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**Role of Blueberry Polyphenols on Markers
of Oxidative Stress, Inflammation and
Hepatic Lipid Accumulation**

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ABSTRACT

Role of Blueberry Polyphenols on Markers of Oxidative Stress, Inflammation and Hepatic Lipid Accumulation

In the last decades, the epidemic of obesity, metabolic syndrome, chronic and degenerative diseases has becoming an emerging health problem in Western Countries with serious economic penalties on health care management.

As a consequence, there is a strong demand for preventive strategies that could be easily applied by the majority of the population. Lifestyle and dietary behavior are both major risk and protective factors in the development and progression of chronic diseases. Preventive approaches acting on dietary habits would be able not only to decrease the risk of developing degenerative diseases but also to reduce the complications associated with aberrant metabolic states or already established disorders. Dietary guidelines generally recommend the consumption of fruits and vegetable rich in bioactive compounds such as berries. Besides the recognized biological properties of bioactives and in particular polyphenols, little is known on their mechanisms of action and potential synergistic or antagonistic effects. This is partially due to the paucity of well-designed dietary intervention studies and to the lack of investigations on the effects of single compounds and their metabolites in appropriate models and conditions.

The objective of the PhD thesis was to study the role of blueberry bioactive compounds in the modulation of markers of oxidative stress, inflammation and hepatic lipid accumulation evaluated through an *in vivo* and an *in vitro* study approach.

The first part of the thesis was focused on the investigation of blueberry polyphenols ability to modulate several biomarkers related to oxidative stress in young smoker volunteers. We previously documented that a single portion of blueberry was able to counteract the impairment of endothelial dysfunction and the increase in blood pressure caused by acute cigarette smoke. On the same volunteers we analyzed, after blueberry intake, the variations in the levels of oxidative stress markers potentially involved in the endothelial dysfunction observed. We did demonstrate neither an increase in antioxidant defense nor a reduction of oxidative stress markers following blueberry consumption. Moreover, we did not document a significant worsening in DNA oxidative damage in peripheral blood mononuclear cells after smoking or an improvement in DNA damage protection after blueberry intake.

The second part of the thesis was performed in the laboratory of Internal Medicine and Metabolic Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and focused on the potential ability of different fractions extracted from a wild blueberry powder rich in ACNs and phenolic acids to counteract lipid accumulation in HepG2 human hepatocytes supplemented with fatty acids (OA, oleic acid and PA, palmitic acid). In particular, we tested ACN and phenolic fractions, as well as the single ACNs and their metabolites in doses achievable through dietary intake. We observed that both ACNs and phenolic fraction were able to reduce significantly lipid accumulation at the concentrations of 0.1 μM and 1 μM compared to OA+PA treatment ($p < 0.05$). Moreover, supplementation with single compounds showed different effects depending on the type of molecule considered suggesting a possible synergistic effect when a mix of compounds is present. No significant effect was observed for any of the metabolites.

In conclusion, through this PhD thesis, important results were added to the analysis and the comprehension of the molecular mechanisms and of the role of blueberry polyphenols in the modulation of markers of oxidative stress and in the prevention of lipid accumulation. The *in vivo* investigation failed to demonstrate that the consumption of a single blueberry portion could modulate markers of oxidative stress and antioxidant defence. The *in vitro* approach supported the potential effect in counteracting lipid accumulation. Further studies are needed to explore the role, the doses and the molecular targets of action of blueberry polyphenols introduced through the diet.

RIASSUNTO

Ruolo dei polifenoli del mirtillo sui marker di stress ossidativo, infiammazione e accumulo epatico di lipidi

Negli ultimi anni la crescente epidemia di obesità, sindrome metabolica e di malattie cronicodegenerative è diventata un problema emergente nei paesi occidentali con un grave impatto economico sul sistema sanitario. Di conseguenza vi è una forte richiesta di strategie preventive facilmente fruibili dalla popolazione. Lo stile di vita e il comportamento alimentare sono senza dubbio fattori coinvolti sia nello sviluppo sia nella progressione delle malattie croniche. Strategie preventive che agiscano sulle abitudini dietetiche potrebbero essere in grado di ridurre il rischio di sviluppare malattie degenerative e le complicanze ad esse associate. Le linee guida per una sana alimentazione raccomandano l'assunzione di frutta e verdura ad alto contenuto di composti bioattivi come ad esempio i frutti di bosco. Oltre alle conosciute proprietà biologiche di questi composti ed in particolare dei polifenoli, poco è noto sui meccanismi d'azione e il loro potenziale sinergico o agonistico. Ciò è in parte dovuto alla mancanza di studi di intervento e di studi sulle proprietà dei singoli composti e dei loro metaboliti valutati in modelli e condizioni sperimentali appropriate.

L'obiettivo della presente tesi di dottorato è stato quello di studiare il ruolo dei composti bioattivi del mirtillo nella modulazione di marker di stress ossidativo, infiammazione e accumulo epatico di lipidi attraverso approcci *in vitro* e *in vivo*.

La prima parte della tesi è stata focalizzata sullo studio della capacità dei polifenoli del mirtillo di modulare alcuni biomarker di stress ossidativo in giovani volontari fumatori. In una sperimentazione precedente era stato dimostrato, che l'assunzione di una porzione di mirtillo era in grado di contrastare il peggioramento della funzione endoteliale e l'aumento della pressione causati dal fumo di una sigaretta. Pertanto, è stato effettuato uno studio sullo stesso gruppo di soggetti per valutare la variazione dei livelli di alcuni marker di stress ossidativo. I risultati non hanno evidenziato né un aumento delle difese antiossidanti, né una riduzione dei marker di stress ossidativo. Inoltre, dopo il fumo non è emerso un aumento significativo del danno al DNA nelle cellule mononucleate separate da sangue periferico, né un miglioramento nella protezione del danno al DNA dopo l'assunzione di mirtillo.

La seconda parte, svolta nel laboratorio di Medicina interna e malattie metaboliche, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, ha avuto come obiettivo lo studio della capacità di frazioni estratte da un liofilizzato di mirtillo selvatico, ricco in antociani e composti fenolici, di ridurre l'accumulo di lipidi in cellule di epatocarcinoma HepG2 supplementate con acidi grassi (OA, acido oleico e PA, acido palmitico). In particolare, sono state testate le frazioni antocianiche e fenoliche, nonché i singoli antociani e corrispondenti metaboliti, in dosi raggiungibili attraverso la dieta. I risultati hanno evidenziato che entrambe le frazioni erano in grado di ridurre l'accumulo di lipidi nelle concentrazioni pari a 0.1 μM and 1 μM rispetto al trattamento con i soli acidi grassi. Inoltre, la supplementazione con i singoli composti mostrava effetti differenti e specifici in funzione del tipo di molecola utilizzata, suggerendo un potenziale effetto sinergico esercitato dai composti presenti nelle frazioni. Nessun effetto significativo è stato ottenuto con i metaboliti.

In conclusione, attraverso questa tesi di dottorato, è stato possibile aggiungere alcuni risultati utili per l'analisi e la comprensione del ruolo dei polifenoli del mirtillo nella modulazione di marker di stress ossidativo e nella prevenzione dell'accumulo epatico di lipidi.

Lo studio *in vivo* non ha permesso di dimostrare una modulazione dei marker di stress ossidativo e di difesa antiossidante a seguito del consumo di una porzione di mirtillo, mentre lo studio *in vitro* ha mostrato un effetto dei bioattivi testati nel contrastare l'accumulo di lipidi. Ulteriori studi saranno sviluppati per esplorare il ruolo, le dosi e i target molecolari di azione dei polifenoli del mirtillo introdotti attraverso la dieta.

ABBREVIATIONS

ABC, adenosine triphosphate-binding cassette;
ACAT, carnitine acylcarnitine translocase;
ACC, acetyl CoA carboxylase;
ACOX, acyl-CoA oxidase;
ACNs, anthocyanins;
AMPK, identified adenosine monophosphate protein kinase;
ApoB, apolipoprotein B;
AST, aspartate aminotransferase;
ALT, alanine aminotransferase;
AI, augmentation index;
AI@75, augmentation index standardized for heart rate of 75 bpm;
ANOVA, analysis of variance;
ALT, alanine aminotransferase;
Ara, arabinoside;
AST, aspartate aminotransferase;
BB, blueberry;
BMI, body mass index;
C3GE, cyanidin 3-glucoside equivalents;
CGA, chlorogenic acid;
ChREBP, carbohydrate response element binding protein;
CI, confidence interval;
COMTs, catechol-O-methyltransferases;
CPT1, carnitine O-palmitoyltransferase 1
CRP, C-reactive protein;
CVD, cardiovascular disease;
Cy, cyanidin;
Cy 3-O-glc, cyanidin 3-O-glucoside;
Cys-Gly, cysteine, cysteinylglycine;
dAix, digital augmentation index;
dAix@75, digital augmentation index normalized for the heart rate;
DiBP, diastolic blood pressure;
DGAT, diacylglycerol acyltransferase;
DMSO, dimethyl sulfoxide;
Dp, delphinidin;
ELOVL6, long chain fatty acid elongase 6;
eNOS, endothelial nitric oxide synthase;
ED, endothelial dysfunction;
EDTA, ethylenediaminetetraacetic acid;
ENDOIII, endonuclease III;
eNOS, endothelial NO synthase;
ELISA, enzyme-linked immunosorbance assays;
FABP, fatty acid binding protein;
FAS, fatty acid synthase;
FFA, free fatty acid;
FBS, fetal bovine serum;
FMD, flow mediated dilation;
FOXA2, forkhead box factor;

FPG, formamidopyrimidine DNA glycosylase;
FRHI, Framingham reactive hyperemia index;
GA, gallic acid;
GAE, gallic acid equivalent;
Gal, galactoside;
GC-MS, gas chromatography–mass spectrometry;
GGT, gamma-glutamyltransferase;
Glc, glucoside;
GPAT, glycerol-3-phosphate acyltransferase;
GSH, reduced glutathione;
GSH-Px, glutathione peroxidase;
GSSG, oxidized glutathione;
GST, glutathione S-transferase;
ED, endothelial dysfunction;
H₂O₂, hydrogen peroxide;
HCC, hepatocellular carcinoma;
HCY, homocysteine;
HDL-C, high density lipoprotein; cholesterol;
HepG2, human hepatocellular liver carcinoma cell line;
HMGCoA, 3- hydroxy-3-methylglutaryl-coenzyme A reductase;
HPLC, high performance liquid chromatography;
HPLC-EC, high performance liquid chromatography- electrochemical detection;
HR, heart rate;
HSL, hormone-sensitive lipase;
hs-CRP, high sensitive C-reactive protein;
HUVEC, human umbilical venous endothelial cells;
ICAM-1, intercellular cell adhesion molecule-1;
IL-6, interleukin-6;
IκB, inhibitor kappa B;
INSIG, insulin-induced gene;
IR, insulin resistance;
LCAD, long-chain acyl CoA dehydrogenase;
LC-DAD-MS/MS; liquid chromatography-diode array detector-mass spectrometry;
LDL-C, low density lipoprotein cholesterol;
LMP, low melting point;
L-PK, L-type pyruvate kinase;
LPS, lipopolysaccharide;
LSD, least significant difference;
MAPK, mitogen-activated protein kinase;
MATE-type multidrug and toxic compound extrusion;
MCAD, medium-chain acyl-CoA dehydrogenase;
MCP-1, monocyte chemoattractant protein-1;
MetS, metabolic syndrome;
mTOR, mammalian target of rapamycin;
MPT, microsomal triglyceride transfer protein;
MUFA, monounsaturated fatty acids;
Mv, malvidin;
NAFLD, nonalcoholic fatty liver disease;
NASH, non-alcoholic steatohepatitis;
NO, nitric oxide;

NF- κ B, nuclear factor kappa B;
NMP, normal melting point;
NRF2, Nuclear factor E2-related factor 2;
OA, oleic acid;
PA, palmitic acid;
PAT, peripheral arterial tone;
PBMC, peripheral blood mononuclear cell;
PBS, phosphate buffer saline;
Pg, pelargonidin;
PGC1, PPAR gamma co-activator 1;
Phe, phenolic acid;
PKA, protein kinase A;
Pn, peonidin;
PNPLA3, patatin-like phospholipase domain-containing 3;
PPAR, peroxisome proliferator-activated recaptor;
PrA, protochatecuic acid;
Pt, petunidin;
PUFA, polyunsaturated fatty acids;
Rham, rhamnosite;
RHI, reactive hyperemia index;
ROS, reactive oxygen species;
SA, syringic acid;
SCAP, nutrient sensors cleavage-activating protein;
SCD, stearoyl-CoA desaturase;
SD, standard deviation;
SDB-F, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate;
SEM, standard error of the mean;
SFAs, total fatty acid especially saturate;
SIK, salt inducible kinases;
SOD, superoxide dismutase;
SPE, solid phase extraction;
SREBP1, sterol regulatory element-binding protein-1;
sVCAM-1, soluble vascular adhesion molecule-1;
SULTs, sulfotransferases;
TCA, tricarboxylic acid;
TAG, tryglicerides;
T2M, type-2 diabetes mellitus
TFA, trifluoroacetic acid;
THP-1, Human acute monocytic leukemia cell line;
TM6SF2, transmembrane 6 Superfamily Member 2
TNF- α , tumor necrosis factor alpha;
TG, triglycerides;
TSC, total serum cholesterol;
UHPLC-MS/MS, ultra high pressure liquid chromatography-mass spectrometry;
UGT, uridine-5 ϕ -diphosphate glucuronosyltransferases;
VICAM-1, vascular cell adhesion molecule-1;
VEGF, vascular endothelial growth factor;
VLDL, very low density lipoprotein;
WB, wild blueberry;
Xyl, xylose;

8-oxo-dG, 8-oxo-2'-deoxyguanosine.

0. PREFACE

Dietary habits and lifestyle are both the major risk and protective factors in the development and progression of degenerative diseases (Mozaffarian et al., 2008; Ignarro et al., 2007; Sofi et al., 2005). Recently much attention has been given by researchers to the study of the potential preventing effect of fruit and vegetable intake in relation to chronic diseases (Hartley et al., 2013; Schwenke et al., 2013; Hung et al., 2004; Bazzano et al., 2003). A particular interest has been focused on the protective role of berries (i.e. blueberries, cranberries, strawberries) for their high content of polyphenols, in particular anthocyanins. The main reason for this interest is first of all the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their potential role in the prevention of various diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases (Manach et al., 2004). Furthermore, polyphenols, which constitute the active substances found also in many plants, modulate the activity of a wide range of enzymes and cell receptors. Within the class of polyphenols, ACNs (anthocyanins) are pigments dissolved in the vacuolar lymph of the epidermal tissues of flowers and fruit, to which they impart a red, blue, or purple color (Mazza et al., 1993). ACNs such as delphinidin, malvidin, petunidin, cyanidin and peonidin, are historically known for their antioxidant activities but they also had a favourable effect in the treatment of some types of neoplasms, lipid reduction, body weight regulation, improved glucose metabolism, blood pressure control, and reduction of chronic inflammation.

Based on these premises the aim of the present PhD thesis was the study of the role of polyphenol-rich foods, in particular blueberries, in the modulation of biomarkers of oxidative stress, inflammation and hepatic lipid accumulation through *in vitro* and *in vivo* approaches.

Thus, the research project can be subdivided into two different activities as described below.

1. Human dietary intervention study: effect of a single portion of blueberry in the modulation of markers of antioxidant defense system and inflammation in young smokers volunteers. To this aim analysis of antioxidant defence and oxidative level were performed on subjects.
2. In vitro study: role of blueberry bioactive compounds extracted from a wild blueberry (WB) freeze-dried powder in the modulation of hepatic lipid accumulation in HepG2 cells. ACN and Phe rich fraction were evaluated for their ability in counteracting lipid accumulation in an *in vitro* model of liver steatosis.

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1. STATE OF THE ART

1.1 Blueberry varieties and characteristics

Consumption of fruits and vegetables is generally recommended in dietary guidelines worldwide; moreover a specific attention is given to the intake of fruits like berries which are rich in nutrients and phytochemicals (Bazzano LA et al., 2003; Lichtenstein AH et al., 2006; Bradbury KE et al., 2014) that could be involved in the prevention of various diseases and disorders. Among soft fruits, berries such as blackberry (*Rubus species*), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium macrocarpon*), red raspberry (*Rubus idaeus*), and strawberry (*Fragaria ananassa*) are the most commercially cultivated in North America. These berries are most commonly consumed fresh, frozen or as processed and derived products, including dried and canned fruits, yogurts, beverages, jams and jellies (Seeram, 2006). In the last year, there has been a growing trend in using berry extracts as ingredients in functional foods and dietary supplements. These extracts may be combined with other colorful fruits, vegetables and herbal extracts. Extracts of fruit from various blackberry, raspberry and gooseberry cultivars have been often investigated for their action as inhibitors of free radicals (Nile SH & Park SW, 2014).

Blueberries of the genus *Vaccinium* are one of the few fruits native in North America (Ebadi, 2006). There are two major blueberry species in the US market: *V. corymbosum* (highbush or cultivated blueberries) and *V. angustifolium* (lowbush or wild blueberries). Highbush blueberries are cultivated in almost all of the North America, while lowbush blueberries are only produced commercially in the Eastern Canada and the Northeastern US. For centuries, blueberries maintained popularity in North America where more than 97% of the total production managed and harvested has been processed. In particular, lowbush “wild blueberry” grows wild, so that commercial blueberry fields are composed of many genetically and phenotypically different clones characterized by phenotypic variation in fruit and leaf color as well as plant height (Kalt W et al., 2001). Usually, lowbush blueberries are first frozen and then used mainly as an ingredient in processed foods. Wild blueberries are characterized by high levels of polyphenols, as well as significant levels of total fibre, fitosterol, vitamin B6, vitamin C, and vitamin K (Vendrame S et al., 2011). Blueberries are also a source of minerals since they contain calcium (150–350 mg per kg FW), potassium (0.5–3.2 g per kg FW) and manganese (12–39 mg per kg FW) (Del Bo’ et al., 2015). These minerals are important cofactors and components of antioxidant enzyme systems (e.g. superoxide dismutase, SOD; glutathione peroxidase, GSH-Px), contributing to antioxidant defense. Blueberry biological properties have been largely related to their high levels and wide diversity of phenolic-type phytochemicals. Both lipophilic (minor) and hydrophilic (major) phytochemicals are found in berries, but it is generally the latter class that has been largely implicated in the bioactivities of these fruits. However, the complementary, additive, and/or synergistic effects resulting from multiple phytochemicals activities found in berry fruits are believed to be responsible for their wide range of observed biological properties rather than these effects being due to a single constituent alone. The structural diversity of blueberry polyphenols could affect their degree of oxidation and the substitution patterns of hydroxylation, their ability to exist as stereoisomers, the glycosylation by sugar moieties and other substituents and the conjugation to form polymeric molecules such as tannins and other derived molecules (Seeram NP, 2014).

In the last years, consumption of blueberries has increased rapidly, largely driven by their putative health benefits, due to their high content of polyphenols, investigated through *in vitro* and *in vivo* studies (Szajdek A, Borowska EJ, 2008; Paredes-Lopez O et al., 2010).

1.2 Blueberries Polyphenols

1.2.1 Chemical structure and classification

Dietary phytochemicals differ widely in composition from various fruits, vegetables, and whole grains, and often have complementary mechanisms to one another. Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability (Li AN et al., 2014).

More than 10,000 different polyphenols have been identified in various plants. The main dietary sources of polyphenols are fruits and beverages. Therefore, it is suggested that in order to receive the greatest health benefits, one should consume a wide variety of plant-based foods daily (Rui HL, 2003). The typical characteristic structure shared by most polyphenols was built from a common intermediate or a close precursor. Firstly, they occurred in conjugated forms, with one or more sugar residues linked to hydroxyl groups, but direct linkages of the sugar to an aromatic carbon also exist. Linkage with other compounds, such as amines, carboxylic and organic acids, lipids and association with other phenols are also common (Li AN et al., 2014). Polyphenols could be divided into different groups by the number of phenol rings that they contain and the basis of structural elements that bind these rings.

Polyphenols are divided into two main categories, namely flavonoids and non-flavonoids, based on the number of phenol rings and the way in which these rings interact.

Flavonoids are polyphenolic compounds comprising 15 carbons with two aromatic rings connected by a three-carbon bridge. The main subclasses of these C₆–C₃–C₆ compounds are the flavones (eg. luteolin, apigenin), flavonols (e.g. quercetin and kaempferol), flavan-3-ols (eg. catechins), isoflavones (eg. genistein), flavanones (eg. hesperetin, naringenin), anthocyanidins (i.e. cyanidin, malvidin, pelargonidin, delphinidin, peonidin, petunidin) and proanthocyanidins (i.e. condensed tannins). Other flavonoid groups that are more minor dietary components are the chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones (Rodriguez-Ramiro I et al., 2015).

The majority of flavonoids occur naturally as glycosides rather than aglycones and the basic flavonoid skeleton can have numerous substituents.

Non-flavonoids may be sub-classified into Phenolic acids and stilbenes. Phenolic acid includes hydroxybenzoic acids (C₆–C₁) and hydroxycinnamic acids (C₆–C₃). Hydroxybenzoic acids (e.g. gallic acid) are found in pomegranate and raspberries. Hydroxycinnamic acids (e.g. caffeic acid) can be found in coffee beans and blueberries. Stilbenes have a C₆–C₂–C₆ structure. Resveratrol which is the main stilbene can be found as *cis* or *trans* isomers as well as conjugated derivatives in grapes and red wine (Rodriguez-Ramiro I et al., 2015).

