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Biomarkers of oxidative stress, inflammation and endothelial function to study the role of blueberry bioactive compounds *in vitro* and *in vivo*

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8. DEVELOPMENT OF A DIETARY INTERVENTION STUDY TO EVALUATE THE EFFECT OF 6-WEEK BLUEBERRY CONSUMPTION ON MARKERS OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL FUNCTION, IN SUBJECTS WITH RISK FACTORS FOR CARDIOVASCULAR DISEASE

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ABSTRACT

Biomarkers of oxidative stress, inflammation and endothelial function to study the role of blueberry bioactive compounds *in vitro* and *in vivo*

Chronic and degenerative diseases, such as cardiovascular disease, are drastically increasing in Western Countries with serious economic penalties on health care management. As a consequence, there is a strong demand for preventive strategies that can be easily applied by the majority of the population. Diet is one of the most important lifestyle factors able to decrease the risk of degenerative diseases but also to reduce the complications associated with aberrant metabolic states or already established disorders. Besides the recognised biological properties of many bioactive compounds, widely distributed in fruit and vegetable, 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, able to demonstrate, through sensitive and reproducible biomarkers, their physiological and biochemical role.

Biomarkers can describe indirectly various levels of exposure, functional effects and pathological endpoints. While there is no known biomarker that bridges the gap between the intake and endpoint, the increase in knowledge regarding functional alterations of specific biomolecules can bring this possibility even closer. In this regard, an ambitious goal should be the development of reference protocols in which the most reliable, accurate and sensitive biosensors and biomarkers are selected in order to study prevention approaches through diet. The objective of the PhD thesis was to study the effect of blueberry and its bioactives in the modulation of biomarkers of oxidative stress, inflammation and endothelial function through *in vitro* and *in vivo* approaches.

In this context, it is important to identify reliable and reproducible biomarkes able to respond to dietary treatment. So, the **first part of the thesis** was focused on the methodological issue with the aim:

1- to test the reproducibility of DNA damage, as marker of oxidative stress, measured by comet assay. In particular two different biomarkers, endogenous and oxidatively induced DNA damage, were evaluated in fresh versus cryopreserved human peripheral blood mononuclear cells (PBMCs) obtained from volunteers included in a 6-week blueberry intervention. This information is critical to assess the suitability of the analysis of these biomarkers in stored biological samples;

2- to test the intra and inter-day reproducibility of the endothelial function, measured as Reactive Hyperemia Index (RHI) in a group of volunteers. RHI was evaluated through Endo-PAT2000, a non-invasive device that exploits biosensor technology to measure endothelial-dependent vasodilation following occlusion of the brachial artery which causes an increased signal of peripheral arterial tone. The reproducibility of this measure is very important to enable the development of adequate experimental protocols and to reduce potential confounding factors that may mask or under/over-estimate the effect of a treatment.

In the whole, the results obtained demonstrated differences in the levels of DNA damage between fresh and cryopreserved PBMCs. In particular, in fresh cells we documented a reduction of endogenous and oxidatively induced DNA damage following 6-week blueberry intervention. Conversely, in cryopreserved cells the protective effect of blueberry was confirmed only when the levels of DNA damage were evaluated as endogenous damage (FPG sensitive sites). Cryopreservation increased the baseline levels of DNA damage with respect to fresh cells but caused an improvement of cell resistance to oxidative stress nullifying the treatment effect.

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Concerning the intra and inter-day reproducibility of RHI, we documented that multiple measurements of endothelial function within the day is related with an increase in RHI response. This transient effect may be attributed to production of vasodilators that may eventually mask improvement of vascular function due to the treatment under study (i.e. overestimation). On the contrary, a low inter-day variability in RHI response was observed.

The **second part of the thesis** was devoted to study the ability of dietary components, in particular blueberry polyphenols, to modulate several biomarkers. Firstly the bioavailability of blueberry bioactive compounds was investigated in two different products; subsequently, acute and chronic studies on subjects exposed to risk factors for cardiovascular disease (i.e. smoking, endothelial dysfunction, etc) were developed to investigate the impact of dietary treatments in the modulation of the biomarkers under study. In particular, markers of oxidative stress (e.g. DNA damage), inflammation (pro-inflammatory cytokines) and endothelial function (nitric oxide production, RHI, arterial stiffness) were evaluated.
We documented that blueberry bioactives, in particular anthocyanins (ACNs), were bioavailable both in blanched and unblanched products even if a higher absorption of ACNs was observed at specific time points after the intake of blanched product. Overall, the different dietary interventions (acute and chronic) performed, demonstrated a significant reduction in the levels of DNA damage. Moreover, the consumption of blueberry had a beneficial effect on endothelial function. In particular, the 6-week blueberry consumption showed a subject-related effect on endothelial function, with half of the volunteers (those with impaired endothelial function or smokers) registering an improvement of RHI. In this regard, the intake of a single portion of blueberry was able to reverse endothelial dysfunction induced by acute cigarette smoking.
No significant effect of long term blueberry intake was observed on markers of inflammation considered.

