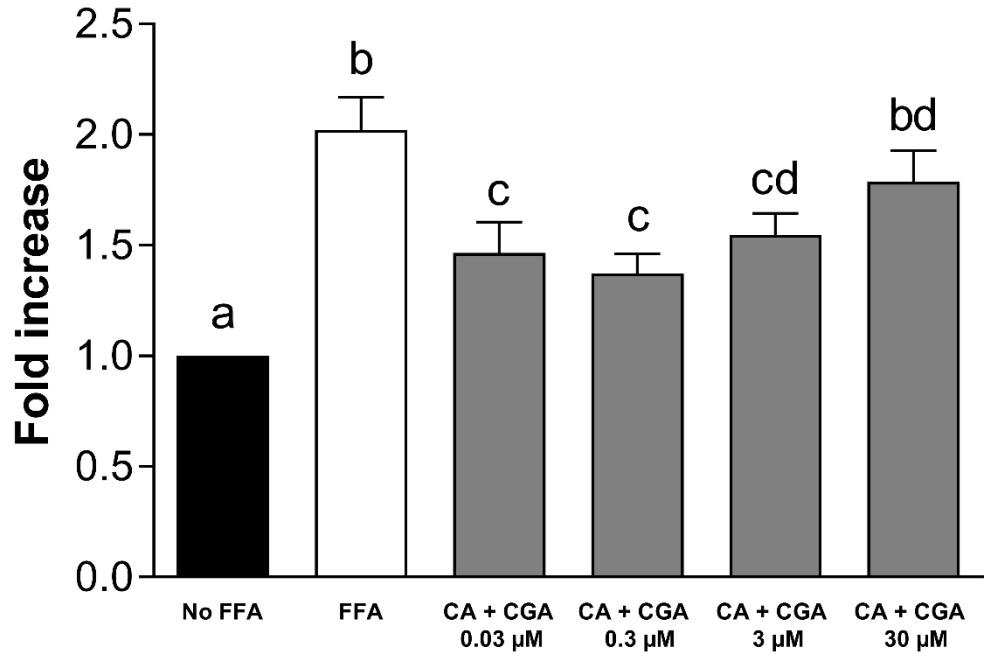

Fold increase
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
c
cd
bd
No FFA
FFA
CA + CGA 0.03 µM
CA + CGA 0.3 µM
CA + CGA 3 µM
CA + CGA 30 µM

Fig. 4

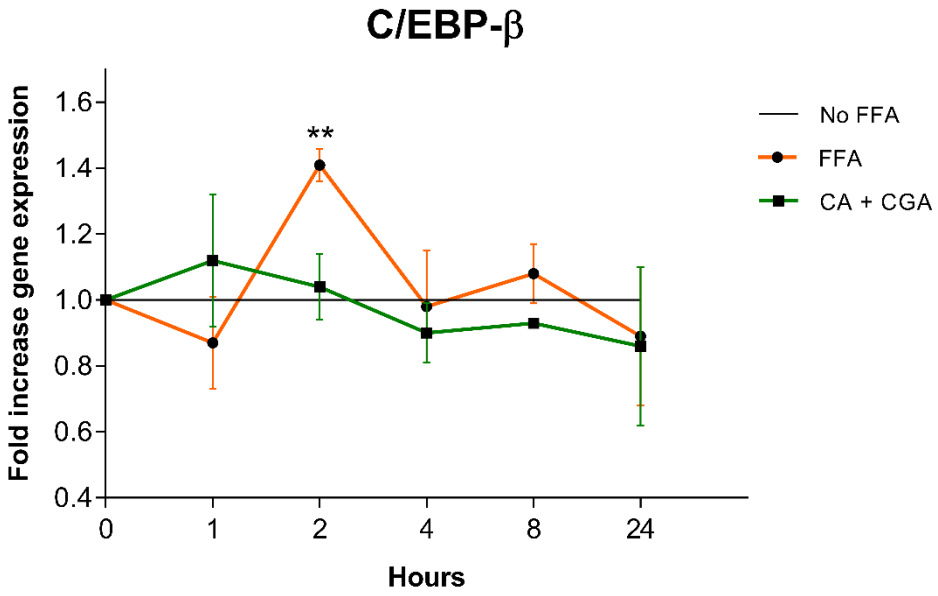
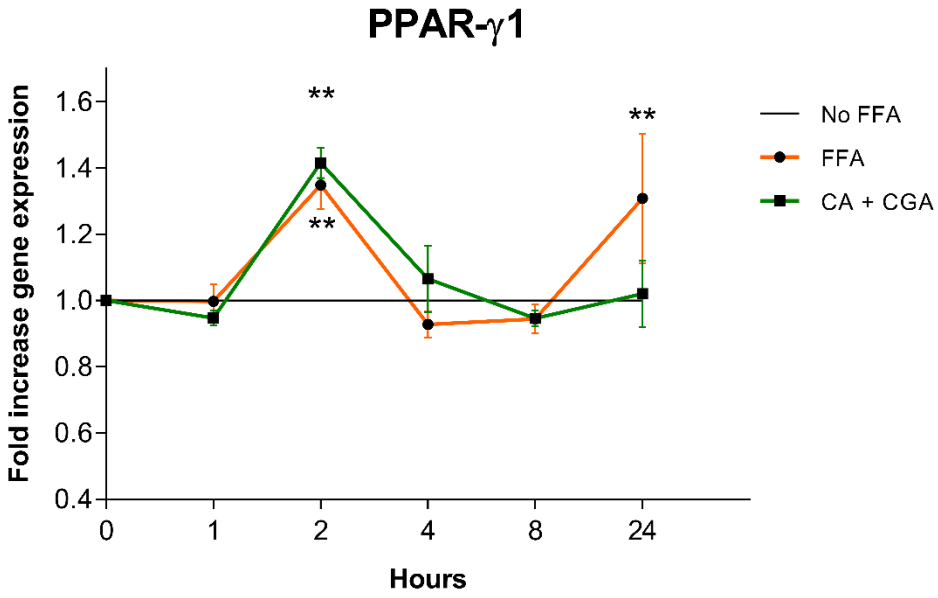

C/EBP-β
Fold increase gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
**
0
1
2
4
8
24
Hours
No FFA
FFA
CA + CGA

Fig. 5


PPAR-γ1
Fold increase gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
**
**
**
0
1
2
4
8
24
Hours
No FFA
FFA
CA + CGA

CHAPTER 2

Effects of anthocyanins and their metabolites on high-fat diet-induced mitochondrial dysfunction and inflammation: *in vivo* and *in vitro*

The second part of the thesis regards the research activity performed at the Department of Nutrition and Environmental Toxicology, University of California (Davis, USA) in which the effect of anthocyanins and metabolites on high-fat diet-induced mitochondrial dysfunction and inflammation was investigated by using in vitro and in vivo models. Specifically, 3T3-L1 preadipocytes cell line was used to study the effect of anthocyanins and their metabolites in the promotion of mitochondrial function and differentiation from white to brown adipocytes. Finally, the effect of these bioactives was also investigated by using an animal model of C57BL/6J mice fed with a high-fat diet.

4.1 INTRODUCTION

4.1.1 High fat diets and mitochondrial function

Mitochondria are dynamic organelles fundamental in cell biology. They are involved in several functions including energy production, regulation of cell death, ion homeostasis and non-shivering thermogenesis **(Osellame et al 2012)**.

Metabolic and environmental stresses, such as high fat (HF) diets, lead to alterations in mitochondrial functionality. In this context, fusion and fission of mitochondria represent essential processes to mitigate harmful consequences. In particular, mitochondrial fusion allows to combine components of partially damaged mitochondria in order to generate functional mitochondria. While, excessively damaged mitochondria are removed thanks to quality control exerted by the fission process. Moreover, fission proteins are also able to induce the generation of new mitochondria in case of necessity. **(Youle et al 2012)**

Through the dynamicity of mitochondria, due to a continuous modeling through fusion and fission, cells are able to address important physiological processes, such as cell division and apoptosis. **(Cerveny et al 2007)**. On the other hand, impairment of mitochondrial function is associated with cardiac and neurodegenerative disorders. **(Whitney et al 2019)**

Different studies showed a negative effect of HF diets on mitochondrial function. Wistar rats fed for 8 weeks with a HF diet observed mitochondrial function impairment through an increase in ROS (Reactive oxygen species) production **(Zalewska et al 2019).** Chronic feeding of albino mice a HF diet resulted in an alteration of mitochondrial function that also led to increases in inflammatory markers **(Jha et al 2019).** C57BL/6J mice fed *ad libitum* with 60% (w/w) lard diet for 4 weeks showed mitochondrial dysfunction through an impairment of mitochondrial ADP sensitivity **(Miotto et al 2018).**

However, there is growing evidence that select polyphenols could be effective in enhancing mitochondrial function. For example, resveratrol supplementation counteracted alterations in mitochondria dynamics in rotenone-treated rats, through the modulation of PGC-1α, OPA1/MFN2 and Fis1/Drp1, main proteins involved in mitochondrial fusion and fission **(Peng et al 2016)**. Nutraceuticals rich in purified cyanidin-3-O-glucoside, 3-O-b-glucosides and delphinidin 3-O-glucoside stabilized fusion and fission processes in H-SY5Y neuroblastoma cells treated with rotenone leading to mitigation of mitochondrial dysfunction **(Parrado-Fernández et al 2016).**

4.1.2 HF diets and browning

The brown adipose tissue is characterized by a high number of mitochondria. It is highly metabolically active since it is able to use glucose and stored triglycerides to generate heat through a process named non-shivering thermogenesis. In fact, the browning of white adipose tissue could represent a relevant approach to manage obesity and metabolic disorders. **(Bartelet et al 2014)**

In contrast, mitochondrial dysfunction in brown adipocytes of obese people causes an accumulation of ectopic fat due to a reduction in energy expenditure and fat oxidation. **(Bournat et al 2010)**

Mice fed with high fat-diet showed lower levels of mitochondria biogenesis biomarkers (such as UCP-1, PGC-1β and ERRα), accompanied by a depletion of mitochondrial mass and structure (e.g. mitofilin, optic atrophy1 and surfeit 1). **(Rong et al 2007)**

Another study highlighted how in obese and diabetic ob/ob mice the epididymal adipose tissues showed a remarkable reduction of mitochondrial populations. **(Wilson-Fritch et al 2004)**

Moreover, HF diets and in a general excess of nutrients lead to the accumulation of fat at different levels, beyond the subcutaneous adipose tissue (which is the largest white adipose tissue depot), such as liver, skeletal muscle, hearth and intestine. This represents a risk factor for metabolic disorders **(Longo et al 2019)**.

On the other hand, under certain conditions, such as cold exposure and β-adrenergic agonists, white adipose tissue goes through a process called browning, in which adipocytes increase the number of mitochondria and acquire thermogenic properties. The latter function is mainly exerted by the uncoupling protein-1 (UCP-1) which is a protein located on the mitochondrial inner membrane able to convert the electrochemical energy into heat (**Lee et al 2019)**. Browning of WAT could be a potential strategy to manage fat accumulation, obesity and related diseases **(Castillo et al 2017)**.

The use of empagliflozin, a drug able to induce adipose tissue browning, in obese C57BL/6J mice fed a HF diet resulted in weight gain suppression, increase of energy expenditure, reduction of obesity-related chronic inflammation and improvement of insulin resistance and hepatic steatosis **(Xu et al 2019).** In C57BL/6N wild-type mice fed with a HF diet for 8 weeks, rescovitine (a cyclin-dependent kinase inhibitor) induced white adipocytes browning and reduced lipogenesis conferring resistance to obesity and ameliorating metabolic profiles **(Wang et al 2016)**.

In addition to drugs, different dietary bioactive compounds demonstrated to have the capacity to induce browning of white adipose tissue, with particular attention to phenolic compounds. A recent review **(Kim et al 2019)** examined the role of resveratrol in browning, concluding the potential strategy to use this natural compound to treat obesity, since it was able to regulate several myokines and adipokines promoting weight loss and mitigating insulin resistance. Apple polyphenols induced thermogenic adaptation in mice fed a HF diet, resulting in lower fat accumulation and reduced inflammation in white adipose tissue **(Tamura et al 2020)**.

4.1.3 HF-diet and inflammation

Overnutrition and excess of ectopic fat leads to systemic and chronic low-grade inflammation. Circulating endotoxin, free fatty acid and inflammatory mediators increase in response to HF diets **(Duan et al 2018)**. In metabolic syndrome induced by HF diets, the NLRP3 inflammasome plays a relevant role. The use of MCC950, an inhibitor of the NLRP3 inflammasome, caused a metabolic improvement in mice fed an obesogenic diet **(Pavillard et al 2017)**. The NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) acts as intracellular sensor by triggering the NLRP3 inflammasome in response to several stimuli such as endogenous danger signals, microbial motifs and environmental irritants. In its regard, the activation of NLRP3 inflammasome leads to the release of different inflammatory mediators, i.e. caspase-1, and IL-1β. One of the events able to induce NLRP3 is mitochondrial dysfunction, the release of mtDNA and mtROS triggers the activation of the inflammasome **(Swanson et al 2019)**.

The inhibition of NLRP3 inflammasome could represent a pivotal therapeutic target since this inflammatory pathway is involved in several diseases such as atherosclerosis, diabetes, metabolic syndrome and neurodegenerative diseases **(Zahid et al 2019)**.

Different studies reported a positive regulation of NLRP3 inflammasome by natural compounds. Epigallocatechin-3-gallate (EGCG), major bioactive polyphenol presents in green tea, inhibited the activation of the NLRP3 inflammasome in New Zealand black/white (NZB/W) F1 lupus-prone mice **(Tsai et al 2011)**. In another study, Sprague-Dawley rats supplemented with quercetin showed a reduction in ROS production and NLRP3 activation **(Jiang et al 2016)**. Resveratrol supplementation for a 4-week period in a rodent model of HF diet-induced obesity resulted in amelioration of metabolic profiles through the inactivation of the NLRP3 inflammasome **(Yang et al 2014)**.

In this context, the aim of this study was to investigate the capacity of ACNs to counteract mitochondrial dysfunction in subcutaneous white adipose tissue (sWAT) as a consequence of HF diet-induced obesity in mice. The capacity of ACNs to promote "beiging" of sWAT, through increased mitochondrial biogenesis and thermogenesis was characterized. In addition, the effects of ACNs on the modulation of NLRP3 inflammasome was evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Materials

3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MA, USA). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies for PGC-1α (sc-517380), UCP-1 (sc-293418), Mfn2 (sc-100560), Nix (sc-166332), Fis1 (sc-376447), OPA1 (sc-393296), Parkin (sc-133167), VDAC1 (sc-390996), HSC-70 (sc-1059), Cytochrome C (sc-7159), PPAR-γ (sc-7273), DRP-1 (sc-101270), citrate synthase (sc390693), PRX1 (sc-293386), PINK1 (sc-517353), MFF (sc-398617), ATP5B (sc-55597) and caspase-1 (sc-56036) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for COX IV (#4844), β-Actin (D6A8) (#12620), pDRP-1 (Ser616) (#3455), NLRP3

(D4D8T) (#15101), NF-κB p65 (D14E12) (#8242), Phospho-NF-κB p65 (Ser536) (93H1) (#3033), p44/42 MAPK (Erk1/2) (137F5) (#4695), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), IKKα (#2682) and Phospho-IKKα/β (Ser176/180) (16A6) (#2697) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for IL-1β (ab9722) and PRDM-16 (ab106410) were purchased from Abcam Inc. (Cambridge, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA, USA). The ECL Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ). Cyanidin, delphinidin, protochatecuic acid, 4-hydroxybenzaldheyde, gallic acid, palmitate, and all other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO).

4.2.2 Animals and animal care

All procedures were in agreement with standards for care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee.6-weeks old healthy male C57BL/6J mice (20–25 g) (10 mice/group) were fed for 14 weeks either: i) a diet containing approximately 10% total calories from fat (Control, C group), ii) a diet containing approximately 60% total calories from fat (lard) (HFD), or the control (C + AC, CA group) and high fat diet (HFD + AC, HFA group) supplemented in the diet with 40 mg AC/kg body weight. Taking into account the translation of the dose from animals to humans **(Reagan-Shaw et al 2007)**, the equivalent dose in human corresponds to 225 mg of AC per day. Moreover, considering that 100 g of raw blueberries provide 240 mg of AC, the quantity could be easily achievable.

AC-containing diets were prepared every two weeks to account for changes in body weight and food intake, and to prevent AC degradation. All diets were stored at −20 °C until use.

Body weight and food intake were measured weekly throughout the study. After 14 weeks on the dietary treatments, mice were euthanized by cervical dislocation. Blood was collected from the submandibular vein into EDTA tubes, plasma was collected after centrifugation at 3,000 x g for 15 min at room temperature. Different adipose tissue pads were collected and weighed. Tissues were flash-frozen in liquid nitrogen and then stored at −80 °C until further analysis.

4.2.3 Cell culture

3T3-L1 preadipocytes were maintained in high glucose DMEM, 10% (v/v) fetal bovine serum (FBS), 50U/mlpenicillin, and 50 μg/ml streptomycin. To induce cell differentiation, 3T3-L1 pre-adipocytes were grown to confluency in culture medium containing 10% (v/v) FBS, and then switched to differentiation medium (DIFM) containing 10% (v/v) FBS added with 20 nM insulin and 1 nM triiodothyronine for 48 h. Adipocyte differentiation was induced by treating cells for 48 h in DIFM further supplemented with 0.5 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, and 0.125 mM indomethacin. After induction, cells were returned to DIFM medium. On day 12 cells were fully differentiated and exhibited massive accumulation of fat droplets. 3T3-L1 adipocytes were incubated in the absence or the presence of 0.5 mM palmitate with or without: cyanidin (CY) (0.1 μM), delphinidin (DEL) (0.1 μM), and 0.5 μM of protocatechuic acid (PCA), 4-hydroxybenzaldeyde (HB), as cyanidin metabolites and gallic acid (GA) as delphinidin metabolite. After 24 h incubation, the cells were collected for subsequent analysis.

4.2.4 Western blot analysis

Tissues and cells were lysed as previously described (**Bettaieb et al., 2014; Vazquez-Prieto et al., 2012**). Aliquots of total homogenates containing 30 μg protein were denatured with Laemmli buffer, separated by reducing 8–15% polyacrylamide gel electrophoresis, and electroblotted onto PVDF membranes. Colored (Biorad Laboratories, Hercules, CA) and biotinylated (Cell Signaling Technologies, Danvers MA) molecular weight standards were ran simultaneously. Membranes were blocked

for 1 h in 5% (w/v) non-fat milk and subsequently incubated overnight at 4°C in the presence of the corresponding primary antibodies (1:1000 dilution) in 5% (w/v) bovine serum albumin in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.1% (v/v) Tween-20. After incubation for 1.5 h at room temperature in the presence of secondary antibodies (HRP conjugated) (1:10,000 dilution) the conjugates were visualized with enhanced chemiluminescence (ECL, Amersham Biosciences) using a Phosphoimager Storm 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ). Data for phosphorylated proteins are presented as phosphorylation level normalized to protein expression.

4.2.5 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC). Fisher least significance difference test was used to examine differences between group means. A P value < 0.05 was considered statistically significant. Figures were prepared using GraphPad Prism 7 (GraphPad Software, CA, USA). Data are shown as mean ± SEM.

4.3 RESULTS

4.3.1 Effect of AC on body weight and fat pad in mice fed a HF diet

AC supplementation for 14 weeks did not affect body weight gain in mice fed a C diet, but caused a significant reduction in body weight in mice fed a HF diet (Fig 1). In particular, the body weight of mice in the group HF + AC was 14.2% lower compared to the body weight of mice fed only a HF diet. Consumption of the HF diet significantly increased the weight of all different isolated fat pads (brown fat, subcutaneous fat, epididymal fat, visceral fat and retroperitoneal fat) compared to mice fed the control diet diet. AC supplementation did not affect the fat pads in mice fed the control diet, except for the brown adipose tissue that observed a statistically significant increase of 30% compared to mice fed only the control diet (213 mg versus 149 mg). AC supplementation in mice fed the HF diet resulted in a significant reduction in visceral

fat (-30.5%), retroperitoneal fat (-33.4%) and brown fat (-31.3%) compared to mice fed only the HF diet.

Table 1 Body and fat pad weight

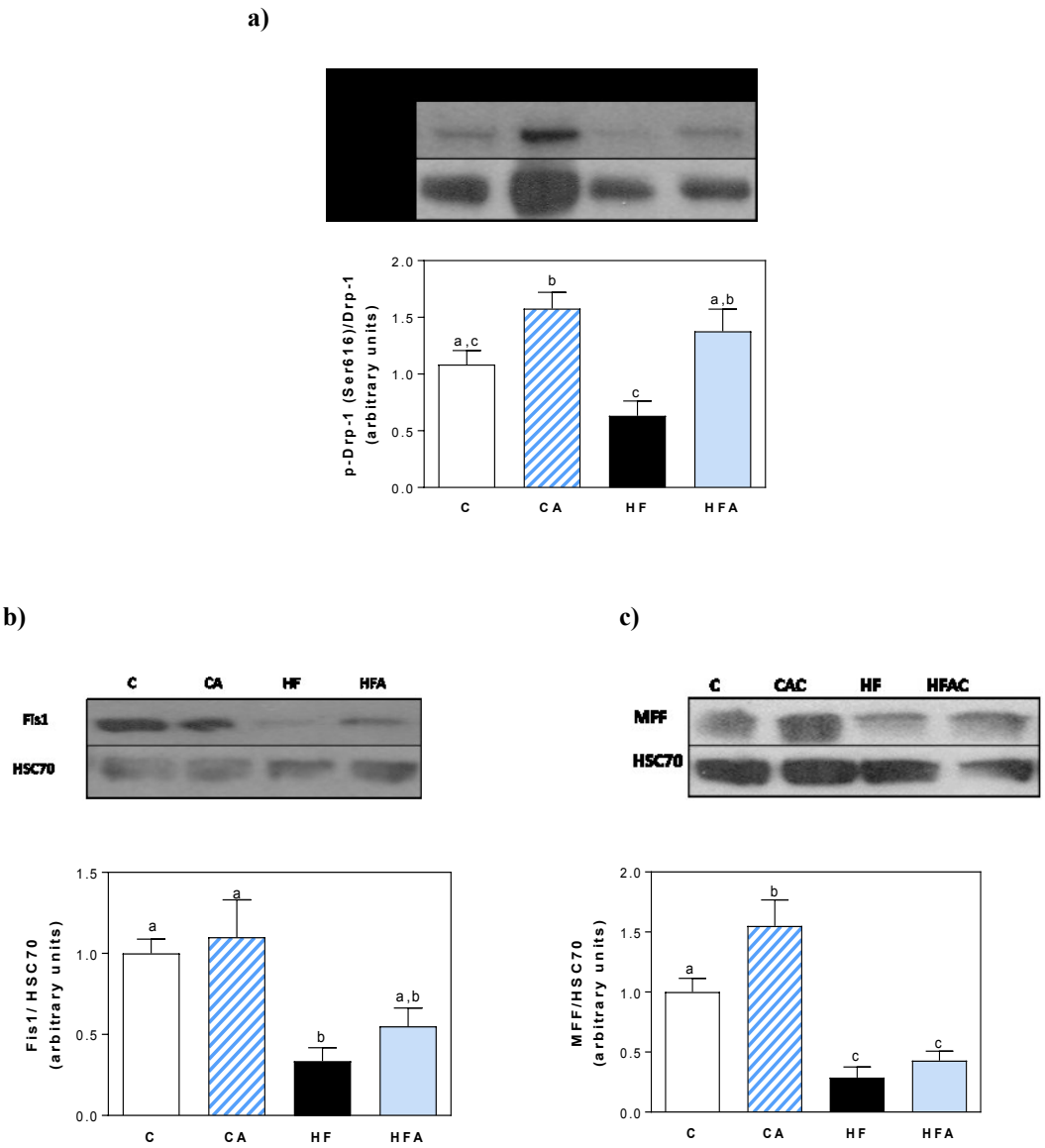
Parameter	Control	HF	HF +AC40	Control + AC40
BW (g)	33.7 ± 0.8^{a}	45.8 ± 0.9^{b}	39.3 ± 2.3^{c}	35.8 ± 1.0^{ac}
Brown fat (mg)	149 ± 12^{a}	259 ± 20^{b}	178 ± 20^{a}	213 ± 24^{b}
Epididymal fat (g)	1.23 ± 0.10^{a}	2.36 ± 0.10^{b}	2.22 ± 0.29^{b}	1.45 ± 0.15^{a}
Visceral fat (g)	0.53 ± 0.10^{a}	1.28 ± 0.12^{b}	0.89 ± 0.12^{c}	0.55 ± 0.07^{ac}
SQ fat (g)	0.98 ± 0.06^{a}	3.54 ± 0.23^{b}	2.71 ± 0.49^{b}	1.59 ± 0.19^{a}
Retroperit. Fat (g)	0.40 ± 0.27^{a}	1.26 ± 0.09^{b}	0.84 ± 0.13^{c}	0.54 ± 0.07^{a}

Body and fat pads weight from mice fed for 14 weeks with the corresponding diets. Values are shown as means ± SE (n = 10). Values having different superscripts are significantly different ($P < 0.05$, one-way ANOVA).