1.2.2 Anthocyanins

Anthocyanins (ACNs) (of the Greek anthos = flower and kianos = blue) are the most important pigments of the vascular plants responsible for the blue, purple and red color of many plant tissues (Del Rio D et al., 2010). They are accumulated in berry skins and also in the flesh of some “teinturier” varieties, from veraison until full maturity, when synthesis stops. ACNs are especially abundant in berries (grape, cherry and plum included) including strawberry, blueberry, bilberry, blackcurrant, cowberry, chokeberry, cranberry and cowberry. Many of the health benefits associated with berry fruit may be due to their high concentrations of ACNs. Anthocyanidin glycosides are generally more stable than the corresponding aglycones, as glycosylation induces intramolecular H-binding within the anthocyanin molecule (Borkowski T et al., 2005). They have several important functions in plants, such as providing protection against harmful UV radiation or plant pigmentation. Anthocyanins are synthesized in the cytosol and delivered into the vacuole, where they are stored as colored coalescences called anthocyanic vacuolar inclusions. Vacuolar uptake may depend on mechanisms mediated by tonoplast transporters or be based on vesicular trafficking. Transporter-mediated uptake may rely on two mechanisms. MATE-type (multidrug and toxic compound extrusion) proteins are localized to the tonoplast and function as vacuolar H⁺-dependent transporters of acylated anthocyanins. ATP-binding cassette proteins are glutathione S conjugate pumps involved in the uptake of glycosylated flavonoids, independent of the presence of an H⁺ gradient (Chen Li et al., 2015). Glutathione S-transferases (GSTs) are thus expected to participate in vacuolar trafficking. They include a member of the GST gene family that has overlapping transcription patterns with anthocyanin accumulation (Gomez C et al., 2009). Although evidence exists for a number of transporters, whether anthocyanins enter the vacuole as single molecules and then aggregate, or whether cytoplasmic vesicles containing coalesced anthocyanins interact with the tonoplast, remains unknown. Type and concentration of anthocyanins differ widely among fruits and vegetables. High intakes of these bioactives can be achieved with regular consumption of berries since they could provide high amounts in a single serving. The average intake of anthocyanins varies by region, season, and among individuals with different social, cultural, and educational backgrounds. Daily consumption of total ACNs has been estimated to be between 3 and 215 mg/day, depending on food choices. Bioavailability of anthocyanin is low and less than 1% of the ingested amount can be absorbed reaching the plasma where the concentration ranges between 10 and 50 nmol L⁻¹ (Fang J, 2014a; Speciale A et al., 2014). However some literature evidence suggest that the apparent low bioavailability of anthocyanins is due to their extensive pre-systemic metabolism, rather than their poor absorption from the gastrointestinal lumen (Fang J, 2014b; Hribar U & Ulrih NP, 2014).

In nature, anthocyanins can be present as monoglycosides (e.g., galactoside, glucoside, and arabinosides) of six aglycones as cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv) that are ubiquitously distributed.

Each aglycone may be glycosylated and/or acylated by different sugars, phenolic acids, and aliphatic acids thus nearly 539 different anthocyanins have been reported from plants (Clifford MN, 2000; Kahkonen MP et al., 2001).

Structurally these compounds are based on the flavilium ion or 2-phenylbenzopyrilium composed of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen and also bonded by a carbon-carbon bond to a third aromatic ring [B]. Their differences are due to the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton, the identity, number and positions at which sugar are attached, the extent of sugar acylation and the identity of the acylating agent. The chemical structure of anthocyanidins varies significantly

depending on the extent of glycosylation and acylation. Commonly they have been found as glycosides of their respective aglycone with the sugar attached at the 3-position on the C-ring or the 5-7-position at the A-ring. Glucose (glc), galactose (gal), arabinose (arab), rhamnose (rham) and xylose (xyl) are the most common sugar substituent.

Anthocyanin structure (e.g., type of aglycone, number and type of glycosyl and acyl moieties) would influence form, distribution and stability, thus affecting their bioavailability, metabolism, or degradation mechanisms.

In solution anthocyanins are in equilibrium with four molecular forms: the flavylium cation, the quinoidal base, the hemiacetal base and chalcone. Primarily, anthocyanin exist as the stable flavylium cation only when $\text{pH} < 2$. The pH changes considerably in the human body from the stomach (1.5) to the intestine surface (5.3), urine (5.75), liver (7.0), and blood (7.40), and, therefore, anthocyanins will thus most probably under physiological conditions occur on different structural forms.

Before passage into the blood stream, aglycones undergo phase II metabolism to form sulfate, glucuronide, and/or methyl metabolites. Once in the portal bloodstream, metabolites rapidly reach the liver, where they can be subjected to further phase II metabolism. Enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion. Anthocyanins absorption could be affected as a consequence of structural changes in response to more-basic pH of the small intestine, the nature of sugar moiety and the acylation levels.

Moreover, functionality of food is usually evaluated on the levels of individual components and their biological activity even if other dietary components of food matrix may influence the absorption and the biological activity of bioactives (Bohn T, 2014). ACNs interact with macromolecules as polysaccharides, tannins, and proteins or with alcohol (Frank T et al., 2003), high-fat diet (Murkovic M et al., 2000) or carbohydrate-rich diet (Nielsen ILF et al., 2003). The final content of bioactives in food could be affected by industrial transforming processes, storage and conservation.

During the last decade, several excellent reviews have illustrated these natural dietary phytochemicals in terms of their absorption (McGhie TK & Walton MC, 2007), metabolism (He J & Giusti MM, 2010), bioavailability and pharmacokinetics (Kay CD, 2006; Welch CR et al., 2008). Dietary guidelines recommendation in increasing consumption of fruits like berries could be explained by health beneficial effects recently documented in many *in vivo* and *in vitro* studies.

1.2.3 Phenolic acids

Phenolic acid are essential for the growth and reproduction of plants and are produced as a response to plant injury by pathogens. Blueberries content depends on the degree of maturity, genetic diversity, preharvest climatic, postharvest storage conditions and processing.

Phenolic acids represent approximately one third of the total intake of plant polyphenols in the human diet (Chalas J et al., 2001). Daily consumption of phenolic acids has been estimated as 25 to 1000 mg (Clifford MN, 1999). They could be found in plant either as free or as bounded compounds linked through ester, ether, acetyl or other bonds. Phenolic acids can be sub-divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids, which are derived from non-phenolic molecules of benzoic and cinnamic acid, respectively. Chemically, these compounds have at least one aromatic ring in which hydrogen is substituted by a hydroxyl group (Rodriguez-Ramiro I et al., 2015). The effectiveness of hydroxycinnamic acids such as caffeic, chlorogenic, sinapic, ferulic, 3-hydroxycinnamic, and 4-hydroxycinnamic acids, as

DNA-cleaving agents in the presence of CuII ions, shows that compounds bearing o-hydroxy or 3,5-dimethoxy groups on phenolic rings were remarkably more effective at causing DNA damage than the compounds bearing no such groups (Roleira FM et al., 2015).

Between phenolic acids, chlorogenic acid (CGA) formed by esterification of caffeic and quinic acids, is one of the most abundant polyphenol in the human diet (Feng R et al., 2005). It has been reported that the level of chlorogenic acid varies between 34.3 and 113.8 mg/100 g in different varieties of highbush and lowbush blueberries (Rodriguez-Mateos A et al., 2012).

CGA is highly bioavailable in nature and data obtained from *in vivo* and *in vitro* experiments highlighted that CGA mostly displays antioxidant and anti-carcinogenic activities (Zang LY et al., 2003; Jin UH et al., 2005; Lee WY & Zhu BT, 2006) even if its anti-inflammatory property has not really been explored so far.

Phenolic acids when ingested in the free form are rapidly absorbed by the small intestine and later conjugated (Scalbert A & Williamson G, 2000). Nevertheless, the chemical structures of the compounds can also influence the conjugation reactions as well as the amount of metabolites formed by the gut microflora in the colon. In human tissues there are no esterase able to release caffeic acid from chlorogenic acid (Plumb et al., 1999). Therefore, the efficiency of absorption of phenolic acids is markedly reduced when they are present in the esterified form rather than in the free forms. Changes in the rate of absorption could also influence health effects displayed by these bioactives.

1.2.4 Bioavailability (absorption, metabolism and bioactivity)

The positive modulation of human health attributed to polyphenols has steadily emerged over the years through different *in vitro/ex vivo*, *in vivo* models and clinical trials. Considerable attention has been paid to the investigation of the absorption, metabolism and bioavailability of polyphenols in the human organism, as an essential step in understanding their biological activity. Bioavailability has been defined in various ways. Sometimes it is simply defined as the proportion of a nutrient or bioactive component that was absorbed from the GIT and it is characterized as plasma concentration (Mason P, 2000). However, the commonly accepted definition of bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Thus, it is not only important to know how much of a nutrient is present in a food or dietary supplement; but even more important to know how much of that present is bioavailable (Srinivasan VS, 2001).

Without knowing the rate and the extent of polyphenols absorption, metabolism and tissue or cell distribution, the comprehension of their role in diseases prevention will be difficult, in particular considering that during absorption and/or metabolism new forms may be generated circulating in blood or in tissues. Molecular studies would be helpful to elucidate physiological role of bioactives. The results would enable to evaluate both their effectiveness in the treatment, the prevention of certain diseases and possible unwanted risks coming from their administration.

Studies showed different rate of absorption, metabolism and bioavailability between ACNs and phenolic acids. Many of these investigations were focused on clarifying mainly ACNs behavior since it has consistently been observed that the type of sugar moiety and the acylation impact on the absorption of ACNs. In this regard, ACNs could be up taken through several mechanisms in three different sites along the gastrointestinal tract: the stomach, the small intestine and the colon.

At the low pH of the stomach, of about pH 1, ACNs occur in their most stable form, as the flavylium cation. Absorption might begin immediately when anthocyanins reach the stomach, and the stomach emptying time will contribute to the extent of the absorption by the mucosa. Gastric absorption seems to be influenced by the glycosidic moiety since rutinoides absorption was significantly lower in comparison to glycosides and galactosides (Matsumoto H et al., 2001) while arabinosides were absorbed better than the corresponding glycosides (Talavera S et al., 2003). Studies in rats (Tsuda T et al., 1999; Vanzo et al., 2008; He J et al., 2009) and human (Passamonti S et al., 2003) have shown that the highest plasma concentrations of anthocyanins could be reached within 10 min to 30 min after their oral intake and disappear from gastrointestinal tract within 4 hours after a meal. ACNs that are not absorbed in the stomach or small intestine may be transferred to the colon. ACNs have been detected both in plasma and urine as intact glycosides since compounds with different molecular size and types of sugar or acylated groups could be absorbed without modifications. Due to their high polarity, ACNs glycosides have been generally considered too hydrophilic for absorption by passive diffusion in the small intestine (Hollman PC, 2004). In order to be absorbed, they need either a specific transporter to transfer the glucoside across the small intestine barrier (Hollman PC et al., 1999); or they have to be hydrolyzed to the aglycone form by enzymes (eg. lactase phloridzin hydrolase, LPH) (Day AJ et al., 2000; Nemeth K et al., 2003) or by colonic bacteria prior to absorption (Rechner AR et al., 2002; Blaut M et al., 2003). The short time from the administration to the absorption by the gastric mucosa indicates an involvement of a transport system responsible for the ACNs rapid transfer. Researchers are still trying to elucidate the role and the functions of different carriers recently pinpointed out. At these regard, some studies have identified the organic anion carrier bilitranslocase as a carrier protein for ACNs absorption. This carrier is expressed not only in the gastric epithelium, but also in the basolateral domain of the liver plasma membrane and in the basolateral membrane of the renal proximal tubules (Elias MM et al., 1990). This transporter is competitively inhibited by most of the ACNs underlying the hypothesis of its possible involvement in their transfer. Zou e al. (2014) have tried to examine the absorption mechanism of Cy 3-O-G in the small intestine across the intestinal brush border, with respect to the role of the glucose carrier SGLT1 and GLUT2 using a Caco-2 cell model. The results showed that Cy 3-O-G could be transported through the Caco-2 cell monolayers in intact glycosidic forms, although the absorption efficiency was relatively low. Since anthocyanins could interfere with the transporters responsible for their own transport, the authors demonstrated that SGLT1 and GLUT2 are probably involved in the intestinal absorption of anthocyanins. The colon, in which ACNs could also be excreted with the bile, might play a very important role in absorption and metabolism of ACNs considering that here these bioactives could also be metabolized by gut microbiota in a range of compounds displaying beneficial health effects different from those of the parent compounds. The first step is the cleavage of the glycosidic bond of monoglycosylated and diglycosylated and even acylated ACNs that could form the less stable aglycones. Deglycosylation by the intestinal microflora is reported to have a fundamental role for the further degradation of aglycones (Fleschhut J et al., 2006).

Anthocyanidin glycosides have been shown to be hydrolyzed by the intestinal microbiota within 20 min–2 h from incubation depending on the sugar moiety (Keppler K & Humpf HU, 2005). After the cleavage of the sugar moiety by the microflora and the hydrolysis of the β -glycoside

bond, different products can be formed, depending on the substituents attached to the B ring of the parent anthocyanin molecule. The microbiota hydrolyzes glycosides into aglycones and metabolizes the aglycones into various aromatic acids which are finally metabolized to derivatives of benzoic acid. The microbial metabolites are absorbed and conjugated with glycine, glucuronic acid, or sulfate. It's noteworthy that despite different fate of individual ACNs in the lower GI tract depending on their structures, some metabolites could be used as markers of ACNs intake. In a study looking at the anthocyanins contents in fecal or cecal samples, the authors observed that losses in the intestinal contents were high for anthocyanin glucosides, moderate for galactosides, and negligible for arabinosides or xylosides (He J et al., 2005). Acylation or deglycosylation enhanced anthocyanin stability. Moreover, ACNs could affect the relative viability of colonic bacterial groups suggesting that these bioactive compounds could be able to modulate and reshape the gut microbial community and may be considered prebiotic compounds.

Absorption of ACNs could also be influenced by other dietary components or by the kind of food matrix. Alcohol, high-fat diet or carbohydrate-rich diets were regarded as possible factors impacting their absorption. When ACNs were given along with a high-fat diet, the maximum ACNs concentration in the plasma was reached 1–2 h later (Murkovic M et al., 2000). A delay was also detected in the peak plasma concentration when ACNs were ingested along with a carbohydrate-rich meal (rice cake) (Nielsen IL et al., 2003). This may indicate that either high-fat or a carbohydrate-rich diet may prolong or delay the transit of ACNs through the GI tract without necessarily affecting their total absorption.

Before passage into the blood stream, the aglycones undergo some degree of phase II metabolism forming sulfate, glucuronide, and/or methylated metabolites through the respective action of sulfotransferases (SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyltransferases (COMTs). There is also efflux of some of the metabolites back into the lumen of the small intestine, and this is thought to involve members of the adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein. MRP-3 and the glucose transporter GLUT2 have also been implicated in the efflux of metabolites from the basolateral membrane of the enterocytes (Manzano S & Williamson G, 2010). Once in the portal bloodstream, metabolites rapidly reach the liver, where they can be subjected to further phase II metabolism and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion (Rechner AR et al., 2002; Piazzon A et al., 2012). These changes in their structures may increase or decrease the bioactivity of the initial compounds.

The gradual release of anthocyanins and derivatives into the bloodstream might be of physiological importance, as this may mean that the biological activity can be exerted over a longer period of time above all under conditions of repeated exposure. These is in agreement with results by Riso et al. (2005), who showed an increment of about 7.8 nmol/L in the total anthocyanin concentration after 21-day intervention with blood-orange juice.

Therefore, detailed knowledge concerning the conjugative and metabolic events and resulting plasma levels following the ingestion of a polyphenol-rich diet is crucial for understanding their bioactivity (Rechner et al., 2002). Despite the large amount of data concerning the bioactivity of ACNs only few studies deal with the bioactive properties of their metabolites, especially as most of these molecules are not commercially available. However, the extensive metabolism that occurs in the liver and kidney changes the metabolic characteristics influencing their accessibility to other tissues.

After oral administration of berries or berry extracts, ACNs were found in the blood stream within minutes. The time required to reach C max ranged from 0.5 to 2 h for plasma, which is much shorter than all other sub-groups of flavonoids (Miyazawa T et al., 1999). In contrast to other flavonoids, the proportion of ACNs absorbed and excreted in the urine as a percentage of the intake is quite small, perhaps much less than 0.1% of intake (Manach C et al., 2002). Wu et

al. (2002) reported in elderly women an urinary recovery of blueberry (*Vaccinium corymbosum*) anthocyanins of 0.004% after intake. To some degree, these low recoveries could be a consequence of anthocyanins undergoing structural rearrangements in response to pH. Maximum plasma levels of total ACNs in the plasma are in the range of 1–100 nmol/L with doses of 0.7–10.9 mg/kg in human studies (Matsumoto H et al., 2001). The clearance of ACNs from the circulation is sufficiently rapid so that by 6 h very little amounts are detected in the plasma (Cao G et al., 2001). However, differences in absorption efficiency depend upon whether are measured the disappearance from the gastrointestinal tract, the appearance in the blood or the excretion in urine. Wu et al. (2006) observed that about 58% of a dose of ACNs disappeared from the gastrointestinal tract within 4 h after a meal as determined by recovery in the duodenum, ileum, cecum and colon despite the extent of disappearance varied considerable for different ACNs (e.g. 98% of the Cy-3-glc and 22% of the Cy-3- sambubioside). Talavera et al. (2003) found that after administration of a high concentration of blackberry ACNs, intact ACNs were observed in plasma from the gastric vein and aorta in few minutes demonstrating that anthocyanin glycosides were quickly and efficiently absorbed from the stomach and rapidly excreted into the bile as intact and metabolized forms. In a recent paper investigating ACNs intestinal absorption with an *in vitro* chamber model, the authors found that the highest absorption of ACNs occurred with jejunal tissue ($55.3 \pm 7.6\%$), minor absorption with duodenal tissue ($10.4 \pm 7.6\%$) and no absorption in tissues from the ileum or colon (Matuschek MC et al., 2006). One of the topics that have recently been mostly investigated is ACNs low bioavailability. This will continue to be difficult to achieve, since ACNs exist in a number of different molecular structures and there are a number of potential metabolites that can be generated both *in vitro* and in the GIT. One approach to help to address this bioavailability issue is the use of isotopically labeled anthocyanins to estimate bioavailability and subsequent transportation, accumulation into various tissue and excretion (Kay et al., 2006).

There are only few studies about absorption, metabolism and bioavailability of the conjugated derivatives of polyphenols, where phenolic acids are included, due to the lack of commercial standards. Glucuronidation and sulfatation conjugation reactions are described to have a significant impact on the bioactivity of phenolic acids. About 30% of chlorogenic acid (CGA) is absorbed in the small intestine, and in healthy subjects with an intact colon, 70% of the ingested chlorogenic acids pass from the small to the large intestine, where it is subjected to the action of the colonic microbiota. The rate of urinary excretion of metabolites was equivalent to 29.2% of intake (Barberan FT et al., 2014).

Many information about how ACNs and phenolic acids are absorbed, how their molecular structures have been modified and which is the contribution given by metabolites to the health benefits are still to be elucidated.

An increased understanding of these aspects will generate the potential for the development and promotion of an increasing in high anthocyanin-containing foods intake such as berry fruits.

1.2.5 Biological properties

The most important difference between food and drugs is that food should provide energy and maintain health, whereas drugs are developed to treat or to prevent diseases, recent studies were focused on the investigation of the food role in the prevention and/or treatment of chronic diseases. At these regard much attention was focused on blueberry polyphenols biological properties. These bioactives are known mainly for their ability to act as antioxidants but their benefits could be even greater since oxidative stress is involved in the etiology of a wide range of chronic diseases.

A large and growing body of evidence shows that berry phytochemicals could have other positive metabolic effects crucial for cardiovascular health such as the ability to promote vasodilatation, to have anti-atherogenic, antithrombotic, and anti-inflammatory effects. Moreover, they could regulate the activities of metabolizing enzymes, modulate nuclear receptors, gene expression, subcellular signaling pathways and repair of DNA oxidative damage (Seeram NP & Heber D, 2006; Seeram NP 2006).

Although the multi-mechanistic actions of berry phytochemicals have been well established through *in vitro* studies, it has only been in the past decade that animal and human studies have significantly deepened the knowledge on the bioavailability, metabolism, tissue distribution, and biological effects of these compounds *in vivo*. In these regards, no clear dose-effect relationship has yet been determined because the effects showed by evidence vary hugely among polyphenols and food sources (Ríos-Hoyo A et al., 2014). Despite their low bioavailability, blueberries polyphenols are extensively metabolized and, *in vivo*, could reach target tissues where they could contribute significantly to the biological effects that have been observed for berry fruits. Levels of berries polyphenols *in vivo* may also be underestimated considering that these compounds could be linked to other molecules. To deepen the knowledge of polyphenols health benefits, research should consider not only aspects related to nutrigenomics (effects of nutrients on the genome, proteome, and metabolome) and nutrigenetics (effects of genetic variation on the interaction between diet and disease) but also to metabolomics (study of chemical processes involving metabolites). Both polyphenols metabolites including glucuronidated, sulfated, and methylated derivatives and the products formed by colonic microbiota may significantly contribute to the health benefits resulting from berries consumption (Seeram NP, 2008).

The study of the numerous beneficial health effects displayed by polyphenols and related compounds has become an important area of human nutrition research.

There is increasing evidence highlighted from studies on human subjects, though still limited, on animal and on cell lines that modest long-term intakes of polyphenol rich foods can have favourable effects on the incidence of cancers and chronic diseases, including cardiovascular disease (CVD), type II diabetes, and impaired cognitive function, which are occurring with increasing frequency in Western population. Examples of their protective activities include reduced incidence and mortality rates of cancer (Doll R, 1990), reduced ischemic heart disease mortality (Armstrong BK et al., 1975), as well as antimutagenic, antimicrobial (Nishino C et al., 1987) and anti-inflammatory- allergic properties (Middleton E & Kandaswami C, 1992; Edenharder R et al., 1993).

These bioactive compounds could either, directly or indirectly, modulate the expression and the activity of cellular targets governing essential pathways shared by different cell types with a wide range of beneficial effects (Kang J et al., 2015). They are able to influence a variety of cell functions including modulation of cell signaling (Malavolta M et al., 2014); alter proliferation and cytotoxicity in cancer cell lines (Mocanu MM et al., 2015); protection of DNA integrity (Acharyya N. et al., 2014); alterations of immune and inflammatory responses; and modifications in cytokine production (Molina N et al., 2015).

Reactive oxygen species (ROS) may be generated from cellular processes and play different roles *in vivo*. The role of ROS could be positive if it is related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signaling and in the synthesis of biologically important compounds. Otherwise, ROS may be very damaging, since they can induce oxidation attacking lipids in cell membranes, proteins in tissues or enzymes and DNA. This oxidative damage is considered to play a causative role in aging and several degenerative diseases associated with it, such as heart disease, cataracts, cognitive dysfunction, and cancer. Humans have antioxidant systems to protect against free radicals. These systems include

some antioxidants produced in the body (endogenous) and others obtained from the diet (exogenous). The first include (a) enzymatic defenses, such as Se-glutathione peroxidase, catalase, and superoxide dismutase, which metabolize superoxide, hydrogen peroxide, and lipid peroxides, thus preventing most of the formation of the toxic HO, and (b) non-enzymatic defenses, such as glutathione, histidine-peptides, the iron-binding proteins transferrin and ferritin, dihydrolipoic acid, reduced CoQ10, melatonin, urate, and plasma protein thiols, with the last two accounting for the major contribution to the radical-trapping capacity of plasma. The various defenses are complementary to each other, since they act against different species at different cellular compartments. However, despite these defense antioxidants (able either to suppress free radical formation and chain initiation or to scavenge free radical and chain propagation), some ROS still escape and can cause damage. Thus, the body antioxidant system is provided also by repair antioxidants (able to repair damage, and based on proteases, lipases, transferases, and DNA repair enzymes). Mechanisms of antioxidant action can include (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen species; and (3) upregulating or protecting antioxidant defenses. Radicals scavenging activity depends on the structure and the substituents of the heterocyclic and B rings. The major determinants for radical-scavenging capability are (i) the presence of a catechol group in ring B, which has the better electron-donating properties and is a radical target, and (ii) a 2, 3- double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. Polyphenols are known mainly for their antioxidant properties acting as radical scavengers (Mileo AM et al., 2016).