The **last part of the thesis** was performed at the University of Copenhagen and focused on the potential antiatherogenic and antiatherosclerotic effect of different fractions extracted from the wild blueberry powder rich in ACNs and phenolic acids. In particular, we tested the ability of the ACN and phenolic fractions, as well as the single ACNs and their metabolites, to reduce lipid accumulation in THP-1 macrophages, and to counteract THP-1 attachment to endothelial cells (HUVEC cell line) following stimulation with pro-inflammatory cytokines.
We observed that both the fractions and single compounds were able to reduce lipid accumulation even if a significant type of treatment/dose interaction was observed. In fact the ACNs fraction was effective at all the doses tested, while the phenolic fraction only at low concentration. Moreover, supplementation with single compounds showed different effects depending on the type of molecule considered suggesting a possible synergistic effect when mix of compounds is present. Regarding the anti-atherosclerotic effect, the ACN fraction was able to reduce THP-1 attachment to HUVEC cells only at the highest dose while low doses of the phenolic fraction was necessary to observe a beneficial effect.

In conclusion, through this PhD thesis it was possible to add important results concerning the suitability and reliability of several biomarkers and biosensors of endothelial function exploited in dietary intervention studies to demonstrate the protective effect of foods/bioactives.
Moreover, the *in vivo* studies developed could supported the beneficial role of blueberry and its bioactives in the modulation of biomarkers of oxidative stress and endothelial function in at risk subjects. In addition, the potential antiatherogenic and antiatherosclerotic effect of blueberry bioactives was supported by the exploited *in vitro* approach. Future *in vitro* studies will focus on the specific mechanisms involved in such modulation.

RIASSUNTO

Biomarkers of oxidative stress, inflammation and endothelial function to study the role of blueberry bioactive compounds *in vitro* and *in vivo*

Le malattie cronico degenerative, come le malattie cardiovascolari, sono drasticamente in aumento nei Paesi occidentali con un grave impatto economico sul sistema sanitario. Di conseguenza vi è una forte richiesta da parte delle istituzioni di strategie preventive facilmente applicabili alla popolazione. La dieta è senza dubbio uno dei più importanti fattori in grado di ridurre il rischio di sviluppare malattie degenerative e le complicazioni ad esse associate. Nonostante le numerose riconosciute proprietà biologiche ascrivibili ai composti bioattivi presenti in frutta e verdura, poco si conosce circa i loro meccanismi d'azione ed i potenziali effetti sinergici o antagonistici. Questa mancanza è in parte attribuita alla carenza di studi di intervento controllati in grado di dimostrare, attraverso l'utilizzo di biomarker sensibili e riproducibili, il ruolo fisiologico e biochimico dei composti bioattivi.

Ad oggi pochi sono i biomarker primari riconosciuti in grado di provare la relazione tra esposizione alla dieta ed endpoint di malattia. Pertanto, l'aumento delle conoscenze in materia di alterazioni funzionali ad opera di molecole biologiche specifiche può contribuire a migliorare le conoscenze a riguardo. Un obiettivo ambizioso dovrebbe essere lo sviluppo di protocolli di riferimento in cui vengano selezionati biosensori e biomarker in grado di rispondere in modo affidabile, accurato e sensibile ad un trattamento dietetico.

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

Pertanto la prima parte della tesi è stata focalizzata su alcuni approcci metodologici con l'obiettivo di:

    1 - testare la riproducibilità del danno al DNA, quale marker di stress ossidativo, attraverso l'utilizzo del saggio comet. A tal riguardo sono stati considerati due diversi biomarker quali la valutazione del danno indotto *ex vivo* mediante acqua ossigenata, e quella del danno endogeno. L'analisi della riproducibilità è stata condotta in campioni di linfociti (PBMC) freschi e congelati ottenuti da volontari in seguito a trattamento per 6 settimane con mirtillo. Questa informazione è importante per stabilire la possibilità di utilizzare questi biomarker in campioni biologici conservati;

    2 - testare la riproducibilità della funzione endoteliale, misurata come indice di iperemia reattiva (RHI), valutata all'interno della stessa giornata e in giornate diverse in un gruppo di volontari. La funzione endoteliale è stata valutata attraverso l'uso del sistema Endo-PAT2000, un dispositivo non invasivo che sfrutta la tecnologia del biosensore per misurare la vasodilatazione endotelio- dipendente del tono arterioso periferico in seguito all'occlusione dell'arteria brachiale. La riproducibilità di questa misura è molto importante al fine di consentire lo sviluppo di adeguati protocolli sperimentali, e di ridurre i potenziali fattori confondenti che possono mascherare, attraverso una sotto/sovrastima l'effetto di un trattamento.

In generale, i risultati ottenuti hanno dimostrato delle differenze nei livelli di danno al DNA tra cellule fresche e congelate. In particolare, nei campioni freschi abbiamo osservato una riduzione dei livelli di danno endogeno ed indotto (mediante acqua ossigenata) in seguito al consumo per sei settimane di mirtillo. Al contrario, nelle cellule sottoposte a congelamento l'effetto protettivo con il mirtillo è stato confermato solo con la valutazione del danno endogeno. Il congelamento ha

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aumentato i livelli basali di danno al DNA, e al tempo stesso ha indotto una maggiore resistenza cellulare al danno ossidativo annullando l'effetto del trattamento con mirtillo.

Per quanto riguarda la riproducibilità della funzione endoteliale (RHI), abbiamo documentato che misurazioni ripetute all'interno della stessa giornata portavano ad un miglioramento della risposta endoteliale funzione. Questo effetto, seppur transitorio, potrebbe essere attribuito alla continua produzione di sostatnze vasodilatatorie che potrebbero mascherare l'effetto di un intervento dietetico.
Una più bassa variabilità della funzione endoteliale è stata invece riscontrata nelle misurazioni eseguite in giornate diverse.