4.3.2 AC improve mitochondrial fission in subcutaneous fat tissue of mice fed a HF diet

Since the proper functioning of DRP-1 (dynamin-related protein), MFF (mitochondrial fission factor) and Fis1 (mitochondrial fission 1 protein) play an essential role in mitochondrial fission **(Kornfeld et al 2018)**, we decided to investigate the expression of these three proteins. There was a trend (p=0.41) for lower p-DPR-1 levels in the HF group compared to the C group **(Fig 1a)**. While, phosphorylated DRP-1 levels were 64.2% higher ($P < 0.05$) in subcutaneous adipose tissue from HFA compared to HFD mice. DRP-1 phosphorylation (Ser616) levels in CA mice were 31.2% higher compared to the C group (Fig 1a). Although, no statistically significant difference was observed between CA and HFA groups. On the contrary, consumption of the HF diet caused a significant ($P< 0.05$) reduction (-66.5%) in subcutaneous adipose tissue Fis1 levels compared to controls **(Fig 1b)**. AC supplementation did not mitigate HF diet-induced decrease in Fis-1 concentration.AC supplementation increased MFF levels in mice fed the control diet (+35.5%) **(Fig 1c)**. The HF diet induced a significant reduction of MFF compared to the CA and C (-72.3% and -71.3% respectively).

Fig. 1 Effect of AC supplementation on subcutaneous adipose tissue mitochondrial fission in HF diet-fed mice: pDRP-1, Fis1 and MFF



Phosphorylation of DRP-1 (Ser616), Fis1 and MFF subcutaneous adipose tissue after 14 weeks on the corresponding diets. Bands were quantified and results for mice fed the control diet supplemented with AC (CA), the HFD (HF), and the HFD supplemented with AC (HFA) were referred to control group values (C). Representative immunoblots are shown. Bar charts represent pDRP-1/DRP-1, Fis1/HSC70 and MFF/HSC70 as means ± SEM from 9 to 10 animals/treatment. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

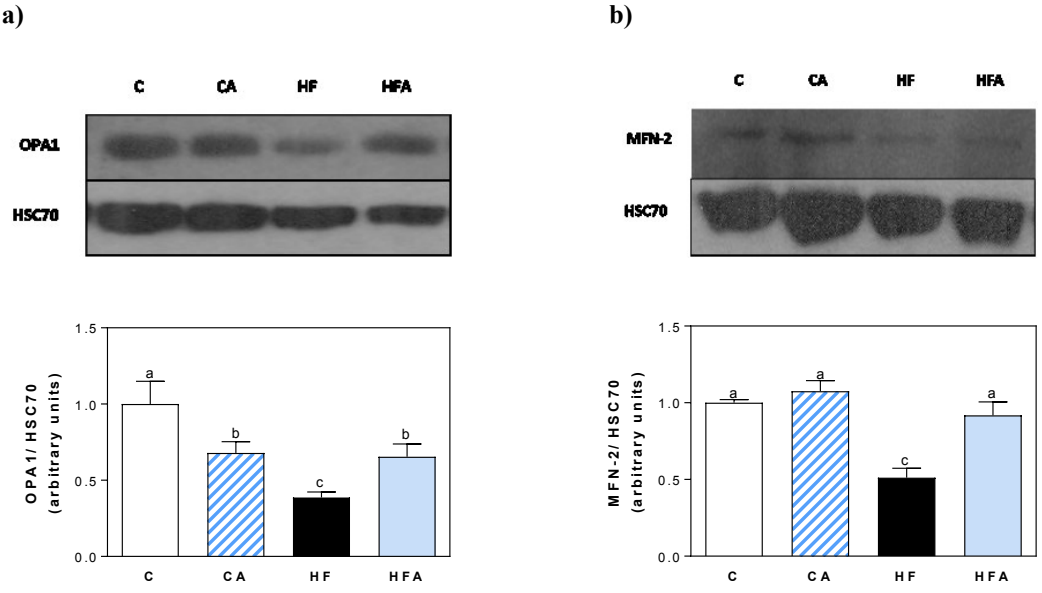
4.3.3 AC improve mitochondrial fusion in subcutaneous fat tissue of mice fed a HF diet

Mitochondrial membrane fusion has a key role in mitochondrial dynamics and functioning. OPA1 (dynamin-like 120 kDa protein) and MFN-2 (mitofusin-2) are required to coordinate mitochondrial fusion **(Song et al 2009)**. Therefore, we investigated the expression of these proteins in our experiments.

In subcutaneous adipose tissue, HF diet feeding caused a 61.3% reduction of OPA1 levels compared to the C group that was partially prevented by AC supplementation. AC supplementation to mice fed the control diet caused a decreased expression of OPA1 **(Fig 2a)**.

In HF diet-fed mice, a reduction of 49% in MFN-2 was observed compared to the C group (**Figure 2b).** The latter effect was mitigated by AC supplementation.

Fig. 2 Effect of AC supplementation on subcutaneous adipose tissue mitochondrial fusion in HF diet-fed mice: OPA1 and MFN-2



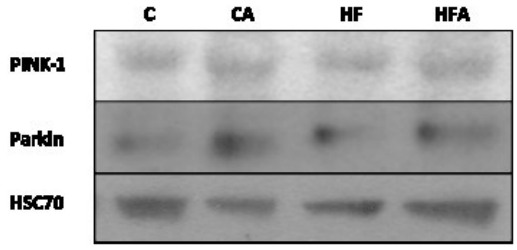
OPA1 and MFN-2 subcutaneous adipose tissue after 14 weeks on the corresponding diets. Bands were quantified and results for mice fed the control diet supplemented with AC (CA), the HFD (HF), and the HFD supplemented with AC (HFA) were referred to control group values (C). Representative

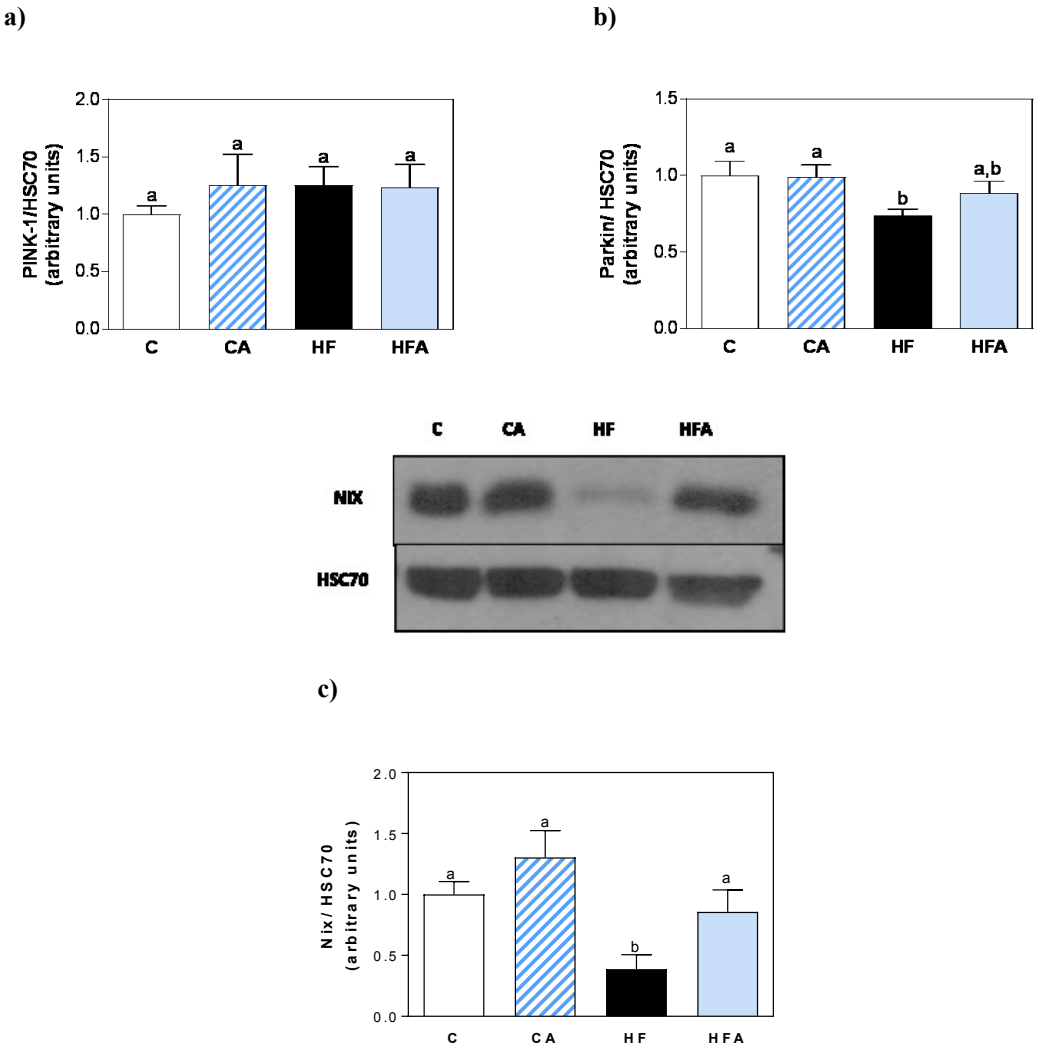
immunoblots are shown. Bar charts represent OPA1/HSC70 and MFN-2/HSC70 as means ± SEM from 9 to 10 animals/treatment. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.3.4 High fat diet consumption is associated with downregulation of proteins that promote quality control of mitochondria and mitophagy

Accumulation of damaged mitochondrial leads to an increased ROS production and oxidative stress that results in mitochondrial dysfunction, triggering a vicious cycle **(Luo et al 2013)**. PINK-1 (Serine/threonine-protein kinase PINK1), Parkin (E3 ubiquitin-protein ligase parkin) and Nix (NIP3-like protein X) work together to ensure a quality control of mitochondria and the eventual autophagy-dependent selective degradation of dysfunctional mitochondria, termed mitophagy **(Park et al 2017)**. PINK-1 levels were similar for all dietary groups. In HF diet-fed mice levels of Parkin and Nix in subcutaneous adipose tissue were lower compared to the C group (-26% and -61%, respectively). AC supplementation did not affect the levels of PINK1 and Parkin in mice fed the contro, diet **(Fig. 3a and 3b)**. AC significantly increased the levels of Nix in mice fed the HF diet (+55%) **(Fig. 3c)**.

Fig. 3 Effect of AC supplementation on subcutaneous adipose tissue mitochondrial quality control and mitophagy in HF diet-fed mice: PINK-1, Parkin and Nix





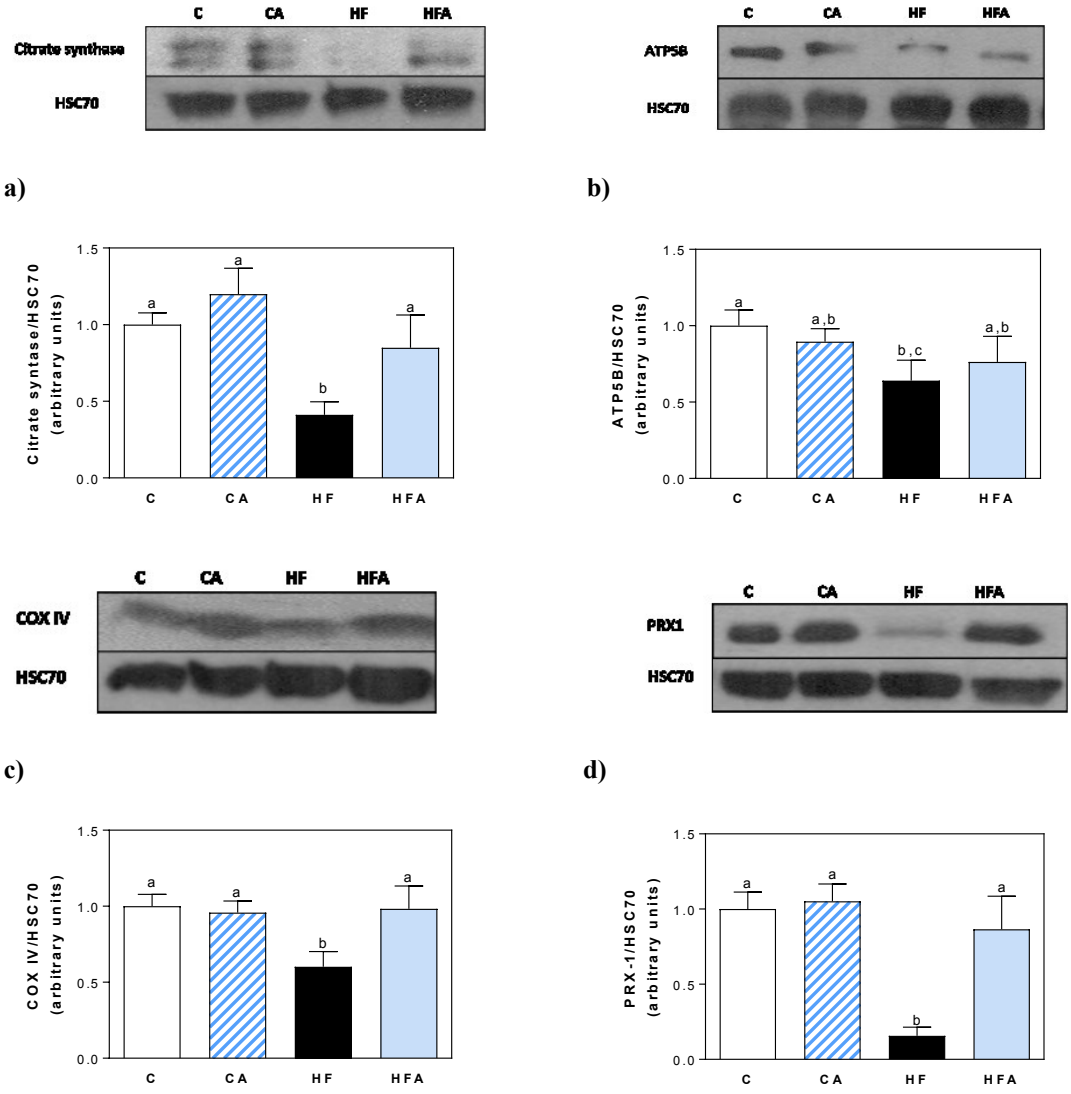
PINK-1, Parkin and Nix subcutaneous adipose tissue after 14 weeks on the corresponding diets. Bands were quantified and results for mice fed the control diet supplemented with AC (CA), the HFD (HF), and the HFD supplemented with AC (HFA) were referred to control group values (C). Representative immunoblots are shown. Bar charts represent PINK-1/HSC70, Parkin/HSC70 and Nix/HSC70 as means ± SEM from 9 to 10 animals/treatment. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.3.5 AC supplementation prevents HF diet-induced decrease in subcutaneous adipose tissue mitochondrial number

We next investigated the potential effects of HF diet consumption and the potential benefit of AC supplementation on mitochondrial number. For this purpose, we measured the expression of proteins that are specifically located at mitochondria: citrate synthase, ATP5B (ATP synthase subunit beta), COX IV (cytochrome c oxidase subunit 4) and PRX1 (Peroxiredoxin 1).

All these proteins, citrate synthase, ATP5B, COX IV and PRX1, were significantly reduced in the subcutaneous adipose tissue of mice fed the HF diet compared to the C diet (-58.8%, -35.9%, -39.8%, -84.3%, respectively) **(Fig. 4)**. AC supplementation mitigated the effects of the HF diet, increasing citrate synthase, COX IV and PRX1 (+51.5%, +39% and +82%, respectively, compared to HF-fed mice).

Fig. 4 Effect of AC supplementation on subcutaneous adipose tissue mitochondrial number in HF diet-fed mice: citrate synthase, ATP5B, COX IV and PRX1



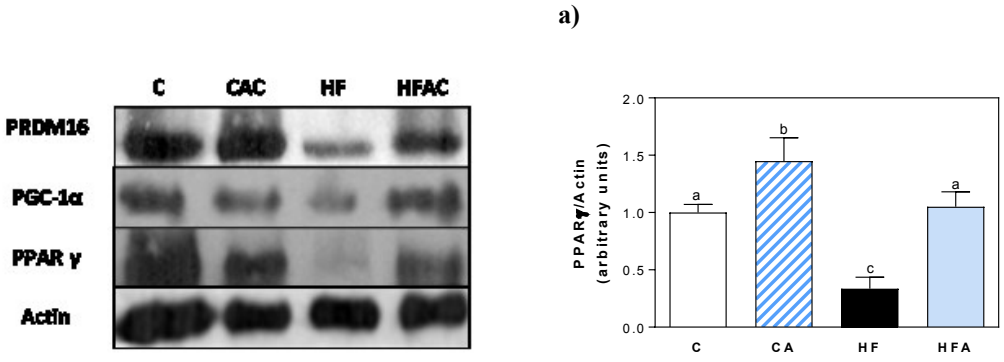
Citrate synthase, ATP5B, COX IV and PRX1 subcutaneous adipose tissue after 14 weeks on the corresponding diets. Bands were quantified and results for mice fed the control diet supplemented with AC (CA), the HFD (HF), and the HFD supplemented with AC (HFA) were referred to control group values (C). Representative immunoblots are shown. Bar charts represent Citrate synthase/HSC70, ATP5B/HSC70, COX IV/HSC70 and PRX1/HSC70 as means ± SEM from 9 to 10 animals/treatment. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.3.6 AC supplementation prevents high fat-induced decrease in mitochondriogenesis and thermogenesis

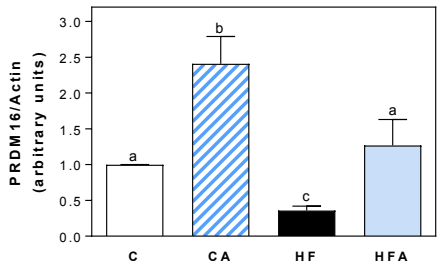
The differentiation of white adipose tissue in brown adipose tissue, also termed browning, and the associated increase in thermogenesis could represent a new therapeutic target against obesity and its related side effects **(Wang et al 2019)**. To this purpose, we decided to investigate the effects of HF diet and AC supplementation on PPAR-γ (peroxisome proliferator-activated receptor gamma) PRDM-16 (histone-lysine N-methyltransferase) PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and UCP-1 (Mitochondrial brown fat uncoupling protein 1), the principal proteins involved in mitochondriogenesis/thermogenesis.

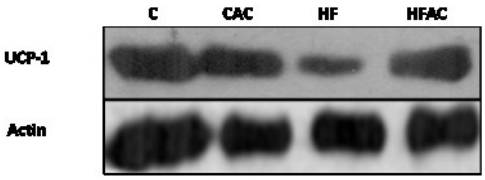
All these proteins, PPAR-γ, PRDM-16, PGC-1α and UCP-1, were significantly reduced in the subcutaneous adipose tissue of mice fed the HF diet compared to the C diet (-66.5%, -64%, -64.5%, -62.8%, respectively) **(Fig. 5)**. AC supplementation in HF diet-fed mice prevented all these decreases. In C diet-fed mice, AC supplementation resulted in significantly higher levels of PPAR-γ, PRDM-16 and PGC-1α (+45%, +141% and +91.4%, respectively). Moreover, the HFA and C groups showed similar levels of PPAR-γ, PRDM-16, PGC-1α and UCP-1 protein expression.