Another mechanism of action described for polyphenols is related to the modulation of endothelial function. The endothelium is essential for maintaining vascular homeostasis. It regulates smooth muscle tone and blood pressure via the synthesis and release of regulatory mediators such as nitric oxide (NO) (Esper RJ, 2006).

Reduction in the bioavailability and/or bioactivity of endothelial-derived NO is thought to result in endothelial dysfunction (ED), a condition characterized by impaired endothelial dependent vasodilation (Esper RJ, 2006). ED contributes to the pathogenesis of atherosclerosis and hypertension, and is considered an independent risk factor for CVD (Bonetti PO et al., 2003). Previous studies have indicated that oxidative stress plays a prominent role in the development of ED, as potent physiological oxidants were shown to reduce NO bioavailability through the formation of ROS, in particular, superoxide (Stocker R et al., 2004). ROS can activate nuclear factor κ B (NF- κ B), a master transcription factor that induces the expression of proinflammatory cytokines, suggesting that oxidative stress can trigger inflammatory responses. The inhibition of NF- κ B is generally thought a useful strategy for treatment of inflammatory disorders. Cellular ROS levels are tightly regulated by endogenous antioxidant systems and exogenous antioxidants. Nuclear factor E2-related factor 2 (NRF2) is central to the endogenous antioxidant defense mechanism as it induces the expression of genes, such as NAD(P)H:quinone oxidoreductase 1, glutamyl cysteine ligase modulatory subunit and glutamyl cysteine ligase catalytic subunit. Alleviation of oxidative stress has been shown to improve ED. Risk factors associated with the metabolic syndrome and Type-2 diabetes mellitus (T2DM), including obesity, glucose intolerance and insulin resistance have been associated with both increased systemic oxidative stress and ED (Deedwania PC, 2003).

In this regard, NO is an essential component in maintaining vascular health, it is formed from L-arginine by the constitutive EC-dependent eNOS (endothelial nitric oxide synthase) in response to several physiological and pathological stimuli of ECs (endothelial cells). NO is a key intravascular and anti-thrombotic factor, but it provokes inflammatory response if converted to peroxynitrite, in the presence of free radicals. There are experiments suggesting that polyphen-

nols can inhibit NO release by suppressing NOS enzymes expression and NOS activity (Stocker et al., 2004; Paixao et al., 2012).

Polyphenols have been also widely studied for their ability to modulate cytokines, the major mediators of local and intercellular communications in immune and inflammatory processes. Inflammation is the normal protective response of the innate immune system to tissue injury or detrimental external stimuli such as pathogens, allergens and other irritants. During a single inflammatory event, a cascade of biochemical events propagates involving the local vascular system, the immune system, and various cells within the injured tissue (Galley HF, Webster NR, 1996). Ideally, the inflammatory response should be rapid in onset, effective (destructive and efficient in clean-up) and self-limiting. Inflammation that persists due to recurrent stimuli or inefficient regulation or resolution of the inflammatory response can cause chronic inflammation. Inflammatory responses are often characterized by the production of pro-inflammatory molecules and cytokines that provide signals between immune cells to coordinate the inflammatory response. As already said, a key player in the induction of inflammation is NF- κ B.

Rahman et al. (2006) suggested that dietary polyphenols could work as modifiers of signal transduction pathways to elicit their beneficial effects. NF- κ B required assistance from other sequence specific transcription factors among the mitogen-activated protein kinase (MAPK). Polyphenols have been demonstrated to modulate MAPK pathway by acting on several steps of the activation cascade and consequently on downstream effectors. These studies strongly proved the idea that polyphenols had the capacity to modulate the immune response and had a potential anti-inflammatory activity. Moreover, concordant results were reported that polyphenols, such as resveratrol and epigallocatechin-3-gallate, *in vivo* had a beneficial effect on energy metabolism in diet-induced obesity and insulin resistance (Stull et al., 2010). This property is relevant since insulin resistance, a hallmark of metabolic disorders, is a risk factor for diabetes and cardiovascular disease.

A critical step in both inflammation and atherosclerosis is the adhesion of circulating monocytes to vascular endothelial cells, which involves vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Studies *in vitro* suggest that flavonoids participate in the prevention and attenuation of inflammatory diseases by decreasing ICAM-1 and VCAM-1 levels (Lotito SB & Frei BD, 2006).

In addition, within polyphenols, both dietary ACNs and hydroxycinnamic acids have been reported to reduce TNF- α -induced up-regulation of various inflammatory mediators such as ICAM-1 or monocyte chemoattractant protein-1 (MCP-1). Therefore, the ability of polyphenols to mediate inflammatory processes is likely to contribute to their antiatherogenic properties. In particular, the effect of flavonoids on the arterial wall due to their estrogenic activity is well known. Flavonoids reduce the risk of cardiovascular diseases (Kris-Etherton PM et al., 2002), decrease serum cholesterol, low-density lipoproteins (LDL) and triglyceride levels (Ricketts ML et al., 2005). Moreover, flavonoids may decrease cardiovascular risk by reducing levels of angiotensin II, a well-known proinflammatory mediator (Naruszewicz M et al., 2007).

Cholorogenic acid and its colonic metabolites such as coumaric acid and dihydroferulic acid [3-(3 ϕ -methoxy-4 ϕ -hydroxyphenyl) propionic acid] showed high antioxidant activity (Josea AA et al., 2007). CGA has been found to act as an antioxidant *in vitro* by directly scavenging a number of ROS. However, despite these antioxidant properties, they were not able to protect human low-density lipoprotein (LDL) oxidation induced by copper and 2,2 ϕ -azobis(2-amidinopropane) dihydrochloride (Josea AA et al., 2007).

It has recently been reported that CGA supplementation may reduce fat accumulation in numerous tissues via inhibitory actions on enzyme regulators involved in lipogenesis (Takatoshi M et al, 2011). Platelet activation and subsequent aggregation play the major role in the pathogenesis of thromboembolic diseases such as myocardial infarction and ischemic heart disease (Hirsh J., 1987). Hyperactive platelets with a high predisposition for activation are known to be apparent

in conditions such as diabetes and heart disease (Hirsh J., 1987). Dihydroferulic acid and 3-(3 ϕ -hydroxyphenyl) propionic acid, chlorogenic acid, and anthocyanin colonic catabolites partly reversed hyper-reactivity of platelets induced by oxidative stress, and could also counteract the negative effects of hormonal stress induced platelet hyper-reactivity (Rechner AR & Kroner C, 2005).

A number of epidemiological studies have investigated the relationship between the intake of fruits and vegetables and cancer incidence. Although various *in vitro* studies conducted using several cancer cell lines originating from the breast, colon, stomach, and prostate have firmly established the anticarcinogenic, antiproliferative, antitumor, and cytotoxic effects of polyphenols (Olsson M et al., 2004; Boivin D et al., 2007; McDougall GJ et al., 2008) very few clinical trials have been conducted regarding the role of polyphenols in cancer prevention, incidence, or mortality. Clinical studies have focused on how polyphenol interventions influence DNA oxidation/damage, which has been correlated with cancer risk (Bjelland S & Seeberg E, 2003). For example, a variety of polyphenol-rich foods have been found to protect against lymphocyte DNA damage, including anthocyanin-rich juice (700 ml of a red mixed berry juice/day; 4 weeks) (Weisel T et al., 2006), blood orange juice (300 ml/day, 3 weeks) (Riso P et al., 2005), purple potato (150 g/day, 6 weeks) (Kaspar KL et al., 2011), and kiwi fruit (500 ml juice, 1 day) (Collins BH et al, 2011). Other trials have reported a decrease in urinary levels of 8-hydroxydeoxyguanosine, which is a biomarker of oxidative DNA damage, following the regular consumption of green tea polyphenols [4 cups/day, 146mg total polyphenols/cup, 4 months; 500/1000mg (poly)phenols/day, 3 months] (Hakim IA et al., 2003; Luo H et al., 2006). The variability of results highlighted from different studies could be due to differences in the intervention, its duration, the health status of the study population, and differences in methodology (Joshipura KJ et al., 2001; Moller P et al., 2003; Thompson HJ et al., 2005; Briviba K et al., 2008; Stoupi S et al., 2012).

A combination of preclinical and epidemiological studies suggests that polyphenols may be effective in reversing neurodegenerative pathology and age-related declines in neurocognitive performance, although at present, a direct association between polyphenols consumption and improvement in neurological health has not been made. The potential of polyphenols to improve neurological health appears to be related to a number of mechanisms, including their ability to interact with intracellular neuronal and glial signaling, to influence the peripheral and cerebrovascular blood flow, and to reduce neuronal damage and losses induced by neurotoxins and neuroinflammation (Small SA et al., 2004; Willis LM et al., 2009; Spencer JP, 2010).

In this context, it is clear that polyphenols, *in vivo*, do not act simply as antioxidants and their numerous beneficial effects are based on more complex and specific actions. Along with medium long-term RCTs, new and more refined molecular approaches will be critical to understand how these molecules interact with human physiological and pathological processes.

Between the class of polyphenols, ACNs have been reported to act as strong antioxidants, inhibit the growth of cancerous cells and inflammation, they could act as vasoprotectors, and have anti-obesity effects (Del Rio D et al., 2013). The majority of evidence, supporting a therapeutic effect of ACNs, emerged from *in vitro* or mechanistic studies since there is still a lack of *in vivo* evidence or human intervention studies (McGhie TK & Walton MC, 2007). Unfortunately studies performed on subjects have shown conflicting results. Chronic intakes (6–8 weeks) of mixed berries, anthocyanin rich tea, and chokeberry and blueberry extracts were able to decrease blood pressure (BP) in hypertensive individuals, myocardial infarction survivors and subjects with markers of the metabolic syndrome (Erlund I et al., 2008,; Basu A et al., 2010; McKay DL et

al., 2010). On the contrary, in other studies chronic intake of anthocyanin-rich foods, such as blueberry and cranberry juice had no modulating effect on BP in healthy individuals, chronic smokers, and people with dyslipidemia, obese subjects, stage 1 hypertensive individuals, and CAD patients (Stull AJ et al., 2010; Dohadwala MM et al., 2011; Ottaviani JL et al., 2011; Hassellund SS et al., 2012). In animal models of hypertension the BP-lowering effect of berry extracts after long-term consumption was confirmed (Elks CM et al., 2011; Wiseman W et al., 2011). Contrasting results on the effect in lowering BP could be due to the different polyphenols profiles of different types of berries (Borges G et al., 2010).

ACNs have different chemical structures and could target several transcription factors involved in different molecular pathways. The beneficial effects observed and documented are not only being due to single ACNs but also to their metabolites. Both of them could play a role in decreasing vascular inflammatory markers, such as cytokines and adhesion and chemoattractant molecules.

Delphinidin suppresses monocyte-EC cell adhesion induced by oxidized LDLs in ECs via the ROS/p38MAPK/NF- κ B pathway (Chen CY et al., 2011). Both delphinidin and cyanidin inhibit platelet-derived growth factor (PDGF) (AB)-enhanced vascular endothelial growth factor (VEGF) production in vascular smooth muscle cells (SMCs) by inhibiting the activation of p38 MAPK and JNK (Oak MH et al., 2006). Additionally, cyanidin-3-*O*-glucoside prevents hypercholesterolemia-mediated EC dysfunction in apolipoprotein E-deficient mice by inhibiting cholesterol and 7-oxysterol accumulation in the aorta and reducing superoxide production (Wang Y et al., 2012). Furthermore, cyanidin-3-*O*-glucoside protected against tumor necrosis factor (TNF α)-induced EC dysfunction by inhibiting ROS production and NF- κ B activation (Speciale A et al., 2010). In vascular SMCs, cyanidin-3-*O*-glucoside inhibited TNF- α -enhanced cell proliferation by inhibiting nitric oxide and oxygen binding (Nox) activator 1 activity (Luo X et al., 2012). In ECs, malvidin-3-glucoside prevented up regulation of EC NOS, inhibition of iNOS and cyclooxygenase-2 expression and inhibited peroxynitrite-enhanced NF- κ B activation (Paixao J et al., 2012).

NF- κ B plays a critical role in the induction of proinflammatory gene expression in response to lipopolysaccharide (LPS). In resting macrophages, NF- κ B is located in the cytosol bounded its inhibitor I κ B α . When macrophages are stimulated by inflammatory agents, I κ B α is phosphorylated and degraded; consequently NF- κ B could enter the nucleus for the induction of proinflammatory gene expression. ACNs markedly attenuated nuclear translocation of NF- κ B subunits p65, indicating that their anti-inflammatory effects is displayed through the inhibition of NF- κ B translocation to the nucleus (Miguel MG, 2011).

Obesity is well recognized as a chronic state of inflammation. Adipose tissue is a dynamic endocrine tissue that produces a host of adipokines with well described effects on metabolism as well as the immune system. Adipose tissue produces and secretes cytokines and chemokines such as leptin, adiponectin and MCP-1, all with metabolic-/immune- modulating functions (Ouchi N et al., 2011). In obesity, the secretion profile of these proteins is altered resulting in elevated pro-inflammatory and reduced anti-inflammatory proteins. Obesity predisposes to glucose intolerance and insulin resistance as excess free fatty acids inhibit glucose uptake in liver and muscle tissues, thereby resulting in elevated glycaemia and insulin levels (Li SY et al., 2009). Intake of a high energy, high fat and high carbohydrate diet/meal increases acute inflammatory stress in both healthy weight and overweight individuals (Calder PC et al., 2011). The prevalence of metabolic syndrome (MetS), CVD, diabetes and Alzheimer's disease is reduced by increased fruit and vegetable intake (Martínez-González MA & Sánchez-Villegas A, 2004; Dauchet L et al., 2009; Fung TT et al., 2009). Recent evidence suggests that berry consumption may improve insulin sensitivity (Stull AJ et al., 2010). Increased level of triglyceride (hypertriglyceridemia) is a major feature of the insulin resistance syndrome. As obesity is strongly associated

with insulin resistance, a reduction in this resistance is important in preventing the development of type-2 diabetes. A high fat diet generally induces hyperglycemia, hyperinsulinemia and hyperleptinemia. Several studies have been performed to evaluate protective properties of polyphenols and ACNs in cell culture and experimental diabetic models (Ghosh D, Konishi T, 2007). Results should be carefully interpreted since these studies involved a wide variety of experimental models of diabetes, duration and type of the treatment and markers of oxidative stress evaluated. Although several *in vitro* and animal studies strongly pointed out ACNs beneficial effects in cardiovascular complications in diabetes, clinical evidence for the supplementation with ACNs and anthocyanin-rich extracts in diabetes is not convincing.

In the last years researchers have deeply improve the knowledge on health beneficial effects of polyphenols and ACNs, but more investigation are warranted in order to provide specific dietary recommendations for berries intake, including optimal dose, delivery mode, timing and frequency of intake in different population sub-groups.

Moreover, it is necessary to identify and study the mechanisms of action and the specific roles not only of polyphenols or ACNs extracts but also of isolated and purified single bioactives components to determine their interaction and to establish their effective concentrations.

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2. AIM OF THE PhD THESIS

The increasing diffusion of chronic and degenerative diseases above all in Western countries is becoming a relevant issue in view of the serious economic penalties related to health care management. In this regard, it is necessary and warranted to investigate possible preventive strategies able to counteract the establishment of some kind of disorders and related pathologies that can be easily applied by the majority of the population. Diet is one of the most important lifestyle factors that could be considered as a target of action in reducing the complications associated with aberrant metabolic states or already established disorders. Intake of fruit and vegetable has been associated to the prevention of chronic diseases due to the presence of phytochemicals. However, little is known on the mechanisms of action and potential synergistic or antagonistic effects of these bioactive compounds. Despite the increase in the results obtained on biological activities of several compounds within the polyphenol class, much remains to be elucidated also through the exploitation of well-designed dietary intervention studies, able to demonstrate their impact *in vivo*. Additionally, few studies investigated the contribution given by single compounds and derived metabolites to the beneficial effects observed.

The objective of the present PhD thesis deals with the evaluation of the role of blueberry bioactive compounds in the modulation of makers of oxidative stress, inflammation and hepatic lipid accumulation evaluated through an *in vivo* and an *in vitro* study approach. Thus, the research project could be divided in two main tasks.

The first task, performed in young smoker volunteers, investigated the effect of a single portion of blueberry intake on markers of antioxidant defense, oxidative stress and nutritional markers. The second task, performed in an *in vitro* model of liver steatosis, investigated the ability of bioactive rich fractions to decrease lipid accumulation.

**3. HUMAN DIETARY INTERVENTION STUDY:
EFFECT OF A SINGLE PORTION OF BLUEBERRY
IN THE MODULATION OF MARKERS OF
ANTIOXIDANT DEFENSE SYSTEM AND
INFLAMMATION IN YOUNG SMOKERS
VOLUNTEERS**

3.1 AIM OF THE STUDY

Cigarette smoking is a source of reactive and toxic substances that are able to promote oxidative stress altering structure and function of DNA, lipids, proteins and carbohydrates. Smoking could also affect the immune system, increasing the levels of pro-inflammatory cytokines, and could cause endothelial dysfunction. Smokers have been found to show, not only, high levels of radicals and inflammatory markers but also low circulating plasma levels of micronutrients and antioxidants. Bioactives present in fruits and vegetables could improve nutritional status and antioxidant protection. in this target group.

In a previous study performed in our laboratory, a human model and a standard protocol were “set up” for studying the effect of smoking on peripheral arterial function. Blueberries are a rich source of bioactive compounds largely investigated for their antioxidant and anti-inflammatory properties. We recently reported that the consumption of a single portion of blueberry counteracted the impairment of endothelial function and the increase in blood pressure, induced by acute cigarette smoke, in young healthy volunteers. To understand the possible mechanisms involved in the protection against endothelial dysfunction we performed a new trial on the same group of subjects. In particular, we analyzed the variations of those biomarkers related to oxidative stress, expected to be negatively modulated by smoking and hypothesized involved in the endothelial dysfunction previously observed in order to identify the potential effect of blueberries on such markers.

3.2 MATERIAL AND METHODS

3.2.1 Subject recruitment

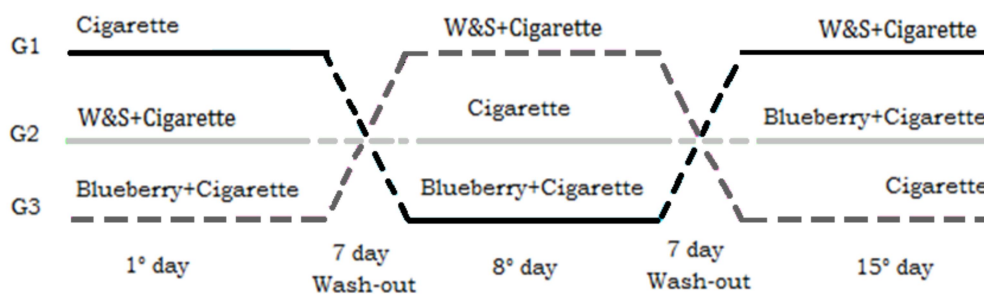
Sixteen healthy male smokers (23.6 ± 2.9 average of age and BMI of 23.0 ± 1.9 kg/m²) were enrolled, from the student population of the University of Milan, to participate in a trial evaluating the effect of fresh-frozen blueberries on peripheral arterial function and arterial stiffness. Volunteers were selected according to smoking habits (about 15 cigarette/day), physical activity (at least 25-30 min per day of brisk walk or jog), alcohol consumption (not more than 10-14 drinks of wine or beer per week) and dietary habits (homogeneous consumption of fruits and vegetables). This was obtained by means of a food frequency questionnaire previously published (Porrini et al., 1995) and revised focusing on berry consumption. Subjects were excluded from the study for the following reasons: hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure > 90 mm Hg), fasting glycaemia (< 5.5 mmol L⁻¹), hypertriglyceridemia (TG ≥ 1.69 mmol L⁻¹) and hypercholesterolemia (total serum cholesterol (TSC) ≥ 5.17 mmol L⁻¹, low HDL cholesterol (HDL-C) < 1.03 mmol L⁻¹, high LDL cholesterol (LDL-C) ≥ 3.36 mmol L⁻¹), endothelial dysfunction (RHI < 1.67) and overweight (BMI ≥ 25 kg/m²). Other exclusion criteria were: history of cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases, traumas of the arms or hand, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations and chronic backache. Subjects were excluded if they were taking any supplement or medications for at least one month before the beginning of the study. Fourteen out of 16 subjects previously enrolled gave their consent to participate in the second step of the trial and to collect blood in order to perform further analysis.

The study was performed in accordance with the ethical standards established in the 2013 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. Moreover, this study was registered at www.isrctn.org as ISRCTN59129089. All participants signed an informed consent form.

3.2.2 Experimental design

Volunteers were selected for a repeated measures crossover study () and randomly assigned to 3 different groups: 1-smoking (S); 2-control treatment (300 mL of water with sugars+ smoking (CS)); 3-blueberry treatment (300 g of blueberry + smoking (BS)).

Fig.9 Randomized cross-over experimental design



G, group; W&S, water+sugar

Each protocol was separated by 7 days of wash-out period. Since sugar seems to affect endothelial function (Ceriello et al., 2008), a drink formulated with water and sugars has been chosen to mimic the glycaemic response that is produced after blueberry consumption. Volunteers followed a polyphenol-free diet 10 days before experimentation. Subjects were deprived of foods with potential vasoactive properties such as chocolate, berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine, red to blue fruits, and green tea. Volunteers abstained also from drinking caffeine-rich beverages and limited coffee to three per day. The day before the experiment and during the trial, breakfast, lunch and dinner were standardized. Breakfast consisted of milk and biscuits (i.e. shortbread) while lunch was composed of two sandwiches (one with cooked ham and cheese and one with raw ham). During dinner, subjects could eat pasta or rice with butter and cheese, and a steak with potatoes and two slices of white bread. The dinner was consumed by 9.00 pm. Only one coffee was allowed at the end of the dinner. No alcoholic drinks or soft drinks were permitted. Meals were standardized to provide adequate energy/macronutrients intake, limiting polyphenols and taking into account Italian dietary habits. All the participants refrained from physical activity from the day before the experiment and did not change their smoking habits (15 cigarette/day; smoking last cigarette at 11.00 pm).