Nella seconda parte della tesi è stata valutato l'effetto in vivo di alcuni componenti della dieta, in particolare i polifenoli del mirtillo.
In primo luogo abbiamo valutato la biodisponibilità dei composti bioattivi in due differenti prodotti di mirtillo (prodotto tal quale e prodotto sottoposto a "blanching"); successivamente, sono stati utilizzati alcuni approcci di intervento in acuto e in cronico per studiare l'effetto dei trattamenti dietetici nella modulazione di biomaker in soggetti esposti a fattori di rischio cardiovascolare (es. fumo, disfunzione endoteliale, ecc.). In particolare sono stati valutati marker di stress ossidativo (es. danno al DNA), infiammazione (citochine pro-infiammatorie), e funzione endoteliale (ossido nitrico, RHI, rigidità arteriosa).
Abbiamo documentato che la componente polifenolica, in particolare gli antociani, erano biodisponibili da entrambi i prodotti di mirtillo, anche se è stato osservato un maggiore assorbimento degli antociani, a specifici tempi, dopo il consumo del prodotto che ha subito il trattamento di blanching. Gli interventi dietetici in acuto e in cronico hanno mostrato una riduzione significativa dei livelli di danno al DNA. Inoltre, il consumo di mirtillo ha avuto un importante effetto nella modulazione della funzione endoteliale. In particolare, in acuto è stato dimostrato che una porzione di mirtillo era in grado di contrastare la disfunzione endoteliale indotta dal fumo di una sigaretta, mentre nello studio in cronico, il consumo per 6 settimane di mirtillo ha mostrato un miglioramento della funzione endoteliale su circa la metà dei soggetti. Nessun effetto significativo è stato invece osservato sui marker di infiammazione valutati.

L'ultima parte della tesi, svolta presso l'Università di Copenhagen, ha avuto come obiettivo la valutazione del potenziale effetto antiaterogenico e antiaterosclerotico di alcune frazioni estratte da un liofilizzato di mirtillo selvatico ricco in antociani e composti fenolici. In particolare, abbiamo testato la capacità delle frazioni (antociani e fenoli), nonché dei singoli antociani e corrispettivi metaboliti, di ridurre l'accumulo di lipidi in cellule THP-1, e di contrastare l'adesione di quest'ultime alle cellule endoteliali (HUVEC) in seguito alla stimolazione con citochine pro-infiammatorie.
I risultati della ricerca hanno evidenziato che entrambe le frazioni ed i singoli composti erano in grado di ridurre l'accumulo di lipidi, anche se tale effetto risultava dipendente dal tipo di trattamento e dalla dose di composto utilizzata. Infatti, mentre la frazione degli antociani risultava efficace a tutte le dosi testate, quella fenolica solo alle basse concentrazioni. Inoltre, la supplementazione con i singoli composti mostrava effetti differenti legati al tipo di molecola utilizzata, suggerendo un potenziale effetto sinergico esercitato dei composti presenti nelle frazioni.
Per quanto riguarda la capacità di ridurre l'adesione dei monociti alle cellule endoteliali, è stato dimostrato che la frazione degli antociani risultava attiva solo alle dosi maggiori, mentre la frazione fenolica alle dosi più basse.

In conclusione, attraverso questa tesi di dottorato è stato possibile aggiungere importanti risultati riguardanti l'adeguatezza e l'affidabilità di biomarker e biosensori utilizzati negli studi di intervento dietetico per dimostrare l'effetto *in vivo* di alimenti / componenti bioattivi.

Dagli studi *in vivo* effettuati emergono risultati positivi circa il ruolo del mirtillo e dei suoi composti bioattivi nella modulazione dello stress ossidativo e della funzione endoteliale nei soggetti esposti a fattori di rischio.

I modelli sviluppati *in vitro* mostrano inoltre il potenziale effetto antiaterogenico e antiaterosclerotico dei composti bioattivi, i cui potenziali meccanismi d'azione saranno oggetto dei prossimi studi.

ABBREVIATIONS

ACNs, anthocyanins;

AI, augmentation index;

AI@75, augmentation index standardized for heart rate of 75 bpm;

ANOVA, analysis of variance;

AOAC, association of official analytical chemists;

ALT, alanine aminotransferase;

Ara, arabinoside;

AST, aspartate aminotransferase;

BAUS, brachial artery ultrasound scanning;

BB, blueberry;

BL-P, blanched purée

BMI, body mass index;

C3GE, cyanidin 3-glucoside equivalents;

CI, confidence interval;

CJ, control jelly;

CRP, C-reactive protein;

CVD, cardiovascular disease;

Cy, cyanidin;

CydG, cyanidin-3,5-diglucoside;

dAix, digital augmentation index;

dAix@75, digital augmentation index normalized for the heart rate;

DiBP, diastolic blood pressure;

DMSO, dimethyl sulfoxide;

Dp, delphinidin**;**

ED, endothelial dysfunction;

EDHF, endothelium-derived hyperpolarizing factor;

EDTA, ethylenediaminetetraacetic acid;

ENDOIII, endonuclease III;

eNOS, endothelial NO synthase;

ELISA, enzyme-linked immunosorbance assays;

FBS, fetal bovine serum;

FMD, flow mediated dilation;