Fig. 5 Effect of AC supplementation on subcutaneous adipose tissue mitochondriogenesis and thermogenesis in HF diet-fed mice: PPAR-γ, PRDM-16, PGC-1α and UCP-1

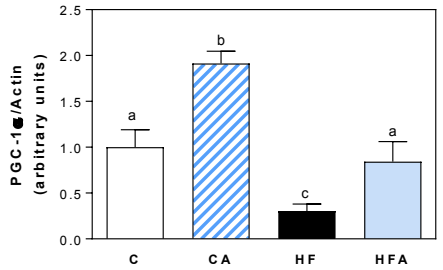


b)

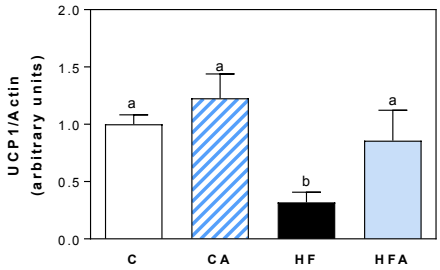




c)



d)

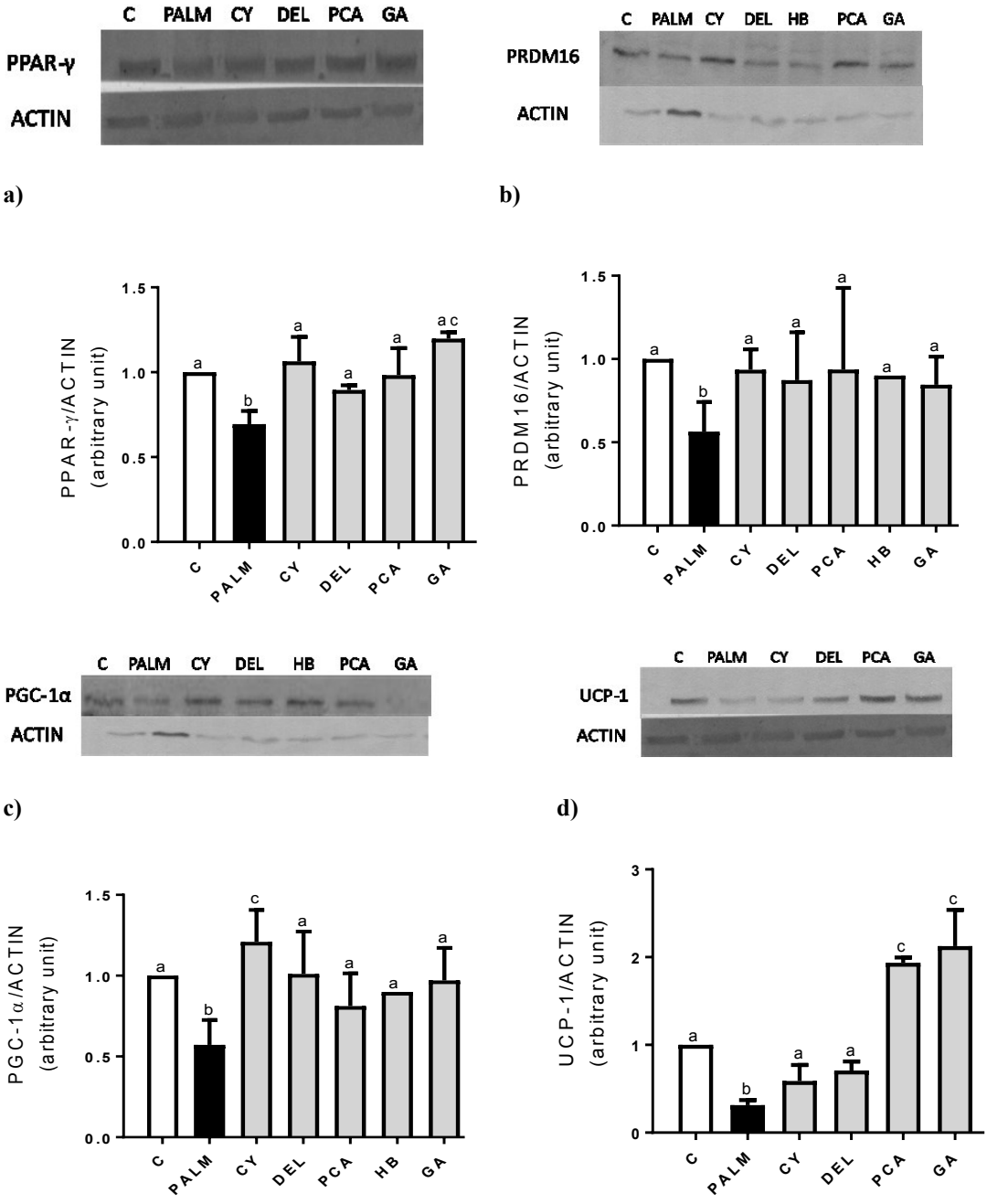


PPAR-γ, PRDM-16, PGC-1α and UCP-1 subcutaneous adipose tissue after 14 weeks on the corresponding diets. Bands were quantified and results for mice fed the control diet supplemented with AC (CA), the HFD (HF), and the HFD supplemented with AC (HFA) were referred to control group values (C). Representative immunoblots are shown. Bar charts represent PPAR-γ /Actin, PRDM-16/Actin, PGC-1α/Actin and UCP-1/Actin as means ± SEM from 9 to 10 animals/treatment. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.3.7 AC and their metabolites improve browning process of 3T3-L1 cells treated with palmitate

Similarly to results obtained HF diet-fed mice, incubation of 3T3-L1 adiposytes in the presence of palmitate determined a statistically significant reduction in PPAR-γ, PRDM-16, PGC-1α and UCP-1 (-30.6%, -43.5%, -42.8%, -68.7%, respectively) **(Fig. 6)**. All phenolic compounds tested (cyanidin-3-O glucoside (CY), delphinidin-3-O glucoside (DEL), protocatechuic acid (PCA), 4-hydroxybenzaldehyde (HB) and gallic acid (GA)) significantly prevented palmitate –induced decreased levels of proteins involved in the browning pathway.

Fig. 6 Effect of phenolic compounds on browning process in 3T3-L1 cells treated with palmitate: PPAR-γ, PRDM-16, PGC-1α and UCP-1

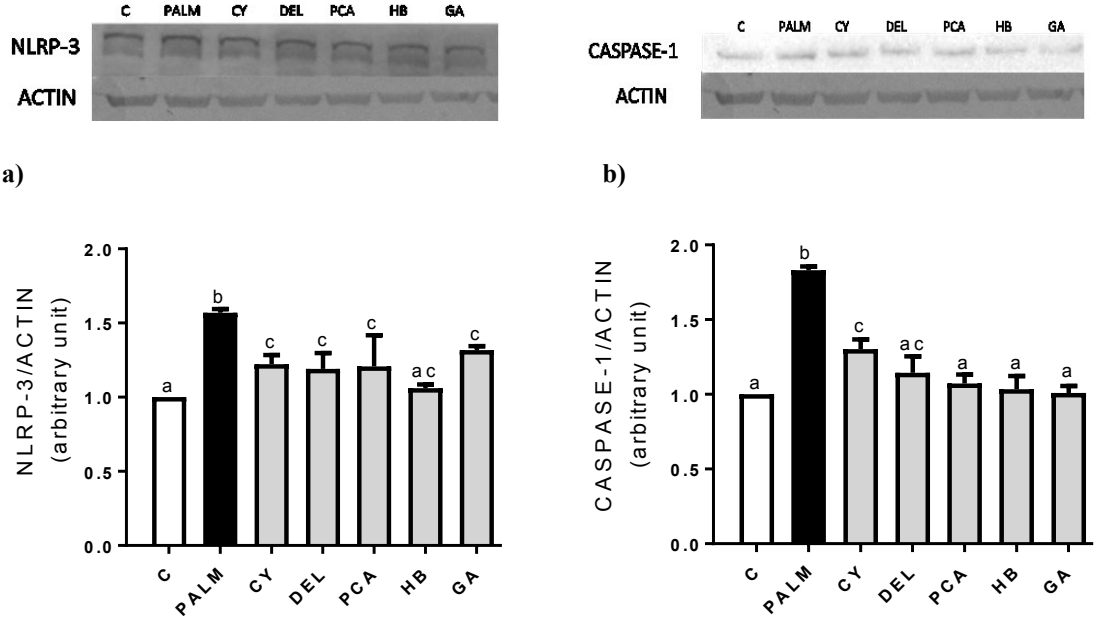


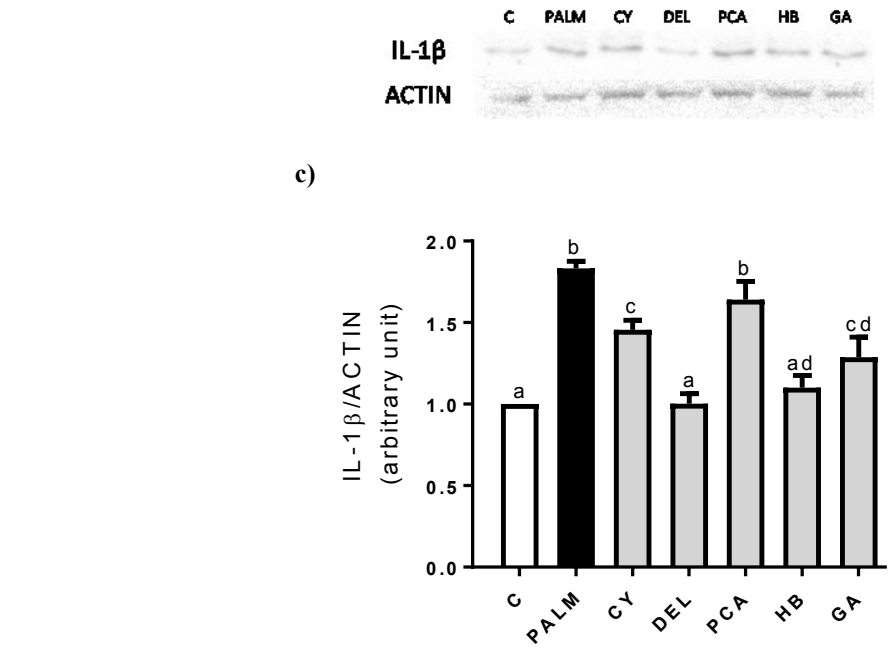
Representative immunoblots are shown. Bar charts represent PPAR-γ /Actin, PRDM-16/Actin, PGC-1α/Actin and UCP-1/Actin as means ± SEM from 3 to 4 independent experiments. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.3.8. AC and their metabolites mitigate NLRP3 inflammasome and inflammatory cascade activation in 3T3-L1 cells treated with palmitate

3T3-L1 preadipocytes treated with palmitate showed a statistically significant increase proteins involved in NLRP-3 inflammasome activation (NLRP-3, caspase-1 and IL-1β; +56.9%, +83% and +83.2%, respectively) **(Fig. 7)** and downstream cascades (phosphorylation of IKK, p65 and ERK1/2; +74.1%, +85% and +90.2%, respectively) **(Fig.8)** compared to non-added cells**.** Most phenolic compounds tested reduced palmitate-mediated increase of all these parameters of inflammation. Only PCA and CY did not show a protective effect against palmitate-induced increase in IL-1β protein levels **(Fig. 7c)** and IKK phosphorylation **(Fig. 8a)**, respectively.

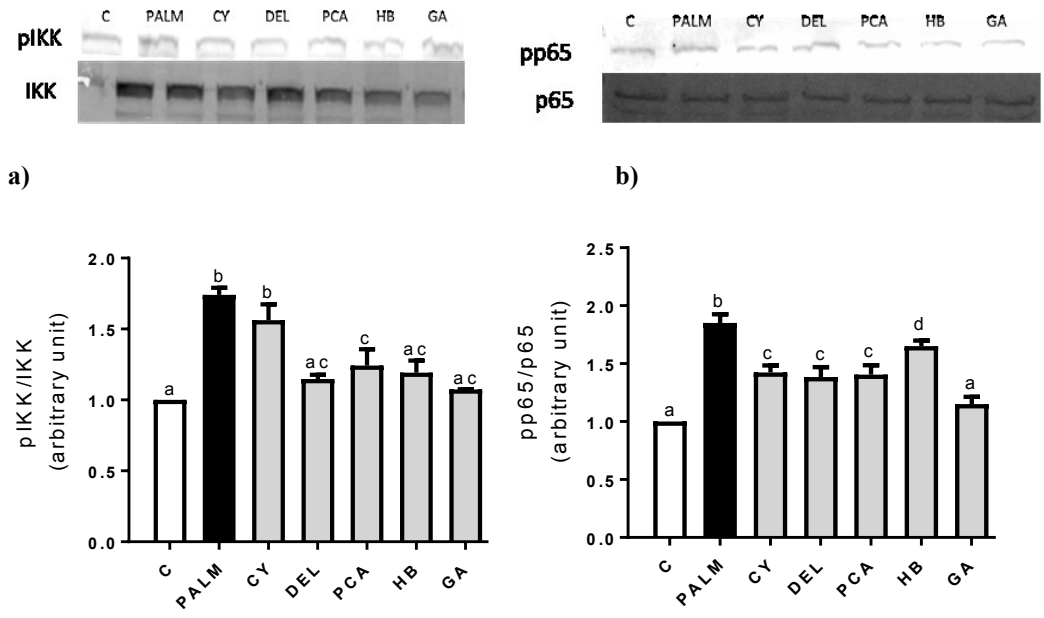
Fig. 7 Effect of phenolic compounds on NLRP-3 inflammasome in 3T3-L1 cells treated with palmitate: NLRP-3, Caspase-1 and IL-1β

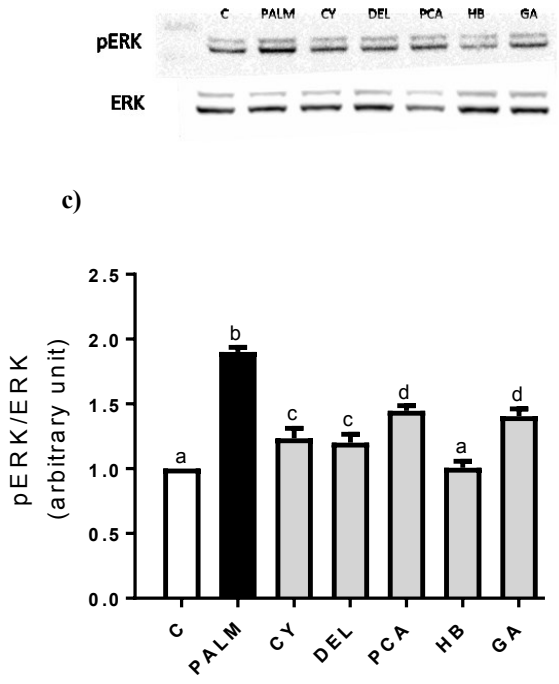




Representative immunoblots are shown. Bar charts represent NLRP-3/Actin, Caspase-1/Actin and IL-1β/Actin as means ± SEM from 3 to 4 independent experiments. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

Fig. 8 Effect of phenolic compounds on inflammatory pathways in 3T3-L1 cells treated with palmitate: pIKK, pp65 and pERK





Representative immunoblots are shown. Bar charts represent pIKK/IKK, pp65/p65 and pERK/ERK as means ± SEM from 3 to 4 independent experiments. Data with different letters are significantly different ($p < 0.05$, one-way ANOVA test).

4.4 DISCUSSION

In the present study, we documented the potential role of AC consumption as an important strategy to mitigate HF-induced obesity and of its co-morbidities in mice through the enhancement mitochondrial dynamics and activation of adipocyte mitochondriogenesis and beiging.

4.4.1 Effect of AC on body weight and fat pad in mice fed a HF diet

Our results show that the consumption of the HF diet for 14 weeks led to body weight gain, which was attenuated by AC supplementation. Similar results were obtained by Wu and colleagues **(Wu et al 2018)** that observed an important reduction in body weight in C57BL/6 mice fed a HF diet and supplemented with blueberry and blackberry anthocyanin (200 mg/kg BW) for 12 weeks. The authors concluded that AC were able to reduce HF diet-induced obesity through a positive regulation of oxidative stress, inflammation and energy expenditure. Also, Esposito and colleagues **(Esposito et al**

2015) documented that daily consumption of black currant powdered extract (32% anthocyanins) for 8 weeks in HF diet-fed C57Bl/6J mice reduced body weight gain. While, in a recent study, cranberry anthocyanins (200 mg/kg BW) were ineffective in reverse body weight gain in mice fed a high fat and high sucrose diet for 13 weeks and subsequently supplemented with cranberry extract for 8 weeks. Despite in question polyphenols fully reversed insulin resistance and hepatic steatosis ameliorating lipid catabolism and inflammation, no beneficial effects were exerted on weight gain and adiposity **(Anhe et al 2017)**.

4.4.2 Effect of AC on mitochondrial dynamics in mice fed a HF diet

Alternatively to several reports, our assumption involves the recovery of mitochondrial function as an approach to counteract obesity and related alterations induced by the excess of nutrients. We found that AC supplementation improved mitochondrial dynamics pathways, fusion, fission, and autophagy in subcutaneous fat tissue of mice fed a HF diet. The evidence regards the effect of phenolic compounds on mitochondrial function is reported by several studies, but mainly targeting apoptosis, membrane potential and the respiratory chain. For instance, delphinidin-3-glucoside and cyanidin-3-glucoside at 40 μM promote state 4 respiration in isolated rat hearth mitochondria reducing cytochrome c **(Skemiene et al 2013).** Gao and colleagues **(Gao et al 2013)** observed that cyanidin (40 μg/ml) was effective in counteracting the loss of mitochondrial membrane potential in cisplatin-induced mitochondrial dysfunction model of HK-2 proximal tubular cells preventing apoptosis. Moreover, pre-treatment with 2 mg/kg BW of cyanidin-3-O-glucoside in C57BL/6 mice subjected to permanent middle cerebral artery occlusion reduces apoptosis-inducing factor release from mitochondria under oxidative stress **(Min et al 2011)**. Only one study in the literature reports effects of anthocyanins on mitochondrial fusion and fission **(Parrado-Fernandez et al 2016)**. In particular, the authors used a nutraceutical rich in cyanidin 3-O-glucoside (20 nM) and delphinidin 3-O-glucoside (30 nM) to treat SH-SY5Y neuroblastoma cells. Their results showed a protective effect of anthocyanins in preventing mitochondrial dysfunction through the modulation of fusion and fission proteins. In line with our findings, they observed a reduction of DRP-1 induced by

anthocyanins. It should be considered that anthocyanins are minimally absorbed at the gastrointestinal tract, so this particular experiment does not reflect a feasible scenario. We evaluated the phosphorylated DRP-1 (pDRP-1) given that the recruitment and the binding to its receptors, MFF and Fis1, requires conformational changes induced by the phosphorylation at the regulation sites **(Lee et al 2016)**. Phosphorylated DRP-1 levels were high in HF diet fed mice supplemented with AC. This results are accompanied by a concomitant increase in MFF and Fis1, both crucial proteins in mitochondrial fission. In contrast, several studies assert that increased levels of DRP-1 phosphorylation are associated to negative effects, such as mitochondrial dysfunction in a cell culture model of Huntington's disease **(Roe et al 2018)**, mitochondrial dysfunction and myocyte death during chronic β-AR (β-adrenergic receptor) stimulation **(Xu et al 2016)** and mitochondrial fragmentation subsequent to TNF-α stimulation in cardiomyocytes **(Shen et al 2018)**. However, a recent review warns about the complexity in interpretation of the effects induced by environmental factors on mitochondrial dynamics. The same compound, but tested at different doses, could lead to a toxic response and a consequent disruption of the balance of fusion and fission, rather than to stress amelioration and adaptive responses (**Meyer et al 2017).** In this context, the increase of proteins involved in mitochondrial fusion and fission pathways observed in our experiment after treatment with AC could mean an improvement of mitochondrial dynamics since these processes are fundamental for rapid and transient morphological adaptations in order to face several cellular processes through mitochondria homeostasis **(Tilokani et al 2018)**. In support of our hypothesis, Peng and colleagues **(Peng et al 2016)** observed a reduction of Drp1, Fis1, OPA1, and MFN2 mRNA and protein levels in Sprague-Dawley rats and PC12 cells (adrenal gland; pheochromocytoma) treated with rotenone to cause mitochondrial dysfunction. Injection of 50 mg/kg BW of resveratrol was capable to increase fusion and fission proteins levels exerting a protective effect on mitochondria. Another study demonstrated the capacity of resveratrol (25-100 μM) to upregulate DPR-1 in in H9c2 cells, but also increase levels of PINK1 and Parkin **(Ren et al 2017)**. The last two proteins did not change in our experiment between HF and HFA groups, while the levels of NIX, another protein related to mitophagy, showed an increase in adipose tissue of mice supplemented with AC. In addition, Robb and co-workers observed a

promotion of mitochondrial fusion by resveratrol (10-20 μM) through increased expression of Mfn-2 in C2C12 myoblasts, PC3 cancer cells, and mouse embryonic fibroblasts resulting in larger and more highly branched mitochondrial networks. Nevertheless, emphasis must be placed on the lack of concerning literature that does not allow to properly compare findings with similar models, compounds and doses.

4.4.3 Effect of AC on mitochondriogenesis and beiging in mice fed a HF diet and 3T3-L1 cells treated with palmitate

Besides the functionality, also the number of mitochondria plays an important role in obesity. In fact, a recent review **(Neidhart et al 2020)** highlights how there is a relationship between mitochondrial number and obesity. In particular, the number of mitochondria is lower in obese subjects and the tissue mainly affected is the adipose tissue. Brown adipose tissue, which is rich in mitochondria, represent a potential therapeutic target against obesity and metabolic disorders since it contributes to increase glucose uptake and energy expenditure **(Leitner et al 2017)**. Not only the activation of brown adipose tissue, but also the browning of white adipose tissue, which has a higher mass compared to the first one, deserves greater focus in order to achieve weight loss and metabolic benefits **(Srivastava et al 2019)**. ATP5B, COX4, citrate synthase and PRX1 are proteins with an important role in mitochondria, which also represent potential biomarkers of mitochondrial content and quality. ATP5B has a key role in ATP synthesis as catalyst and it maintains the electrochemical proton gradient during oxidative phosphorylation **(Bozgeyik et al 2014)**. COX IV represents the last enzyme of mitochondrial respiratory chain, responsible for the establishment of the mitochondrial electrochemical gradient through the catalysis of electrons transfer from reduced cytochrome c to molecular oxygen at the level of inner mitochondrial membrane **(Hatefi et al 1985)**. Citrate synthase is involved in the first step of Krebs cycle catalyzing the formation of citrate from acetyl CoA and oxaloacetate and it is one of the most commonly used biomarkers of mitochondrial content **(Larsen et al 2012)**. PRX1 protects mitochondria against oxidative stress by converting hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively **(Greetham et al 2009)**.

Our results show that the HF diet caused a reduction of ATP5B, COX4, citrate synthase and PRX1

compared to mice fed a control diet. Except for ATP5B, AC supplementation increased the level of these proteins in HF diet fed mice, suggesting a potential protective role exerted by these dietary bioactive compounds in mitigating the effects of nutrients excess on obesity and related metabolic disorders. Although, to the best of our knowledge, no studies investigated the effect of anthocyanins on our biomarkers of mitochondrial content, in support of our findings Lambert and colleagues **(Lambert et al 2018)** observed an increased activity of mitochondrial enzymes citrate synthase and COX in obese rats supplemented for 8 weeks with grape polyphenols (50 mg/kg/d). Also Ballmann and co-workers **(Ballmann et al 2017)** demonstrated that control diet fortified with 0.2% quercetin improved mitochondrial protein content in Mdx/Utrn+/- mice after an 8-month period of supplementation. In particular, quercetin was able to induce a higher protein level of mitochondrial protein marker, e.g. citrate synthase. In an *in vitro* model of human neurons, 2h treatment with 10 μM EGCG increased the activity of cytochrome c oxidase and ATP synthesis, exerting a potential protective role against mitochondrial dysfunction **(Castellano-Gonzalez et al 2016)**. Such high concentrations of EGCG, which are far from achievable in any human tisuue beyond the gastrointestinal tract lumen, limits the value of this study. With respect to PRX1 and ATP5B, no comparison of our results can be discussed since no other studies were performed in order to evaluate the role of dietary bioactive compound on these two proteins in the context of mitochondrial content and quality.

In terms of biomarkers related to the browning of white adipose tissue such as PPAR-γ, PRDM-16, PGC-1α and UCP-1, a stronger evidence is provided about the role of polyphenols, both *in vitro* and *in vivo* **(Zhang et al 2019)**. We observed an increase in the protein levels of PPAR-γ, PRDM-16, PGC-1α and UCP-1 in our models of C57BL/6J mice fed a high fat diet plus anthocyanin extract and 3T3-L1 adipocytes treated with single anthocyanins and their plasma metabolites. The high fat diet in mice and the analogous treatment of adipocytes with palmitate, induced a reduction those key proteins involved in the differentiation of white adipose tissue to brown adipose tissue, on the other hand anthocyanins could play a positive role in the modulation of obesity and metabolic health through the modulation of the browning process. Similar

to our findings, You and colleagues **(You et al 2017)** noticed an increase of UCP1, PGC-1α, Sirt1 and PPARα in obese C57BLKS/J-Leprdb/Leprdb (db/db) male mice after providing cyanidin-3-glucoside dissolved in water (1 mg/mL) for 16 weeks, resulting in an increased energy expenditure and improved glucose homeostasis control. C3H10T1/2 mesenchymal stem cells treated with 10 μg/ml of mulberry and mulberry wine extracts rich in cyanidin-3-glucoside showed higher expression levels of thermogenic genes such as UCP-1, PGC-1α, and PRDM-16 **(You et al 2015)**. Again, this study disregards the limited bioavalability of anthocyanins, and their metabolism by the microbiota to metabolites (e.g. PCA and GA) that are absorbed and appear in plasma at much larger concentrations than the parent compounds. For this reason, our experiments in 3T3-L1 adipocytes were carried out at low concentrations and including the main cyanidin and delphinidin metabolites present in plasma. In addition to studies conducted testing the effect of anthocyanins, also different classes of polyphenols showed to positively regulate mitochondrial biogenesis as discussed in a recent review by Silvester and co-workers **(Silvester et al 2019)**. However, different doses and models used among studies and lack of evidence in human make difficult to extrapolate nutritional recommendations.