The day of the experiment, fasted overnight subjects came to the facilities of the Division of Human Nutrition and consumed a light breakfast (providing about 200 kcal) consisting of milk and biscuits (i.e. shortbread). Subjects were allocated into three groups for a repeated measures 3-armed randomized-controlled study: S- Smoking treatment; BS- Blueberry treatment (300 g of blueberry) + Smoking; CS- Control treatment (300 mL of water with sugar) + Smoking. Blueberries and control drink were consumed 90 min after breakfast to avoid possible interference between milk proteins and absorption of polyphenols. Each subject received all the three treatments separated by 7 days of wash-out period. The cigarette, containing approximately 6 mg of Tar by volume, 0.5 mg of nicotine and 0.9 mg of carbon monoxide, was smoked 100 min after blueberry or control consumption. This protocol was selected by considering previous observations on the detrimental effect of smoking on peripheral arterial function (20 min after smoking) and the beneficial effect observed on endothelial function at 120 min from the intake of blueberries. We hypothesized that the beneficial effects on endothelial function could be related to the kinetics of absorption of polyphenol compounds that occurred up to 120 min from the blueberries consumption. Blood was collected at baseline (before blueberries intake) and 20 min after smoking (120 min after blueberry/control treatment). Additional blood samples were collected at 60, 90, 120 min after smoking (respectively 180, 210, 240 min after blueberry/control treatment) and after 24 h from the intervention.

3.2.3 Preparation of blueberry and control treatment

A single batch of fresh blueberries (*Vaccinium corymbosum* L. "Brigitta") were purchased, processed by Individually Quick Freezing technique (Thermolab, Codogno, Italy), and stored at -20°C until use. On the study day, 300 g of frozen blueberries (previously thawed at + 4°C overnight) was consumed by the volunteers. The control treatment was prepared by suspending the same amount of sugars provided by blueberry in 300 mL of water. No bioactive compounds were added to the control.

3.2.4 Variables under study

The improvement of reactive hyperemia index (RHI), measured by a non-invasive plethysmographic method, it was considered as the primary endpoint. The other variables under study were: markers of antioxidant defense [aminothiol redox state such as glutathione (GSH), cysteine, cysteinylglycine (Cys-Gly), homocysteine (Hcy) in their reduced and oxidized forms], nutritional markers (vitamin C, folate, and vitamin B12), marker of oxidative stress (endogenous and oxidatively-induced DNA damage), urea, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT). Moreover, screening for triglycerides, total cholesterol, LDL and HDL-cholesterol, glucose and C-reactive protein was performed at the recruitment stage.

3.2.5 Separation of plasma, serum and peripheral blood mononuclear cells

Blood samples were collected into vacutainers containing heparin or K-EDTA as anticoagulant for plasma, or silicon for serum. Plasma was separated within 30 min after collection while serum within 1 h by centrifugation at $1000 \times g$ for 15 min at 4°C . Samples were aliquoted and stored at -80°C until analysis. Peripheral blood mononuclear cells (PMBCs) were obtained from 100 μL of whole blood gently mixed in micro tubes with 900 μL cold RPMI-1640 medium. Then, 100 μL Histopaque-1077 was carefully added to the bottom of the tube and centrifuged at $200 \times g$ for 4 min at room temperature. Cells were collected and washed in 1 mL PBS solution. The samples were then centrifuged for 10 sec at $5000 \times g$ at room temperature to pellet the cells. Pellets were resuspended in PBS and immediately used for the analysis.

3.2.6 Evaluation of the biochemical parameters

A general laboratory biochemical assessment was performed in serum including evaluation of hepatic function (AST, ALT and GGT), lipid profile [triglycerides (TAG), total serum cholesterol (TSC), and HDL-cholesterol] and glucose [20]. All these parameters were determined using standard laboratory methods. LDL cholesterol was calculated using the Friedewald's method ($\text{LDL} = \text{total cholesterol} - (\text{HDL} + 1/5 \text{ TG})$).

3.2.7 Evaluation of aminothiols, urea and urate in plasma and serum

Plasma reduced and total aminothiols (Cys, Cys-Gly, Hcy, GSH and GSSG) were determined as described below.

Plasma reduced aminothiols were determined by prompt acidification with 10% trichloroacetic acid (1:1, v/v), protein precipitation, and sample derivatization with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), a specific derivatization reagent for -SH groups. Plasma total aminothiols were instead measured after a reducing step with tri-n-butylphosphine, followed by sample derivatization with the same agent described above. Thiol concentrations were determined by isocratic high-performance liquid chromatography (HPLC; Varian, Surrey, UK) on a Discovery C18 column ($250 \times 4.6 \text{ mm I.D}$, Supelco, Sigma-Aldrich) and eluted with a solution of 0.1 mol/L potassium dihydrogenphosphate-acetonitrile (92:8, v/v), pH 2.1, at a flow rate of 1 mL/min, as previously described (21, 22). Fluorescence intensities were measured with excitation λ at 385 nm and emission λ at 515 nm, using a JASCO fluorescence spectrophotometer.

Urea is evaluated by an UV enzymatic method based on its hydrolyzation to ammonia and CO₂ in presence of urease, followed by the conversion of ammonia, 2-oxoglutarate and NADH in glutamate and NAD. The decrease in NADH absorbance at 340 nm during the time is proportional to urea concentration. Uric acid levels were measured by an enzymatic colorimetric method (Cobas Integra Uric Acid Cassette; Roche Diagnostics, Indianapolis, IN) on an autoanalyzer (Cobas Integra 400; Roche Diagnostics).

3.2.8 Evaluation of nutritional markers in plasma and serum

The analysis of vitamin C in plasma was performed on 100 µL plasma (fresh sample in duplicate) to which 100 µL MPA (10 %) solution was added. Samples were vortexed, centrifuged at 2200 × g for 2 min and the supernatant (50 µL) was immediately analyzed by HPLC analysis. The chromatographic system consisted of a model 510 system pump (Waters Corp., Milford, MA, USA), a 5mm Atlantis C18 column (250 x 4.6mm internal diameter; Waters, Dublin, Republic of Ireland) and detection was achieved at 245 nm (UV-Vis detector Varian 9050; Varian Inc., Palo Alto, CA, USA). Samples were eluted (1.4 mL min⁻¹) with a mobile phase of 0.1% formic acid. Chromatographic data were acquired by a Millennium 4.0 Workstation (Waters Corp) (23).

Folate concentrations were determined by electrochemiluminescent immunoassay (Folate III) using Cobas immunoassay analyzers (Roche). Briefly, serum samples were incubated with the folate pretreatment reagents and with the ruthenium labeled folate binding protein to form a folate complex which is dependent upon the analyte concentration. Streptavidin-coated microparticles and folate labeled with biotin were added to form a ruthenium labeled folate binding protein-folate biotin complex. The entire complex was bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was then aspirated into the measuring cell where the microparticles -were magnetically captured onto the surface of the electrode. Unbound substances were washed away and application of a voltage to the electrode induced-chemiluminescent emission which was measured by a photomultiplier. Results were determined via a calibration curve.

Vitamins B12 levels were measured by a competitive test principle using intrinsic factor specific for this vitamin. As folate assessment, also Vitamin B12 evaluation was performed with electrochemiluminescence immunoassay (ECLIA) using Cobas immunoassay analyzers (Roche).

3.2.9 Evaluation of FPG-sensitive sites and H₂O₂-induced DNA damage in PBMCs

The levels of endogenous oxidized DNA bases in PBMCs were determined as FPG-sensitive sites; the protein detects 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. Cells were suspended with low melting point agarose (1.5% w/v) in Tris-acetate EDTA buffer, pH 7.4, at 37°C and immediately pipetted (104 cells per gel) into frosted glass microscope slide (Richardson Supply Co., London, UK) precoated with a layer of 1% (w/v) normal melting point agarose similarly prepared in Tris-acetate EDTA buffer. Slides were placed in lysis buffer (2.5 M NaCl, 0.1M Na₂EDTA, 10 mM Trizma, 1% TRITON x-100, 1% dimethyl sulfoxide, 1% N-Lauroylsarcosine sodium salt, pH 10) for 1 h at 4°C in the dark. One slide was treated with FPG enzyme (100 ng mL⁻¹, for 45 min at 37°C) while the other slide acted as control (24, 25). For the determination of cell resistance against oxidatively-induced DNA damage cells were treated with H₂O₂ (500 µmol L⁻¹ in PBS) or control PBS

solution for 5 min, and placed in lysis buffer for 1 h at 4°C in the dark (25). Slides from both the treatments (FPG-sensitive sites and H₂O₂-induced DNA damage) were placed and left for 40 min in the electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, 40 min at 4°C in the dark). Electrophoresis was performed at 1.1 V/cm² for 20 min. Slides were successively neutralized (0.4 M tris-HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 µg mL⁻¹), washed in PBS, drained, and covered with cover slips (25). One hundred comets from the two gels of each slide were electronically captured using an epifluorescence microscope attached to a high sensitivity CCD video camera and to a computer provided with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). The levels of DNA damage were calculated as percentage of DNA in tail. For each sample, the percentage DNA in tail of control cells (i.e. cells not treated with H₂O₂ or with FPG) was subtracted from the percentage DNA in tail of H₂O₂-treated or FPG incubated cells, respectively (25).

3.2.10 Evaluation of peripheral arterial function and arterial stiffness

Peripheral arterial function (RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index) and arterial stiffness (dAIx, digital augmentation index; dAIx@75, digital augmentation index normalized by considering a heart rate of 75 bpm) in the small finger arteries were assessed by a non-invasive plethysmographic method (Endo-PAT2000, Itamar Medical Ltd., Caesarea, Israel). Data previously obtained were correlated with markers of nutritional status, antioxidant defense and oxidative stress.

3.2.11 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) for continuous variables and frequency for categorical variables. Data were tested for normality of distribution by the Kolmogorov–Smirnov test. Variables that were not normally distributed were logarithmically transformed.

A repeated measures analysis of variance (ANOVA) was used to evaluate the effect of treatment (as between-subjects factor) and time (as within-subject factor) on the levels of biochemical and functional parameters under study. Moreover the interaction between treatment and time was considered to unravel the effect of S, BS and CS treatments over time. To correct for multiple comparison, we used the Benjamini and Yekutieli false discovery rate method: statistical significance was calculated using the formula $p = \alpha / \sum(1/i)$, where $\alpha = 0.05$, i ranges from 1 to N and N represents the number of comparisons (26).

All analyses were performed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA). A two-tailed P value <0.05 was considered statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 Baseline characteristics of the study population

Baseline characteristics of the fourteen subjects enrolled in the study are reported in Table 1. Lipid profile (TAG, TSC, LDL-C and HDL-C), glucose, blood pressure, endothelial function (normal RHI value >1.67), body mass index (BMI) and all other biochemical parameters were in the normal range. Six subjects showed a moderate hyperhomocysteinemia (range 17.6-33.8 $\mu\text{L/L}$) with plasma total homocysteine (Hcy-pt) values $\geq 15 \mu\text{mol/L}$. Moreover, three subjects had folate levels (range 7.48-9.3 nmol/L) below 10 nmol/L suggesting folate deficiency, while two subjects were borderline.

Table 1. Baseline characteristics of the study population (N = 14)

Variables	Value
Age (y)	22.41 ± 0.73
Body weight (kg)	72.72 ± 1.90
BMI (kg/m ²)	22.80 ± 1.93
Glucose (mmol/L)	3.96 ± 1.03
TG (mmol/L)	1.18 ± 0.50
TSC (mmol/L)	4.21 ± 0.73
HDL-C (mmol/L)	1.40 ± 0.42
LDL-C (mmol/L)	2.26 ± 0.60
ALT (U/L)	18.92 ± 8.77
AST (U/L)	19.00 ± 3.28
GGT (U/L)	16.07 ± 12.47
Vitamin C (µmol/L)	57.66 ± 19.83
Vitamin B12 (pmol/L)	288.49 ± 96.39
Folate (nmol/L)	13.21 ± 4.43
GSH_pt	8.53 ± 2.30
GSH_pr	0.42 ± 0.25
GSSG	8.11 ± 2.29
Cys-Gly_pt	33.18 ± 6.41
CysGly_pr	1.14 ± 0.48
Cys_pt	220.50 ± 53.86
Cys_pr	4.52 ± 0.88
Hcy_pt	15.82 ± 8.66
Hcy_pr	0.068 ± 0.031
Uric acid	5.29 ± 0.88
Urea	33.14 ± 6.49
CRP (mg/dL)	0.09 ± 0.11
RHI	2.20 ± 0.29
FRHI	0.62 ± 0.31
dAIx (%)	-8.21 ± 8.73
dAIx@75 (%)	-19.07 ± 8.75
FPG-sensitive sites (% DNA in tail)	18.85 ± 7.29
H ₂ O ₂ -induced DNA damage (% DNA in tail)	39.75 ± 6.51

Data are expressed as mean ± SD. BMI, body mass index; TG, triglycerides; TSC, total serum cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; GSH_pt, glutathione total; GSH_pr, glutathione reduced; GSSG, glutathione oxidized; Cys-Gly_pt, cysteinylglycine total; Cys-Gly_pr, cysteinylglycine reduced; Cys_pt, cysteine total; Cys_pr, cysteine reduced; Hcy_pt, homocysteine total; Hcy_pr, homocysteine reduced; CRP, C-reactive protein; RHI, reactive hyperemia index; FRHI, Framingham reactive hyperemia index; dAIx, digital augmentation index; dAIx@75, digital augmentation index standardized for heart rate of 75 bpm; FPG, formamidopyrimidine DNA glycosylase; H₂O₂, hydrogen peroxide.

In the present study, we tried to elucidate the effect of the same portion of blueberries on markers of oxidative stress and antioxidant defense that we expected to be modulated after smoking and that could explain the protective effect against endothelial dysfunction following blueberries consumption. For these reasons, we enrolled the same subjects to test the hypothesis that blueberries could affect several markers of oxidative stress and antioxidant defense.

3.3.2 Composition and characteristics of the blueberry portion and control drink

Blueberries provided about 27g of sugars (16.4g of fructose and 10.6g of glucose), and approximately 309 mg of ACNs (malvidin-galactoside, delphinidin-galactoside, petunidin-galactoside and malvidin-arabinoside were the dominant compounds), 856 mg of total phenolic acids and 2.4 mg of ascorbic acid. The control drink provided the same amount and type of sugars but no bioactive compounds (Table 2).

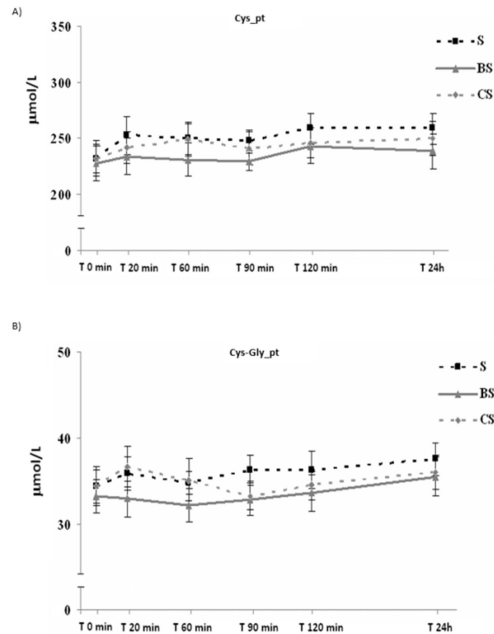
Table 2 . Blueberry portion and control drink composition

	Blueberry	W&S (Control-drink)
Blueberry (g)	300	--
Water (g)	--	300
Sugars (g/300g)		
Fructose	16.4±0.3	16.4
Glucose	10.7±0.6	10.7
Total phenol compounds (mg/300g)	727.2±6.3	--
Total anthocyanins (mg/300g)	348.3±20.9	--
Vitamin C (mg/300g)	2.4±0.06	--

3.3.3 Effect of treatment on plasma levels of aminothiols

The levels of aminothiols in plasma for each treatment measured at baseline (time 0 min) and after 20, 60, 90, 120 min and 24 h from smoking are reported in Figure 1. On the whole, repeated measures ANOVA did not show significant effects of treatment and of interaction time x treatment, but revealed a significant effect of time (after correction for multiple testing) for plasma circulating levels of total (pt) Cys (P for time = 0.007, P for interaction = 0.889, P for treatment = 0.673; Figure 1) and Cys-Gly_pt (P = 0.010; P for interaction = 0.540, P for treatment = 0.408; Figure 1) that increased following smoking treatment. No significant effect was documented for Cys_pr (P for time = 0.078, P for interaction = 0.847, P for treatment = 0.990), Cys-Gly_pr (P for time = 0.694, P for interaction = 0.993, P for treatment = 0.469), GSH_pr (P for time = 0.606, P for interaction = 0.735, P for treatment = 0.443), Hcy_pt (P for time = 0.121, P for interaction = 0.804, P for treatment = 0.987), and Hcy_pr (P for time = 0.060, P for interaction = 0.791, P for treatment = 0.908) after each treatment.

Fig.1 Plasma levels of aminothiols after treatment



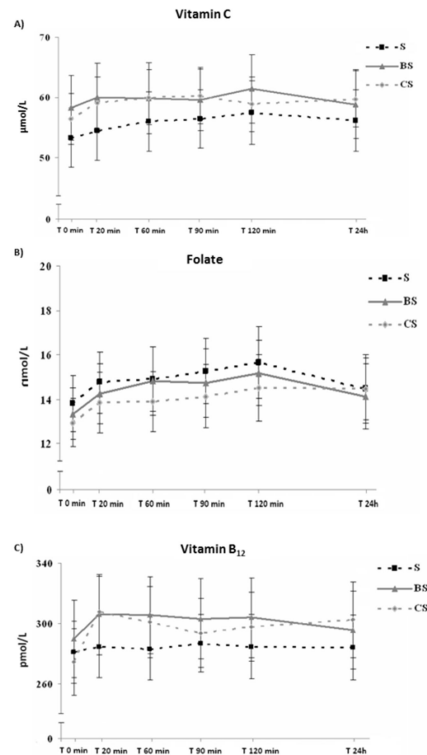
In the present study, the levels of aminothiols were in the normal range and comparable with those of nonsmokers probably due to the young age of the volunteers. Smoking strongly influences the levels of the sulphur-containing aminoacids glutamylcysteine, cysteinylglycine and GSH (Moriarty S et al., 2003). Moriarty et al. (2003) reported that smokers have low levels of GSH and cysteine compared to nonsmokers showing that tobacco smoke exposure is responsible for a decrease in plasma cysteine levels. About the effect of acute cigarette smoke on plasma cysteine levels, only Tsuchiya et al. (2002) documented a significant decrease in plasma cysteine levels 5 min after cigarette smoke even if the levels of cysteine returned to baseline within 60 min from smoking. Gamma-glutamyl transpeptidase, the only enzyme of the cycle located on the outer surface of plasma membrane, plays a key role in GSH homeostasis by catabolizing extracellular GSH and providing cysteine for its synthesis (Dhonukshe-Rutten et al., 2009). We theorized that the consumption of blueberry could increase GSH levels explaining the improvement in the endothelial function previously observed, since GSH could retain and transport the nitric oxide (vasodilator agent) and preserve the endothelial cell barrier function (Dhonukshe-Rutten et al., 2009). Evidence from *in vitro* and *in vivo* studies support a beneficial effect of berries (e.g blueberries, cranberries) in the modulation of GSH levels (Weisel et al., 2006; Spormann et al. 2008; Slemmer JE et al., 2013; Coban J et al., 2014; Coban J et al., 2015). Spormann et al. showed (2008) that 4-week red fruit juice intake (200 mL/day, containing a mix of berries) increased plasma circulating levels of GSH and decreased GSSG in a group of hemodialysis patients. Weisel et al. (2006) reported that 4-week

intervention with fruit/berries juice (700 mL/day) increased blood levels of GSH in healthy subjects. We observed that neither acute cigarette smoking nor blueberry intake affected GSH plasma levels, whereas an increase of cysteine_{pt} and cysteine-glycine_{pt} levels was observed following smoking treatment. We may hypothesize that this increase might be due to a mechanism of cell protection against oxidative stress induced by smoking. Reduced forms of aminothiols and homocysteine did not vary significantly following the three treatments.

3.3.4 Effect of treatment on serum and plasma levels of nutritional markers

Figure 2 reports the levels of dietary markers (vitamin C, folate and vitamin B12) measured in plasma and serum, for each treatment, at baseline (time 0 min) and after 20, 60, 90, 120 min and 24 h from smoking. On the whole, repeated measure ANOVA did not show significant effect of treatment and of interaction time x treatment, but revealed a significant effect of time (after correction for multiple testing) for blood circulating levels of vitamin C (P for time = 0.003, P for interaction = 0.502, P for treatment = 0.829; Figure 2), folate (P for time <0.001, P for interaction = 0.642, P for treatment = 0.642; Figure 2) and vitamin B12 (P for time <0.001, P for interaction = 0.051; P for treatment = 0.879; Figure 2) that increased following all the three treatments.

Fig.2 Plasma levels of dietary markers after treatment



Evidence showed that smokers compared with nonsmokers have lower circulating concentrations of vitamin C, carotenoids and folate (Alberg AJ, 2002; Vardavas CI et al., 2008; Mouhamed Haj D et al., 2011). In our group of volunteers the concentrations of vitamins were in the normal range with the exception of some subjects that reported low serum levels of folate. It's important to consider that the effects of acute smoking on concentrations of vitamins and antioxidants have been poorly investigated. Tsuchiya et al. (2002) documented a significant reduction in the levels of ascorbic acid and uric acid in the first 5 min after smoking but this reduction was only transient and the concentrations returned to baseline levels within 60 min. Dietary intervention studies with berries documented a significant increase for the levels of vitamin C (Møller P et al, 2004; Wilms LC et al., 2007; Spormann TM et al., 2008) even if have failed to positively affect plasma/serum concentrations of carotenoids, folate and vitamin B12 (Duthie SJ et al., 2006; Riso P et al., 2013). Our results are surprising and in contrast with the literature (Tsuchiya M et al., 2002) since we observed an increase of ascorbic acid, folate and vitamin B12 along time for each treatment, while no significant effect was observed for uric acid. We may theorize that the increase of vitamins, in particular vitamin C and folate, might be due to an antioxidant protection/repair mechanism against oxidative stress induced by smoking.