FPG, formamidopyrimidine DNA glycosylase;

FRHI, Framingham reactive hyperemia index;

GAE, gallic acid equivalent;

Gal, galactoside;

GC-MS, gas chromatography–mass spectrometry;

GGT, gamma-glutamyltransferase;

Glc, glucoside;

GSH, reduced glutathione;

GSH-Px, glutathione peroxidase;

GSSG, oxidized glutathione;

GST, glutathione S-transferase;

H_2O_2, hydrogen peroxide;

HDL-C, high density lipoprotein; cholesterol;

HepG2, human hepatocellular liver carcinoma cell line;

HPLC, high performance liquid chromatography;

HPLC-EC, high performance liquid cromatography- electrochemical detection;

HR, heart rate;

hs-CRP, high sensitive C-reactive protein;

Abbreviations

HUVEC, human umbilical venous endothelial cells;

ICAM-1, intercellular cell adhesion molecule-1;

IL-6, interleukin-6;

IkB, inhibitor kappa B;

IQF, individually quick frozen;

IS, internal standard;

IsoPs, isoprostanes;

LC-DAD-MS/MS; liquid chromatography-diode array detector-mass spectrometry;

LDL-C, low density lipoprotein cholesterol;

LMP, low melting point;

LPS, lipopolysaccharide;

LSD, least significant difference;

MAP, monomeric anthocyanin pigment;

MDA, malonildialdehyde;

MMP, metalloproteinases;

MPA, metaphosphoric acid;

Mv, malvidin;

NB-P, unblanched purée

NO, nitric oxide;

NF-κB, nuclear factor kappa B;

NMP, normal melting point;

Ogg1, 8-Oxoguanine DNA glycosylase (Ogg1);

PAT, peripheral arterial tone;

PBMC, peripheral blood mononuclear cell;

PBS, phosphate buffer saline;

PC, polymeric color;

Abbreviations

Pn, peonidin;

PMA, phorbol 12-myristate 13-acetate;

Pt, petunidin;

RHI, reactive hyperemia index;

ROS, reactive oxygen species;

SD, standard deviation;

SEM, standard error of the mean;

SOD, superoxide dismutase;

SPE, solid phase extraction;

sVCAM-1, soluble vascular adhesion molecule-1;

TFA, trifluoroacetic acid;

THP-1, Human acute monocytic leukemia cell line;

TNF-α, tumor necrosis factor alpha;

TG, triglycerides;

TPC, total phenolic content;

TSC, total serum cholesterol;

UHPLC-MS/MS, ultra high pressure liquid chromatography-mass spectrometry;

VEGF, vascular endothelial growth factor;

WB, wild blueberry;

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

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0. PREFACE

Lifestyle and dietary habits are considered the major risk factors in the development and progression of degenerative diseases (Mozaffarian et al., 2008; Ignarro et al., 2007; Sofi et al., 2005). Several epidemiological studies showed an inverse association between the intake of fruit and vegetable and chronic diseases (Hartley et al., 2013; Schwenke et al., 2013; Hung et al., 2004; Bazzano et al., 2003). This can be attributed to the high content of nutrients and non-nutritive food constituents that could play an important role in the disease prevention. In fact, long-term, large-scale, population-based cohort studies have found that high levels of blood vitamin E, vitamin C, folate and carotenoids were associated with a lower risk of cardiovascular disease (CVD) (Stradling et al., 2013; Wang et al., 2013; Riccioni et al., 2012; Núñez-Córdoba et al., 2011; Verhaar et al., 2002). In addition, regular consumption of dietary fiber, particularly from cereal and vegetable sources, may improve CVD health through multiple mechanisms including lipid reduction, body weight regulation, improved glucose metabolism, blood pressure control, and reduction of chronic inflammation (Cho et al., 2013; Bernstein et al., 2013; Satija & Hu 2012). Also polyphenols, widely distributed in foods such as tea, coffee, cocoa, olive oil, and berries have been associated to a reduction of chronic and CVD (Estruch et al., 2013; Del Rio et al., 2013; Di Castelnuovo et al., 2013; Tresserra-Rimbau et al., 2013; Lou-Bonafonte et al., 2012; Ros 2012; Chong et al., 2010). Historically, biological actions of polyphenols have been attributed to antioxidant activities, but recent evidence suggests that immunomodulatory and vasodilatory properties of polyphenols may also contribute to CVD risk reduction (Santhakumar et al., 2013; Wallace 2011; Visioli & Davalos 2011; Scalbert et al., 2005).

On the base of these premises, it is widely supported the recommendation of consuming five or more servings of fruit and vegetable per day (Heimendinger et al., 1996). This information provides important basis to judge the preventive potential of a diet rich in fruit and vegetable. In fact, exploring the role of diet and more specifically the effect of nutrients and non-nutrients on the metabolic processes of disease is critical in designing public health directives for population approaches to preventing diseases.

Biomarkers are useful tools used in nutrition science to estimate the effect of nutrition interventions on health outcomes, when these are not directly measurable. However the availability and development of more "direct" and non-invasive biomarkers and biosensors is required.

Biomarkers are defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu & Tavel 2010). Biomarkers such as those of DNA damage and repair, lipid and protein oxidation, inflammation, have been used in animal and human studies in order to evaluate the effects of different treatments both for preventive and therapeutic purposes.