4.4.4 Phenolic compounds mitigate NLRP3 inflammasome and inflammatory cascade in 3T3-L1 cells treated with palmitate

Mitochondrial dysfunction, through an increase in ROS production and subsequent release of mtDNA due to apoptosis, induces the activation of the NLRP3 inflammasome. Downstream, the triggering of NF-κB activation amplifies the inflammatory response leading to a worsening of mitochondrial dysfunction and igniting a vicious cycle **(Lopez-Armada et al 2013)**.

In the present study, we found that palmitate (500 μM) induces higher protein levels of NLRP3, caspase-1, IL-1β, phosphorylation of the NF-κB cascade components IKK and p65 and of p44/42 MAPK (ERK1/2) in 3T3-L1 adipocytes This represents a potential danger signal able to determine mitochondrial dysfunction through the triggering of inflammatory cascades. On the other hand, the tested anthocyanins and their metabolites were capable of reducing the detrimental effects of palmitate. Moreover,

these results were obtained using physiologic concentrations of polyphenolic compounds **(Kuntz et al 2015)**. Other researchers stated the capacity of palmitate to induce lipid overload in 3T3-L1 adipocytes and consequent inflammation through NF-κB activation, i.e. IKK phosphorylation and p65 nuclear translocation, that was inhibited by a dietary supplement consisting of 17 purified ACNs isolated from bilberries and blackcurrant. Pretreatment of 3T3-L1 adipocytes with ACN extract (10 and 20 μg/ml) for 24 hours inhibits PA -induced NF-κB inflammatory pathway activation **(Muscarà et al 2019)**. The beneficial effect of anthocyanins on NLRP3 inflammasome was also demonstrated by Jin and colleagues **(Jin et al 2018)**. They treated ARPE-19 human retinal pigment epithelial cells with 4-hydroxyhexenal in order to induce the activation of NLRP3 inflammasome and observed a protective effect of cyanidin-3-glucoside (50 and 100 μM) in reducing NLRP3 inflammasome-related gene expressions (NLRP3, IL-1β, IL-18, and caspase-1), moreover the activity of caspase-1 and IL-1β release were decreased. In another cell model, represented by RAW264.7 macrophages, Wang and co-workers **(Wang et al 2017)** reported that blueberry extract rich in anthocyanins was able to inhibit gene expression of NLRP3, IL-1β, caspase-1, TNF-α and IL-6 after LPS stimulation. They also demonstrated that LPS stimulation, through ROS production, induces mitochondrial dysfunction and subsequent activation of NLRP3 pathway. The latter was inhibited by the blueberry extract that exerted anti-inflammatory potential **(Wang et al 2017)**. Although, several studies reported a protective effect of anthocyanins on inflammatory processes, our data showed for the first time the effect of single anthocyanin and their metabolite on NLRP3 inflammasome in 3T3-L1 pre-adipocytes, highlighting a potential mechanism of action through which these compounds could mitigate HF-induced low-grade inflammation and relative adverse consequences such as mitochondrial dysfunction.

4.5 CONCLUSION

In conclusion we demonstrated that AC prevented HF-mediated inhibition of the pathway leading to mitochondria biogenesis, and thermogenic respiration. Moreover, HF-induced obesity impairs mitochondria fission and fusion, which can be in part

mitigated by AC intake. In addition, palmitate-mediated activation of NLRP3 inflammasome and NF-κB pathway was counteracted by anthocyanins and their metabolites in 3T3-L1 pre-adipocytes.

The current findings suggest that consumption of select AC could be an important strategy to mitigate HF-induced obesity and its co-morbidities via activation of adipose tissue browning and through the regulation of mitochondrial dynamics. These results are relevant since the beneficial effects are obtained by using AC and their metabolic derivatives at physiologically relevant concentrations.

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CHAPTER 3

Protective effects of (poly)phenol-rich foods and dietary patterns in human studies

5.1 INTRODUCTION

The third part of the thesis regards the role and the effects of (poly)phenols in humans. In particular, it has been investigated through a systematic review of human intervention studies the role of berries (as source of phenolic compounds) in the modulation of markers of vascular function. In addition, the effect of a polyphenol-rich diet (including berries) on markers of oxidative stress and vascular function was investigated in a group of older subjects with increased intestinal permeability.

The utilization of *in vitro* models in nutritional research has a pivotal role to identify molecular pathways and mechanisms of action involved in the biological effect of dietary compounds. However, what happens in cellular models does not always reflect the real situation in vivo, where a biological system interacts and affects a potential dietary component. Thus, observational studies and, above all, clinical trials represent a further fundamental step in order to validate the beneficial effect of dietary bioactive compounds. However, it is important to underline that the study of the protective role of polyphenols *in vivo* is extremely complex by considering the large number of factors that can affect individual response to a given treatment or in a specific environment and condition. While there are many observations suggesting a relationship between polyphenol intake (total or single classes) and different health related outcomes, a recent review we have performed demonstrated that available data are still not

sufficient and accurate enough to define a dietary recommendation **(Del Bo' et al 2019).** Specifically, we documented an overall protective effect of several polyphenol classes on CV risk events, mortality and other outcomes of health status. This association was already observed with a total flavonoid intake higher than 500 mg/day while it was estimated a mean intake of total polyphenols (evaluated considering Folin-Ciocalteau data) of about 900 mg/day. However, the large variability in terms of study design, but also the considerable limitations related to the methods used to estimate polyphenol intake demonstrated how it is difficult to determine a reference intake for this class of dietary bioactive compounds. In addition, despite the importance of observational studies for the evaluation of possible associations between bioactives and human health they appeared not suitable to establish a cause-effect relationship. Consequently, only well designed intervention studies remain essential to identify dietary requirements capable to exert a specific effect, modulating the targeted outcome through a definite amount consumed in a particular population. In addition, when considering dietary recommendations, it is not only a problem of "dose" of bioactives but also of the characteristics of the food source providing them. In fact, it is highly demanded the demonstration of the role of polyphenol rich foods in the modulation of specific markers. In this regard, berries have been largely investigated as protective foods for their high content of anthocyanins which have been suggested to affect numerous body functions including vascular function. In a recent systematic review (**Martini et al., 2020**) we have shown that the overall evidence resulting from human intervention studies suggest a protective role of berries on vascular function. Although different results could depend on time of exposure, the type and dose of berry, but also the biomarkers analyzed. Furthermore, polyphenols are recognised for other numerous biological activities, such as antimicrobial, antiproliferative, antioxidant, and anti-inflammatory functions. An important aspect is that their effects can be exerted at both intestinal and systemic levels. In this context, it has been hypothesized a role for some polyphenols in the modulation of intestinal permeability. In this regard, we have conducted a perspective (**Bernardi et al 2019)** aimed to evidence the impact of polyphenols on intestinal permeability, through the recap of results from *in vitro* and *in vivo* studies. Moreover, gaps and future directions were highlighted in order to proceed more accurately in this research field. It is important to stress how gut barrier

play a pivotal role in the maintenance of human health and the abnormal increase of intestinal permeability could represent the underlying mechanisms of several pathological conditions. To this regard, diet represents a valid strategy to positively modulate intestinal permeability, in particular through the effect exerted by phenolic compounds. Data from animal models and *in vitro* studies suggestes the capacity of some polyphenols to increase the expression of tight junction proteins and to reduce the release of different pro-inflammatory cytokines. While, from human studies it appears a connection between polyphenols, gut barrier integrity and the capacity of these dietary bioactive compounds to modulate the microbiota and the immune system. These activities could also contribute to the mitigation of several function dysregulation (such as cardiometabolic and vascular functions) which seem to have a direct or indirect connection with gut barrier impairment. Thus the potential of polyphenols in the modulation of intestinal permeability can be fundamental to provide a rationale for the impact of these bioactives in the modulation of oxidative stress and inflammation.

Current literature about berries and their role in vascular function

Cardiovascular diseases represent one of the most studied groups of chronic and degenerative conditions investigated in the field of polyphenol research. In this context, it was performed a systematic review on the role of berries on vascular function.

Systematic Review
5.2 Role of Berries on Vascular Function: A Systematic-Review of Human Intervention Studies

(Post-print of published paper; DOI: 10.1093/nutrit/nuz053)

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Article type: Special Article

ABSTRACT

Context Berries are a source of polyphenols with recognised health-promoting activities. **Objective** The aim of this systematic review is to provide evidence of short and long-term benefits of berries on vascular function. **Data sources** Human intervention studies were collected from PubMed and Scopus databases **Data extraction** After selection, 22 randomized-controlled trials were included and analyzed. Most of them were performed in healthy subjects or individuals with cardiovascular risk factors. **Results** The overall results seem to suggest a protective role of berries on vascular function even if dependent on time of exposure, type and dose of berry and biomarkers analysed. Flow mediated dilation and reactive hyperaemia index (markers of vascular reactivity) improved following short-term interventions, while pulse wave velocity and augmentation index (markers of arterial stiffness) only after medium-long term studies. **Conclusions** In conclusion, the current evidence suggests that berries, at physiological relevant doses, may have a role in the modulation of vascular function and stiffness. High-quality human intervention trials are encouraged in order to strengthen these findings and to better elucidate the mechanisms involved in such modulation.

Keywords: berries; (poly)phenols; endothelial function; vascular function; intervention studies; systematic review

INTRODUCTION

Berries represent a wide group of blue, purple or red small-sized and highly perishable fruits. Blueberry, cranberry, currant, raspberry and blackberry are the most common varieties of berries consumed around the world1. Berries are an important source of (poly)phenols, including anthocyanins (ACNs), proanthocyanidins, flavonols, flavones, flavan-3-ols, flavanones, isoflavones, stilbenes, lignans and phenolic acids2,3.

Berry consumption has been associated with a reduced all-cause mortality4. Moreover, in the last few years numerous epidemiological and clinical studies documented the protective effects of berries against many non-communicable chronic diseases, with some focusing on cardiovascular diseases (CVDs)5–7 which remain the leading causes of death worldwide8. The development of CVDs is often accompanied by a decline in vascular health and function. The endothelium represents an important part of the vasculature, by covering the inner surface of the blood vessels, and acting by controlling the flow of nutrients and non-nutrients, the passage of the fluids into the tissues, and the secretion of vasoactive substances, such as the vasodilator nitric oxide (NO) and vasoconstricting molecules like endothelin-1.

Common biomarkers for the evaluation of vascular health include blood pressure (BP), arterial stiffness and vascular reactivity. Vascular reactivity can be assessed through endothelium-dependent (i.e. acetylcholine) or independent (i.e. nitroglycerin) mechanisms9,10. The main methods for vascular reactivity assessment are flow-mediated dilation (FMD) and peripheral arterial tonometry/reactive hyperaemia index (PAT/RHI). FMD is considered the gold standard non-invasive ultrasound technique, measuring vasodilation at the level of the brachial artery following a standard occlusion11,12. EndoPAT is a plethysmographic technique able to measure pressure changes in the finger tips caused by a 5 min occlusion of the brachial artery13. Among other measures, arterial stiffness can be measured through pulse wave velocity (PWV), which directly measures point-to-point pulse wave transit time, and pulse wave analysis (PWA), which uses the pulsatile waveform shape to make assumptions about arterial haemodynamics. The stiffness can be also quantified through the augmentation index

(AIx), defined as the difference between the second and first systolic peak expressed as percentage of the pulse pressure14.

Some systematic reviews and meta-analyses of observational and randomized controlled trials (RCT) reported a relationship between the consumption of polyphenols and polyphenol-rich foods and modulation of vascular function markers such as AIx, PWV, FMD and RHI15–17. Other direct and/or indirect biomarkers of vascular function include serum concentrations of inflammatory markers, adhesion molecules, lipids and lipoproteins, oxidized LDL-C, and clotting factors18. A meta-analysis of RCTs, performed by Huang et al.19, has shown that berry consumption may significantly reduce the levels of LDL-C, BP, fasting glucose, and tumour necrosis factor-α (TNF-□) supporting their potential contribution on cardiovascular health.

Based on these premises, the aim of this systematic review is to summarize the research findings of RCTs investigating the effect of berry consumption on markers of vascular function, in order to elucidate their potential role in CV health. The current systematic review exclusively focuses on studies (both acute and chronic interventions) performed with berries and berry products, differentiating it from other recent works.

METHODS

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement20, and included all relevant PRISMA checklist items. A review protocol has not been published and this review has not been registered with any systematic review database.

Eligibility Criteria (Inclusion and Exclusion Criteria)

Studies were included in the present review if they investigated the effect of berry consumption on one or more markers of vascular function in humans. The studies present in literature had to adhere to the following criteria to be considered in the review process: i) to be randomised-controlled trials that provided results on both acute (i.e. single dose supplementation) or chronic berry consumption and ii) to provide a

characterization of the berry polyphenolic content. Conversely, exclusion criteria were: i) the presence of a combination of berries with other foods (because the beneficial effect could not be attributed specifically to berries) and ii) the fact of being published in a language different from English and with no accessible translation. No restrictions for the characteristics of subjects (e.g. age, gender, health condition) were considered.

A more detailed list of criteria for eligibility in this systematic review has been summarized in Supplementary Table 1, by following the PICOS (Population, Intervention, Comparison, Outcome, Study design) format21.

Search strategy and study selection

A systematic literature search was conducted using PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and Scopus (http://www.scopus.com) databases on December 2017 (updated October 2018). For completeness, searches were augmented by screening the bibliographies of relevant review articles. The search had no limit ranges for year of publication. Three search themes were considered: terms related to berry (e.g. strawberry, blueberry, cranberry) were combined with terms related to outcomes (e.g. pulse wave capacity, flow mediated dilation, arterial stiffness) and population type (e.g. human, volunteers, patients) to identify all potentially relevant literature published (further information on the search strategy in Appendix S1). No language or other restrictions were set in the literature search. The identification process is illustrated in Figure 1.

Study selection and data collection process

Two reviewers (MM and DM) independently abstracted data from studies eligible for inclusion. Disagreement between reviewers was resolved through consultation with a third independent reviewer (CDB) to reach a consensus.

The following data were extracted from each study: name of first author, country, registered trial number, sample size at recruitment and enrolment stages, inclusion/exclusion criteria, study design, dietary products used during the interventions, and vascular function outcomes.

Risk of Bias in Individual Studies

Risk of bias in individual studies and across the studies was assessed independently by two review authors (DA and DM) following the criteria of the Cochrane Handbook for Systematic Reviews of Interventions 5.1.022. The following parameters for each component's rating were considered to produce the resulting scores: 1) Selection Bias. Sequence generation and allocation concealment; 2) Performance Bias. Blinding of participants and personnel; 3) Detection Bias. Blinding of outcome assessment; 4) Attrition Bias. Incomplete outcome data; 5) Reporting Bias. Selective reporting; 6) Other Bias. All the scores were assessed as "Low risk of bias", "High risk of bias", or "Unclear risk of bias" if insufficient details about these parameters were reported in the study22. All disagreements were resolved by consensus with a third review authors (CDB).

RESULTS

Study selection

The study selection process is shown in Figure 1. A total of 880 records were identified from the database search (PubMed and Scopus), while no additional papers were found by hand searching. After removing 179 duplicate articles, 701 studies were screened and 671 were discarded based on title and abstract. After the full-text reading of the remaining 30 eligible papers, a total of 9 records were further excluded were excluded because i) the study had no placebo/control food (n= 3), ii) berries were provided along with other food components (n= 1), iii) the fed products were not fully characterized for their (poly)phenol content (n= 5). At the end of the selection process, 22 RCTs were considered for qualitative analysis.

Study characteristics

The main characteristics of the 22 included studies are reported in Tables 1 and 2. Out of these 22 works, 11 dealt with acute interventions23–33and 9 with chronic interventions34–42, while 2 studies43,44 investigated the effect of both acute and chronic berry consumption.

The current review summarizes the main findings obtained evaluating the effect of different berries (mainly blueberries, cranberries, strawberries and blackcurrants) on vascular function. The berries were provided as raw fruits, drinks/smoothies, or extracts in capsules. The (poly)phenol content was dependent on the type of berry and on the administered portion (250-300 g for raw fruits, 250-1000 mL/day for drink/smoothie and 600 mg for capsules). The main outcome variables measured were FMD and RHI for the vascular reactivity, and PWV and AIx for the arterial stiffness.

Risk of bias of the studies

Risks of bias within individual studies and across the studies are shown in Supplementary Figure 1 and Supplementary Figure 2, respectively. Results showed the blinding of participants and personnel (performance bias) and blinding of outcome assessment (detection bias) to represent the highest risks of bias.

Acute studies

Table 1 reports the main results obtained in 13 short-term studies23-33,43,44 performed with berries on FMD (n=5), RHI (n=5) and other markers (n=3) of vascular function. Alqurashi and colleagues23 have shown that the intake of 200 g of açai smoothie significantly increased FMD at 2 h (+1.4%; $p=0.001$) and at 6 h (0.8%; $p<0.001$) post-consumption in healthy overweight men. Similarly, Rodriguez-Mateos et al.32, found that the consumption of 3 pieces/buns of blueberry baked products and/or a blueberry drink (equivalent to 240 g of fresh blueberry) increased FMD after 1, 2, and 6 h from the intake. A significant improvement in FMD occurred after the consumption of the two items, at 1 h for the drink and 2 h for the baked products (up to +2.6%). In another study, the same authors reported that post-acute consumption of 5 cranberry juices (450 mL each) containing different amounts of (poly)phenols (409, 787, 1238, 1534, and 1910 mg), significantly augmented FMD 1, 2, 4, 6, and 8 h from the intervention33. FMD gradually increased in a time- (spiking at 4 h) and dose-dependent manner, with maximum effect after the intake of 1238 mg total polyphenols (about +2.5%)

While, Istas et al.29 showed that the intake of two different portions (200 and 400 g) of red raspberries (containing 201 or 403 mg of total polyphenols, respectively) improved FMD at 2 h (+1.6% and +1.2%, respectively) and 24h (+1.0% and +0.7%, respectively) in a group of 10 healthy subjects, so not finding a dose-response relationship.

Regarding RHI, 3 studies reported a significant increase in this outcome measure. Del Bo' et al.27 found that 300 g of blueberries counteracted an impairment in RHI (−4.4 ± 0.8%, $p<0.01$) and improved Framingham (f) RHI (fRHI, +28.3 ± 19.2%, $p<0.0001$) in a group of healthy smokers with normal endothelial function (2 h post consumption). In another study, the authors documented that the same 300 g blueberry portion increased RHI values in smokers (+35.2 ± 7.5%, $p=0.02$) and in non-smokers (+54.8 ± 8.4%, $p=0.01$) with endothelial dysfunction26. Finally, Flammer et al.44 observed a significant increase ($p=0.01$) in RHI (from 1.7 ± 0.4 to 2.0 ± 0.6; about +18%) at 1 h after cranberry juice consumption (2x 230 mL) in subjects with peripheral endothelial dysfunction and cardiovascular risk factors.

Conversely, two studies did not report significant effects following short-term interventions with berries. Del Bo' et al.28 showed that a portion of blueberry purée (300g) did not affect vascular reactivity, measured as RHI, after 1 h from the intake in a group of young healthy volunteers with normal peripheral arterial function (RHI>1.67) A similar result was also documented by Jin and colleagues30, following the intake of 250 mL of blackcurrant juice (20%), in a group of healthy subjects. The investigators measured vascular reactivity by laser Doppler imaging in response to acetylcholine (endothelial dependent) and sodium nitroprusside (endothelial independent), testing the effect after 2 h from the intake of the juice30.

Regarding PWV and AIx, the studies included in this systematic review failed to observe any significant effect on these markers following berry intervention25–27,31,33.

Chronic studies

Table 2 shows the results obtained in 11 medium-long term interventions34-44 providing results on the effect of berries on markers of vascular function. Khan et al.38showed a significant increase (p=0.022) in FMD (from 5.8±3.1 to 6.9±3.1%; about +19%) after 6-week consumption of blackcurrant juice (1 L/day, providing 815 mg of total polyphenols) in healthy subjects. Similarly, Stull et al.41reported a significant improvement in RHI after a 6-week intake of two blueberry smoothies (45 g freeze-dried blueberry powder, providing about 800 mg total polyphenol) in subjects with metabolic syndrome. The results showed a greater effect of blueberry versus placebo (0.32 ± 0.13 versus -0.33 ± 0.14, respectively; $p = 0.0023$). On the other hand, 4 studies reported no significant effect on RHI or FMD35,39,43,44. Djurica et al.43 found that 1-week consumption of 50 g freeze-dried strawberry powder (equivalent to 500 g of fresh strawberries, with pelargonidin-3-glucoside as main phenolic compound) did not improve RHI in overweight/obese adolescents. Flammer et al.44 showed that the intake of cranberry juice (2×230 mL/day) over 4 months had no effect on RHI in subjects with peripheral endothelial dysfunction and cardiovascular risk factors. Similarly, Riso et al.39 could not demonstrate an effect on RHI after a 6-week intervention with a wild blueberry drink (250 mL/day, providing 475 mg of anthocyanins) in subjects with cardiovascular risk factors. These results were consistent with Dohadwala et al.35 in which a 4-week cranberry supplementation (480 mL/day, providing 94 mg ACNs and 835 mg total polyphenols) did not affect FMD in subjects with coronary artery disease.

With respect to arterial stiffness, 6 out of 7 studies showed a positive modulation of PWV and AIx following berry intervention. Feresin et al.36 reported that the intake of 240 mL of a strawberry drink (providing 25 g/day of freeze-dried powder, equivalent to approximately 1.5 cups of fresh strawberries) for 8 weeks significantly decreased brachial-artery pulse wave velocity (baPWV) and femoral-artery pulse wave velocity (faPWV) in pre- and stage 1-hypertensive postmenopausal women (-0.73 m s-1, $p = 0.03$ and -0.55 m s-1, $p = 0.02$, respectively). Similarly, Johnson et al.37 (2015) observed a significant reduction in baPWV (from 1,498±179 cm/sec to 1,401±122 cm/sec; at about 6.5%; $p<0.05$) following 8-week consumption of a blueberry drink (providing 22 g/day of freeze-dried blueberry powder) in a comparable population. Dohadwala et al.35 documented a significant reduction in carotid-femoral pulse wave

velocity (crPWV; from 8.3 ± 2.3 m/s to 7.8 ±2.2 m/s; at about -6%; P=0.003), but not carotid-radial pulse wave velocity (cfPWV), after a 4-week intake of cranberry juice (480 mL/day) in subjects with coronary artery diseases. Significant findings were also documented for total peripheral resistance (TPR) and AIx. For example, 1-week consumption of New Zealand blackcurrant extract (600 mg/day) reduced TPR (-16%; $p<0.05$) in healthy males, both at rest and during exercise performance34. Similar results were also observed following 1-week intake of 6 g/day of New Zealand blackcurrant powder in well trained endurance athletes (TPR, -25%; P=0.003)42. Ruel et al.40 observed a decrease in AIx (−10.8% ± 6.4%; $p<0.0001$) following 4-week intervention with cranberry juice (500 mL/day, providing 400 mg of total polyphenols) in obese men, while Riso et al.39 reported no significant effect on AIx after 6-week intervention with wild blueberry drink (250 mL/day, providing 475 mg of anthocyanins) in subjects with cardiovascular risk factors.