3.3.5 Effect of treatments on the levels of background strand breaks, FPG-sensitive sites and H₂O₂-induced DNA damage

The evaluation of DNA damage in PBMCs, determined by comet assay, is used as a biomarker of oxidative stress in dietary intervention studies (Collins BH et al., 2001; Møller P et al, 2004; Riso P et al., 2009). At these regard, it is noteworthy that variation in cell storage and in the protocol chosen can significantly affect results. Cell cryopreservation is one of the possible methods of cell storage, the majority of data on DNA damage is derived from analysis performed on cryopreserved cells even if no mention of storage process is made. We recently performed, for the first time, a comparison between DNA damage measured as FPG-sensitive sites and H₂O₂-induced in fresh versus cryopreserved PBMCs obtained from individual participating in a dietary intervention study (Del Bo' C et al., 2015). Results demonstrated that sample cryopreservation significantly increased DNA damage prominently in control cells (without FPG or H₂O₂ treatment) and in FPG-treated cells. Increased level of DNA damage indicated that some DNA breakage has occurred during cryopreservation, including mechanical injury due to crystal formation (Table 3).

Table 3 Background DNA damage in fresh and cryopreserved PBMCs

DNA damage	Before WB	After WB	Before PL	After PL
Background DNA damage (% DNA in tail, EB)				
Fresh PBMCs (N= 18 subjects)	6.4 ± 1.5 ^A	6.6 ± 1.1 ^A	6.3 ± 1.4 ^A	6.1 ± 1.6 ^A
Cryopreserved PBMCs (N=18 subjects)	12.8 ± 7.4 ^B	12.6 ± 8.5 ^B	14.1 ± 7.9 ^B	11.9 ± 6.5 ^B
Background DNA damage (% DNA in tail, PBS)				
Fresh PBMCs (N= 16 subjects)	6.1 ± 1.1 ^A	6.4 ± 1.1 ^A	6.1 ± 1.3 ^A	6.5 ± 1.4 ^A
Cryopreserved PBMCs (N=16 subjects)	10.5 ± 5.0 ^B	9.7 ± 4.4 ^B	11.8 ± 6.0 ^B	10.1 ± 4.2 ^B

Legend: Data are expressed as mean ± SD. WB, wild blueberry; PL, placebo; PBS, phosphate buffer saline; EB, endonuclease buffer

^(A,B)Data with different letters within the same row are significantly different ($P \leq 0.05$)

In this study, for the evaluation of FPG-sensitive sites and H₂O₂-induced DNA damage after the three different treatments administered, we preferred to analyze fresh blood samples just after the collection from volunteers.

Results of the levels of strand breaks, FPG-sensitive sites and H₂O₂-induced DNA damage evaluated along time for each treatment are reported in Table 4. Overall, repeated measure ANOVA did not show significant effect of treatment, of time, and of interaction time x treatment for the levels of DNA damage also after correction for multiple testing.

Table 4 Effect of smoking (S), blueberry + smoking (BS) and control + smoking (CS) treatment on background and H2O2-induced DNA damage, FPG-sensitive sites in PBMCs (n=14)

T	Time	Background SBs (%DNA in tail, EB)	FPG-sensitive sites (% DNA in tail)	Background SBs (%DNA in tail, PBS)	H2O2-induced DNA damage (%DNA in tail)
S	T 0 min	6.03 ± 0.25	15.9 ± 1.4	6.05 ± 0.21	40.2 ± 1.5
	T 20 min	6.09 ± 0.14	20.8 ± 2.6	5.90 ± 0.20	39.2 ± 1.9
	T 24 h	6.37 ± 0.28	17.8 ± 1.5	5.91 ± 0.20	39.6 ± 1.4
BS	T 0 min	6.37 ± 0.18	23.5 ± 2.7	5.86 ± 0.20	44.4 ± 2.0
	T 20 min	6.54 ± 0.16	18.6 ± 1.7	5.75 ± 0.21	40.4 ± 1.4
	T 24 h	6.24 ± 0.19	19.6 ± 2.3	6.23 ± 0.17	44.6 ± 1.5
CS	T 0 min	5.69 ± 0.20	16.5 ± 1.2	5.87 ± 0.25	42.4 ± 1.2
	T 20 min	5.90 ± 0.20	20.1 ± 1.7	5.93 ± 0.26	41.6 ± 1.2
	T 24 h	5.72 ± 0.15	16.8 ± 1.8	5.73 ± 0.19	42.2 ± 1.4
2P	for time	0.517	0.450	0.756	0.288
	for interaction	0.389	0.060	0.412	0.681
	for treatment	0.060	0.298	0.753	0.077

Legend: ¹Data are expressed as mean±SEM.

²P values correspond to the time, the treatment and the interaction between treatment and time in the overall ANOVA (SPSS Inc, Chicago, IL, USA).

T, treatment; S, smoking treatment; BS, blueberry treatment + smoking; CS, control treatment + smoking; SBs, strand breaks; PBS, phosphate buffer saline; EB, endonuclease buffer; FPG, formamidopyrimidine DNA glycosylase; H₂O₂, hydrogen peroxide.

Several observational studies from Italy, Turkey, Greece, France, Poland and Scotland have shown that chronic smoking is associated with high levels of DNA damage (Duthie SJ et al., 1996; Lebailly P et al., 1998; Piperakis S et al., 1998; Sardas S et al., 1998; Palus J et al. 1999). Few studies examined the effect of acute cigarette smoking on the levels of DNA damage, Kiyosawa et al. documented that smoking two consecutive cigarettes in 10 min increased the levels of 8-hydroxy-2'-deoxyguanosine in healthy male volunteers (Kiyosawa H et al., 1990). Likewise, the role of berries in the modulation of endogenous and oxidatively-induced DNA damage has been poorly investigated (Del Bo' C et al., 2015). We previously found a significant reduction in the levels of FPG-sensitive sites and H₂O₂-induced DNA damage following 6-week wild blueberry intake (Riso P et al., 2013). On the contrary, Duthie et al. (2006) reported a lack of protective effect on Endo III-sensitive sites and DNA oxidative damage after 2-week cranberry intervention. Wilms et al. (2007) documented no significant effect on the levels of H₂O₂-induced DNA damage after 4-week consumption of blueberry/apple juice, while Møller et al. (2004) observed a significant increase in FPG-sensitive sites after 3-week blackcurrant intervention. Our results showed that acute cigarette smoking did not increase the levels of

FPG-sensitive sites and H₂O₂-induced DNA damage in our subjects at 20 min from smoking although the decrease in endothelial function was observed at the same time. We could hypothesize that the lack of effect of smoking on DNA damage could be related to: the short time of exposure to toxic compounds, the insufficient dose of cytotoxic substances able to induce DNA oxidative damage (the stressor used was only one cigarette), or the compensatory increase in DNA repair mechanisms. Moreover, the consumption of the portion of blueberries provided to our smoker volunteers did not affect the levels of FPG sensitive sites and H₂O₂-induced DNA damage even if, in another study, the same portion of blueberry (300 g) was able to improve DNA resistance to oxidative damage in a group of nonsmoker subjects (Del Bo' C. et al, 2013).

3.3.6 Effect of treatments on serum levels of urea, uric acid, ALT, AST and GGT activity

The concentrations of urea, uric acid, AST, ALT and GGT in serum, for each treatment, evaluated at baseline (time 0 min) and after 20, 60, 90, 120 and 24 h from cigarette smoking are presented in Table 5. Overall, repeated measure ANOVA did not show significant effects of treatment and of interaction time x treatment, but underlined a significant effect of time (after correction for multiple testing) for urea concentration (P for time = 0.001), ALT activity (P for time = 0.001) and GGT activity (P for time = 0.011), while no significant modulation was observed for uric acid (P for time = 0.179) and AST activity (P for time = 0.054).

Table 5 Effect of smoking (S), blueberry + smoking (BS) and control + smoking (CS) treatment on urea and uric acid concentration, and liver and renal function activity in serum (n=14)1

	Time	Urea ($\mu\text{g/dL}$)	Uric acid ($\mu\text{g/dL}$)	AST (U/L)	ALT (U/L)	GGT (U/L)
S	T 0 min	33.43 \pm 2.45	5.39 \pm 0.25	18.86 \pm 0.99	19.71 \pm 2.41	16.50 \pm 3.14
	T 20 min	31.86 \pm 2.12	5.29 \pm 0.24	18.57 \pm 1.10	19.93 \pm 2.95	16.93 \pm 3.48
	T 60 min	31.79 \pm 2.25	5.31 \pm 0.23	17.93 \pm 0.92	19.43 \pm 2.65	16.43 \pm 3.37
	T 90 min	31.36 \pm 2.29	5.33 \pm 0.26	18.71 \pm 1.12	20.71 \pm 3.78	17.57 \pm 3.89
	T 120 min	31.43 \pm 2.32	5.25 \pm 0.24	18.29 \pm 1.00	19.27 \pm 2.88	16.50 \pm 3.34
	T 24 h	30.79 \pm 2.12	5.21 \pm 0.23	18.36 \pm 1.20	20.50 \pm 3.76	17.14 \pm 3.94
BS	T 0 min	30.36 \pm 1.66	5.26 \pm 0.20	18.50 \pm 0.92	18.86 \pm 2.49	15.57 \pm 3.36
	T 20 min	26.64 \pm 1.35	5.23 \pm 0.19	18.71 \pm 1.14	19.79 \pm 4.03	17.29 \pm 3.79
	T 60 min	27.79 \pm 1.51	5.20 \pm 0.17	18.14 \pm 1.04	20.00 \pm 4.04	16.57 \pm 3.92
	T 90 min	27.86 \pm 1.38	5.18 \pm 0.18	18.50 \pm 0.92	18.86 \pm 3.84	16.93 \pm 3.66
	T 120 min	26.71 \pm 1.27	5.14 \pm 0.19	17.71 \pm 1.01	17.57 \pm 2.72	16.43 \pm 3.33
	T 24 h	28.57 \pm 1.75	5.37 \pm 0.13	17.43 \pm 0.94	17.79 \pm 3.06	16.50 \pm 3.47
CS	T 0 min	31.43 \pm 2.19	5.11 \pm 0.19	18.64 \pm 1.28	19.00 \pm 2.44	15.79 \pm 3.27
	T 20 min	28.79 \pm 1.75	5.16 \pm 0.16	19.43 \pm 1.32	20.00 \pm 3.89	17.64 \pm 3.97
	T 60 min	29.14 \pm 1.64	5.09 \pm 0.15	18.93 \pm 1.28	18.79 \pm 3.16	17.00 \pm 3.75

	T 90 min	28.29 ± 1.60	5.04 ± 0.17	18.21 ± 1.14	17.21 ± 2.37	16.36 ± 3.15
	T 120 min	28.50 ± 1.75	5.09 ± 0.17	18.86 ± 1.23	18.21 ± 2.83	16.50 ± 3.22
	T 24 h	28.57 ± 1.78	5.29 ± 0.14	18.14 ± 1.01	18.21 ± 2.75	16.50 ± 3.36
	for time	0.001	0.179	0.054	0.001	0.011
2P	for interaction	0.577	0.254	0.408	0.363	0.414
	for treatment	0.383	0.820	0.953	0.866	0.998

Legend: ¹Data are expressed as mean±SEM.

²P values correspond to the time, the treatment and the interaction between treatment and time in the overall ANOVA (SPSS Inc, Chicago, IL, USA).

T, treatment; S, smoking treatment; BS, blueberry treatment + smoking; CS, control treatment + smoking; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

Little is known about the relationship between smoking and liver damage evaluated through GGT, ALT and AST activity. Most of the studies have shown no positive association between smoking and ALT or AST (Tajima K et al., 1998), while a significant association between smoking and GGT activity has been reported (Whitehead et al., 1996). High levels of GGT are associated with an inflammatory and oxidative stress status (Lee DH et al., 2004; Bo S et al., 2005). Some in vitro and in vivo studies reported the protective effects of berries against liver damage (Lee DH et al., 2004; Bo S et al., 2005; Wang YP et al., 2010; El-Gengaihi SE et al., 2013; Zhao M et al. 2015). Our results are in agree with literature Tajima K et al., 1998) and our previous finding (Riso P et al., 2013) since no effect on liver (evaluated through GGT, ALT and AST activity) was observed after one cigarette smoking and the consumption of a portion of blueberries.

3.4 CONCLUSION

We previously demonstrated that acute cigarette smoking caused endothelial dysfunction in a homogeneous group of young smoker volunteers and blueberry intake was effective in counteracting this impairment. In our study we did not document an increase in the antioxidant defense and a reduction of oxidative stress markers, following the consumption of a single blueberry portion, since we did not detect a significant modulation of aminothiols, vitamins and uric acid after blueberry intake. Moreover, we did not observe neither a significant worsening in DNA oxidative damage after smoking nor an improvement in DNA damage protection after blueberry intake. In conclusion, the consumption of a single blueberry portion failed to modulate markers of oxidative stress and antioxidant defense system examined in our experimental conditions. This investigation could not help to understand which mechanisms are implicated in the modulation of endothelial function previously observed. Further studies are needed to highlight the target of action of blueberry bioactive compounds responsible for the protection against endothelial dysfunction.

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4. IN VITRO STUDY: ROLE OF BLUEBERRY BIOACTIVE COMPOUNDS EXTRACTED FROM A WILD BLUEBERRY (WB) FREEZE-DRIED POWDER IN THE MODULATION OF HEPATIC LIPID ACCUMULATION IN HEPG2 CELLS

PREMISE AND RATIONALE

4.1 Hepatic lipid accumulation and pathophysiology of NAFLD

Obesity has become an emerging health problem through the 20th and 21st centuries. Following the epidemic of obesity, nonalcoholic fatty liver disease (NAFLD), also known as hepatic steatosis, has become the leading cause of liver disease in western countries (Bellentani S et al., 2010; Lazo M et al., 2013; Li Z et al., 2014). The prevalence of NAFLD in the general population is growing worldwide: 44% in the USA, 33% in Europe and 25% in Italy (Petta S et al., 2015). NAFLD is a disease of all ages, and has been reported in children as young as 2 years of age (Nobili V et al., 2015). Given the increasing prevalence of this pathology, adequate knowledge regarding the disease is becoming important for physicians and patients. Current efforts have answered some questions to elucidate the mechanism and causes of the disease, but much questions remain unknown. NAFLD is an umbrella term used to describe the histological spectrum ranging from simple steatosis, defined by a concentration of hepatic triglycerides (TGs) exceeding 5% of liver weight without alcohol abuse, to nonalcoholic steatohepatitis (NASH) (Kleiner DE et al., 2005; Day C, 2006).

In non-alcoholic steatohepatitis (NASH), liver steatosis is associated with hepatic inflammation, hepatocellular ballooning and fibrosis which may be indistinguishable from alcoholic steatohepatitis. Simple steatosis generally represents a benign condition. However about 25% of patients affected by steatosis can progress to NASH and fibrosis. Finally, NASH may evolve to cirrhosis and then to end-stage liver failure or hepatocellular carcinoma (HCC) (Onykwere CA et al., et al., 2015).

The pathogenesis of non-alcoholic liver steatosis and NASH appears multifactorial and many mechanisms have been proposed as possible causes of fatty liver infiltration (Schuppan D, Schattenberg JM, 2013). The association of steatosis with a number of different clinical conditions has been suggested. Common metabolic diseases, such as hyperlipidaemia, type 2 diabetes and central obesity, which define the Metabolic Syndrome, are well established cardiovascular risk factors and have been associated to both benign liver steatosis and progressive NASH (Clark JM, 2006). Moreover, NAFLD is now considered as the hepatic manifestation of the Metabolic Syndrome (Lonardo et al., 2015). Since NAFLD is usually associated with insulin resistance, treatment of comorbidity should be regarded as of paramount importance in the management of these patients (Yki-Järvinen H, 2015). Several therapeutic interventions have been proposed such as weight reduction, insulin sensitizer agents, lipid lowering drugs, antioxidants supplementations as vitamin E and treatment of vitamin D3 deficiency (Onykwere CA et al., 2015). The use of statins in NAFLD patients with dyslipidemia can improve liver function tests, as well as steatosis. Furthermore, statins seem to be safe in NAFLD/NASH patients with dyslipidemia. However, there is a lack of evidence for the use of statins in the treatment of NASH patients without dyslipidemia (Tziomalos K et al., 2015). It is noteworthy that until now there are no effective therapies for NAFLD apart from lifestyle modifications as diet and increasing physical activity. Ongoing research efforts are focused on understanding the underlying pathobiology of hepatic steatosis with the intention of identifying novel therapeutic targets.

The complex pathophysiology of NAFLD could be explained by the two-hit hypothesis. Firstly, the accumulation of fatty acids and TAG in hepatocytes leading to steatosis could be caused by different mechanisms as increased hepatic delivery and uptake of fatty acid (FA) associated with increased lipolysis in adipose tissue and/or increased intake of dietary fat; decreased FA oxidation; increased hepatic de novo lipogenesis; decreased hepatic lipid export via very low density lipoprotein VLDL (Tilg H & Moschen AR, 2008; Berlanga A et al., 2014).

The inability to regulate lipid partitioning leads to the second hit, whereby an overwhelmed FA β -oxidation produces mitochondrial dysfunction which increases reactive oxygen species resulting in sustained oxidative stress and a depletion of the antioxidant defences (Gupte AA et al., 2013; Serviddio G et al., 2013). FA intermediates and a compromised oxidative status activated Kupffer cells producing inflammatory mediators leading to the progression from benign steatosis to NASH (Schuppan D et al., 2010).

More recently, this theory was challenged by a 'multiple hits' hypothesis, where multiple extra- and intra-hepatic signals have been identified as possible causes (Marra F & Lotersztajn S, 2013). Lipotoxicity is not necessarily linked to triglycerides accumulating within the liver, as some free fatty acids (FFA; palmitate and other lipotoxic intermediates) were shown to be more hepatotoxic than triglycerides (Ricchi M, et al., 2009). Moreover, the liver is targeted by signals from other tissues, including adipose tissue, the gut and its microbiota (Tilg H & Moschen AR, 2010).

It is noteworthy that also genetic factors give an important contribution to the establishment of steatosis as it has emerged from familiar and epidemiological studies. Genome-wide association studies have defined genetic determinants of steatosis (Macaluso FS et al., 2015). The rs738409 C > G sequence variant in Patatin-like phospholipase domain-containing 3 (PNPLA3), encoding for the I148M protein variation, has been identified as a major determinant of interindividual and ethnicity-related differences in hepatic fat content (Romeo S et al., 2008; Dongiovanni P et al., 2013). Recently, the Transmembrane 6 Superfamily Member 2 (TM6SF2) E167K variant has been shown to increase susceptibility to progressive NAFLD (Dongiovanni P et al., 2015). More genetic variants have to be investigated for the comprehension of their specific contribution to the development of steatosis and for the identification of new therapeutic targets.

4.1.1 Normal liver processing of lipids

The liver plays a major role in lipid metabolism, importing free FAs (FFAs) and manufacturing, storing, and exporting lipids (Fig.14); derangements in any of these processes can lead to the development of NAFLD. FAs are involved in many important cellular events, such as synthesis of cellular membranes, energy storage, and intracellular signaling pathways. However, chronically elevated FFAs can disturb diverse metabolic pathways and induce insulin resistance (IR) in many organs. At these regard hepatic fat accumulation has been strongly associated with IR (Privitera G et al., 2015).

Under physiological conditions, TG synthesis is stimulated by an excess of FFAs. The TGs can then be stored as lipid droplets within hepatocytes or secreted into the blood as VLDL. Plasma FFA is generated by white adipocytes via lipolysis, which is induced by beta adrenergic receptor agonists such as catecholamine under fasting conditions (Arner P, 2005). This process involves the regulation of protein kinase A (PKA)-dependent phosphorylation and activation of hormone-sensitive lipase (HSL), a key rate limiting enzyme in the lipolysis, to promote this

pathway. Insulin under feeding conditions could reverse this pathway limiting the liberation of FFA and rather inducing *de novo* lipogenesis in this tissue. Lipolysis is hyper activated in adipocytes, resulting in the increase in plasma FFA (Delarue J & Magnan C, 2007). The main plasma membrane transporters for FFA are fatty acid transporter protein (FATP), caveolins, fatty acid translocase (FAT)/CD36, and fatty acid binding protein (FABP). In mammals 6 members of FATPs have been founded, they contain a common motif for fatty acid uptake and fatty acyl-CoA synthetase function (Doege H & Stahl A, 2006). Among family members, FATP2 and FATP5 are highly expressed in the liver, and utilized as major FATPs for the normal physiological context. Caveolins, which are important proteins in the TG synthesis under obesity, consist of three protein family members termed caveolins 1, 2, and 3, and are founded in membrane structures called caveolae, important for protein trafficking and the formation of lipid droplets (Fernandez MA et al., 2006). FAT/CD36 is a transmembrane protein that accelerates FA uptake via facilitated diffusion (Silverstein RL & Febbraio M, 2009). Normally, this protein is not highly expressed in the liver, but is enhanced by diet-induced obesity (Berger E et al., 2015). The hepatic expression of CD36 was positively correlated with hepatic TG contents in NAFLD patients, underscoring the potential importance of this transporter in this pathology. FABPs are cytosolic lipid binding proteins that facilitate intracellular transport of FFAs (Makowski L & Hotamisligil GS, 2005). Among 9 isoforms, FABP1 and FABP5 are highly expressed in the liver but the expression of FABP4 and FABP5 in the liver was correlated with hepatic fatty infiltration in NAFLD patients (Westerbacka J et al., 2007). Further studies are necessary to integrate roles of these fatty acid transporters in the hepatic FFA uptake under both physiological and pathological conditions. Moreover, TG accumulation in the cytoplasm of hepatocytes, as the hallmark of NAFLD, arises from an imbalance between lipid acquisition (FA uptake and *de novo lipogenesis*) and removal (mitochondrial FA oxidation and export as a component of VLDL particles) (Berlanga A et al., 2014).