However, the choice of a biomarker able to respond to a dietary treatment is not easy. In particular it is important to think about the purpose of the study and the biologic relationship of the biomarker with dietary intake. The current state of knowledge should be evaluated not only for selecting an appropriate biomarker, but also in assessing how to evaluate the biomarker appropriately.

As of today, there is a lack of scientific consensus in the validation of markers in nutrition research and this represents one of the difficulties encountered in dietary intervention studies but also in the preparation of health claim dossiers for the functional foods.

The aim of the present PhD thesis is to study the role of polyphenol-rich foods, in particular blueberries, in the modulation of biomarkers of oxidative stress, inflammation and endothelial function through *in vitro* and *in vivo* approaches.

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

1- selection and application of biomarkers and biosensors of oxidative stress and endothelial function to test the feasibility, sensitivity, and reproducibility following a short term dietary treatment. In particular, it will be assessed the reproducibility of the levels of cell

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DNA damage (evaluated as endogenous and oxidatively induced damage by comet assay), as marker of oxidative stress. Moreover, it will be test the feasibility, sensitivity and intra/inter-day reproducibility of the reactive hypermia index, as marker of endothelial function, evaluated by mean of an innovative and noninvasive biosensor named Endo-PAT2000.

2- Study of the bioavailability of blueberry bioactive compounds and their role in the modulation of several makers. In particular:
- absorption and excretion of bioactive compounds (i.e. ACNs) from different blueberry products in a group of young human volunteers;
- development of acute studies to evaluate the effect of a single portion of blueberry in the modulation of markers of oxidative stress, inflammation and endothelial function in **young non-smoker** and **smoker** volunteers;
- development of a dietary intervention study to evaluate the effect of 6-week blueberry consumption on markers of oxidative stress, inflammation and endothelial function, in **subjects with risk factors for cardiovascular disease**.

3- Role of the potential antiatherogenic and antiatherosclerotic effect of different polyphenol-rich fractions through an *in vitro* approach. To this aim, two different cells models will be used: human monocytic (THP-1) cell line, and human umbilical venous endothelial cells (HUVECs). The first model will be used to evaluate the capacity of polyphenol-rich fractions to reduce lipid accumulation (antiatherogenic effect); while the second one to assess whether polyphenol-rich fractions will be able to reduce THP-1 cells adhesion to HUVECs following stimulation with pro-inflammatory cytokines (antiatherosclerotic effect).

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1 STATE OF THE ART

1.1 Biomarker definition

The term biomarker was coined for the first time in 1989, but only in the 1998 a National Institutes of Health panel standardized the definition of biomarker or biological marker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological process, or pharmacologic responses to a therapeutic intervention*"(Biomarkers Definitions Working Group 2001).

Biomarkers may have the greatest value in early efficacy and safety evaluations such as *in vitro* and *in vivo* studies, and early-phase clinical trials in order to establish "proof of concept." Although much progress has been made to identify potential biomarkers, the challenge still remains to validate them not only by a biological but also from an analytical point of view. A marker is termed ''*validated*'' if it ''*has been demonstrated by robust statistical methods to be associated with a given clinical endpoint (prognostic markers), to predict the effect of a therapy on a clinical endpoint (predictive markers), or to be able to replace a clinical endpoint to assess the effects of a therapy (surrogate markers)*'' (Buyse et al., 2010). The ideal biomarker should be measurable, highly sensitive, specific, reliable, accessible, standardized, dependable, cost effective, and easily interpretable by clinicians (May &Wang 2008).

Biomarkers can be either biochemical, physiological, anatomical or physical; they can describe indirectly various levels of exposure, functional effects and pathological endpoints. A biomarker may be measured on a biological sample (as blood, urine, or tissue), it may be a registration obtained from a person (e.g. blood pressure), or it may be an imaging test (such as echocardiogram) (Ramachandran 2006). Biomarkers may have several other applications in disease detection and monitoring of health status. They can be used to select high-risk people or targeted populations for preventive interventions, specific screening, and early diagnostic strategies (Mayeux 2004). Furthermore, biomarkers may provide important information concerning the pathogenesis of a disease or appear to be useful in risk stratification, in disease diagnosis, or in monitoring therapy; many others may be risk factors themselves, representing therefore potential targets of therapy (Mayeux 2004).

1.2 Classification of biomarkers

In the clinical practice, three types of biomarkers are distinguished (Biomarkers Definitions Working Group 2001).

Type 0 biomarker: a marker of the natural history of a disease and correlates longitudinally with knows clinical indices.

Type I biomarker or clinical endpoints: a marker that captures the effects of a therapeutic intervention in accordance with its mechanism of action. Clinical endpoints are the most credible characteristics used in the assessment of the benefits and risks of a therapeutic intervention in randomized clinical trials.

Type II biomarker or surrogate endpoint: surrogate endpoints are a subset of biomarkers used to replace a clinical endpoint. They are closer to the exposure/intervention of interest and may be easier to relate casually than more distant clinical events. A surrogate endpoint should predict clinical benefit on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence.

The term surrogate endpoint applies primarily to endpoints in therapeutic intervention trials. In addition, the same biomarkers utilized as surrogate endpoints in clinical trials are often extended to clinical practice in which disease responses are similarly measured. The application of biomarkes as

surrogate endpoints in a clinical research trial is possible, but it requires the specification of the clinical endpoints to substitute, as well as the characteristics of population and disease state in which the substitution is being made.