Potential risks of bias

The highest risks of bias concerned blinding of participants as well as the blinding of the outcome assessment during the conduction of RCTs. For the latter, very few studies declared the non-blindness, while in most studies personnel blinding was unstated. Allocation has been particularly considered and described in the various studies included in this review. There were high risks of bias when it was impossible to render the control undistinguishable from the berry product. Despite being randomized controlled trials, the randomization processes have been scarcely described in the papers, mainly due to poor or absent explanation of how the randomization was produced. However, it is worth to note that the blindness of the randomization process in dietary interventions is sometimes difficult to achieve, due to the risk of an unbalanced allocation with consequent impact on data reliability.

DISCUSSION

There is a clear interest in the exploitation of berries and derived products for their potential role in cardiovascular health, with a specific focus on vascular function. RCTs are considered the gold standard for ascertaining a causal relationship between intervention and effect of a treatment. The effect of polyphenol-rich foods in the modulation of vascular reactivity has been evaluated in several intervention studies but few systematic reviews and meta-analyses summarized their effects. In some cases, the effects were inconsistent for the measured markers of vascular function45–47. This discrepancy could be due to the inclusion of studies having heterogeneous characteristics and reporting high risk of bias. Moreover, most of the studies were focused on bioactives and bioactive-rich foods in general, making the specific effects of berries very difficult to be identified. Conversely, the present review exclusively considered studies performed with berries and berry products, selected on the basis of quality criteria, and included only RCTs performed either in acute and chronic interventions. The complete picture obtained through this work points at an improvement in FMD and RHI (markers of vascular reactivity) following acute berry interventions. Some of the studies linked the observed effects to the increase in plasma circulating levels of berry bioactive constituents27 while others by their circulating gut/liver phenolic metabolites. 29

Only one study showed a dose-response relationship between the intake of berries and vascular reactivity33 while 4 studies did not report significant effects following berry consumption24,28,30,43. These results may be due to the characteristics of the studied population (i.e. healthy individuals without specific risk factors and with normal endothelial function at basal levels). Moreover, matrix effect, potentially reducing the availability and/or impact of berry bioactives, small portions (even if more realistic) of berries, and time of evaluation/measurement of vascular function due to the rapid clearance rate and poor absorbance of polyphenols may all have been diluting factors in the framework of the final evidence.

An additional source of variability among acute studies can be related to the study protocol adopted and the characteristics of the test meals. Some studies provided berries and/or berry products (whole fruits or drink) alone or within/together with a

high-fat23,31 or a high-carbohydrate meal24, and it is recognized that foods and food matrix may positively or negatively affect polyphenol bioavailability48. Moreover, the consumption of high-fat and/or high-carbohydrate meals may transiently increase post-prandial triglycerides and glycaemia with a negative effect on endothelial function49. These important variables/aspects could have affected the results obtained in the studies.

Regarding the effects observed in medium-long term interventions, no clear favourable effects of berry products on vascular reactivity markers have been found, in line with the systematic review of Heneghan and coworkers50 that showed an effect only in 3 out of 7 studies. The discrepancy between short and long-term studies in terms of vascular reactivity (RHI and FMD) is intriguing and may be attributed to the complexity of the mechanisms involved in the maintenance of the vascular system function. For example, Dohadwala and coworkers35 reported that changes in nitric oxide mediated vascular reactivity can occur rapidly following a dietary intervention and this has been related to the "acute" absorption of food bioactives and/or their metabolites (e.g. able to directly/indirectly affect nitric oxide production). This underlines that necessarily critical factors affecting the evaluation of vascular function include the experimental design (e.g. in terms of timing of measurements), the targeted mechanism (e.g. nitric oxide production), and also the characteristics of the markers used to evaluate vascular reactivity. In fact, the markers available may provide different information depending on the study protocols (acute vs. chronic intervention). For example, short term studies can provide information on the direct modulatory effect of the absorbed bioactives (i.e. supporting biological plausibility) conversely, in the long term approach generally the exposure to the food bioactives is absent or limited (due to the active and rapid clearance of phenolic compounds even when consumed regularly). In this context, is not surprising the lack of effect underlined in chronic studies where the measurement is performed about 12 h after the last intake of the bioactives. Moreover, the type of markers used may affect the results depending on the actual targeted measurement.

Also the large heterogeneity of the enrolled groups of volunteers among the different RCTs (i.e. healthy subjects, individuals with cardiovascular risk factors or complications), also in terms of vascular function levels, could have affected the results

obtained. Moreover, it cannot be excluded that the duration of the intervention was insufficient to exert a beneficial effect involving these specific target groups of population. An additional source of variability can be related to the form and the way through which berries have been provided. Some studies provided berries as raw fruit, others as a beverage, a smoothie, a sweet cake (i.e. muffins), alone or in combination with a meal. Moreover, berries could have been provided in addition to the habitual diet (resulting in an increased energy intake) or as substitutes of other foods normally consumed that are thus being displaced from the diet (isocaloric condition). The lack of the food that has been replaced may be important in determining the final effect on vascular function, although it is quite difficult to ascertain the magnitude. Moreover, the differences in berry administration may have played an important role in the results obtained, since the quality of a meal in terms of energy, macro and micronutrients intake may affect the vascular response. Also, considering that polyphenol intake may represent a confounding factor, subjects were often asked to maintain their usual diet and to refrain from the consumption of berries and other foods throughout the study period. Despite this, only few studies provided data about the actual dietary intake34,41 and the energy intake during the intervention and between treatments was rather constant. Conversely, no information about the actual intake of polyphenols was provided.

Moreover, it is worth noting that the synergistic effects of other coexisting substances in berry foods such as vitamin C51, fiber52, potassium53 and magnesium54 may play a role in determining the improvement on vascular function.

Arterial stiffness has been recognized as a determinant of pulse pressure and elasticity of the blood vessels. The loss of elasticity of the artery walls reduces its compensatory ability to absorb the pulsatile energy and the wave propagation effects that influence peripheral wave reflection. This inability for compensatory response results in a gradual increase in blood pressure with age, leading to the development of isolated systolic hypertension and cardiovascular risk. Numerous intervention and observational studies have examined the relationship between polyphenols/polyphenol-rich foods and arterial stiffness. In a cross-sectional study, Jennings and colleagues15 showed that high intake of anthocyanins and flavones were inversely associated with low arterial stiffness (measured as PWV) across extreme

quintiles of intake in women. Successively, Lilamand and colleagues17 assessed the relationship between flavonoids intake and arterial stiffness, measured as PWV, analysing 16 intervention and 2 cross-sectional studies. Four intervention trials reported a significant decrease of arterial stiffness after a flavonoid-based intervention, while the observational studies showed a significant association between high flavonoid consumption and low arterial stiffness. A recent systematic review and meta-analysis of RCTs showed an improvement in arterial stiffness following anthocyanin supplementation47. The effects were more evident on PWV after acute intake, while the results on AIx were not univocal following both acute and chronic interventions. In the present systematic review, we documented that short-term interventions with berries failed to modulate PWV and AIx, as well as stiffness and reflation indexes (Table 1), in line, at least in part, with those previous observations. Conversely, medium-long term interventions suggest an improvement of these markers (Table 2), in accordance with results reported in the review of RCTs by Heneghan and coworkers50. A potential explanation of the different findings between short and medium/long term trials may be attributed to the type of subjects enrolled and to the duration of the treatment. In fact, the short-term interventions were performed in healthy subjects, and it is plausible that substantial variations in arterial stiffness over a short follow-up are unlikely to be observed in individuals without vascular dysfunction. In addition, also the type of marker analyzed (e.g. PWV versus AIx) and its high variability among subjects could have played a crucial role in the obtained results. It is noteworthy that most of the studies did not consider arterial stiffness as a primary outcome, and that the trials were mostly underpowered for arterial stiffness evaluation. For this very reason, future studies should be specifically designed to ascertain the effect of berries on this specific marker.

Strengths and limitations of the study

Caution should be used when interpreting results or drawing conclusions on vascular effect of berries due to the high heterogeneity among studies in terms of type and dose of berries, administration source (i.e. whole fruit, juice drink or capsules), amount of provided polyphenols, and their bioavailability. Although the inclusion of different types of berries may represent a strength, the different composition of berries in terms of the type and quantity of phenolic compounds may be among the most important

factors influencing the in vivo effects of berries on vascular function. It is well known that berry fruits contain different anthocyanin profiles55,56 and, for this reason, it is not always easy to compare study results because most of them differ for the referring standard compounds in anthocyanin intake, i.e. cyanidin- and peonidin-derivatives as major ACNs in cranberries, while pelargonidin predominates in strawberries6. Another weakness was the lack of good quality information about the bioavailability of anthocyanins and related metabolic products. Actually, after ingestion, anthocyanins metabolic fate is deeply influenced by pH and gut microbiota activities57. It is well ascertained that, similarly to other phenolic compounds, anthocyanins have a limited bioavailability, lower than 15%58. This is influenced by their interaction with several gut microbial strains and the subsequent phase II metabolism at enterocyte and hepatocyte level, leading to the production of several different metabolites, including phenylpropionic and phenylacetic acids58–60. A very limited number of studies provided information about the circulating amount of these metabolites in in vivo berry-related studies61. Moreover, it is not always easy to link the biological effects of berry consumption to anthocyanin gut microbial derivatives, as they are also the results of the degradation metabolism of several other phenolic compounds62.

Other critical aspects are represented by the study design (acute versus chronic intervention and parallel versus crossover design), the duration of the intervention, subjects' characteristics, and sample size. Most of the studies were performed on healthy subjects, so that the inclusion in the analysis of trials involving volunteers with risk factors or diseases may have increased the heterogeneity of the results, making it difficult to draw any unequivocal conclusion. Moreover, some studies, despite being sufficiently powered, randomized and controlled, were performed in small groups of subjects and, for this reason, the results obtained have to be considered as preliminary and deserve further investigations.

The use of different methods and the lack of standardized procedures and gold standard methodologies for the assessment of vascular function outcomes could be another potential critical point. For example, positioning of the cuff (upper versus lower arm), duration of brachial artery occlusion, and timing for the detection of peak hyperaemia still differ among investigators. This information is missing in the papers analysed, but it is clear that the different experimental conditions adopted may have had a role on the

modulation of nitric oxide dependent and independent vasodilation mechanisms63, and affect the results obtained.

Finally, it is important to underline that the search strategy applied in this systematic review excluded other direct and indirect markers of vascular function (e.g. circulating adhesion molecules, cytokines, interleukins, and blood pressure), which, in some studies, have been used to improve the understanding of the obtained results.

Potential mechanisms of action involved in the modulation of vascular function

One of the main hypothesized mechanisms of action of polyphenols consists of the activation of endothelial nitric oxide synthase (eNOS)/NO/cyclic guanosine mono phosphate (cGMP) signalling pathway involved in vasodilation. Once activated, NO stimulates soluble guanylate cyclase in the vascular smooth muscle cells by releasing cGMP, a second messenger, which induces the smooth muscle cells of the vessel to relax64,65. In addition, polyphenols have been shown to increase post-prandial release of the active glucagon-like peptide 1, a major intestinal hormone that stimulates glucose-induced insulin secretion from β cells, upregulate endothelial nitric oxide synthase expression and increase endothelial nitric oxide synthase phosphorylation, resulting in improved production of NO and thus endothelium-dependent relaxations66,67.

Beside polyphenols, also insulin response may positively affect vascular function, since the binding to its receptor on endothelial cells seems to activate the eNOS pathway and, thus, the vasodilation process68. These processes are usually very fast and have been identified as potential mechanisms of action in the short-term studies.

Other putative mechanisms through which polyphenols may affect vasodilation, in the short and medium-long term interventions, involved the regulation of vascular redox signalling. In this regard, berry components may activate nuclear factor E2-related factor 2-antioxidant/xenobiotic response element signalling pathway, which represents the major mechanism in cellular defence against oxidative and electrophilic stress64. Furthermore, polyphenols may modulate pro-inflammatory pathways by inhibiting reactive oxygen species and the redox-sensitive transcription of nuclear factor-kappa

B, involved in gene expression of several pro-inflammatory cytokines, chemokines, adhesion molecules, inducible nitric oxide synthase, cyclooxygenase 2 and cytosolic phospholipase 2, all playing an important role in the regulation of NO production and modulation of vascular function69.

CONCLUSIONS

In conclusion, despite the numerous limitations and confounding factors present in the reviewed studies, the overall results of this systematic review seem to suggest a potential positive effect of berries in the modulation of vascular function. In particular, the effects were observed for FMD and RHI in short-term studies and for PWV and AIx in medium-long term ones suggesting that differences in biomarker modulation may depend on the time of exposure to the dietary interventions and/or to the experimental protocol of the study. Future research using appropriate study designs that consider current knowledge gaps and combine the use of different biomarkers are consequently highly recommended. Further RCTs in different and well characterized target groups of volunteers should be performed in order to strengthen the evidence on the efficacy of such treatments on vascular health and function, and perhaps to shed more light on the mechanisms underneath these effects. In this regards, studies on the structure-activity relationship of berry-polyphenols and/or their metabolic products could help understanding the potential mechanisms through which these compounds interact and positively affect the vascular system. Despite very difficult to estimate, most of the studies have shown an improvement of vascular function for doses of berries higher than 200 g (providing at least 600-700 mg of total polyphenols). This data should be considered indicative and dose- and time-dependent studies would be desirable to better identify the portion of berries (and related polyphenol amount) eliciting a beneficial effect on vasodilation. This information could be useful for the development of new products with vasoactive properties and possibly able to maintain vascular health and reduce the incidence of CVDs, also depending on identified target groups.

ACKNOWLEDGEMENTS

Authors' contribution

DM, DA wrote the first draft of the manuscript. MM with DM made the literature search, reviewed the abstracts of the studies selected, and prepared the tables. CDB acted as a third independent reviewer and improved the manuscript. DDR, PR and MP critically revised the scientific contents and improved the quality of the manuscript.

Acknowledgements

We are grateful to Mr. Giorgio Luzzini for its contribution in the initial literature search, and to Dr Chris I.R. Gill for his critical comments on the manuscript.

The authors are grateful for support granted by Ministero delle Politiche Agricole, Alimentari, Forestali e del Turismo (MIPAAFT) and the work was performed within the framework of the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL) MaPLE and VALID projects.

Funding and sponsorship

No external funding was received.

Declaration of interest

The authors report no conflicts of interest arising from the present research and its publication.

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FIGURE CAPTIONS

Figure 1. Flowchart of the study selection process

Supplementary Figure 1. Risk of bias summary: review authors' judgments about each risk of bias item for each included study**.**

Supplementary Figure 2. Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

Table 1. Characteristics of the study design, population, products, and outcomes of the considered acute intervention studies investigating the effect of berry consumption on one or more markers of endothelial function.

REF.	STUDY DESIGN	STUDY POPULATION	BERRY INTERVENTION	CONTROL/ PLACEBO INTERVENTION	OUTCOME VARIABLES	MAIN FINDINGS
Alqurashi *et al.* (2016)[25], UK	Randomized, crossover, controlled, double-blind	n=23 healthy nonsmoker males (mean age 46 ± 1.9 y; mean BMI 27.6 ± 0.4 kg/m^2)	200 g of açai smoothie (AS, 150 g acai pulp + 50 g banana) Composition per serving: Total Polyphenols 694 mg (493 mg ACN, 173.6 mg GA, 9.6 mg quercetin, 9.3 mg CGA); Total carotenoids: 179.3 mg	200 mg control smoothie (CS) (50 g banana matched for fat) Composition per serving: Total phenolics <10 mg; Total carotenoids: 0 mg;	FMD up to 6 h after consumption	↑FMD at 2 h and 6 h after AS, but not CS consumption
Castro-Acosta *et al.* (2016)[28], UK	Randomized, crossover, controlled, double-blind	n=22 subjects (13 M, mean age 45.4±13.7y, mean BMI 25.5±3.8 kg/m2)	200 mL of three different blackcurrant drinks Composition per 200 mL: Total phenolics: 460, 810 and 1596 mg, respectively (total ACN 131, 322, 599 mg); vitamin C <0.5 mg	Placebo drinks matched for astringency by adding tannins Composition per 200 mL: Total phenolics 207 mg (total ACN 46 mg); vitamin C <0.5 mg	DVP-SI, DVP-RI up to 2 h after consumption	=DVP-SI and DVP-RI compared to baseline for all blackcurrant and placebo drinks
Del Bo' *et al.* (2017)[26], Italy	Randomized, crossover, controlled	Study 1: n=12 nonsmokers males with peripheral arterial dysfunction (mean age 24.2±1.2y; mean BMI 22.5±1.2 kg/m^2) Study 2: n=12 smoker males, mean age 24.5±1.9y, mean BMI 22.9±1.1kg/m^2)	Study 1: 300 g of thawed blueberry (BB) Study 2: blueberry treatment + smoking (BS) Composition per serving: Total phenolics 856 mg (309 mg ACN, 30 mg CGA); Vitamin C: 2.4 mg	Study 1: 300 mL of water matched for sugar (C) Study 2: Smoking (S); Smoking + Control treatment (SC) Composition: n.d.	RHI, dAIx, dAIx@75 2h after consumption	Study 1: ↑RHI; =dAIx and dAIx@75 Study 2: ↑RHI; =dAIx and dAIx@75
Del Bo' *et al.* (2014)[27], Italy	Randomized, cross-over, controlled	n=16 healthy male smokers (mean age 23.6±0.7y, mean BMI 23.0±0.5kg/m^2)	300 g BB + smoking Composition per 100 g: Total phenolics 242.4 mg (116.1 mg ACN, 30.1 mg CGA), Vitamin C 0.8 mg	1) smoking 2) 300 mL of water with sugar + smoking Composition per 100 g: Total phenolics: 0 mg; vitamin C 0 mg	RHI, fRHI, dAIx, dAIx@75 2h after consumption	↑RHI and fRHI; =dAIx and dAIx@75

Del Bo' *et al.* (2013)[28], Italy	Randomized, crossover, placebo-controlled	n=10 healthy nonsmoker males (mean age 20.8 ± 1.6y, mean BMI 22.5 ± 2.1 kg/m^2)	300 g of homogenized BB Composition per 100 g: Total phenolics: 242.4mg (30.1mg CGA, 116.1mg ACN); Vitamin C: 0.8 mg	200 g Control Jelly (CJ) (20g of gelatine matched for sugars in 200mL of water) Composition per 100 g: Total phenolics 0 mg; Vitamin C: 0 mg	RHI by EndoPAT 1h after consumption	= RHI after either BB or CJ consumption
Djurica *et al.* (2016)[43], USA	Randomized, crossover, controlled, double-blind	n=25 overweight or obese males (mean age 16 y; mean BMI: not clear)	50 g of freeze-dried strawberry powder (FDSP) Composition per 50 g FDSP: pelargonidin-3-glucoside 198.5 mg, 15.31 mg procyanidin B1, 12.52 mg catechin and other phenolics)	50 g control powder (CP) matched for energy content and sugars Composition per 50 g: Total phenolics 0 mg	RHI & fRHI by PAT 1h after consumption	= RHI and fRHI after either FDSP or CP consumption
Flammer *et al.* (2013)[44], USA	Randomized, placebo-controlled, double-blind, parallel	n=69 subjects with endothelial dysfunction and CV risk factors - Placebo group (n=37, 11M, 2 smokers; mean age 51.4±15.1y, mean BMI 27.2±5.5 kg/m^2) - Cranberry juice (CBJ) group (n=32, 20M, 1 smoker, mean age 44.8±17.5y, mean BMI 27.7±5.9 kg/m^2)	2x230mL CBJ Composition per mL: Total phenolics 1740 µg (151 µg ACN, 2662 µg total proanthocyanidins)	2x230 mL placebo beverage matched for sugars Composition per mL: Total phenolics n.d.	RHI by EndoPAT 1h after consumption	↑ RHI after either CBJ and placebo beverage, no difference between the two groups; =AIx
Istas *et al.* (2018)[29], UK	Randomized, crossover, controlled, double blind	n=10 healthy males (mean age 27±3y, mean BMI 23±2kg/m2)	592 mL of drinks containing 200 or 400 g of frozen raspberries in water. Composition per serving: Total polyphenols 201 and 403 mg (164 and 328 mg ACN), Vitamin C 0.105 g	592 mL of placebo drink matched for micro- and macronutrient to the 400 g raspberry drink	FMD up to 24 h after consumption	↑FMD at 2h after consumption
Jin *et al.* (2011)[30], UK	Randomized, crossover, placebo-controlled, double-blind	n=20 healthy subjects (11 F/9 M, mean age 44.5±13.3y, mean BMI 23.81±2.46 kg/m^2)	250mL of 20% blackcurrant juice (BCJ) Composition per 100 mL: 81.5 mg PAs, 12.2 mg delphinidin, 8.0 mg cyanidin; Vitamin C 10.2 mg	250mL of control drink Composition per 100 mL: <10 mg PAs; Vitamin C: 0 mg	LDI measures of vascular reactivity in response to acetylcholine (endothelial dependent) and sodium nitroprusside (endothelial	=Endothelium dependent and independent vasodilation

					independent) 2h after BCJ consumption	
Richter *et al.* (2017)[31], USA	Randomized, crossover, placebo-controlled	n=30 nonsmokers, overweight or obese subjects (13F, mean age 28.1±2.7y, mean BMI 31.4±0.8kg/m^2; 17M, mean age 28.2±2.0y, mean BMI 31.3±0.6kg/m^2)	40 g FDSP with a high-fat (50 g total fat) meal Composition per 40 g: 158.76 mg pelargonidin-3-glucoside and other phenolics; Vitamin C 229 mg	40 g CP with a high-fat meal Composition per 40 g: Total phenolics not available; Vitamin C 0.196mg	AP, Ai@75, PWV up to 4 h after the meal	↓AP, Ai@75 after both FDSP and Cpcompared to baseline at 2 and 4 h =PWV
Rodriguez-Mateos *et al.* (2016)[33], Germany	Randomized, crossover, controlled, double blind	n=10 healthy males (mean age 24±2y, mean BMI 24±2 kg/m^2)	Five different CBJ Composition per serving: Total polyphenols 409, 787, 1238, 1534, and 1910 mg, respectively (6.8-32.3 mg ACN; 14.5-76.9 mg flavonols; 12.8-59.2 mg PAs)	Control drink matched for macro and micronutrients Composition per serving: Total polyphenols 2.9 mg (2.7 mg PAs)	FMD (%), PWV (m/s), AIx (%) up to 8 h after consumption	↑FMD at 1,2 4 6 and 8 h after consumption (max at 4h) with maximal effects for the drink containing 1238 mg total polyphenols; =AIx and PWV
Rodriguez-Mateos *et al.* (2014)[32], Germany	Randomized, crossover, controlled	n=10 healthy males (mean age 27 ± 1y, mean BMI 25 ± 0.8 kg/m^2)	a) Three baked products containing 34 g BB powder in (BB bun) Composition per bun x3: Total polyphenols: 637 mg (196 mg total ACN, 140 mg total procyanidins, 221 mg CGA) b) 34 g BB powder dissolved in 500 mL water (BB drink) Composition per 500 mL: Total polyphenols: 692 mg (339 mg total ACN, 111 total procyanidins, 179 mg CGA)	Control baked products (control bun) matched for macro and micronutrients Composition: n.a.	FMD up to 6 h after consumption	↑FMD at 1, 2 and 6h after consumption (max at 1h for BB drink and at 2h for BB bun)
Rodriguez-Mateos *et al.* (2013)[25], UK	Two randomized, crossover, controlled, double-blind	Study 1: n=10 healthy males (mean age 27 ± 1.3y, mean BMI 25±0.8 kg/m^2) Study 2: n=11 healthy males (mean age	Study 1: three different BB drinks Composition per serving: total polyphenols 766, 1278, and 1791 mg (310-724 mg ACN; 137-320 mg procyanidin; 273-637 mg	Studies 1 and 2: Control drink matched for macro and micronutrients Composition per serving: Total	Study 1: FMD; PWV; AIx; DVP up to 6 h after consumption Study 2: FMD 1 h after consumption	Study 1: ↑FMD but not at 4 h; =PWV; AIx; DVP; Study 2: ↑FMD dose-dependent to ≤766 mg

	27 ± 1.0y, mean BMI 22±0.9 kg/m^2)	CGA); Vitamin C 4-9.5 mg Study 2: five different BB drinks Composition per serving: total polyphenols 319, 639, 766, 1278, and 1791 mg (129-727 mg ACN; 57-320 mg procyanidin; 114-637 mg CGA); Vitamin C 1.7-9.5 mg	polyphenols 0 mg; Vitamin C: 6.8 mg

Legend: ACN: anthocyanins; AIx: augmentation index; AP: augmentation pressure; AS: açai smoothie; BB: blueberry; BCJ: blackcurrant juice; BMI: body mass index; BS: blueberry + smoking; C: control; CBJ: cranberry juice; CGA: Chlorogenic acid; CJ: control jelly; CP: control powder; CS: control smoothie; CV: cardiovascular; dAIx: digital augmentation index; dAIx@75: dAIx normalized by considering a heart rate of 75 bpm; DVP: digital volume pulse; F: females; FDSP: freeze dried strawberry powder; FMD: flow mediated dilation; fRHI: Framingham reactive hyperaemia index; GA: gallic acid; LDI: laser Doppler imaging; M: males; PAs: phenolic acids; PAT: peripheral arterial tonometry; PWV: pulse wave velocity; RHI: reactive hyperaemia index; RI: reflection index; S: smoking; SC: smoking + control; SI: stiffness index.