In order to control the progression of NAFLD, it is important to understand the regulatory mechanisms of lipid accumulation in the human liver. Recently, investigations identified adenosine monophosphate protein kinase (AMPK) as a central regulator of cellular metabolism and as a possible key enzyme in the regulation of liver physiology. AMPK consists of heteromeric complexes with a catalytic α subunit and regulatory β and γ subunits. AMPK is able to sense the energy status of the cell in a variety of different ways (Hardie DG et al., 2015). Moreover, AMPK is able to regulate metabolism through its effects on glucose homeostasis, protein synthesis, lipid and oxidative metabolism. When activated AMPK decreases lipogenesis and stimulates mitochondrial fatty acid oxidation. The former task of this enzyme is accomplished by phosphorylating 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA) and acetyl CoA carboxylase 1 (ACC1). Inactivation of HMG-CoA reductase blocks the conversion of HMGCoA to mevalonate and causes the inactivation of ACC1 leading to a reduction in the ability to synthesize fatty acids. Decreased synthesis in malonyl CoA upregulated the action of carnitine O-palmitoyltransferase 1 (CPT1) increasing fatty acid oxidation (Hardie DG et al., 2015). Noteworthy, AMPK has also been shown to decrease transcription of lipogenic genes through its inhibition of sterol regulatory element-binding protein-1c (SREBP1c) (Foretz M et al., 2005). Given its ability to sense and regulate the metabolic state of the cell, modulation of AMPK has become an important therapeutic target in NAFLD. Various compounds already used for the treatment of type 2 diabetes have been shown to have effects on the AMPK pathway. Several evidences showed that polyphenols such as resveratrol (Baur JA, et al., 2006) and epigallocatechin gallate are able to activate AMPK (Lin CL et al., 2007).

4.1.2 De novo lipogenesis pathways

De novo lipogenesis is an integrated metabolic pathway that embraces glycolysis (conversion of glucose to acetyl-CoA), biosynthesis of saturated fatty acid followed by desaturation, and the formation of TG. Key rate limiting enzymes in the process include glucokinase and liver-type pyruvate kinase in the glycolysis, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in the fatty acid synthesis, long chain fatty acid elongase 6 (ELOVL6) and stearoyl-CoA desaturase (SCD) in the formation of monounsaturated fatty acids, and glycerol-3-phosphate acyltransferase (GPAT), lipins, and acyl-CoA: diacylglycerol acyltransferase (DGAT) in the formation of TG (Towle HC et al., 1997).

FAS is a rate-limiting enzyme in the fatty acid biosynthesis and catalyzes the last step. ACC governs both a key rate-limiting step in the fatty acid biosynthesis and fatty acid oxidation since it synthesizes malonyl-CoA, the inhibitor for CPT1 (Munday MR, 2002).

Two major transcription factors for lipogenesis, SREBP-1c and carbohydrate response element binding protein (ChREBP), are involved in the transcriptional activation of genes encoding aforementioned rate-limiting enzymes in the lipogenesis and have been associated with increased *de novo lipogenesis* in NAFLD. SREBP-1c is a member of SREBP family that control transcriptional regulation of lipid metabolism (Horton JD et al., 2002). As ER-bound precursors, full-length SREBPs reside in the ER by using its transmembrane domain in the middle. Transport of SREBPs from the ER to the Golgi apparatus is mediated in part by nutrient sensors cleavage-activating protein (SCAP) and insulin-induced gene (Insig), and the mature form of SREBPs is generated by two consecutive proteolytic cleavages. SREBP-1c can be further activated by mammalian target of rapamycin (mTOR) pathway while it can be inhibited by PKA, AMPK, and salt inducible kinases (SIKs) (Porstmann T et al., 2008). ChREBP was first identified as a regulator for the hepatic glycolysis since it activates the transcription of L-type pyruvate kinase (L-PK) gene, and was later shown to be involved in the regulation of other lipogenic genes in the pathway (Uyeda K & Repa JJ, 2006). It has been suggested a possible regulation of ChREBP by AMPK even if this hypothesis is still to be verified.

4.1.3 Fatty acid oxidative pathways

Fatty acid β oxidation in mitochondria is a process to shorten the fatty acids into acetyl-CoA, which can be later converted into ketone bodies (beta hydroxybutyrate or acetoacetate) or can be incorporated into the tricarboxylic acid (TCA) cycle for the full oxidation (Eaton S et al., 1996a). To initiate the process, fatty acyl-CoAs should be transported across the mitochondrial membranes by activity of a couple of CPTs. Fatty acyl-CoAs are converted to fatty acyl-carnitines by CPT1 in the mitochondrial outer membrane and translocated into the intermembrane space. Fatty acyl-carnitines are then transported across the mitochondrial outer membrane by carnitine acylcarnitine translocase (ACAT). CPT2 converts fatty acyl-carnitines to fatty acyl-CoAs for the fatty acid β oxidation inside the mitochondrial matrix. The first step involves the beta dehydrogenation of the acyl-CoA ester by chain length-specific acyl-CoA dehydrogenases (e.g. VLCAD, LCAD, and MCAD). 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase are subsequently involved in the fatty acid β oxidation process to complete the conversion of acyl-CoA ester into acetyl-CoA (Eaton S et al., 1996b).

Under fasting conditions, fatty acid β oxidation is enhanced through the inactivation of ACC and the increased expression of β oxidation genes. Peroxisome proliferator-activated receptor (PPAR) alpha and its co-activator PPAR gamma co-activator 1 (PGC-1) alpha are critical in enhancing the expression of target genes including CPT1, long-chain acyl CoA dehydrogenase

(LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and acyl-CoA oxidase (ACOX) (Reddy JK & Hashimoto T, 2001). During starvation the expression of these genes could also be increased by AMPK activity. The clinical implication of impaired mitochondrial β oxidation on the progression of NAFLD is not conclusive. Further study is necessary to delineate the role of fatty acid β oxidation on hepatic lipid accumulation and the progression of NAFLD.

4.1.4 TG secretion

In the liver, TG secretion is realized through the formation of VLDL (Hussain MM et al., 2003). VLDL consists of hydrophobic core lipids containing TGs and cholesterol esters, which is covered by hydrophilic phospholipids and apolipoprotein B (apoB) 100. ApoB 100 is a liver-specific protein that is critical in the VLDL assembly, while apoB 48 in the intestine is associated with chylomicron formation. The VLDL assembly process occurs initially in the rough ER during the translation and translocation of the apoB 100, resulting in the formation of a partially lipidated apoB 100. Then, these molecules, the pre-VLDL, have been transported into the Golgi for the maturation, and subsequently released from the liver via exocytosis. Hepatic steatosis was reported in patients carrying mutations in apoB 100 (hypobetalipoproteinemia) and in microsomal triglyceride transfer protein (MTP) (abetalipoproteinemia) underlying the importance of these proteins in the lipid homeostasis in humans (Tanoli T et al., 2004).

The action of insulin has been identified of critical importance in the regulation of VLDL assembly and secretion, since this hormone could inhibit the transcription of MTP through the regulation of phosphatidylinositol 3 kinase/Akt. Akt phosphorylates and inhibits transcription factor FoxA2, a critical forkhead box factor for activating expression of MTP at the transcription level (Koo SH & Montminy M, 2006). Under insulin resistance, perturbation of this process results in hypertriglyceridemia due to increased TG secretion. However, prolonged exposure of the liver to FFA would promote ER stress and other oxidative stress in the liver, leading to the degradation of apoB 100, decrease in the VLDL secretion, and worsening of hepatic steatosis (De Wit NJW et al., 2012).

4.2 Dietary NAFLD management

Currently therapies to manage NAFLD are based on clinical recommendations on lifestyle modification as increasing physical activity and achieving a gradual weight loss through energy restriction diet. Exercise and improving diet content could decrease steatosis and reduce hepatic inflammation and hepatocellular injury (Harrison SA et al., 2009; Promrat K et al. 2010; Sullivan S et al., 2012). Generally most of the recommendations are focused on reducing the intake of total fatty acid especially saturate (SFAs) and trans fatty acid and fructose. A substantial role in the etiology of NAFLD is played by excessive intake of fructose caused by the high consumption of nonalcoholic beverages among subjects in developed countries. Fructose intake causes the development of fatty liver through stimulation of *de novo lipogenesis* (Ma J et al., 2015).

Several epidemiological studies demonstrated that diets rich in fruit and vegetables could be helpful for the prevention of chronic diseases including NAFLD. This could be attributed to the high content of bioactive compounds largely investigated for their numerous beneficial properties (Musso G et al., 2003). Recommendations were also focused on enhancing intake of polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and long-chain n-3 fatty acids. We have recently reviewed the evidence on their mechanisms of action emerged from *in vitro*, *in vivo* studies and clinical trials of bioactive compounds. Several studies were focused

on vitamin E and vitamin D role in preventing NAFLD since vitamins are known to have anti-oxidant and anti-inflammatory properties (Dongiovanni P et al., 2015). These vitamins have demonstrated that their supplementation could possibly have a beneficial role even if dosages and mechanisms are still to be clarified. Simultaneously either mineral as copper, selenium or iron were supposed to give a significant contribution in the development and treatment of NAFLD since most patients have decay in their homeostasis (Dongiovanni P et al., 2015).

NAFLD is a disease still to be fully investigated since it could have different approaches for its prevention and treatment. More studies are needed to highlight the molecular mechanisms leading to fat accumulation, oxidative balance impairment and liver fibrosis. Food bioactive compounds are able to modulate the activation of genes involved in lipogenesis, fibrogenesis, lipid peroxidation and inflammation and they could be a new therapeutic approach for liver diseases.

4.2.1 Polyphenols and NAFLD

In the last years growing interest has been shown on the role of polyphenols in relation to different chronic pathologies including liver diseases. Several *in vitro* and *in vivo* studies investigated polyphenols properties demonstrating potential beneficial effect related to NAFLD despite having few clinical evidences in treatment of this pathology. Consumption of polyphenols-rich bayberry juice reduced the levels of oxidative (i.e. protein carbonyl groups), inflammatory (i.e. TNF α and interleukin-8) and apoptotic (i.e. tissue polypeptide-specific antigen and cytokeratin-18 fragment M30) biomarkers in young individuals with NAFLD (Guo H et al., 2014). Another study demonstrated that an *Hibiscus sabdariffa L.* extract rich in polyphenols (1.43% of flavonoids, 2.5% anthocyanins and 1.7% phenolic acid), administered in capsules, was able to decrease body weight, serum FFAs and to improve liver steatosis in overweight subject (Chang HC et al., 2014).

Flavonoids seem to target different pathways, they inhibit lipogenic (ACC, SREBP-1, FAS, LXR α) and increase lipolytic enzymes (AMPK, PPAR α , CPT-1), improve antioxidant defenses acting as effective scavengers and had anti-inflammatory properties inhibiting NF κ B pathway (Van De Wier B, et al., 2015).

It's important to considered that dietary studies invariably involve supplementation of whole foods, not individual components; therefore, it seems logical that examination of the ensuing protective roles that follow consumption of fruits and vegetables should involve studies in which the complete profile of the different bioactives present in foods would be examined and tested in the proportions in which they are supplemented. This would highlight the possible competition kinetics for absorption and synergistic interactions. In particular, within the class of polyphenols, anthocyanins seem to be promising for the numerous beneficial effects that have been described. Despite their low absorption and rapid metabolism, regular intake of ACNs may ameliorate hyperglycaemia, modulate endothelial function, and decrease inflammation (Valenti L et al., 2013). Moreover, they seemed able to prevent oxidative stress (Suda I et al., 2008), modulate lipid metabolism and fat deposition (Zhang PW et al., 2015) in different tissues including the liver. Till now, only few clinical studies on humans are available and they diverge for ACNs source, doses and clinical features of patients. Suda et al. 2008 showed an effect of ACN intake (200 mg acylated ACNs from purple sweet potato) in the reduction of liver enzymes (e.g. gamma-glutamyltransferases) in subjects with borderline levels of one or more hepatic markers. In a recent study, NAFLD patients received either purified ACNs (320 mg/d) derived from bilberry and black currant or placebo for 12 weeks (Zhang PW et al., 2015). Individuals receiving ACNs showed a decrease in plasma alanine aminotransferase, cytokeratin-18 fragment and myeloperoxidase, and an overall improvement of insulin resistance

(Zhang PW et al., 2015). Several *in vitro* studies, performed mainly in HepG2 cells supplemented with oleic acid and/or palmitic acid, highlighted three different mechanisms of action by which ACNs could prevent the progression of liver dysfunction/damage: inhibition of lipogenesis (i.e. reducing SREBP1c), promotion of lipolysis (i.e. inducing PPAR α activity with activation of AMPK pathway) and reduction of oxidative stress (i.e. induction of antioxidant enzymes). Mulberry ACNs (0.1, 0.3, 0.5 mg/mL) supplementation in HepG2 reduced lipogenesis (SREBP-1, FAS, ACC and A-FABP), cholesterol biosynthesis (SREBP-2 and HMGCR) and TG biosynthesis, and enhanced fatty acid β -oxidation (PPAR α and CPT-1) (Chang JJ et al., 2013). Cyanidin 3-*O*-glucoside reduced cellular lipid concentration in HepG2 cells by rewiring the expression of genes involved in lipid metabolic pathway as PPAR α (Jia Y et al., 2013), whereas in primary mice hepatocytes it decreased intracellular ROS production acting as free radical scavenger and enhanced PI3K/Akt activation (Jiang X et al, 2014). Considering the variety of ACNs food source, dosages and experimental model used, it's evident that more investigations are needed to deeply characterize the molecular mechanisms used.

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4.4 AIM OF THE STUDY

Considering the pandemic diffusion of liver diseases as NAFLD, it's becoming relevant elucidate the development of this pathology and the strategy for its prevention and treatment. In previous studies, performed in our lab, two bioactive rich fractions extracted from a wild blueberry (WB) freeze-dried powder could be able to counteract lipid accumulation (Del Bo' C et al., 2015) and have immunomodulatory effect in two different cellular models (Taverniti V et al., 2014). HepG2 human hepatocytes supplemented with fatty acids has largely been used in studies as an *in vitro* model for the evaluation of lipolysis and lipogenesis markers modulation. Firstly, we identified the most meaningful *in vitro* model and the best experimental condition for investigating blueberry polyphenols properties in the modulation of makers of lipid accumulation. Secondly, we evaluated the ability of ACNs and phenolics rich fraction in the reduction of lipid accumulation in the *in vitro* model selected. Moreover, in order to evaluate the contribution of WB single compounds we further tested the effect of Delphinidin 3-*O*-glucoside, Cyanidin 3-*O*-glucoside and Malvidin 3-*O*-glucoside and their metabolites, Gallic acid, Protocatechuic acid and Syringic acid on the same cell model.

4.5 MATERIAL AND METHODS

4.5.1 Separation and characterization of different bioactive fractions extracted from wild blueberry (WB) powder

The freeze-dried WB powder was used to extract three different fractions providing the main WB bioactives: the water-soluble fraction (containing mainly sugars and organic acids, WS fraction), the ethyl acetate-soluble fraction (containing mainly chlorogenic acid, Phe fraction), and the methanol-soluble fraction (containing mainly anthocyanins, ACN fraction). Extraction was performed following the method described by Wrolstad et al., 2005 with a few modifications. Briefly, the WB powder was suspended in water, sonicated for 10 min, and centrifuged at 3000g for 10 min. Fraction separation from the supernatant was obtained through a solid-phase extraction (SPE) cartridge (Strata-X 300 mg/3 mL, Phenomenex, Torrance, CA, USA). Three milliliters of supernatant was loaded, and the elution of WS, Phe, and ACN fractions was carried out respectively with 0.01 N HCl (5 mL), ethyl acetate (10 mL), and methanol (5 mL) containing 0.1% HCl. The fractions were dried under vacuum with a rotavapor (RC Jouan 10, Jouan, Winchester, VA, USA) at 20 °C for ACN, at 40 °C for Phe, and at up to 60 °C for the WS fraction. The residues were dissolved in methanol acidified with HCl (0.05 mM) for the ACNs, methanol for the Phe, and water for the WS fractions. The solutions were analyzed for the content of ACNs, Phe, sugars, and organic acids and stored at -20 °C until use.

4.5.2 Analysis of the ACNs and Phe-rich fraction

Analysis was performed with a liquid chromatographic system, which consisted of an Alliance model 2695 (Water, Milford, MA, USA) equipped with a model 2998 photodiode array detector (Waters). The separation was carried out through a C18 Kinetex column (150 × 4.6 mm, 2.6 μm, Phenomenex) at 45 °C with a 1.7 mL min⁻¹ flow rate. The eluents were (A) 1% H₃PO₄ and (B) acetonitrile/water (35:65, v/v). The elution gradient was linear as indicated: 0–15 min, 14% B; 15–25 min, from 14 to 20% B; 25–35 min, from 20 to 32% B; 35–45 min, from 32 to 50% B; 45–48 min, from 50 to 90% B; 90% for 3 min. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 nm (ACNs) and 320 nm (Phe). Calibration curves ranging from 2 to 50 μg mL⁻¹ were obtained for Cy-, Dp-, Pt-, Pe-, and Mv-3-O-glc, Cy- and Pt-3-O-gal, and Pt-3-O-ara and chlorogenic acid. For the ACNs, the working solution was diluted from the stock solution with methanol acidified with 0.1% TFA. Each analysis was carried out in duplicate. The identification of single ACNs was confirmed by LC coupled to electrospray ionization–mass spectrometry (ESI-MS) as already described by Del Bo' et al.⁹ Briefly, the mass spectrometer operated in positive full-scan mode in the range of 200–800 Da. The capillary voltage was set to 3.5 kV, the cone voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350 °C. Data were acquired by Masslynx 4.0 software (Micromass, Beverly, MA, USA).

4.5.3 Preparation of the pure anthocyanins and metabolites

A stock solution of standards of Mv, Cy, and Dp-3-O-glc, as well as their correspondent metabolic products as SA, PrA, and GA, respectively, was prepared. These ACNs were chosen because they were the compounds absorbed and detectable in plasma after consumption of a single portion of blueberry as recently reported (Del Bo' C et al., 2012). Lyophilized standards (10 mg) were dissolved in 10 mL of acidified methanol (HCl 0.05 mM). Aliquots (1 mL) of stand-

ard were concentrated under nitrogen and subsequently quantified by spectrophotometric analysis. Standards were then stored at -20°C until use.

4.5.4 HepG2 Red Oil O Staining

Human HepG2 hepatoma cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), 1% Penicillin Streptomycin and 1% L-GLN in a humidified atmosphere with 5% CO_2 at 37°C . HepG2 cells were plate in 6 wells plate to reach approximately the concentration of 1×10^6 cells/well. Cells were incubated for 48 hours at 37°C , 5% CO_2 . In order to evaluate the possible effect of blueberry bioactive compound on lipid accumulation, HepG2 cells were supplemented with a combination of free fatty acids: palmitic acid (PA, saturated C16:0) plus oleic acid (OA, n-9 mono-unsaturated, C18:1, cis). Different concentrations of PA and OA were used (0.08 mM, 0.8 mM and 1.66 mM) to identify the lower FFAs concentration showing a significant increase in lipid accumulation compared to untreated cells (0.08mM) without toxic effects. HepG2 supplemented with FFAs were incubated with 0.01 μM , 0.1 μM and 1 μM of WB fractions, standards and metabolites for 24 hours. For lipid visualization and quantification through Oil Red O staining, after being washed 3x with PBS, cells were fixed with 10% formalin, stained with 0.7% Oil Red O solution absorbance was then read at 540 nm. The increase of absorbance is proportional to lipid accumulation.

4.5.5 Statistical analysis

Data were analyzed by t-test. Differences were considered significant at $p \leq 0.05$.

4.6 RESULTS AND DISCUSSION

4.6.1 Characterization of Wild blueberry fractions

The two extracts obtained from WB powder were analyzed in order to evaluate the content of Phe and ACNs. The fractions composition is reported in Table 6.

Total ACN content was 42.9 ± 0.75 mg/mL. Sixteen different ACNs were identified in the extract, the dominant compounds were: malvidin glucosides (about 24,4 mg/mL; 22,7% of the total ACN amount), delphinidin glucosides (about 11,4 mg/mL; 10,6% of the total ACN amount) and cyanidin glucosides (about 7,4 mg/mL; 6,9% of the total ACN amount), while petunidin and peonidin glucosides represented about 0,6% and 4,7% of the total ACNs, respectively. No phenolic compounds and sugars were detected in the ACN fraction. In the Phe fraction, chlorogenic acid was the main phenolic compound (11.1 ± 1.68 mg/ml), while only traces of caffeic and ferulic acids were found. No sugars were detectable.

Table 6 Characterization of ACN fraction.

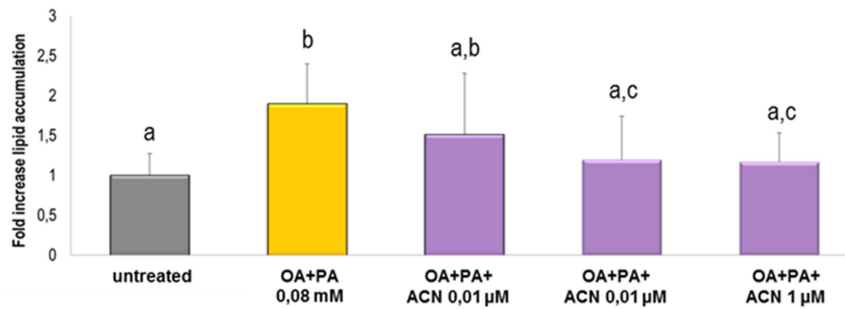
COMPOUNDS	Extracted mg mL ⁻¹	%
Delphinidin 3-gal	8,6	8,0
Delphinidin 3-glc	11,4	10,6
Cyanidin 3-gal	4,8	4,5
Delphinidin 3-ara	4,1	3,8
Cyanidin 3-glc	7,4	6,9
Petunidin 3-gal	2,1	2,0
Cyanidin 3-ara	4,7	4,4
Petunidin 3-glc	0,6	0,6
Peonidin 3-gal	9,4	8,8
Delphinidin	1,2	1,1
Petunidin 3-ara	2,0	1,9
Peonidin 3-glc	5,0	4,7
Malvidin 3-gal	14,3	13,3
Peonidin 3-ara	0,5	0,5
Malvidin 3-glc	24,4	22,7
Malvidin 3-ara	5,4	5,0
Delphinidin 3-glc-ac	0,3	0,3
Malvidin 3-glc-ac	1,0	0,9
TOTAL	42,9± 0,75	

*Data are reported as mean ± standard deviation.
glc, glucoside; gal, galactoside; ara, arabinoside*

4.6.2 Effect of ACN and Phe rich fractions on lipid accumulation in HepG2 hepatocytes cells

The results, obtained in three different experiments in which each condition was tested in triplicate, have shown a significant increase in intracellular lipid accumulation after oleic acid plus palmitic acid treatment (0.08 mM) compared to the untreated cells (fold increase value: untreated cells=1, OA+PA treatment=2.8; $p<0.05$). Moreover ACN-rich fraction lead to a significant reduction in hepatic lipid accumulation at the concentration of 0.1 and 1 μ M compared to OA+PA treatment (fold increase value: untreated cells=1, OA+PA treatment=1.9, ACN 0.1 μ M=1.2, ACN 0.1 μ M=1.1 $p<0.05$) (Fig 3).