Although all surrogate endpoints can be considered biomarkers, very few have been clinically validated to the status of surrogate endpoint. In the cardiovascular area, for example, FDA's Center for Food Safety and Applied Nutrition recognizes three surrogate endpoints when evaluating the scientific evidence for a potential health claim and qualified health claim: blood pressure, (plasma and serum) total cholesterol, and low-density lipoprotein (LDL) cholesterol (NIH-FDA 1999).

It should be mentioned that when studying the effect of dietary treatments on disease prevention, type I or type II biomarkers are generally selected and they need to be both significant endpoints (generally intermediate endpoint) related to a pathological condition (i.e. need to have biological plausibility) but also be sensitive enough to detect the modulatory effect, that is generally small, following dietary interventions if compared with pharmacological treatments. This consideration is fundamental and limits sometime the availability of adequate biomarkers to demonstrate preventive roles.

1.3 Biological markers in cardiovascular research

Multiple biological pathways have been implicated in the etiology of chronic and degenerative disease such as CVD, including, but not limited to oxidative stress and inflammation (May &Wang 2008). The recognition of biological markers may help in monitoring the efficacy of treatments, and facilitate the development of pharmacological tools for those subjects at risk of CVD. So, the most promising biomarkers are those that appear to relate closely with the pathophysiological process of the disease. However, due to the complexities of CVD pathogenesis, there is no single biomarker available to estimate absolute risk of future cardiovascular events. In addition, not all biomarkers are equal; the functions of many biomarkers overlap, some offer better prognostic information than others, and some are better suited to identify/predict the pathogenesis of particular cardiovascular events. Here, are reported some of the biomarkers of oxidative stress, inflammation and endothelial function/dysfunction widely used in the research to predict CVD.

1.3.1 Biomarkers of oxidative stress

Oxidative stress is defined as impairment between antioxidant and pro-oxidant species that generates a stress response in cells leading to metabolic dysregulation and alteration in cell signaling and other cellular functions, which are involved in the pathogenesis of various disease states (Betteridge 2000).

Since oxidative stress centrally contributes to CVD, it is extremely important to translate this knowledge into the characterization and identification of biomarkers able to detect the oxidative stress (Lee et al., 2012; Betteridge 2000).

Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chloride species (RCS) in the microenvironment; and molecules of the antioxidant system that change in response to increased redox stress (Roberts & Sindhu 2009).

DNA, lipids (including phospholipids), proteins and carbohydrates are examples of molecules that can be modified by excessive ROS, RNS and RCS *in vivo*.

1.3.1.1 Oxidative DNA damage

Numerous studies have shown that oxidative DNA damage links pathogenically to a variety of aging-associated degenerative diseases such as cancer and CVD (Wu et al., 2004).

Genomic damage can be caused by a variety of physical and chemical agents such as ultraviolet and ionizating radiation, xenobiotics and endogenous ROS that accumulate in cells due to natural metabolic processes. DNA damage occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. Common types of DNA damage are: base loss, base deamination, base alkylation, base dimerization, base oxidation and single/double strand breakage (Saul & Ames 1986). Nuclear and mitochondrial DNA from tissue and blood lymphocytes is usually the site of oxidation damage. The formation of ROS inside the cell can lead to oxidized DNA bases, apurinic/apyrimidinic sites or DNA strand breaks. Among all purine and pyridine bases, guanine is the most prone to oxidation. The most common oxidized base lesion is the highly mutagenic 8-oxo-2'-deoxyguanosine (8-oxo-dG) (Cooke et al., 2001) which can be removed from the 8-oxoGua:Cyt base pair by 8-Oxoguanine DNA glycosylase (Ogg1). If 8-oxoGua is not removed from the DNA, this lesion is mutagenic because of mispairing with adenine. Moreover, 8-oxo-dG is unstable and can react with compounds such as peroxynitrate to even more mutagenic lesions. Urinary 8-OHdG, in particular, has been measured most frequently to indicate the extent of oxidative damage because it is noninvasive and technically less involved (Cooke et al., 2001); but 8-OHdG is also a biomarker reflecting the balance between oxidative damage and repair rate (Wu et al., 2004).

The methods used to estimate 8-oxodG include high performance liquid cromatography-electrochemical detection (HPLC-EC), gas chromatography–mass spectrometry (GC-MS), LC-MS/MS, antibody-based immunoassays, ^{32}P-post-labelling and enzyme-linked immunosorbance assays (ELISA) (Cooke et al, 2006), direct enzymic detection by using bacterial glycosilases and endonuclease enzymes (Collins et al, 1993). Urinary 8-OHdG does not directly reflect DNA oxidation within cells, but it could be used in combination with direct measurements of oxidative damage to DNA. However, a reduction in the excretion could result from either reduced formation of oxidised DNA bases (beneficial) or reduced repair of such damage (detrimental) (Ferguson et al, 2006).

[32]P-post-labelling procedures allow the detection of both 8-oxodG and DNA adducts, but are time-consuming methods (time-span for the analysis is several days) and often characterised by lack of specificity or formation of DNA damage during sample preparation (Cooke et al, 2006).

Levels of 8-oxodG detected by means of ELISA assays are often higher than those obtained by HPLC-based methods, thus they do not represent a good method of detection (Cooke et al, 2006).