Table 2. Characteristics of the study design, population, products, and outcomes of the considered chronic intervention studies investigating the effect of berry consumption on one or more markers of endothelial function.

REF.	STUDY DESIGN	STUDY POPULATION	DURATION OF INTERVENTION	BERRY INTERVENTION	CONTROL/ PLACEBO INTERVENTION	OUTCOME VARIABLES	MAIN FINDINGS
Cook *et al.* (2017)[34], UK	Randomized, double-blind, crossover	n = 13 healthy males (mean age 25±4 y; mean BMI 25±3 kg/m^2)	1 week	600 mg/day (2 x 300 mg capsule) of New Zealand blackcurrant (NZBC) extract Composition per capsule: 105 mg ACN	600 mg/day (2 × 300 mg capsule) of cellulose Composition: n.d.	Total peripheral resistance	↓total peripheral resistance at rest after NZBC (-25%) and during sustained isometric contraction at 15,30,45,60,90,105 and 120 s
Djurica *et al.* (2016)[43], USA	Randomized, controlled, double-blind, cross-over	n=25 overweight or obese males (mean age 16 y; mean BMI: not clear)	1 week	50 g/day of freeze-dried strawberry powder (FDSP) Composition per 50 g: Pelargonidin-3-glucoside 198.5 mg, 15.31 mg Procyanidin B1, 12.52 mg Catechin and other phenolics)	50 g control powder (CP) matched for energy content and sugars Composition: Total phenolics 0 mg	RHI & fRHI by PAT	= RHI and fRHI after either FDSP or CP consumption
Dohadwala *et al.* (2011)[35], USA	Randomized, double-blind, crossover, placebo-controlled	n=44 subjects with stable coronary artery disease - CBJ first, n=22 (15 M, mean age 61±11y; mean BMI 30±5 kg/m^2) - Placebo (PJ) first, n=22 (15 M, mean age 63±9y, mean BMI 29±4 kg/m^2)	4 weeks	480 mL/day cranberry juice (CBJ) Composition per serving: Total polyphenols 835 mg (94 mg ACN)	480 mL/day PL juice drink matched for calories and sensory characteristics Composition: n.d.	Carotid-radial PWV, carotid-femoral PWV, FMD (%), lnPATratio	↓carotid-femoral PWV after CBJ = FMD (upper arm), lnPATratio, carotid-radial PWV
Feresin *et al.* (2017)[36], USA	Randomized, controlled, double-blind, parallel	n=60 postmenopausal females with pre- or stage-1 hypertension - Control group, n=20 (mean age 58±1y, mean BMI 32.1±0.7kg/m^2) - Intervention group 1, n=20 (mean age 61 ±1y, BMI 31.0±1.0 kg/m^2) -	8 weeks	Intervention group 1: 25 g/day FDSP + 25 g/day of placebo powder Intervention group 2: 50 g/day of FDSP Composition per 25 g FDSP: 99.22 mg pelargonidin-3-glucoside, 7.70 mg procyanidin B1, 6.26 mg catechin and other phenolics	50 g of PL powder Composition: Total phenolics 0 mg	Brachial-ankle and femoral-ankle PWV	↓brachial-ankle PWV and femoral-ankle PWV after 25 g but not 50 g of FDSP. No treatment effect

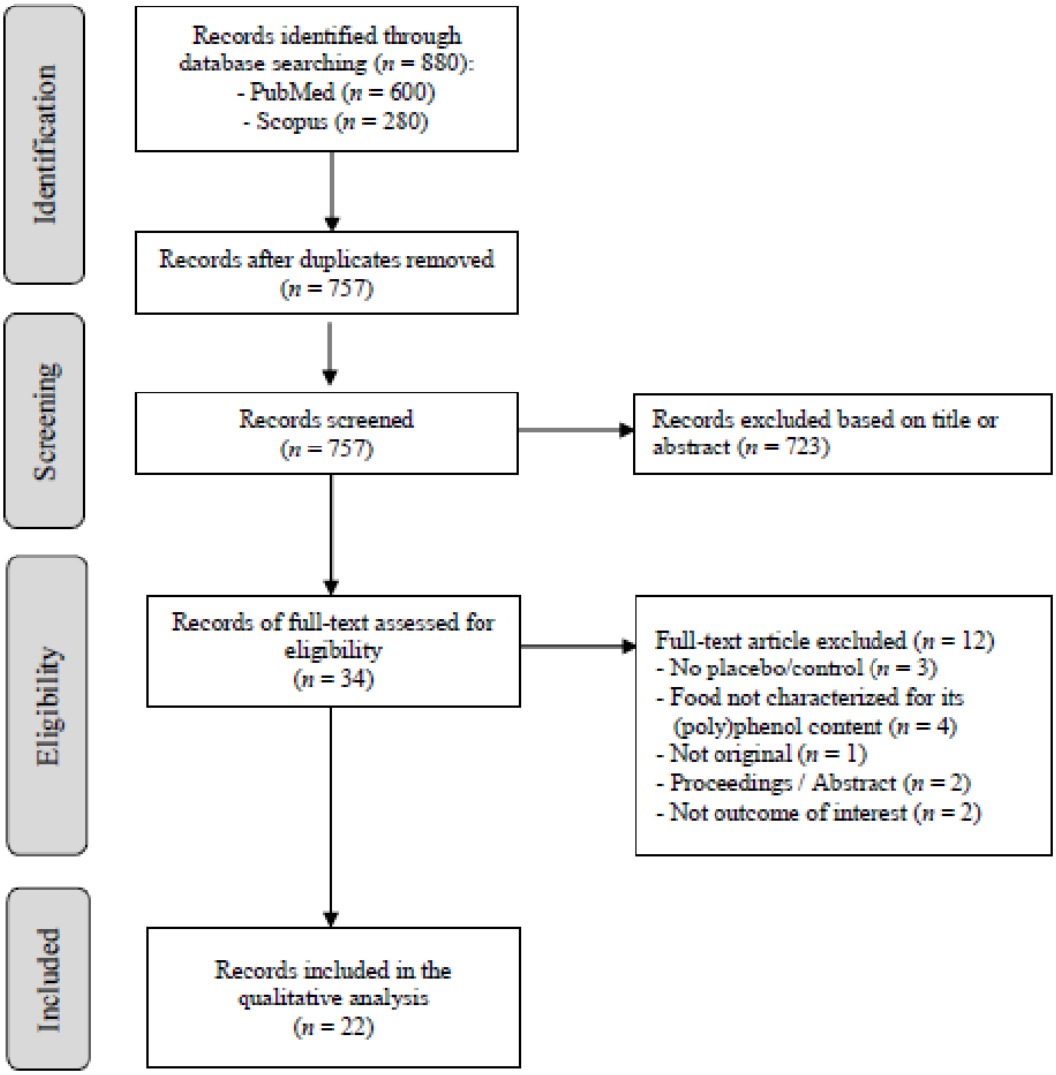
		Intervention group 2, n=20 (mean age 59±1y, mean BMI 32.7±1.1 kg/m^2)					
Flammer *et al.* (2013)[44], USA	Randomized, placebo-controlled, double-blind, parallel	n=69 subjects with endothelial dysfunction and CV risk factors - Placebo group, n=37 (11M, mean age 51.4±15.1y, mean BMI 27.2±5.5 kg/m2) - Cranberry juice (CJ) group, n=32 (20M, mean age 44.8±17.5y, mean BMI 27.7±5.9 kg/m^2)	4 months	2x230mL CBJ Composition per mL: Total phenolics 1740 µg (151 µg total ACN, 2662 µg total proanthocyanidins)	2x230 mL PL beverage matched for sugars Composition: n.d.	RHI	= RHI after either CBJ and PL, no difference between the two groups
Johnson *et al.* (2015)[37], USA	Randomized, controlled, double-blind, parallel	n=48 light smoker subjects with pre-hypertension - Intervention group, n=25 (mean age 59.7±4.58y, mean BMI 30.1±5.94 kg/m^2) -Placebo group, n=23 (mean age 57.3±4.76y, mean BMI 32.7±6.5 kg/m^2)	8 weeks	22 g/day of freeze-dried BB powder Composition per serving: Total phenolics 844.58 mg (469.48 mg ACN), vitamin C 2.27 mg	22g/day of macronutrient-matched CP Composition: Total phenolics 0 mg, vitamin C 0 mg	Carotid-femoral and brachial-ankle PWV	↓brachial-ankle PWV after blueberry but non control =carotid-femoral PWV

Khan *et al.* (2014)[38], UK	Randomized, double-blind, placebo-controlled, parallel	n= 66 healthy subjects - Placebo group, n= 21 (15 M, mean age 51±8y; mean BMI 28.9 ± 6.5kg/m^2) - Intervention group 1, n=22 (15 M, mean age 55±10y, mean BMI 28.4 ± 5.4kg/m^2) - Intervention group 2, n=21 (13 M, mean age 51±11y; mean BMI 29.2 ± 6.9 kg/m^2)	6 weeks	- Intervention group 1: 1 L/day low blackcurrant juice (BCJ, 4 x 250 mL) Composition per 100 mL: Total polyphenols 27.3 mg (4 mg ACN), vitamin C 1.1 mg - Intervention group 2: 1 L/day high BCJ (4 x 250 mL) Composition per 100 mL: Total polyphenols 81.5 mg (14.3 mg), vitamin C 10.2 mg	1 L of flavored water (4x250 mL) Composition: n.d.	FMD	↑FMD after high BCJ, but not after low BCJ, compared to placebo
Riso *et al.* (2013)[39], Italy	Randomized, controlled, crossover	n=18 healthy males with one risk factor for CVD (mean age 47.8±9.7y, mean BMI 24.8±2.6 kg/m^2)	6 weeks	250 mL/day Wild BB drink (25g of BB powder in 250 mL of water) Composition per 25 g powder: 375 mg ACN, 127.5 mg CGA	250 PL drink/day matched for sensory characteristics Composition: n.d.	RHI, fRHI, AIx, AI@75	=RHI, fRHI, AIx, AI@75
Ruel *et al.* (2013)[40], Canada	Randomized, controlled, double-blind crossover	n=35 healthy overweight men (mean age 45±10y, mean BMI 28.3 ± 2.4 kg/m^2)	4 weeks	500 mL/day CBJ (4x125mL) Composition per 500 mL: Total polyphenols 400 mg (20.8 mg ACN, 296 mg proanthocyanidins), vitamin C 128 mg	PL-juice matched for sensory characteristics Composition per 500 mL: Total polyphenols 156 mg (20.8mg ACN, 296 mg proanthocyanidins), vitamin C 128 mg	Resting AIx, AIx salbutamol, AIx GTN, global endothelial function	= resting AIx, AIx salbutamol, AIx GTN and global endothelial function after CBJ compared to placebo, but ↓ within-group resting AIx, AIx salbutamol and global endothelial function after CBJ, and ↓ within-group resting AIx and ↑ within-group AIx salbutamol and AIxGNT in subjects with MetS

Stull *et al.* (2015)[41], USA	Randomized, double-blind, placebo-controlled, parallel	n=44 non-smokers with MetS - Intervention group, n=23 (11 M, mean age 55±2y, mean BMI 35.2±0.8kg/m^2) - Placebo group, n=21 (5 M, mean age 59±2y, mean BMI 36.0±1.1kg/m^2)	6 weeks	Two smoothies /day (2 x 12-oz yogurt and skim milk-based smoothie with 22.5 g of freeze-dried BB powder) Composition per smoothie: Total phenolics 773.6 mg (290.3 mg ACN), vitamin C 2.7 mg	Two smoothies /day (2 x 12-oz yogurt and skim milk-based smoothie without BB powder) Composition per smoothie: Total phenolics n.d, vitamin C 0 mg	RHI	↑RHI after the intervention compared to placebo
Willems *et al.* (2015)[42], UK	Randomized, controlled, double-blind crossover	n=13 triathletes (8 M, mean age 38±8y, mean BMI 23±2 kg/m^2)	1 week	6 g/day Sujon NZBC dissolved in water Composition per serving: 138.6 mg ACN, vitamin C 49 mg	250 mL/day BCJ Composition per serving: 3-4 mg ACN, 32 mg vitamin C	Total peripheral resistance	↓ total peripheral resistance after NZBC compared to control (-16%)

Legend: ACN: anthocyanins; AIx: augmentation index; AIx@75: AIx normalized by considering a heart rate of 75 bpm; BB: blueberry; BCJ: blackcurrant juice; BMI: body mass index; CBJ: cranberry juice; CGA: Chlorogenic acid; CP: control powder; CV: cardiovascular; CVD: cardiovascular disease; F: females; FDSP: freeze dried strawberry powder; FMD: flow mediated dilation; fRHI: Framingham reactive hyperaemia index; GTN: glyceryl trinitrate; lnPAT: natural logarithm of the peripheral arterial tonometry index; M: males; MetS: metabolic syndrome; NZBC: New Zealand blackcurrant; PJ: placebo juice; PL: placebo; PWV: pulse wave velocity; RHI: reactive hyperaemia index.

Figure 1

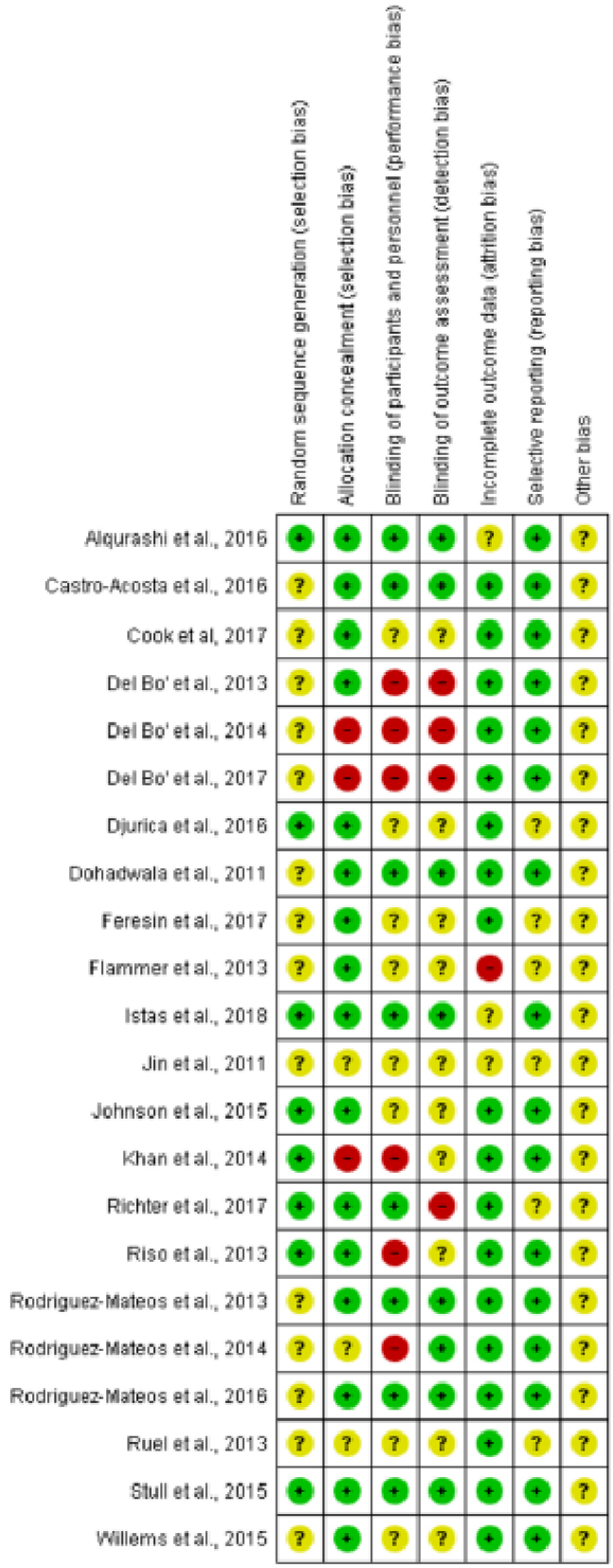

Identification
Screening
Eligibility
Included
Records identified through database searching (n = 880):
- PubMed (n = 600)
- Scopus (n = 280)
Records after duplicates removed (n = 757)
Records screened (n = 757)
Records excluded based on title or abstract (n = 723)
Records of full-text assessed for eligibility (n = 34)
Full-text article excluded (n = 12)
- No placebo/control (n = 3)
- Food not characterized for its (poly)phenol content (n = 4)
- Not original (n = 1)
- Proceedings / Abstract (n = 2)
- Not outcome of interest (n = 2)
Records included in the qualitative analysis (n = 22)

Supplementary Table 1: PICO criteria for the inclusion of the intervention studies[21].

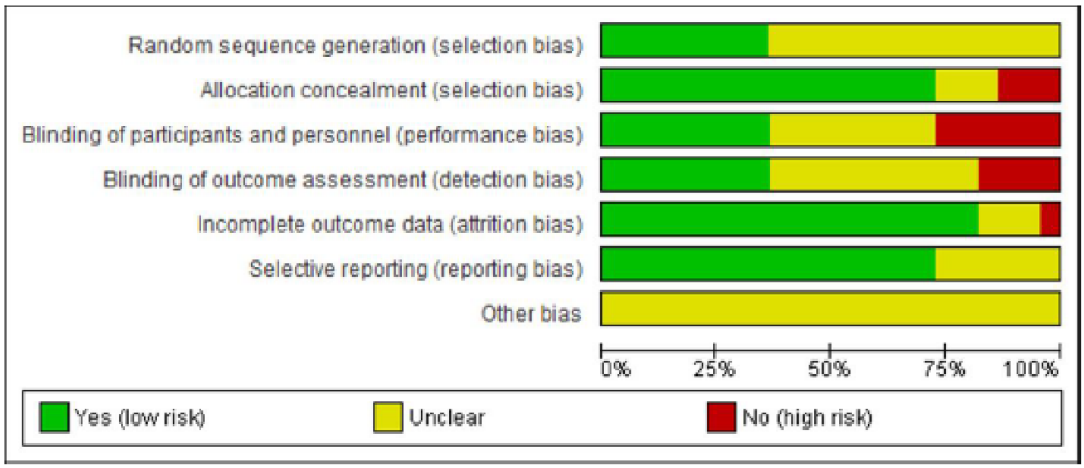
Parameter	Criteria
Population (P)	Not hospitalized children, adolescents or adults, regardless of the age, BMI and health/pathological status.
Intervention (I)	Dietary intervention studies involving the consumption of berries, regardless of the supplied form (raw, juices, supplements, etc.), not in combination with other foods which may overlap the effects.
Comparison (C)	Control group (berries totally or partially excluded, totally or partially substitute with other fruits/supplements).
Outcome (O)	Endothelial dysfunction, such as RHI (Reactive Hyperaemia Index), Aix (Augmentation index), PWV (Pulse Wave Velocity) and FMD (Flow Mediated Dilation).
Study design (S)	Inclusion: Randomized controlled trials Exclusion: Non-randomized controlled trials; Retrospective, prospective, or concurrent cohort studies; Cross sectional studies; Case reports; Editorials

[21] Lichtenstein AH, Yetley EA, Lau J. Application of systematic review methodology to the field of nutrition. J Nutr. 2008;138:2297-2306

Supplementary Figure 1



Supplementary Figure 2



5.3 Effect of a polyphenol-rich diet on markers of oxidative stress and endothelial function in older subjects with intestinal permeability: the MaPLE project

The last part of the research was performed as part of the activities developed within the MaPLE project (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly), funded within the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL). The project aim was to investigate benefits of a polyphenol-enriched dietary pattern on intestinal permeability in older subjects. Polyphenols were selected since they can exert both direct and indirect effects with specific regard to their impact on the host microbial ecosystem. In addition, there was specific interest in understanding if and how such intervention could improve other critical conditions with specific regard to inflammation, oxidative stress and vascular function very often compromised in the older subjects.

In the context of the MaPLE project, my main contribution was to investigate the impact of the dietary intervention on biomarkers of DNA damage and vascular function in older subjects enrolled in the study.

5.3.1 MATERIALS AND METHODS

5.3.1.1 Setting and subjects' recruitment

The MaPLE trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy), an institution including residential care and independent residences for older subjects. The setting was selected in order to enable a significant control of most of the experimental variables affecting dietary intervention studies. More details are reported in **Guglielmetti et al. (2020)**. Subjects selection was performed in collaboration with physicians and staff at OIC Foundation, based on medical examination and the evaluation of drug therapies. The final eligibility was defined according to the inclusion and exclusion criteria reported below.