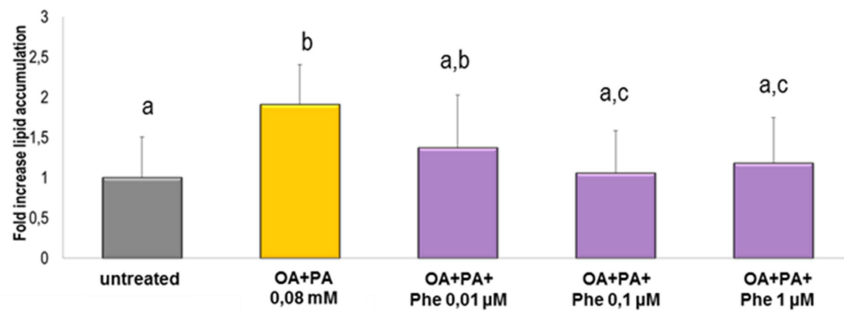
Fig. 3 Effect of ACNs fraction on lipid accumulation in HepG2 hepatocytes. Data are reported as fold increase in lipid accumulation with respect to the control cells without fatty acids. Results are expressed as mean±standard deviation.



OA, oleic acid; PA, palmitic acid; ACN, anthocyanin
a,b,c Data with different letters are significantly different ($P \leq 0.05$)

Fig. 4 Effect of Phe fraction on lipid accumulation in HepG2 hepatocytes. Data are reported as fold increase in lipid accumulation with respect to control cells without fatty acids. Results are expressed as mean±standard deviation.

Phe-rich fraction resulted in a significant reduction of hepatic lipid accumulation at the concentration of 0.1 and 1 µM compared to OA+PA treatment (fold increase value: untreated cells=1, OA+PA treatment=1.9, Phe 0.1 µM=1.1, Phe 0.1 µM=1.2 ($p < 0.05$) (Fig.4).



OA, oleic acid; PA, palmitic acid; Phe, phenolic
a,b,c Data with different letters are significantly different ($P \leq 0.05$)

Primary cultures of human hepatocytes are the optimal *in vitro* model for studying determinants of NAFLD. However their widespread use is limited by logistical factors such as liver samples availability. The main alternative model is the human hepatocyte-derived cell line, HepG2. Palmitic (16:0) and oleic (18:1n-9) acids are the most abundant FA in the liver of both normal subjects and NAFLD patients and have been used (generally in a bovine serum complex) to induce lipid accumulation in HepG2 and reproduce the key cellular features of human NAFLD (Gomez-Lechon MJ et al., 2007). Evidence highlighted through *in vitro* studies hypothesized

three possible mechanisms of action by which ACNs and phenolic acids could prevent the progression of liver dysfunction/damage: inhibition of lipogenesis (i.e. reducing SREBP1c activity with activation of AMPK pathway), promotion of lipolysis (i.e. inducing PPAR α activity with activation of AMPK pathway) and reduction of oxidative stress (i.e. induction of antioxidant enzymes) (Valenti L et al., 2013; Van De Wier B et al., 2015).

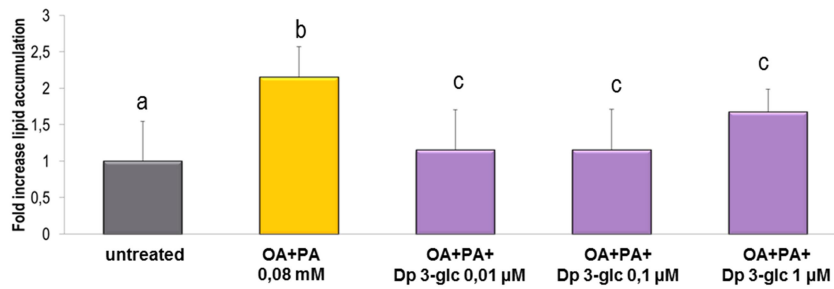
Our results seem in accordance with those shown by several studies investigating the role of extracts from ACN-rich foods (e.g. sweet potatoes, berries and oranges) (Hwang YP et al., 2011; Peng CH et al., 2011; Salamone F et al., 2012; Chang JJ et al., 2013; Santhakumar AB et al., 2013; Vendrame S et al., 2013) since ACNs and phenolic-rich fractions have led to a significant reduction in lipid accumulation for the middle and the highest concentrations tested, showing to have the same effect. However, it is noteworthy that the majority of these studies have used supra-physiological doses of compounds, with little consideration given to human equivalent doses. The investigation of bioactives biological effect using doses achievable through dietary intake or supplementation was one of our major tasks. At these regard, we documented in plasma of human volunteers, after blueberry intake, an ACNs concentration of 0.01 μ M (10 nmoli/litro-0,005 μ g/mL) (Del Bo' C et al., 2013). Moreover, Czank et al. 2013 found concentration of cy 3-glc-labeled of \sim 0.1 μ M (100 nmoli/litro-0,05 μ g/mL) and protocatechuic acid concentration of \sim 1 μ M (1000 nmoli/litro-0,5 μ g/mL) in serum of volunteers after isotopically labeled cyanidin-3-glucoside intake.

Thus, this is the first study evaluating the effect of an anthocyanin-rich and a phenol-rich fraction extracted from a blueberry powder in HepG2 cells in doses reachable through a usual dietary intake.

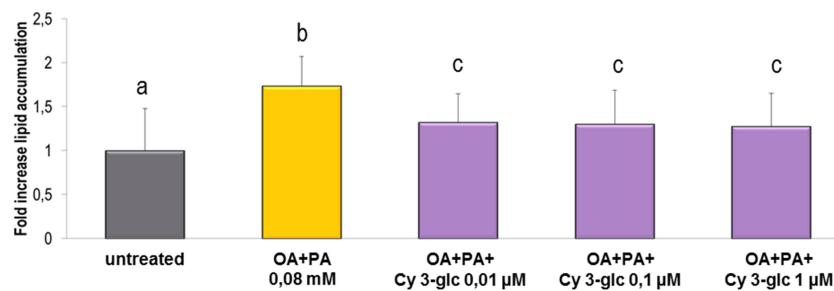
4.6.3 Effect of single anthocyanin compounds on lipid accumulation in HepG2 hepatocytes cells

The results, obtained in three different experiments in which each condition was tested in triplicate, have shown a significant reduction in hepatic lipid accumulation in samples treated with delphinidin 3-*O*-glucoside (fold increase value: untreated cells=1, OA+PA treatment=2.1, Dp 3 *O*-glc 0.01 μ M=1.1 Dp 3 *O*-glc 0.1 μ M =1.1, Dp 3 *O*-glc 1 μ M =1.7 p <0.05) and cyanidin 3-*O*-glucoside (fold increase value: untreated cells=1, OA+PA treatment=1.7, Cy 3-*O*-glc 0.01 μ M=1.3 Cy 3 *O*-glc 0.1 μ M =1.3, Cy 3 *O*-glc 1 μ M =1.3 p <0.05) compared to OA+PA treatment. No significant effect was observed for Mv 3-*O*-glc and for any of the metabolites tested.

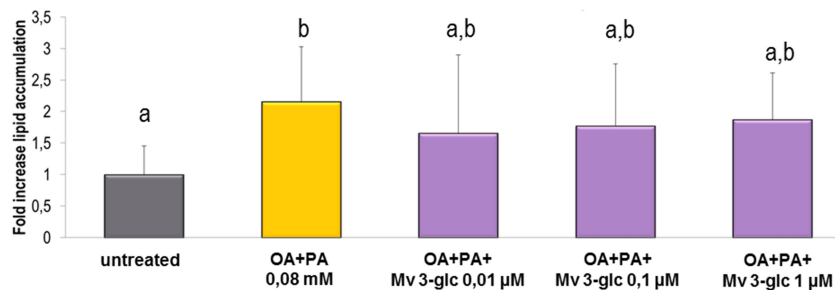
Fig.5 Effect of Dp 3-O-glc, Cy 3-O-glc, Mv 3-O-glc on lipid accumulation in HepG2 hepatocytes. Data are reported as fold increase in lipid accumulation with respect to the control cells without fatty acids. Results are expressed as mean±standard deviation.



OA, oleic acid; PA, palmitic acid; Dp 3-O-glc, delphinidin 3-O-glucoside
a,b,c Data with different letters are significantly different ($P \leq 0.05$)



OA, oleic acid; PA, palmitic acid; Cy 3-O-glc Cyanidin 3-O-glucoside
a,b,c Data with different letters are significantly different ($P \leq 0.05$)

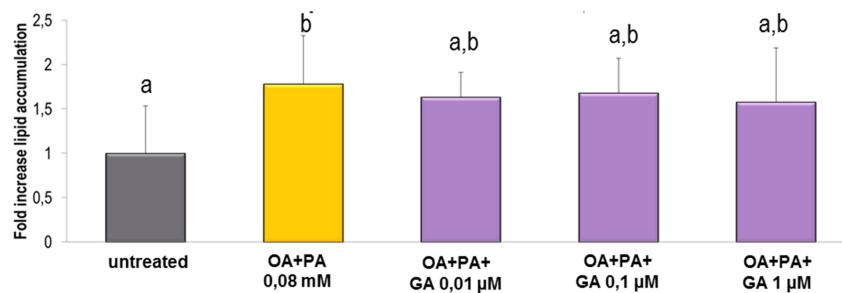


OA, oleic acid; PA, palmitic acid; Mv 3-O-glc malvidin 3-O-glucoside
a,b,c Data with different letters are significantly different ($P \leq 0.05$)

Recent studies evaluating the effect of single ACNs have shown conflicting results since each compound could modulate different molecular targets. In the present study we have analyzed

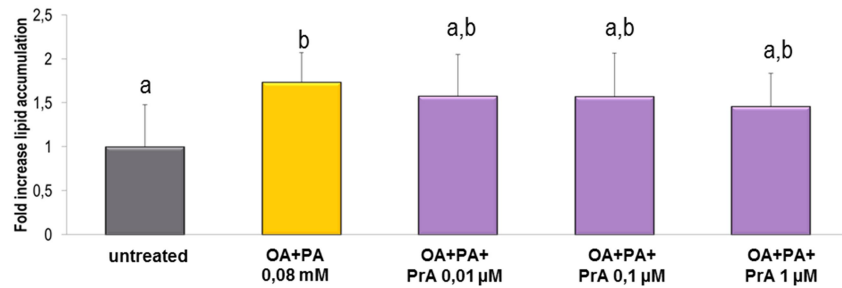
the most representative ACNs (Mv, Dp and Cy 3-glc) in the WB fraction used and, as we recently documented, the only ACNs absorbed in the human plasma following blueberry intake (Del Bo' C et al., 2012). Very few studies have tried to investigate the contribution given by single compounds to the inhibition of lipid deposition observed in *in vitro* models with contrasting results. In a recent study performed in our lab, Del Bo' demonstrated that both Dp 3-glc and Mv 3-glc were able to reduce lipid deposition in TPH-1 macrophages, used as cell model, especially at low doses as we found in HepG2 cells. Contrary to the results exposed by Del Bo' et al. (2015), we showed a significant effect in lipid reduction for Dp 3-glc, Cy 3-glc but not for Mv 3-glc. Our data are in agreement with those found in a recent study in which Dp 3-glc and Cy-3-glc were identified as active compounds whereas Mv-3-glc exhibited a negative effect despite the doses used were much higher than ours (Wang Y et al., 2015). These results may be due to the biological properties of different ACNs that confer different degrees of inhibition toward lipid deposition in HepG2 cells. From a structural point of view, flavonoids with a 3'4'-odihydroxyl group in the B-ring inhibited lipid over-accumulation. The activation of AMPK can lead to regulation of a number of downstream targets (3-hydroxy-3-methyl-glutaryl CoA reductase and acetyl coenzyme A carboxylase) involved in lipid metabolism. ACNs can attenuate hepatic lipid accumulation and stimulate lipolysis in HepG2 cells by activating AMPK and suppressing lipogenic enzymes (Hwang YP et al., 2011; Wei X et al., 2011; Chang JJ et al., 2013). Another possible mechanism for attenuating the hepatic steatosis effect of ACNs is related to the modulation of the c-Jun N-terminal kinase/forkhead box O1 signaling pathway and the related inflammatory adipocytokines. Overall, reducing lipogenesis and oxidative stress in hepatocytes may become new effective targets in the pathogenesis and therapy of NAFLD (Wang Y et al., 2015).

Fig. 6 Effect of Gallic acid, Protocatechuic acid, Syringic acid on lipid accumulation in HepG2 hepatocytes. Data are reported as fold increase in lipid accumulation with respect to the control cells without fatty acids. Results are expressed as mean±standard deviation

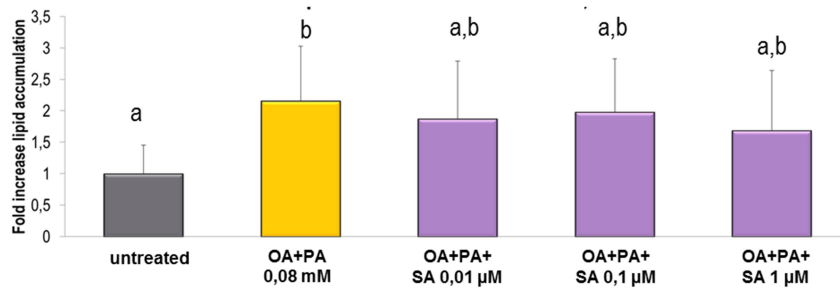


OA, oleic acid; PA, palmitic acid; GA, gallic acid

^{a,b,c} Data with different letters are significantly different ($P \leq 0.05$)



OA, oleic acid; PA, palmitic acid; PrA protocatechuic acid
^{a,b,c} Data with different letters are significantly different ($P \leq 0.05$)



OA, oleic acid; PA, palmitic acid; SA syringic acid
^{a,b,c} Data with different letters are significantly different ($P \leq 0.05$)

The biological properties of ACNs have nearly always been studied *in vitro* by using their native form, which appears quite inappropriate because of their *in vivo* extensive and rapid bio-transformation after ingestion. Only a small part of dietary ACNs are adsorbed, and large amounts of the ingested compounds are likely to enter the colon as previously discussed (see 1.2.4). It has been established that the intestinal microbiota plays a key role in the metabolism of anthocyanins. After ingestion, anthocyanins can be hydrolyzed by intestinal glycosidase and the resulting aglycones are further metabolized in the large intestine to other breakdown metabolites (Rimbach G et al., 2012). In a weak alkaline environment, ACNs can also degrade their constituent phenolic acids, namely, malvidin, cyanidin, and delphinidin to form syringic, protocatechuic, and gallic acids, respectively. In our study any of the metabolite tested were able to lead a reduction in lipid accumulation, whereas in a previous study Del Bo' et al (2015) demonstrated that syringic and gallic acids decreased lipid accumulation in TPH-1 macrophages in all the concentrations tested while protocatechuic acid was effective only for some concentrations (Del Bo' C et al., 2015). Differently, metabolites were not active in our *in vitro* model of liver steatosis, this could be due in part to the low concentrations tested. Future *in vitro* and *in vivo* studies are warranted to confirm these and other results present in literature and to provide support for the intake of ACNs-rich products as an achievable, safe, and inexpensive adjunct therapy to inhibit the development of hepatic steatosis.

4.7 CONCLUSION

It is widely recognized that the role of bioactive compounds present in foods such as polyphenols could be crucial for setting up strategies in the prevention or treatment of chronic diseases. NAFLD is one of the leading causes of liver disease and it is settled by an unhealthy eating behavior and lifestyle. Until now there are no approved drugs and the main clinical recommendation is improving dietary pattern and increasing physical activity. It is evident the requirement of data demonstrating cause-effect relationship and the molecular mechanisms leading to fat accumulation, oxidative balance impairment and liver fibrosis. The supplementation of the diet with bioactives rich foods is an inexpensive, nontoxic therapy that may results in overall health improvement, reducing the risk of chronic liver diseases. The few data present in literature and our results have showed that polyphenols-rich foods can be promising for the prevention of NAFLD and its complications. Three different independent pathways have been hypothesized as target of actions: inhibition of lipogenesis by reducing Srebp1c, promotion of lipolysis by induction of PPAR α activity, and reduction of oxidative stress. It is evident that additional studies are required to clarify the molecular mechanisms and the single compounds contribution through *in vitro* and *in vivo* models. Moreover, well-designed intervention studies are needed to explore the role, the doses and the extent of the benefits of anthocyanins and phenolic acids introduced through the diet.

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APPENDIX 1 COPIES OF ABSTRACTS OF PAPERS

- Taverniti V, Fracassetti D, Del Bo' C, **Lanti C**, Minuzzo M, Klimis-Zacas D, Riso P, Guglielmetti S. Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells. *Journal of Agriculture and food chemistry* 2014, 62, 8346–8351.
- Del Bo' C, Fracassetti D, **Lanti C**, Porrini M., Riso P. Comparison of DNA damage evaluated through the comet assay in fresh versus cryopreserved peripheral blood mononuclear cells obtained from a dietary intervention study. *Mutagenesis* 2015 Jan;30(1):29-35.
- Dongiovanni P, **Lanti C**, Gatti S, Rametta R, Recalcatti S, Fracanzani AL, Maggioni M, Riso P, Cairo C, Fargion S, Valenti L. High fat diet subverts hepatocellular iron uptake determining dysmetabolic iron overload. *PLoS ONE* 2015 10(2): e0116855.
- Del Bo' C, Porrini M, Campolo J, Parolini M, **Lanti C**, Klimis-Zacas D, Riso P. A single blueberry (*V. corymbosum*) portion does not affect markers of antioxidant defense and oxidative stress in healthy volunteers following cigarette smoking. *Mutagenesis* 2015.
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Immunomodulatory Effect of a Wild Blueberry Anthocyanin-Rich Extract in Human Caco-2 Intestinal Cells

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ABSTRACT: Intestinal inflammation is a natural process crucial for the maintenance of gut functioning. However, abnormal or prolonged inflammatory responses may lead to the onset of chronic degenerative diseases, typically treated by means of pharmacological interventions. Dietary strategies for the prevention of inflammation are a safer alternative to pharmacotherapy. Anthocyanins and other polyphenols have been documented to display anti-inflammatory activity. In the present study, three bioactive fractions (anthocyanin, phenolic, and water-soluble fractions) were extracted from a wild blueberry powder. The Caco-2 intestinal model was used to test the immunomodulatory effect of the above fractions. Only the anthocyanin-rich fraction reduced the activation of NF- κ B, induced by IL-1 β in intestinal epithelial Caco-2 cells. Specifically, concentrations of 50 and 100 μ g mL⁻¹ decreased NF- κ B activation by 68.9 and 85.2%, respectively ($p \leq 0.05$). These preliminary results provide further support for the role of food bioactives as potential dietary anti-inflammatory agents.

KEYWORDS: wild blueberry, anthocyanins, Caco-2 cells, NF- κ B, luciferase reporter system

INTRODUCTION

Inflammation is a nonspecific immune response consisting of complex reactions as the body defends itself against injuries that are encountered daily by host cells such as the mucosal surface of the gastrointestinal tract. The orchestration of these responses guarantees both effective protection against harmful agents and maintenance of homeostasis. The nuclear factor κ B (NF- κ B) pathway is an immunological route through which inflammation processes are triggered and regulated. The NF- κ B transcription factor exists in an inactive state in the cytosol of cells; upon its activation and translocation into the nucleus, the induction of genes involved in immune and pro-inflammatory responses is induced, leading to the expression of cytokines and chemokines, adhesion molecules, and inflammatory enzymes.¹

In some individuals, or in particular conditions, the above system fails to suppress the inflammatory processes once activated, leading to exacerbated responses and to the onset of pathological consequences such as inflammatory bowel diseases (IBD). Usually, effective therapies are mainly based on pharmacological interventions, which are often connected with adverse side effects. Therefore, the potential of utilizing dietary strategies to prevent and/or reduce the inflammatory state may represent a safer and affordable tool.

Polyphenols are a class of phytochemicals that have been shown to modulate inflammatory processes.² In vitro and in vivo studies report that pure polyphenolic molecules, but also plant extracts, can interfere with immunological pathways and the production of inflammatory mediators.³

The anti-inflammatory abilities of polyphenols have been partially attributed to a direct action on the host immune system. It is reported that these bioactives could affect different

intracellular pathways in the intestinal mucosa by triggering specific immune responses.⁴ In fact, diverse phenolic compounds (e.g., kaempferol, quercetin, genistein, luteolin) have been documented to exert their effects through the attenuation of pro-inflammatory cytokines.^{5,6} Consequently, it has been proposed that such compounds may decrease the risk of individuals to develop chronic inflammation.⁴ Specifically, previous investigations (both in vitro and in mouse models) documented that polyphenols can exert modulatory effects in the presence of different inflammatory stimuli.²

Anthocyanins (ACNs) are pigments of the polyphenol class that confer red to blue color to several common fruits and vegetables, which have been shown to exert different health-promoting properties as documented in both in vitro and in vivo studies.^{7–12} Apart from their antioxidant activity,¹³ ACNs play a key role in promoting host health through diverse mechanisms: from improvement of lipid profiles, vasomotor tone, and blood pressure to the modulation of detoxifying enzymes^{7–10} and inflammation.^{11,12,14}

ACNs are poorly absorbed,¹⁵ even if new evidence suggests higher bioavailability than previously reported.¹⁶ Consequently, most of them reach the colon, where their concentrations, as well as that of other polyphenols, can reach high concentrations, justifying a beneficial effect against IBD.¹⁷

Thus, the present study aims to investigate the effect of different bioactive fractions, obtained from an ACN-rich wild

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Original Manuscript

Comparison of DNA damage by the comet assay in fresh versus cryopreserved peripheral blood mononuclear cells obtained following dietary intervention

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Abstract

Endogenous and oxidatively induced DNA damage, as evaluated by the comet assay, are widely used as biomarkers of oxidative stress in numerous dietary intervention studies. This analysis can be performed on fresh peripheral blood mononuclear cells (PBMCs) or on cryopreserved cells. However, information pertaining to the effects of cryopreservation on DNA damage is often missing, and this may be crucial in studies in which samples are analysed before and after intervention. The purpose of this study was to compare DNA damage in fresh versus cryopreserved PBMCs obtained from subjects following a 6-week intervention with wild blueberry drink or placebo drink. Fresh and 12-month-stored PBMCs were analysed for formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites and H₂O₂-induced DNA damage. The levels of FPG-sensitive sites were significantly higher in the cryopreserved compared with the fresh cells ($P < 0.001$), while H₂O₂-induced DNA damage was significantly lower after storage ($P < 0.001$). Both the fresh and cryopreserved samples showed reductions in FPG-sensitive sites following the wild blueberry treatment (fresh PBMCs: from 12.50 ± 5.61% to 9.62 ± 3.52%, $P = 0.039$; cryopreserved PBMCs: from 22.7 ± 6.1% to 19.1 ± 7.0%, $P = 0.012$). In contrast, the decrease in H₂O₂-induced DNA damage observed in the cryopreserved cells masked the protective effect of the wild blueberry drink documented in the fresh samples (fresh PBMCs: from 44.73 ± 7.46% to 36.34 ± 9.27%, $P < 0.001$; cryopreserved PBMCs: from 25.8 ± 4.6% to 23.9 ± 4.6%, $P = 0.414$). In conclusion, our results suggest that FPG-sensitive sites, and more importantly, H₂O₂-induced DNA damage could be significantly modified following the long-term storage of samples obtained from individuals participating in a dietary intervention study. Because storage may affect the assessment of the protective role of diet against DNA damage as a marker of oxidative stress, further research is needed.