The single cell gel electrophoresis (comet assay) represents a relatively simple technique that allows the measure of DNA single- and double-strand breaks, oxidised bases (purines and pyrimidines), as well as resistance to *ex vivo* treatment by hydrogen peroxide or ionising radiation at the level of single cell (McKelvey-Martin et al., 1993).

The measure of oxidized bases can be performed through the use of specific enzymes that are universal in eukaryotes and prokaryotes. For example, E. coli express formamidopyrimidine DNA glycosylase (FPG) for base excision repair of oxidised guanines and this enzyme is often used for detection of such lesions by means of comet assay. The comet assay has also been used to assess DNA repair as rejoining of single- strand breaks and, more recently, to evaluate repair of 8-oxodG (Smart et al., 2006).

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1.3.1.2 Oxidative damage to lipids

Lipids are susceptible targets of oxidation because of their molecular structure often abundant in reactive double bounds. Oxidized LDL, lipid hydroperodixe, malonildialdehyde (MDA), and isoprostanes (F2-IsoP) are products of lipid peroxidation. Compared to others, F2-IsoP are chemically stable end-products and for this reason are the most well studied markers (Ho et al., 2013).

F2-IsoPs are a series of prostaglandin F2a-like compounds produced *in vivo* by non-enzymatic peroxidation of arachidonic acid, esterified in phospholipids and then subsequently hydrolysed to their free acid form by the platelet activating factor acetylhydrolase (Ho et al., 2013).

IsoPs are released from the cell membrane into circulation by phospholipases (Stafforini et al., 2006), and can be quantified in all human tissues and biological fluids, including plasma, urine, cerebrospinal and broncho-alveolar lavage fluid.

Levels of IsoPs in plasma and urine samples have been shown to correlate with *in vivo* oxidative stress in a number of animal and human studies (Fam & Morrow 2003). IsoPs are elevated in association with risk factors such as hyperhomocysteinemia, hypercholesterolaemia, diabetes mellitus, obesity, cigarette smoking (Morrow 2005), as well as myocardial ischaemia/reperfusion (Delanty et al., 1997).

F2-IsoP levels are elevated in human atherosclerotic lesions compared with normal vascular tissue (Gniwotta et al., 1997), and may participate in the actual pathogenesis of atherosclerosis through effects on vasoconstriction, platelet aggregation, and proliferation of vascular smooth muscle cells (VSMCs) (Fam & Morrow 2003).

IsoPs can be measured using GC/MS, LC/MS, ELISA, and radioimmunoassay in plasma and urine samples (Musiek et al., 2005). Moreover, commercial immunoassay kits for IsoPs have been developed; they are cheap and easy to use but have variable performance and results correlate poorly with mass spectrometric techniques (Smith et al., 2011), which still remain the gold standard tecniques for IsoP quantification (Morrow 2005).

Oxidative damage to lipids (i.e. lipid peroxidation) could also be obtained *in vivo* by measuring oxidized low density lipoprotein (LDL) particles. This may be evaluated in blood using immunological methods (i.e. antibodies) with appropriate specificity. Phosphatidylcholine hydroperoxidases measured in blood or tissue by HPLC is also an acceptable marker of lipid peroxidation (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011).

Thiobarbituric acid reactive substances, MDA, high density lipoprotein (HDL)-associated paraoxonases, conjugated dienes, breath hydrocarbons, auto-antibodies against LDL particles and *ex vivo* LDL resistance to oxidation have been proposed, but they are not reliable *in vivo* markers of lipid peroxidation. However, some of these markers can be used as evidence, for example in addition to measurements of F2-IsoP and *in vivo* LDL oxidation, only if appropriate tecniques are used for their analysis. In this regard, concentrations of MDA in blood or tissue must be assessed by HPLC (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011).

1.3.1.3 Oxidative damage to proteins

Proteins are a major target for biological oxidants as a result of their abundance and high rate constants for reaction with many species. Reactions can occur with both the side chains and backbone, with the extent of attack at particular sites dependent on multiple factors. In some cases, damage is limited to specific residues, whereas with other species (e.g., hydroxyl radicals), damage is widespread and nonspecific (Ho et al., 2013).

Direct measurements of oxidative damage to proteins *in vivo*, such as measurements of specific products of amino acid oxidation in proteins after hydrolysis, can be obtained by means of HPLC-

MS. Measures of protein oxidation by-products (protein carbonyls) by using conventional assays, such as colorimetric or ELISA methods are usually susceptible to interferences by molecules other than proteins, and could only be used in combination with at least one direct marker of oxidative damage to proteins *in vivo* if assessed directly in blood or tissue (Ho et al., 2013; Stoner et al., 2013).

Measure of tyrosine nitration have been used as indicator of oxidative stress since the accumulation reflects a loss of balance between oxidant formation and antioxidant defence mechanisms (Peluffo & Radi 2007).

Nitrotyrosine originates as tyrosine in both a free and protein-bound form. The protein-bound form that is involved in atherosclerosis is attached to LDL. This molecule is then nitrated to form the biologically active nitrotyrosine, and occurs in a highly efficient manner in human serum (Gaut et al., 2002). Once modified, the nitrated form of LDL is collected and consumed by macrophages via phagocytosis. The end product of this degradation is the deposition of cholesterol and foam cells during the plaque development.