To be included in the trial, the subjects had to be $\geq$ 60 years old, with an adequate nutritional status, a good cognitive status, good functional autonomy, and with an

increased intestinal permeability (evaluated as serum zonulin level concentrations). Exclusion criteria included: having Celiac disease, advanced stage of chronic diseases such as cirrhosis, renal insufficiency (dialysis), severe Chronic Obstructive Pulmonary Disease (COPD) or severe cardiovascular disease (heart failure class III or IV NYHA - New York Heart Association). Moreover, subjects with malignant tumours that required treatment in the previous 2 years were excluded as well as those treated with antibiotics in the last month before the intervention period.

5.3.1.2 Intervention

The full study protocol of the MaPLE trial is reported in **Guglielmetti et al 2020.** The study consists in a single-blind, randomized, controlled, cross-over trial (polyphenol-rich diet versus control diet) performed in a group of older individuals ($\geq$ 60 y). Each intervention period (polyphenol-rich diet, PR-diet and control diet, C diet) was 8 weeks long and separated by an 8-week wash-out. The control and polyphenol-rich diets were similar in energy and nutrients. During the C diet participants consumed their habitual diet, while during the PR-diet food and beverages low in polyphenols consumed during the C diet were replaced with three portions/day of polyphenol-rich food/beverages (e.g. Renetta apple and purée, dark chocolate in the form of powder or callets, berries and related products, blood orange and juice, pomegranate juice and green tea). PR-diet provided a mean of 724 mg/day of total polyphenols estimated by Folin-Ciocalteu analysis. Thus, the total polyphenol intake in the intervention diet, i.e. including the menu plus the PR-foods, was roughly doubled compared to the C-diet.

At the beginning and at the end of each intervention period all participants underwent physical and general condition examinations (i.e. height, weight, blood pressure and clinical signs). In addition, biological samples were collected for the analysis of metabolic and functional markers.

5.3.1.3 Anthropometrical evaluations

Height and weight were measured according to Lohman et al international guidelines (**Lohman et al 1992**); body mass index (BMI) was calculated according to the formula – weight (kg)/height (m2). Reference scores were defined according to international guidelines (**Lohman et al 1992**).

5.3.1.4 Blood sampling and serum collection

After an overnight fast, blood samples were drawn in Vacutainer tubes containing silicone gel for serum and maintained at room temperature for at least 30 min. Serum was then obtained by tube centrifugation (1400 g x 15 min, 4°C), splitted in small aliquots into specific vials and stored at -80°C until analysis.

5.3.1.5 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated by using Histopaque 1077 density gradient, according to the procedure reported by **Del Bo' et** (**2015**). Different batch of isolated PBMCs was then diluted into medium constituted by fetal bovine serum, RPMI 1640 medium and dimethyl sulfoxide (50:40:10), and stored at -80°C until analysis. The analysis of DNA damage, as a marker of oxidative stress, was performed on PBMCs after a rapid thawing at 37°C and washing with fresh RPMI medium and cold phosphate buffer saline. For the analysis, both endogenous and oxidatively-induced DNA damage was performed by comet assay (**Del Bo' et al 2015**).

5.3.1.6 Marker of oxidative stress: analysis of DNA damage by comet assay

The evaluation of endogenous DNA damage was carried out enzymatically by the use of formamidopyrimidine DNA glycosylase (FPG-sensitive sites) able to detect the oxidized purines (mainly 8-oxo-7,8-dihydroguanine). A description of the specific steps and conditions in the protocol has been previously reported (**Del Bo' et al 2015**). In brief, a solution of low melting point agarose was added to PBMCs suspension, mixed and rapidly spotted onto GelBond Film precoated with normal melting point. Coverslips were added on top of the slides and left to solidify for a few minutes at 4°C.

After solidification, coverslips were removed and slides were transferred into a lysis buffer for 1 h at 4°C in the dark. Then, slides were washed 3 times in a cold buffer and treated. One slide was treated with a solution of FPG enzyme, while the other slide (control) with a buffer without FPG. Samples were then incubated at 37°C for 45 min. Successively, the slides were transferred in a horizontal electrophoresis tank containing an alkaline electrophoresis buffer for 40 min at 4°C in order to favour DNA unwinding and then, electrophoresis was performed (25 V, 300 mA; for 20 min.). Finally, samples were washed in a neutralizing buffer for 15 min at 4°C in the dark and dried in ethanol for 2 h. Oxidatively induced DNA damage was performed according to the procedure previously reported (**Del Bo' et al 2015**). Briefly, two GelBonds Film containing cell suspension were prepared for each subject: one was treated with hydrogen peroxide for 5 min at room temperature in the dark; the other was treated for 5 min with a buffer solution (control slide). Following the oxidative treatment, slides were immersed in a lysis buffer for 1 h at 4°C in the dark, and then transferred in a horizontal electrophoresis tank and treated as reported for the endogenous DNA damage. Ethidium bromide was used for the staining process. Fifty comets per gel (100 comets per condition) were scored using an epifluorescence microscope and analysed with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). The levels of DNA damage were calculated as tail intensity (% DNA in tail).

5.3.1.7 Markers of vascular function: evaluation of vcam and icam serum levels

Serum samples at each time point were used to quantify vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) using an ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy). After competitive treatment with antibodies and fluorophore, fluorescence was quantified using a TECAN Infinite F200 plate reader. A 4-parameter algorithm was used to create the standard curve and to calculate serum concentrations.

5.3.1.8 Statistical analysis

Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM corp., Chicago, IL) was used for statistical analysis. The Kolmogorov-Smirnov test was applied to verify the normal distribution of the variables. One-way ANOVA was performed to ascertain differences at baseline between men and women in terms of sVCAM-1, sICAM-1 and DNA damage. In order to exclude a carry-over effect, ANOVA with the treatment sequence (PR-diet *versus* C-diet or vice versa) as the independent factor was carried out. Successively, as none was detected, data were analysed by ANOVA for repeated-measures design with treatment (PR-diet vs C-diet) and time (before and after each treatment) as dependent factors. Potential gender differences were also considered. The level of significance was set at $p \leq 0.05$. Post hoc analysis of differences between treatments was assessed by the least significant difference (LSD) test with $p \leq 0.05$ as level of statistical significance. All results were expressed as mean ± standard deviation (SD).

5.3.2 RESULTS

5.3.2.1 Baseline characteristics of the subjects

In **Table 1** are reported the characteristics at baseline of the 51 subjects enrolled and that completed the entire intervention study, while this data stratified by sex is shown in **Table 1a and Table 1b**. Age ranged between 60 and 98 years old with a median value of 77 years old. Age distribution was comparable in men and women while a high inter-individual variability was observed for BMI (IQR: 22.5;30.7) and sVCAM-1 (IQR: 628.0;1327.1).

Table 1 - Baseline characteristics of subjects selected for the study

Variables	Median (IQR)	Mean ± SD
Age (y)	77 (70;87)	78.0 ± 10.3
Body weight (kg)	73.6 (62;83)	73.1 ± 14.0
BMI (kg/m^2)	25.7 (22.5;30.7)	26.8 ± 5.5

sVCAM-1 (ng/mL)	967.9 (628.0;1327.1)	1239 ± 1683
sICAM-1 (ng/mL)	51.4 (43.9;65.4)	55.6 ± 20.5
net-H_2O_2-induced DNA damage (% DNA in tail)	27.2 (22.0;34.5)	29.1 ± 11.49
net-FPG sensitive sites (% DNA in tail)	17.1 (11.7;26,8)	20.2 ± 11.32

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

BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H2O2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 1a - Baseline characteristics of women selected for the study

Variables	Median (IQR)	Mean ± SD
Age (y)	80 (74;88)	80 ± 9.39
Body weight (kg)	65.1 (74;88)	68.58 ± 13.25
BMI (kg/m^2)	25.7 (22.1;30.8)	26.96 ± 5.5
sVCAM-1 (ng/mL)	973.82 (841.2;1448.1)	1452.9 ± 2207.32
sICAM-1 (ng/mL)	50.53 (42.78;63.05)	53.23 ± 14.6
net-H_2O_2-induced DNA damage (% DNA in tail)	28.5 (21.9;34.7)	28.97 ± 11.62
net-FPG sensitive sites (% DNA in tail)	14.3 (9.0;20.17)	14.79 ± 7.58

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

BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H2O2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 1b - Baseline characteristics of men selected for the study

Variables	Median (IQR)	Mean ± SD

Age (y)	73.5 (74;88)	74.5 ± 10.8
Body weight (kg)	78 (69.9;88.4)	79.0 ± 13.2
BMI (kg/m^2)	26 (23.7;28.6)	26.6 ± 5.7
sVCAM-1 (ng/mL)	947.1 (561.0;1227.2)	956.4 ± 572.0
sICAM-1 (ng/mL)	52.7 (44.5;69.6)	58.8 ± 24.2
net-H_2O_2-induced DNA damage (% DNA in tail)	24.9 (19.0;38.2)	28.4 ± 11.6
net-FPG sensitive sites (% DNA in tail)	16.5 (9.4;27.1)	18.6 ± 10.3

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

BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H2O2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

5.3.2.2 Effect of intervention on DNA strand breaks, Net-FPG-sensitive sites and Net- H_2O_2 induced DNA damage in PBMCs

The effect of MaPLE intervention on the levels of DNA strand breaks, Net-FPG-sensitive sites and Net- H_2O_2 induced DNA damage in PBMCs is reported in **Table 2.** On the whole, ANOVA by considering time, treatment and time *x* treatment interaction did not show significant differences in the levels of DNA damage, apart from an effect of treatment (p=0.030) for the levels of Net-FPG-sensitive sites that showed an apparent increase after C-diet but not PR-diet. A further analysis by considering the contribution of gender (**Table 2a, Table 2b**) was performed, but no significant effect was observed.

5.3.2.3 Effect of intervention on sVCAM-1, sICAM-1 levels in serum samples

The effect of MaPLE intervention on sVCAM-1, sICAM-1 levels in serum samples is reported in **Table 2.** On the whole, ANOVA by considering time, treatment and time *x* treatment interaction did not show significant differences in the levels of sVCAM-1,

sICAM-1. A further analysis by considering the contribution of gender (**Table 2a, Table 2b**) was performed, but no significant effect was observed.

Table 2 - Effect of 8-week intervention with PR-diet and C-diet on anthropometric, functional markers and oxidative stress markers in the whole group of subjects

Variables (n = 51)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
sVCAM-1 (ng/mL)	980.4 ± 527.8	1037.4 ± 683.9	1319.9 ± 1713.2	1094.4 ± 703.0	0.095	0.462	0.197
sICAM-1 (ng/mL)	54.9 ± 20.5	59.9 ± 28.8	57.9 ± 23.8	55.7 ± 22.8	0.665	0.352	0.600
net-H_2O_2-induced DNA damage (% DNA in tail)	29.1 ± 11.5	30.2 ± 9.7	28.2 ± 9.0	28.5 ± 9.5	0.235	0.507	0.650
net-FPG sensitive sites (% DNA in tail)	20.2 ± 11.3	21.5 ± 10.6	19.9 ± 10.4	22.4 ± 10.8	0.765	0.030	0.577

All data are expressed as mean ± standard deviation (SD). Data with $P<0.05$ are significantly different. T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction.

PR, polyphenol-rich diet; C, control diet; BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H_2O_2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 2a - Effect of 8-week intervention with PR-diet and C-diet on anthropometric, functional markers and oxidative stress markers in women

Women (n = 29)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
sVCAM-1 (ng/mL)	1025.1 ± 499.2	1097.8 ± 562.6	1609.0 ± 2172.6	1250.3 ± 773.9	0.066	0.467	0.208
sICAM-1 (ng/mL)	56.6 ± 18.7	59.9 ± 25.0	55.9 ± 20.8	54.1 ± 20.1	0.336	0.200	0.121
net-H_2O_2-induced DNA	29.7 ± 10.2	30.3 ± 8.0	27.6 ± 9.0	27.2 ± 8.3	0.071	0.931	0.678

damage (% DNA in tail)							
net-FPG sensitive sites (% DNA in tail)	19.5 ± 8.7	20.5 ± 9.5	19.6 ± 10.5	21.0 ± 10.2	0.838	0.226	0.889

All data are expressed as mean ± standard deviation (SD). Data with P<0.05 are significantly different. T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction.

PR, polyphenol-rich diet; C, control diet; BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H_2O_2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 2b - Effect of 8-week intervention with PR-diet and C-diet on anthropometric, functional markers and oxidative stress markers in men

Men (n = 22)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
sVCAM-1 (ng/mL)	921.6 ± 569.6	957.8 ± 824.6	939.0 ± 653.2	888.8 ± 547.8	0.737	0.920	0.724
sICAM-1 (ng/mL)	56.6 ± 23.0	60.0 ± 33.7	60.4 ± 27.7	57.9 ± 26.2	0.695	0.886	0.305
net-H_2O_2-induced DNA damage (% DNA in tail)	28.3 ± 13.1	30.1 ± 11.8	29.0 ± 9.1	30.0 ± 10.9	0.888	0.391	0.812
net-FPG sensitive sites (% DNA in tail)	21.0 ± 14.1	22.7 ± 11.8	20.3 ± 10.5	24.0 ± 11.5	0.832	0.075	0.516

All data are expressed as mean ± standard deviation (SD). Data with P<0.05 are significantly different. T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction.

PR, polyphenol-rich diet; C, control diet; BMI, body mass index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; H_2O_2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

5.3.3 DISCUSSION

In the context of the MaPLE study, the polyphenol-rich diet was able to reduce serum zonulin levels, a widely recognized surrogate marker of intestinal permeability, in the older subjects enrolled in the study (**Del Bo' et al., under review**). On the contrary, here it was demonstrated that the PR-diet failed to positively modulate markers of oxidative stress (i.e. H_2O_2-induced DNA damage and FPG-sensitive sites), and vascular function (sVCAM-1, sICAM-1). The role of polyphenols in counteracting oxidative stress has been widely studied both *in vitro* and *in vivo* reporting a possible direct and/or indirect positive contribution of polyphenols in its modulation. In this regard, in our laboratory we performed several human intervention studies in which the role of polyphenols and/or polyphenol-rich foods was investigated considering their impact on DNA damage as oxidative stress markers. For example, in a previous publication, one portion (300 g) of blueberries, providing 348 mg of ACNs and 90 mg of chlorogenic acid, significantly reduced the levels of H_2O_2-induced DNA damage (-18%) but not the levels of endogenous DNA damage, 1 hour after consumption compared to control, in a group of young healthy subjects (**Del Bo' et al 2013**). In another study, it was demonstrated that 6-week intervention with a drink containing 25 g freeze-dried powder of wild blueberries (Vaccinium angustifolium), providing 375 mg of ACNs, was able to significantly reduce the levels of endogenously oxidized DNA bases (from 12.5 ± 5.6 % to 9.6 ± 3.5 %, $p \leq 0.01$) and the levels of H_2O_2-induced DNA damage (from 45.8 ± 7.9 % to 37.2 ± 9.1 %, $p \leq 0.01$) compared to the placebo drink, in subjects with CVD risk factors (**Riso et al 2013**). The different results observed among studies could be attributed to the different target group of population analyzed (i.e. young subjects, adults with CVD risk, older subjects). Also, the type of intervention in terms of food administered, could have contributed to the different results observed. Other potential factors could relate to the sample size and the extent and duration of the intervention. However, our observations are in line with those found in other studies with polyphenol-rich foods. For instance, Giovannelli and coworkers did not observe a significant protective activity towards oxidative DNA damage, assessed through Comet assay, after 1 month of high-proanthocyanidin red de-alcoholised wine (500 mL/die, proanthocyanidin dose = 7 mg/kg b.w.) *versus* low-proanthocyanidin rosé de-alcoholised wine (500 mL/die,

proanthocyanidin dose = 0.45 mg/kg) in 21 post-menopausal women (**Giovannelli et al 2011**). Also Ramirez-Tortosa and colleagues were not able to observe any protective effect on DNA damage after providing a polyphenol-rich dessert to elderly subjects. In particular, twenty-two subjects (six men and sixteen women) (average ages 78.88) consumed 200 g of dessert containing 224.5 mg/kg of anthocyanin for a period of 2 weeks (**Ramirez-Tortosa et al 2004**).

Another important aspect in elderly is the increase in oxidative stress and the subsequent augment of DNA damage levels, due to an imbalance between antioxidant mechanisms and pro-oxidant species production (**Liguori et al 2018**). The accumulation of DNA damage leads to phenotypic consequences and functional decline in different organs associated with aging-related diseases, such as cancer, cardiovascular and neurodegenerative diseases (**Kirkwood et al 2005**). Moreover, oxidative stress and DNA damage are closely associated with blood vessels homeostasis. The functional decline of vasculature is accelerated in condition of DNA damage accumulation and defective DNA repair. Excessive ROS production leads to changes in vascular cell biology, determining endothelial dysfunction, inflammation and vascular remodeling which are the main contributors of vascular aging and related diseases (**Bautista-Nino et al 2016**; **Weseler et al 2010**). Despite a growing evidence regarding the protective role of polyphenols on DNA damage and vascular dysfunction, further studies focused on molecular mechanisms are needed due to the complex bioavailability and metabolism of phenolic compounds.

Regarding the biomarkers of vascular function, such as sVCAM-1, sICAM-1, and more in general risk factors of cardiovascular diseases, in literature there is growing evidence, coming from recent systematic reviews, about the potential beneficial effect of certain polyphenols (**Weaver et al 2020, Martini et al 2020, Rangel-Huerta et al 2015, Shrime et al 2011**). However, in the current study, it was not possible to demonstrate a modulation of vascular function biomarkers following a polyphenol-rich diet. In accordance with our results, Martınez-Lopez and colleagues observed no effect on the levels of VCAM-1, ICAM-1 in healthy and moderately hypercholesterolemic volunteers after 4-week consumption of two servings per day (7.5 g per serving) of a soluble cocoa product (providing 45.3 mg flavanols per day) in milk compared with consuming only milk (**Martınez-Lopez et al 2014**). Also, Williams et al were not able to demonstrate the

capacity of red wine (4 mL/kg, corresponding to 2 to 3 glasses), rich in polyphenols, to reduce VCAM-1 and ICAM-1 levels compared to a white wine in thirteen men (aged 48 to 70 years) with angiographic evidence of CAD **(Williams et al 2004)**. Similar results were also reported in a previous study following 6-week supplementation with wild blueberry (**Riso et al., 2013**). While a significant reduction was observed following supplementation with 4 cups of freeze-dried strawberry beverage (50 g freeze-dried strawberries approximately 3 cups fresh strawberries) compared to equivalent amounts of fluids (controls, 4 cups of water) daily for 8 weeks in twenty-seven subjects with metabolic syndrome (**Basu et al 2010**). Discrepancy between results, could be attributable to the large inter-individual variability for the vascular markers and this may have precluded the observation of significant effects of the treatment versus control diet. Moreover, it is reported that levels of both VCAM-1 and ICAM-1 are different among the population, in particular they are higher in older subjects with respect to younger individuals and it can affect the impact of dietary bioactive compounds on these markers **(Herrera et al 2010, Richter et al 2003)**.

In conclusion, results obtained on the efficacy of a polyphenol-rich dietary pattern on biomarkers of oxidative stress and vascular function, in our experimental conditions, can not document a protective effect demonstrated on these markers. However, these results require a careful interpretation since human intervention studies that assessed the effect of polyphenols on our biomarkers in elderly population are quite few. Thus, a comparison with existing literature is difficult and could be misleading if using data obtained on different target populations. Based on that, we cannot be certain about the inefficacy of the polyphenol-rich diet on oxidative stress and endothelial function, but rather it is plausible to wonder if the biomarkers adopted are adequate in our older people. In fact, the actual problem related to markers is the complex interpretation of their modulation that can be different depending on several factors such as the intervention, the model, but also in the same model it is possible to obtain dissimilar results in the light of the target population. Further, well designed clinical trials are still needed in order to confirm or reverse the results observed in the current study, also taking into consideration different biomarkers that could be more affected by a dietary treatment with a polyphenol-rich food.

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GENERAL CONCLUSION AND FUTURE PERSPECTIVES

Research on the protective effect of diet and food bioactives on host metabolism and functions is extremely complex and no one single study (both in vitro or in vivo) can be able to provide definitive conclusions on their efficacy due to the large number of factors affecting their impact. However, the availability of reliable biomarkers that are sensitive, specific, accurate, precise, repeatable and reproducible, as well as the ability to identify and select those biomarkers that can be helpful for the evaluation of a hypothesized effect is fundamental to advance this research field.

In the case of polyphenols, their extensive and complex absorption, metabolism and bioavailability need to be taken into account. For this reason, several phenolic compounds and metabolites have been tested in the experiments performed during this PhD. Moreover, many biomarkers were utilized and different types of models were adopted, passing through cell lines, mice and humans.
Despite the different approaches and markers considered, overall it is possible to assert that, as reported in literature, it is confirmed that polyphenols and their metabolic derivatives possess numerous biological effects. The use of a polyphenol extract in an animal model and the supplementation of different cell models with pure compounds have enabled us to elucidate which compounds were involved in the modulation of inflammation and vascular function and their molecular mechanisms of action. Most of the observed effects were obtained using physiologically relevant concentration, presuming an effective protective effect of these compounds introduced through the diet.

The protective activity (i.e. against oxidative stress and vascular dysfunction) was less evident in the dietary intervention study on older volunteers. This is not surprising by considering the increased complexity of human being, the high inter-individual variability and the uncertainty of the optimal dose/time of intervention. Finally, such

studies are generally developed to demonstrate the impact on a primary endpoint that in our study was actually significantly modulated thanks to the intervention.

In conclusion, in the next future, a better understanding of the bioavailability of polyphenols is necessary to clarify which compounds effectively arrive to target cells and tissues starting from parental compounds present in foods. This knowledge could explain different results obtained between *in vitro* and *in vivo* models. Finally, it should be emphasized as also appropriate biomarkers of dietary exposure would be helpful to enable the assessment of dietary intake, avoiding the limits associated with the traditional questionnaires. Furtherly, the characterization of biological pathways is essential to better study the molecular mechanisms of action through which these compounds exert their biological activity. Consequently, in conclusion, *in vitro* mechanistic studies, but above all well-designed randomized controlled trials, are needed to achieve newer insights aimed to develop dietary recommendations for the maintenance of an optimal health status.

APPENDICES

7.1 PUBLISHED PAPERS:

- Modulation of Adhesion Process, E-Selectin and VEGF Production by Anthocyanins and Their Metabolites in an In-Vitro Model of Atherosclerosis. **M Marino**, C Del Bo, M Tucci, D Klimis-Zacas, P Riso, M Porrini Preprints 2020

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

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Received: 10 January 2020; Accepted: 25 February 2020; Published: 28 February 2020

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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 [1]. In particular, activated endothelial cells release numerous cell surface adhesion molecules such as vascular cell

- Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin and adhesion of monocytes to endothelial cells. C Del Bo, **M Marino**, P Riso, P Møller, M Porrini Chemico-biological interactions 300, 49-55 2019

Chemico-Biological Interactions 300 (2019) 49–55

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin and adhesion of monocytes to endothelial cells

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ARTICLE INFO

Keywords:
Anthocyanins
Metabolites
E-selectin
VCAM-1
Cell culture
Atherogenesis

ABSTRACT

This study investigated the capacity of an anthocyanin-rich fraction (ACN-RF) from blueberry, single anthocyanins (cyanidin, delphinidin and malvidin-3-glucoside; Cy, Dp and Mv-3-glc) and related metabolites (protocatechuic, gallic and syringic acid; PrA, GA and SA) to resolve an inflammation-driven adhesion of monocytes (THP-1) on endothelial cell (HUVECs) and secretion of cell adhesion molecules E-selectin and vascular cell adhesion molecule 1 (VCAM-1).