Introduction

The comet assay is a rapid and simple technique for the evaluation of DNA damage in all types of eukaryotic cells and tissues. This assay has been used in various human biomonitoring studies to investigate the effects of exposure to pollutants, environmental contaminants (1–3) and bioactive constituents of diet and/or food (4–10).

For the comet assay, cells are immobilised in a bed of low-melting-point (LMP) agarose. Following gentle cell lysis, samples are treated with alkali to unwind and denature the DNA and hydrolyse sites of damage, and electrophoresis is then performed. Cells are successively stained with a fluorescent DNA intercalating dye and visualised under a microscope by epifluorescence (11). This assay allows for the detection of several classes of DNA alterations, including

RESEARCH ARTICLE

High Fat Diet Subverts Hepatocellular Iron Uptake Determining Dysmetabolic Iron Overload

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Abstract

Increased serum ferritin associated with mild hepatic iron accumulation, despite preserved upregulation of the iron hormone hepcidin, is frequently observed in patients with dysmetabolic overload syndrome (DIOS). Genetic factors and Western diet represent predisposing conditions, but the mechanisms favoring iron accumulation in DIOS are still unclear. Aims of this study were to assess the effect a high-fat diet (HFD) on hepatic iron metabolism in an experimental model in rats, to further characterize the effect of free fatty acids on iron metabolism in HepG2 hepatocytes *in vitro*, and to assess the translational relevance in patients with fatty liver with and without iron accumulation. Despite decreased uptake of dietary iron, rats fed HFD accumulated more hepatic iron than those fed regular diet, which was associated with steatosis development. Hepatic iron accumulation was paralleled by induction of ferritin, in the presence of preserved upregulation of hepcidin, recapitulating the features of DIOS. HFD was associated with increased expression of the major iron uptake protein Transferrin receptor-1 (TfR-1), consistently with upregulation of the intracellular iron sensor Iron regulated protein-1 (IRP1). Supplementation with fatty acids induced TfR-1 and IRP1 in HepG2 hepatocytes, favoring intracellular iron accumulation following exposure to iron salts. IRP1 silencing completely abrogated TfR-1 induction and the facilitation of intracellular iron accumulation induced by fatty acids. Hepatic TfR-1 mRNA levels were upregulated in patients with fatty liver and DIOS, whereas they were not associated with liver fat nor with inflammation. In conclusion, increased exposure to fatty acids subverts hepatic iron metabolism, favoring the induction of an iron uptake program despite hepatocellular iron accumulation.

Original Manuscript

A single blueberry (*Vaccinium corymbosum*) portion does not affect markers of antioxidant defence and oxidative stress in healthy volunteers following cigarette smoking

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Abstract

We previously reported that a portion of blueberries reversed endothelial dysfunction induced by acute cigarette smoking. Since smoking-induced endothelial dysfunction is associated with a condition of oxidative stress, we evaluated whether the observed effect was mediated by modulation of markers of oxidative stress and antioxidant defence. Fourteen out of 16 male healthy smokers previously enrolled, participated in a three-armed randomized controlled study with the following experimental conditions: smoking treatment (one cigarette); blueberry treatment (300 g of blueberries) + smoking (one cigarette); control treatment (300 ml of water with sugar) + smoking (one cigarette). The cigarette was smoked 100 min after blueberry/control/water consumption. Each treatment was separated by 1 week of washout period. Plasma vitamin (C, B₁₂ and folate) and aminothiols concentrations, endogenous [formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites] and oxidatively induced DNA damage (resistance to H₂O₂-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were measured at baseline and 20, 60, 90, 120 min and 24 h after smoking. On the whole, analysis of variance did not show a significant effect of treatment on the modulation of markers of oxidative stress and antioxidant defence but revealed an effect of time for plasma concentrations of vitamin C ($P = 0.003$), B₁₂ ($P < 0.001$), folate ($P < 0.001$), total cysteine ($P = 0.007$) and cysteine-glycine ($P = 0.010$) that increased following the three treatments after smoking. No significant effect of treatment was observed for the levels of FPG-sensitive sites ($P > 0.05$) and H₂O₂-induced DNA damage ($P > 0.05$) in PBMCs. In conclusion, the consumption of a single blueberry portion failed to modulate markers of oxidative stress and antioxidant defence investigated in our experimental conditions. Further studies are necessary to elucidate this finding and help clarifying the mechanisms of protection of blueberries against smoking-induced endothelial dysfunction.

Nutritional therapy for nonalcoholic fatty liver disease

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Abstract

Following the epidemics of obesity, nonalcoholic fatty liver disease (NAFLD) has become the leading cause of liver disease in western countries. NAFLD is the hepatic manifestation of metabolic syndrome and may progress to cirrhosis and hepatocellular carcinoma. To date, there are no approved drugs for the treatment of NAFLD, and the main clinical recommendation is lifestyle modification, including increase of physical activity and the adoption of a healthy eating behavior. In this regard, studies aimed to elucidate the effect of dietary interventions and the mechanisms of action of specific food bioactives are urgently needed.

The present review tries to summarize the most recent data evidencing the effects of nutrients and dietary bioactive compounds intake (i.e., long-chain PUFA, Vitamin E, Vitamin D, minerals and polyphenols) on the modulation of molecular mechanisms leading to fat accumulation, oxidative stress, inflammation and liver fibrosis in NAFLD patients.

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Keywords: Nonalcoholic fatty liver disease (NAFLD); Food bioactives; Molecular mechanisms; *In vitro* studies; Animal models; Clinical trials

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), also known as *hepatic steatosis*, is defined by liver fat deposition in the absence of excessive alcohol intake [1]. Following the epidemics of obesity, NAFLD has become the leading cause of liver disease (prevalence, 20–34%) [2,3], and it is epidemiologically associated with the metabolic syndrome and insulin resistance (IR) [4–6]. NAFLD is an umbrella term used to describe a histological spectrum ranging from simple steatosis, defined by a concentration of hepatic triglycerides (TGs) exceeding 5% of liver weight, to nonalcoholic steatohepatitis (NASH) characterized by hepatocellular damage, lobular necroinflammation and fibrogenesis [7,8]. NASH may evolve to cirrhosis and then to end-stage liver failure or hepatocellular carcinoma [9,10]. Genetic variants play a major role in disease predisposition [11] by interacting with nutritional and other environmental factors, typically hypercaloric diet and lack of physical activity. To date, there are no approved drugs for the treatment of NAFLD, and the main clinical recommendation as an initial step is lifestyle modification.

Systematic reviews on the role of specific nutrients and phytochemicals on NAFLD and related outcomes have recently been published [12,13]. In this review, we will specifically focus on the

mechanism by which selected macro-/micronutrients and food bioactives exert a beneficial effect on the hepatic outcomes of NAFLD.

2. Pathophysiology of NAFLD

Fatty liver results from an imbalance between TG accumulation and removal and represents the safest way to store free fatty acids (FFAs) in the liver [6,14]. Excess hepatocellular TG derives from several sources including dietary fatty acids, increased peripheral lipolysis due to adipose tissue IR and elevated hepatic *de novo* lipogenesis due to hyperinsulinemia. Indeed, the major determinant of NAFLD is systemic IR [4,15]. Reduction of lipid secretion through very low-density lipoproteins (VLDL) and a decreased fatty acids oxidation are also involved in hepatic fat accumulation [5].

The development of NASH has been explained by the occurrence of multiple so-called “second-hits” leading to the activation of inflammation in the context of hepatic steatosis [16,17]. The initial hit leading to the development of fatty liver renders hepatocytes susceptible to other multiple hepatotoxic insults including (a) peroxidation; (b) oxidative stress secondary to free radicals produced during β - and ω -oxidation of FFAs; (c) inflammation triggered by endotoxin engaging Toll-like receptor-4 in Kupffer cells (KCs) and hepatocytes due to increased intestinal permeability; (d) qualitative and quantitative changes in gut microbiota [18,19]; (e) hepatic stellate cells (HSCs) activation; and (f) mitochondrial dysfunction. All these conditions lead in the end to inflammation, cellular damage and activation of fibrogenesis [20].

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APPENDIX 2 TITLE OF PAPERS SUBMITTED

- Dongiovanni P; Ruscica M; Ferri N; Macchi C; **Lanti C**; Rametta R; Maggioni M; Fracanzani AL; Badiali S; Fargion S; Magni P; Valenti L. Liver fat accumulation is associated with circulating PCSK9 levels. Hepatology, 2016. Paper submitted.

APPENDIX 3 COPIES OF ABSTRACTS OF ORAL COMMUNICATIONS AND POSTERS

- **Lanti C**, Taverniti V, Fracassetti D, Del Bo' C, Riso P, Porrini M, Klimis-Zacas D, Guglielmetti S. Anthocyanins from Wild Blueberry exert in vitro immunomodulatory properties. *Societa' Italiana di Nutrizione Umana, Annual Meeting, Firenze, October 21-22, 2013.*
- Dongiovanni P, **Lanti C**, Gatti S, Rametta R, Recalcatti S, Fracanzani AL, Maggioni M, Riso P, Cairo C, Fargion S, Valenti L. High fat diet subverts hepatocellular iron uptake determining dysmetabolic iron overload. *European Iron Club meeting, Verona, September 11-14, 2014.*
- **Lanti C**, Dongiovanni P, Riso P, Valenti L. Effect of blueberry bioactive compounds on hepatic lipid accumulation in HepG2 cells: preliminary data. *Societa' Italiana di Nutrizione Umana, Annual Meeting, Roma, October 20-21, 2014.*
- Deon V, Del Bo' C, **Lanti C**, Porrini M, Campolo J, Klimis-Zacas D and Riso P. A single portion of blueberries can affect peripheral arterial tone in young smokers with endothelial dysfunction: preliminary observations and perspectives. *Societa' Italiana di Nutrizione Umana, Annual Meeting, Roma, October 20-21, 2014.*
- Dongiovanni P; Ruscica M; Ferri N; Macchi C; **Lanti C**; Rametta R; Maggioni M; Fracanzani AL; Badiali S; Fargion S; Magni P; Valenti L. Liver fat accumulation is associated with circulating PCSK9 levels. *Monothematic conference AISF 2015: "Nash: malattia epatica, oncologica e cardiovascolare", Modena, October, 8-10 2015.*
- Del Bo' C, Deon V, **Lanti C**, Porrini M, Campolo J, Klimis-Zacas D, Riso P. Effect of a single portion of blueberries on markers of vascular function and oxidative stress in young smokers: a randomized-controlled trial. *4th Food Omics: Food to life, Cesena, October 8,9 2015.*
- Dongiovanni P, **Lanti C**, Ciappellano S, Riso P, Valenti L. Role of blueberry bioactive compounds in the reduction of hepatic lipid accumulation in an *in vitro* model. *Societa' Italiana di Nutrizione Umana, Annual Meeting, Firenze, December 2-4, 2015.*

Anthocyanins from Wild Blueberry (*Vaccinium angustifolium*) exert *in vitro* immunomodulatory effect

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Background: Research on berries is increasing considering their significant content in bioactive compounds such as polyphenols, especially anthocyanins (ACNs). These compounds have been demonstrated to possess antioxidant, anti-inflammatory and immunomodulatory effects.

Aim: The present study aimed to investigate the effect of three different fractions (ACN, phenolic and water soluble fractions) obtained from a wild blueberry (WB) powder on immune responses in intestinal epithelial Caco-2 cells and U937 human macrophages, in presence of pro-inflammatory molecules.

Methods: Caco-2 cells were transfected with the plasmid pNiFty2-Luc containing a promoter with five NF- κ B-binding sites followed by the firefly luciferase reporter gene *luc*. The effect of WB fractions was evaluated in the presence of IL-1 β , a pro-inflammatory cytokine. Caco-2 cells were supplemented with 25, 50 and 100 μ g/m of the water soluble fraction and 5, 25, 50 and 100 μ g/mL of ACN and of the phenolic fractions. Immunomodulatory activity for each fraction was monitored in real time by quantification of bioluminescence with a luminometer. Human U937 macrophages were supplemented with three different concentration of the ACN fraction (1, 10 and 25 μ g/mL) in presence of LPS to evaluate the modulation of the pro-inflammatory cytokine TNF- α expression by means of q-PCR.

Results: The ACN fraction reduced the activation of NF- κ B induced by IL-1 β in Caco-2 cells in a dose-dependent manner. Specifically, the concentration of 50 and 100 μ g/mL decreased the bioluminescence by 68.9% and 85.2% respectively ($p \leq 0.05$). Neither the phenolic nor the water soluble fractions showed a modulation in the activation of NF- κ B. In U937 macrophages only the highest concentration of ACN fraction had a significant effect in reducing the LPS-dependent induction of TNF- α .

Conclusion: Only the ACN fraction displayed anti-inflammatory properties by decreasing the activation of NF- κ B in presence of IL-1 β in Caco-2 cells and exerting a dampening effect towards LPS-induced TNF- α production in immune cells. Further experiments will be focused on the identification of single compounds and metabolites possibly involved in the anti-inflammatory effect (s) of WB.

Effect of blueberry bioactive compounds on hepatic lipid accumulation in HepG2 cells: preliminary data.

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Background: Non alcoholic fatty liver disease (NAFLD) defined by excess lipid accumulation in the liver, is the hepatic manifestation of metabolic syndrome. Lifestyle and diet represent both protective and risk factors in NAFLD development. Therefore, in the last years many studies investigated the effect of bioactive compounds present in fruit and vegetables on fatty liver. It has been reported that anthocyanins (ACNs) decrease hepatic lipid accumulation and may counteract hepatic inflammation. Nevertheless, the mechanism of action of these compounds and their impact on NAFLD has yet to be fully determined.

Aim: The present study aimed to investigate the effect of two bioactive fractions (ACNs and phenolic acids) extracted from a freeze-dried wild blueberry powder (WB) on lipid hepatic accumulation in HepG2 hepatocytes cultured with fatty acids. In order to evaluate the contribution of WB single compounds we further tested the effect of Delphinidin 3-*O*-glucoside and its metabolite, Gallic acid, on the same cell model.

Methods: HepG2 cells were supplemented with a combination of free fatty acids: palmitic acid (PA, saturated) plus oleic acid (OA, n-9 mono-unsaturated). Different concentrations were used (0.08 mM, 0.8 mM and 1.66 mM). We next used 0.08 mM which was the lower FFAs concentration showing a significant increase in lipid accumulation compared to untreated cells. HepG2 supplemented with FFAs were incubated with 0.01 μM, 0.1 μM and 1 μM of ACN fraction, phenolic fraction, Delphinidin 3-*O*-glucoside and gallic acid for 24 hours. For lipid visualization and quantification HepG2 cells were stained with Oil Red O and absorbance was read at 540 nm.

Results: Lipid accumulation was significantly reduced by ACN fraction at the concentration of 0.1 and 1 μM and by phenolic fractions at the concentrations of 0.01 and 0.1 μM ($p < 0.05$). Also Delphinidin 3-*O*-glucosid lead to a significant reduction of intracellular lipids at the concentrations of 0.01 and 0.1 μM ($p < 0.05$) whereas Gallic acid had no effect.

Conclusion: These results show that ACNs fractions, phenolic fractions and Delphinidin 3-*O*-glucoside could reduce lipid accumulation in HepG2 cells supplemented with FFAs. Further experiments will be aimed to investigate the mechanisms of action of these compounds and their possible clinical application.

A single portion of blueberries can affect peripheral arterial tone in young smokers with endothelial dysfunction: preliminary observations and perspectives

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Background: Cigarette smoke adversely affects vascular function by promoting endothelium injury via reactive oxygen species and nitric oxide dysregulation. Emerging evidence suggests an important role of dietary factors in modulating endothelial function. In particular, blueberries (*Vaccinium corymbosum*) appear to have beneficial effects on peripheral arterial dysfunction induced by acute cigarette smoking in young healthy volunteers.

Objective: The aim of this study is to investigate the possible effect of one portion of blueberries on peripheral arterial function in young smokers with endothelial dysfunction.

Methods: The study involves 16 male smokers with endothelial dysfunction. Subjects are randomized in a 3-armed controlled study with the following 3 experimental conditions: - smoking (S) treatment (one cigarette); - blueberry treatment (300 g of blueberry) + smoking (BS); - control treatment (300 mL of water with glucose and fructose) + smoking (CS). One week of wash-out period is scheduled between each treatment. Blood pressure (BP), heart rate (HR), and peripheral arterial function (reactive hyperemia index, RHI) are measured before and 20 min after smoking by using finger plethysmography method (Endo-PAT2000). Results obtained are elaborated by analysis of variance (ANOVA). Post-hoc analysis of differences between treatments is assessed by the least significant difference (LSD) test with $p \leq 0.05$ as level of statistical significance.

Results and Conclusion: Elaboration of preliminary data on 5 subjects showed that smoking does not affect RHI, BP and HR in subjects with endothelial dysfunction differently from what observed in smokers with normal endothelial function. However, ANOVA revealed a significant difference in the effect of the 3 treatments on RHI ($p= 0.01$). In particular, BS and CS treatments improved RHI index with respect to S treatment ($+30.9 \pm 24.2\%$ BS vs S, $p= 0.0055$; $+25.3 \pm 27.2\%$ CS vs S, $p= 0.014$). On the contrary, no effect was observed for systolic and diastolic BP, and HR ($p= 0.80$, $p= 0.75$ and $p= 0.94$, respectively). Further analysis of data on the whole group of subjects will help clarifying the protective effect of wild blueberry consumption in subjects with endothelial dysfunction

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Effect of a single portion of blueberries on markers of vascular function and oxidative stress in young smokers: a randomized-controlled trial

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The consumption of berries (e.g. strawberries, cranberries and blueberries) has been shown to improve vascular function [1], inflammation [2] and oxidative stress [3].

The aim of the present study was to investigate the effect of a single blueberry portion on markers of arterial function and oxidative stress in a group of smoker volunteers.

Sixteen healthy male smokers were recruited for a randomized controlled crossover study. Three types of conditions were assessed: 1-smoking treatment (S); 2-control treatment (300 mL of water with sugar + smoking (CS); 3-blueberry treatment (300 g of blueberry + smoking (BS). Each treatment was separated by one week of wash-out period. Peripheral arterial function (reactive hyperemia index, RHI) was measured before and 20 min after smoking. Plasma aminothiols concentrations, endogenous (formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites) and oxidatively induced DNA damage (resistance to H₂O₂-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were measured at baseline and 20, 60, 90, 120 min and 24 h after smoking. Blueberry consumption, but not the control drink, counteracted the impairment of RHI ($-4.4 \pm 0.8\%$ BS treatment vs $-22.0 \pm 1.1\%$ S treatment, $p < 0.01$) after acute cigarette smoking. Analysis of variance did not show a significant effect of treatment on the modulation of plasma aminothiol concentrations and the levels of FPG-sensitive sites ($P > 0.05$) and H₂O₂-induced DNA damage ($P > 0.05$) in PBMCs, but revealed an effect of time for plasma concentrations of total cysteine ($P = 0.007$) and cysteine-glycine ($P = 0.010$) that increased following the three treatments.

In conclusion, the consumption of a single blueberry portion counteracted the impairment of endothelial function but failed to modulate markers of oxidative stress. Further studies are necessary to confirm this finding and help clarifying the mechanisms of protection of blueberry against endothelial dysfunction.

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Role of blueberry bioactive compounds in the reduction of hepatic lipid accumulation in an *in vitro* model.

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Background: Nonalcoholic fatty liver disease (NAFLD) defined by excess lipid accumulation in the liver in the absence of excess alcohol intake, is the hepatic manifestation of the metabolic syndrome. Till now the main clinical recommendation for the prevention and treatment of NAFLD is lifestyle modification. In the last years studies have tried to understand the effect of bioactive compounds present in fruit and vegetables on fatty liver. It has been described that polyphenols decrease hepatic lipid accumulation and counteract hepatic inflammation. Nevertheless, their mechanism of action has yet to be fully determined.

Aim: To investigate the effect of two bioactive fractions (ACNs and phenolic acids) extracted from a freeze-dried wild blueberry powder (WB) on lipid hepatic accumulation in HepG2 hepatocytes cultured with fatty acids. In order to evaluate the contribution of WB single compounds we further tested the effect of Delphinidin 3-*O*-glucoside, Cyanidin 3-*O*-glucoside and Malvidin 3-*O*-glucoside and their metabolites, Gallic acid, Protocatechuic acid and Syringic acid on the same cell model.

Methods: HepG2 cells were supplemented with a combination of free fatty acids: palmitic acid (PA, saturated) plus oleic acid (OA, n-9 mono-unsaturated) 0.08 mM, which was the lowest FFAs concentration inducing a significant increase in lipid accumulation compared to untreated cells without toxic effects. HepG2 supplemented with FFAs were incubated with 0.01 μM, 0.1 μM and 1 μM of the two WB fractions, standards and metabolites for 24 hours. For lipid visualization and quantification HepG2 cells were stained with Oil Red O and absorbance was read at 540 nm.

Results: Lipid accumulation was significantly reduced by ACN and phenolic fractions at the concentration of 0.1 and 1 μM ($p < 0.05$). Also Delphinidin 3-*O*-glucoside and Cyanidin 3-*O*-glucoside led to a significant reduction of intracellular lipids at all the concentrations tested ($p < 0.05$) whereas Malvidin 3-*O*-glucoside and all ACNs metabolites had no effect. The reduced intracellular lipid accumulation was independent of AMPK pathway activation and it was not mediated by inhibition of SREBP1c dependent lipogenic program.

Conclusion: These results show that ACNs fractions, phenolic fractions, Delphinidin 3-*O*-glucoside and Cyanidin 3-*O*-glucoside, but not ACNs metabolites, reduce lipid accumulation in HepG2 cells supplemented with FFAs.

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