The adhesion of THP-1 to HUVECs was induced by tumour necrosis factor α (TNF-α, 100 ng mL^{-1}). Subsequently, ACN-RF, single ACNs and metabolites (from 0.01 to 10 μg mL^{-1}) were incubated for 24 h. The adhesion was measured in a fluorescence spectrophotometer. E-selectin and VCAM-1 were quantified by ELISA. No toxicological effects were observed for the compounds and the doses tested. ACN-RF and Mv-3-glc reducedTHP-1 adhesion at all the concentrations with the maximum effect at 10 μg/ml (−60.2% for ACNs and-33.9% for Mv-3-glc). Cy-3-glc decreased the adhesion by about 41.8% at 10 μg mL^{-1}, while PrA and GA reduced the adhesion of THP-1 to HUVECs both at 1 and at 10 μg mL^{-1} (−29.5% and −44.3% for PrA, respectively, and −18.0%and −59.3% for GA, respectively). At the same concentrations a significant reduction of E-selectin, but notVCAM-1 levels, was documented. No effect was observed following Dp-3-glc and SA supplementation. Overall, ACNs and metabolites seem to resolve, in a dose-dependent manner, the inflammation-driven adhesion of THP-1 to HUVECs by decreasing E-selectin concentrations. Interestingly, Mv-3-glc was active at physiologically relevant concentrations.

1. Introduction

Anthocyanins (ACNs) are a group of abundant and widely consumed flavonoids providing the red, blue, and violet colours in fruit- and vegetable-based food products. The dietary intake of ACNs is up to 9-fold higher than that of other dietary flavonoids. Epidemiological studies have found an inverse association between the consumption of ACNs and risk of cardiovascular diseases [1–6]. Their role in prevention of cardiovascular disease is strongly linked to the protection against oxidative stress and inflammation [7–10]. Atherosclerosis is the main underlying cause of cardiovascular disease in humans. The early stage, i.e. atherogenesis, is characterized by activation of endothelial cells to express cell adhesion molecules and recruit monocytes. This process is identical to the vascular responses to tissue inflammation, which resolves when the underlying cause of inflammation (e.g. an invading infectious agent) has been removed. However, the prolonged inflammatory milieu in early atherosclerotic foci stimulates the transformation of monocytes foam cell [11].

It has been shown that ACNs prevent endothelial cell dysfunction by modulating the expression and activity of several enzymes involved in nitric oxide production [12,13]. Furthermore, recent evidence suggests that ACNs can down-regulate the expression of adhesion molecules and prevent the adhesion of monocytes to endothelial cells challenged by pro-inflammatory cytokines [12,14]. The absorption of ACNs is low (< 1%), but most of them are rapidly transformed by human gut to metabolic products, reaching a plasmatic concentration much higher than that of parental ACNs, indicating their contribution in the biological activity observed should be considered [15]. We have reported

Abbreviations: ACN-RF, anthocyanin-rich fraction; Cy-3-glc, cyanidin-3-glucoside; Dp-3-glc, delphinidin-3-glucoside; GA, gallic acid; HUVEC, humbelical vein endothelial cells; Mv-3-glc, malvidin-3-glucoside; PrA, protocatechuic acid; SA, syringic acid; THP-1, human monocytic cells; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1

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https://doi.org/10.1016/j.cbi.2019.01.002
Received 31 July 2018; Received in revised form 12 December 2018; Accepted 2 January 2019
Available online 03 January 2019
0009-2797/

- Role of berries in vascular function: a systematic review of human intervention studies. D Martini,

M Marino, D Angelino, C Del Bo, D Del Rio, P Riso, M Porrini. Nutrition reviews 2019

Lead Article

Role of berries in vascular function: a systematic review of human intervention studies

Daniela Martini, Mirko Marino, Donato Angelino, Cristian Del Bo', Daniele Del Rio, Patrizia Riso, and Marisa Porrini

Context: *Berries are a source of polyphenols with recognized health-promoting activities. Several studies suggest that consumption of berries may improve vascular function.* ***Objective:*** *The aim of this systematic review is to provide evidence of short- and long-term benefits of berries on outcomes of vascular function.* ***Data Sources:*** *Human intervention studies were collected from PubMed and Scopus databases.* ***Study Selection:*** *Studies were eligible if they investigated the effects of acute or chronic berry consumption on one or more markers of vascular function in humans and provided a characterization of the berry polyphenolic content. Only randomized controlled trials were included, and studies were excluded if berries were combined with other foods.* ***Data Extraction:*** *After selection, 22 randomized controlled trials were included and analyzed, most of which were performed in healthy individuals or patients with cardiovascular risk factors.* ***Results:*** *The overall results seem to suggest a protective role of berries in vascular function, likely dependent on the time of exposure, the type and dose of berry, and the biomarkers analyzed. Flow-mediated dilation and reactive hyperemia index (markers of vascular reactivity) improved following short-term interventions, while pulse wave velocity and augmentation index (markers of arterial stiffness) improved only after medium- to long-term intervention.* ***Conclusions:*** *Current evidence suggests that berries, at physiological relevant doses, may have a role in the modulation of vascular function and stiffness. High-quality human intervention trials are encouraged in order to strengthen these findings and to better elucidate the mechanisms involved in such modulation.*

INTRODUCTION

Berries represent a wide group of blue, purple, or red small-sized and highly perishable fruits. Blueberry, cranberry, currant, raspberry, and blackberry are the most common varieties of berries consumed around the world[1]. Berries are an important source of phenols and polyphenols, including anthocyanins, proanthocyanidins, flavonols, flavones, flavan-3-ols, flavanones, isoflavones, stilbenes, lignans, and phenolic acids.[2,3]

Berry consumption has been associated with reduced all-cause mortality.[4] Moreover, in recent years, numerous epidemiological and clinical studies have documented the protective effects of berries against many noncommunicable chronic diseases, with some focusing on cardiovascular disease,[5–7] which remains

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Key words: berries, endothelial function, intervention studies, (poly)phenols, systematic review, vascular function.

doi: 10.1093/nutrit/nuz053

- Systematic review on polyphenol intake and health outcomes: is there sufficient evidence to define a health-promoting polyphenol-rich dietary pattern? C Del Bo, S Bernardi, **M Marino**, M Porrini, M Tucci, S Guglielmetti. Nutrients 11 (6), 1355 2019

nutrients

MDPI

Review

Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?

Cristian Del Bo' [1,†], Stefano Bernardi [1,†], Mirko Marino [1], Marisa Porrini [1], Massimiliano Tucci [1], Simone Guglielmetti [1], Antonio Cherubini [2], Barbara Carrieri [2,3], Benjamin Kirkup [4], Paul Kroon [4], Raul Zamora-Ros [5], Nicole Hidalgo Liberona [6,7], Cristina Andres-Lacueva [6,7] and Patrizia Riso [1,*]

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Received: 18 April 2019; Accepted: 3 June 2019; Published: 16 June 2019

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

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

- Overview of human intervention studies evaluating the impact of the mediterranean diet on markers of DNA damage. C Del Bo, **M Marino**, D Martini, M Tucci, S Ciappellano, P Riso, M Porrini Nutrients 11 (2), 391 2019

nutrients

MDPI

Review

Overview of Human Intervention Studies Evaluating the Impact of the Mediterranean Diet on Markers of DNA Damage

Cristian Del Bo' [1,*], Mirko Marino [1], Daniela Martini [2], Massimiliano Tucci [1], Salvatore Ciappellano [1], Patrizia Riso [1,†] and Marisa Porrini [1,†]

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Received: 29 December 2018; Accepted: 11 February 2019; Published: 13 February 2019

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Abstract: The Mediterranean diet (MD) is characterized by high consumption of fruits, vegetables, cereals, potatoes, poultry, beans, nuts, lean fish, dairy products, small quantities of red meat, moderate alcohol consumption, and olive oil. Most of these foods are rich sources of bioactive compounds which may play a role in the protection of oxidative stress including DNA damage. The present review provides a summary of the evidence deriving from human intervention studies aimed at evaluating the impact of Mediterranean diet on markers of DNA damage, DNA repair, and telomere length. The few results available show a general protective effect of MD alone, or in combination with bioactive-rich foods, on DNA damage. In particular, the studies reported a reduction in the levels of 8-hydroxy-2′–deoxyguanosine and a modulation of DNA repair gene expression and telomere length. In conclusion, despite the limited literature available, the results obtained seem to support the beneficial effects of MD dietary pattern in the protection against DNA damage susceptibility. However, further well-controlled interventions are desirable in order to confirm the results obtained and provide evidence-based conclusions.

Keywords: Mediterranean diet; DNA damage; DNA repair; telomere length; dietary intervention study

1. Introduction

Oxidative stress is a condition characterized by an imbalance between formation of reactive oxygen species (ROS) and antioxidant defense mechanisms. Overproduction of ROS can cause oxidative damage to lipids, proteins, and DNA [1]. The integrity and stability of DNA is essential to life and for the maintenance of normal cell functions. The most common types of stressors, apart from oxidative species, include chemical agents, ultraviolet/ionizing radiation, and xenobiotics that can contribute to DNA damage and to formation of base deamination, base alkylation, base dimerization, base oxidation, and single/double strand breakage [1]. The resulting DNA damage, if not properly repaired, can increase risk of mutagenesis and bring to the onset or the development of numerous degenerative diseases including cardiovascular diseases (CVDs), diabetes mellitus, Alzheimer's disease, and cancer [2–4]. Chronic oxidative stress has also been reported as a critical mechanism involved in telomere shortening [5]. Telomeres consist of long stretches of TTAGGG-DNA repeats associated with specific proteins and located at the end of chromosomes. They are involved in

- Polyphenols and intestinal permeability: rationale and future perspectives. S Bernardi, C Del Bo', **M Marino**, G Gargari, A Cherubini. Journal of agricultural and food chemistry 2019

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Perspective

Cite This: J. Agric. Food Chem. 2020, 68, 1816−1829

pubs.acs.org/JAFC

Polyphenols and Intestinal Permeability: Rationale and Future Perspectives

Stefano Bernardi,[†,‡] Cristian Del Bo',[†,‡] Mirko Marino,[‡] Giorgio Gargari,[‡] Antonio Cherubini,[§] Cristina Andrés-Lacueva,[∥,⊥] Nicole Hidalgo-Liberona,[∥,⊥] Gregorio Peron,[∥,⊥] Raúl González-Dominguez,[∥,⊥] Paul Kroon,[#] Benjamin Kirkup,[#] Marisa Porrini,[‡] Simone Guglielmetti,[‡] and Patrizia Riso*[,‡]

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

KEYWORDS: *polyphenols, intestinal permeability, in vitro studies, animal studies, human studies*

INTRODUCTION

Over the last 10 years, there has been significant research effort to investigate the central role of gut function and properties in the promotion of human health and/or the development of several pathological conditions.

The intestine is the main organ involved in the absorption of nutrients and water, and it is the largest area of contact with environmental factors. It contains a large number of specialized immune cells that can coordinate with defensive responses that prevent or counteract exposure of the host and its immune system to luminal antigens of different origins (e.g., microbial and dietary origin).[1]

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

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

While the absorption and/or transport of nutrients (i.e., sugars, amino acids, vitamins, fatty acids, and minerals) occur through specific transporters or membrane channels (transcellular path),[3] a complex system of junctions crucial for the transport between adjacent cells [i.e., tight junction (TJ), gap junction (GJ), adherens junction (AJ), and desmosomes] constitutes the paracellular path.[4]

TJs have a composite molecular structure consisting of multiple protein complexes (with more than 50 proteins identified) that include a series of transmembrane tetraspan proteins, named occludin, claudins, and tricellulin, able to develop fibrils crossing the membranes and creating a connection with adjacent cell proteins. In addition, single-span transmembrane proteins are included and are mostly

Special Issue: Food Bioactives and Health

Received: April 16, 2019
Revised: June 21, 2019
Accepted: June 24, 2019
Published: June 24, 2019

DOI: 10.1021/acs.jafc.9b02283
J. Agric. Food Chem. 2020, 68, 1816−1829

- Potassium bromate as positive assay control for the Fpg-modified comet assay. Peter Møller, Damian Muruzabal, Tamara Bakuradze, Elke Richling, Ezgi Eyluel Bankoglu, Helga Stopper, Sabine AS Langie, Amaya Azqueta, Annie Jensen, Francesca Scavone, Lisa Giovannelli, Maria Wojewódzka, Marcin Kruszewski, Vanessa Valdiglesias, Blanca Laffon, Carla Costa, Solange Costa, João Paulo Teixeira, **Mirko Marino**, Cristian Del Bo, Patrizia Riso, Sergey Shaposhnikov, Andrew Collins. Mutagenesis 2020

Mutagenesis, 2020, 35, 341–347
doi:10.1093/mutage/geaa011
Original Manuscript
Advance Access publication 22 April 2020

OXFORD

Original Manuscript

Potassium bromate as positive assay control for the Fpg-modified comet assay

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Received 22 January 2020; Editorial decision 24 March 2020; Accepted 27 March 2020.

Abstract

The comet assay is a popular assay in biomonitoring studies. DNA strand breaks (or unspecific DNA lesions) are measured using the standard comet assay. Oxidative stress-generated DNA lesions can be measured by employing DNA repair enzymes to recognise oxidatively damaged DNA. Unfortunately, there has been a tendency to fail to report results from assay controls (or maybe even not to employ assay controls). We believe this might have been due to uncertainty as to what really constitutes a positive control. It should go without saying that a biomonitoring study cannot have a positive control group as it is unethical to expose healthy humans to DNA damaging (and thus potentially carcinogenic) agents. However, it is possible to include assay controls in the analysis (here meant as a cryopreserved sample of cells i.e. included in each experiment as a

7.2 SUBMITTED PAPERS:

- A Mix of Chlorogenic and Caffeic Acid Counteracts Lipid Accumulation and Downregulates C/EBPß and PPAR-γ1 gene expression in Macrophages.
  Mirko Marino*, Cristian Del Bo'*, Massimiliano Tucci, Giacomo Mantegazza, Valentina Taverniti, Peter Møller, Patrizia Riso, Marisa Porrini. European Journal of Nutrition

- A polyphenol-rich dietary pattern improves intestinal permeability, evaluated as serum zonulin levels, in older subjects: the MaPLE randomized controlled trial. Cristian Del Bo, Stefano Bernardi, Antonio Cherubini, Marisa Porrini, Giorgio Gargari, Nicole Hidalgo-Liberona, Raùl Gonzàlez-Domìnguez, Raul Zamora-Ros, Gregori Peron, **Mirko Marino**, Letizia Gigliotti, Mark S Winterbone, Benjamin Kirkup, Paul A Kroon, Cristina Andres-Lacueva, Simone Guglielmetti, Patrizia Riso. Clinical Nutrition

- Impact of 12-month cryopreservation on endogenous DNA damage in whole blood and isolated mononuclear cells evaluated by comet assay
  Mirko Marino, Letizia Gigliotti, Peter Møller, Marisa Porrini, Patrizia Riso, Cristian Del Bo'. Scientific Reports

  Intervention Studies on Dietary Polyphenols over the last 20 Years: A Review of Registered Trials tracking Past Efforts and Possible Future Directions. **Mirko Marino**, Cristian Del Bo', Daniela Martini, Marisa Porrini, Patrizia Riso. Critical Reviews in Food Science and Nutrition

7.3 POSTER AND ORAL PRESANTIONS:

- Redox regulation of intestinal epithelial permeability: 3. E Cremonini, D Iglesias, **M Marino**, P Oteiza Free Radical Biology and Medicine 139 2019

- Anthocyanidins promotes beiging of white adipose tissue in mice fed a high fat diet: 150. C Rodriguez-Lanzi, E Cremonini, **M Marino**, D Iglesias, A Mastaloudis. Free Radical Biology and Medicine 139 2019

- High fat diet-induced obesity alters mitochondrial dynamics in mouse white adipose tissue: effects of anthocyanins: 41. E Cremonini*, **M Marino***, D Iglesias, C Fraga, P Oteiza Free Radical Biology and Medicine 139 2019

- Role of caffeic and chlorogenic acid in the modulation of cellular fatty acid uptake / **M. Marino**, M. Tucci, V. Taverniti, P. Riso, M. Porrini, D. Klimis-Zacas, C. Del Bo'. - In: PROCEEDINGS OF THE NUTRITION SOCIETY. - ISSN 0029-6651. - 79:OCE2(2020 Jun 10). ((Intervento presentato al 13. convegno European Nutrition Conference, FENS 2019: 15–18 October 2019, Malnutrition in an Obese World: European Perspectives tenutosi a Dublin (Ireland) nel 2019.

- A randomised controlled trial to assess the impact of a polyphenol-enriched diet on intestinal permeability in the elderly: The MaPLE study / S. Bernardi, S. Guglielmetti, C. Del Bo', **M. Marino**, G. Gargari, A. Cherubini, C. Andres-Lacueva, N. Hidalgo, P. Casas-Agustench, M. Winterbone, A. Narbad, P. Kroon, P. Riso. ((Intervento presentato al 8. convegno International Conference on Polyphenols and Health (ICPH) tenutosi a Quebec City nel 2017.

- Effect of caffeic and chlorogenic acid in the modulation of lipid accumulation in THP-1-derived macrophages / C. Del Bo', **M. Marino**, M. Tucci, P. Riso, M. Porrini. ((Intervento presentato al 3. convegno World Congress on Nutrition and Dietetics tenutosi a Prague nel 2019.

- Protective Effect of Gut Phenolic Metabolites Against Inflammation and Atherosclerosis: an in vitro Approach / C. Del Bo', **M. Marino**, L. Battisti, A. Moreletti, M. Porrini, S. Ciappellano. - In: ATHEROSCLEROSIS SUPPLEMENTS. - ISSN 1567-5688. - 32(2018 Jun 09), pp. 127-128. ((Intervento presentato al 18. convegno International Symposium on Atherosclerosis tenutosi a Toronto nel 2018.

- Ruolo di acido caffeico e clorogenico nella modulazione dell'accumulo dei lipidi in un modello di aterogenesi / **M. Marino**, M. Porrini, J. Tadini, M. Tucci, P. Riso, C. DEL BO' - In: Congresso nazionale SINU: book of abstracts[s.l] : SINU, 2018 Nov 20. - ISBN 9788897843313. - pp. 6-6 ((Intervento presentato al 39. convegno SINU tenutosi a Napoli nel 2018.

- Role of phenolic acids in the modulation of monocytes adhesion to endothelial cells and vascular adhesion molecules in a TNF-α stimulated pro-inflammatory environment / C. DEL BO', **M. Marino**, A. Moreletti, P. Riso, M. Porrini. ((Intervento presentato al 2. convegno International conference on food bioactives & health held tenutosi a Lisbon nel 2018.

- In vitro approach to evaluate the role of gut phenolic metabolites in the modulation of inflammation and atherosclerosis / C. DEL BO', **M. Marino**, P. Riso, K. Dorothy, M. Porrini. ((Intervento presentato al 5. convegno Foodomics Conference "Foodomics 2018 : From Data to Knowledge" tenutosi a Cesena nel 2018.

- Studio in vitro per valutare il ruolo di antociani e metaboliti nella modulazione del processo infiammatorio ed aterogenico / C. DEL BO', **M. Marino**, P. Riso, D. Klimis-Zacas, M. Porrini. ((Intervento presentato al 38. convegno Congresso Nazionale SINU tenutosi a Torino nel 2017.

7.4 PARTECIPATION TO TRAINING SCHOOLS:

"NutRedOx" in Molecular Nutrition at Universidade Lusófona, Lisbon, Portugal in the context of the NutRedOx COST action CA 16112

"Carotenoid bioavailability" at the University of Newcastle (UK) in the context of the EUROCAROTEN COST action CA 15136

"Functional and omics analysis of carotenoid interventions" at the University of the Balearic Islands in Palma de Mallorca (Spain) in the context of the EUROCAROTEN COST action CA 15136

7.5 SUPPLEMENTARY DIDACTIC:

Tutorship (ex art.45):

Course of "Nutrizione Applicata"

Professor Patrizia Riso

16 hours

7.6 TRAINING COURSES AND SEMINARS:

- Training school: “NutRedOx” in Molecular Nutrition at Universidade Lusófona, Lisbon, Portugal in the context of the NutRedOx COST action CA 16112

- Training school: “Carotenoid bioavailability” at the University of Newcastle (UK) in the context of the EUROCAROTEN COST action CA 15136

- Training school: “Functional and omics analysis of carotenoid interventions” at the University of the Balearic Islands in Palma de Mallorca (Spain) in the context of the EUROCAROTEN COST action CA 15136

- Training course: “UC Laboratory Safety Fundamentals Refresher”. 2019-02-24.

- Training course: “PPE: Personal Protective Equipment”. 2019-03-02.

- Training course: “Animal care and use”. 2019-03-31.

- Training course: “Mendeley per la gestione di bibliografie” 08-06-2018

- Training course: “Revisioni sistematiche con meta-analisi: metodi statistici ed interpretazione dei risultati” 22-05-2018”

- Training course: “PubMed” 10-05-2018

- Training course: “Citometria a flusso, cell sorting e applicazioni” 28-06-2018

- Seminar: “Diet for immunotherapy enhancement against tumors” 25-05-2018

- Seminar: “Research integrity” 15-06-2018

- Supplementary didactic: “Approcci esploratici all’analisi statistica di dati sanitari”. Prof. Carlo La Vecchia. October 8-9-10, 2018.

- Seminar: “Enhancement of antifungal activity of essential oils for food contact materials made from plants”. September 11, 2018. Aula biochimica, Via Celoria, 2.

- Seminar: "Ellagic acid, curcumin and epigallocatechin gallate in the control of intestinal inflammation". 2019-02-06.

- Seminar: "Differential effects of short-term ketogenic, high fat and fructose-enriched diets on metabolic parameters in mice". 2019-02-14.

- Seminar: "Epigallocatechin-3-gallate suppresses pancreatic cancer growth

through ROS-dependent and independent effects". 2019-02-21.

- Seminar: "The ERK signaling cascade in bile-induced Caco-2 monolayer permeabilization: prevention by (-)-Epicatechin and NADPH oxidase inhibitors". 2019-02-28.

- Seminar: "Effects of dietary (-)-Epicatechin on cognition and hippocampal neuroinflammation in high-fat diet-induced obese mice". 2019-03-08.

- Seminar: "An Introduction to Harmine and Pancreatic Cancer". 2019-03-14.

- Seminar: "Zinc deficiency decreases HO-1 mediated response to DA induced oxidative stress in IMR-32 neuroblastoma cells" 2019-03-21.

- Seminar: "Zinc and brain redox signaling". 2019-04-23.

- Seminar: "Exploring multiple combination strategies for pancreatic cancer". 2019-04-30.

- Seminar: "Effects of (-)-Epicatechin on high-fat diet (HFD)-induced hippocampal inflammation and impaired memory and learning in obese mice". 2019-05-07.

- Seminar: "Combined effects of gestational Phthalate Exposure and zinc deficiency on developmental neurogenesis". 2019-05-14.

- Seminar: "Impact of high-fat diet on pancreatic protein expression and its relationship to pancreatic cancer". 2019-05-20.

ACKNOWLEDGEMENTS