Several animal studies have linked nitrotyrosine to inflammation (Gaut et al., 2002; Fernandez et al., 2003; Bro et al., 2003). In humans, nitrotyrosine formation has been observed in vascular and myocardial tissue in both healthy individuals and those with CVD (Bertesaghi et al., 2007). However, nitrotyrosine levels are raised in patients with diabetes mellitus (Zou et al., 2004), atheromatous plaques (Pennathur et al., 2004), and in patients with peripheral arterial disease (Da Ros et al., 2003), coronary artery disease (Shishehbor et al., 2003), and myocardial infarction (Feng et al., 2001).

Free nitrotyrosine (3-NO $_2$ -Tyr) can be measured by MS/MS coupled with GC or HPLC as the current gold standard technique (Duncan 2003).

Other ways of quantifying protein nitration are immunocytochemical and immunohistochemical assays based on either monoclonal or polyclonal anti 3-NO $_2$ -Tyr antibodies.

1.3.2 Biomarkers of inflammation

Inflammation is the process of innate immunity in response to physical, physiological and/or oxidative stress and is associated with activation of Nuclear Factor kappa B (NF-κB) signaling pathway, a transcription factor and key mediator in the activation of genes involved in the inflammatory process (Stoner et al., 2013). Generally, NFκB is retained in an inactive form in the cytoplasm by an inhibitor molecule, inhibitor kappa B (IκB) (Hoesel & Schmid 2013). When a pro-inflammatory stimulus or oxidative event occurs, the inhibitor molecule becomes phosphorylated and induces the degradation and liberation of the NF-κB complex in its active form that induce the expression of several inflammatory mediators such as cytokines and chemokines (tumor necrosis factor alpha, TNFα; interleukins, IL-1, IL-6 and IL-8), and adhesion molecules (vascular cell adhesion molecule-1, VCAM-1; intercellular adhesion molecule-1, ICAM-1) (Ho et al., 2013). Once produced, cytokines are rapidly trapped by neighbouring cells via their high-affinity receptors. Measuring the levels of circulating cytokines is not necessarily a perfect surrogate endpoint. Nevertheless, a variety of blood inflammatory markers have been shown to predict future CV risk. Among them, C-reactive protein (CRP), IL-6 and TNF-α are the major inflammatory markers used to predict CVD (Stoner et al., 2013).

1.3.2.1 Tumor necrosis factor alpha

TNF-α is a cytokine with a wide range of pro-inflammatory activities (Vassalli 1992). It is primarily produced by macrophages, but it can derive also by a broad variety of cell types including lymphoid cells, endothelial cells, mast cells, cardiac myocytes, and adipose tissue (Beutler &Cerami 1989). Large amounts of TNF-α are released in response to lipopolysaccharide (LPS), other bacterial products and IL-1. In the skin, mast cells appear to be the predominant source of pre-formed TNF-α,

which can be released upon inflammatory stimulus. TNF-α may influence the atherosclerotic process both by causing metabolic perturbations and by increasing the expression of cellular adhesion molecules. TNF-α induces the expression of chemokines (Rollins et al., 1990), and enhances the production of cytokines and growth factors (Libby et al., 1986). TNF-α also stimulates new vessel formation and induces features characteristic of developing atheroma.

Moreover, disturbances in the TNF-α metabolism have been implicated in metabolic disorders, such as obesity and insulin resistance (Hotamisligil 1994). Increased plasma concentrations of TNF-α have been found in patients with premature, coronary artery disease (Skoog et al., 2002), acute myocardial infarction (Prondzinsky et al., 2012), and peripheral arterial disease (Botti et al., 2012).

1.3.2.2 Interleukin-6

IL-6 is a pleiotropic cytokine produced by a variety of cells including fibroblasts, endothelial cells, mononuclear phagocytes, neutrophils, hepatocytes, lymphocytes (T and B) and neural tissue-neurons (Abeywardena et al., 2009). IL-6 is implicated in the generation and propagation of inflammation by acting on a wide range of tissues influencing cell growth and differentiation, including angiogenesis and also re-vascularization (Barnes et al., 2011). IL-6 also mediates the synthesis and secretion of CRP (Casas et al., 2008), and it plays an important role in the instability of vulnerable plaque and in the endothelial cell activation resulting in an increase in the expression of adhesion molecules (ICAM-1, VCAM-1), release of chemokines (IL-8) (Barnes et al., 2011). Chronically elevated IL-6 levels are common in patients with CVD (Coles et al., 2007), contributing to cell damage, oxidative stress, blood clotting and atherothrombotic events (Yudkin et al., 2000). IL-6 levels are also elevated in patients with stable coronary artery disease (Jha et al., 2010), and acute miorcardial infarction (Kinugawa et al., 2012).

1.3.2.3 C-reactive protein

CRP and high sensitive (hs)-CRP are the most well-known inflammatory markers (Casas et al., 2008). The production of CRP occurs primarily in the liver, by the hepatocytes as part of the acute phase response upon stimulation by IL-6, and to a lesser degree by TNF-α and IL-1ß, originating at the site of inflammation. Among other effects, CRP has been shown to enhance the expression of ICAM-1, VCAM-1, E-selectin in endothelial cells (Pasceri et al., 2001; Pasceri et al., 2000).

CRP has been shown in prospective cohort and case-control studies to be a reliable measure of underlying systemic inflammation and a strong predictor of future cardiovascular events.

In patients with unstable angina, CRP concentrations have been demonstrated as predictors of recurrent cardiac instability (Biasucci et al., 1999). Similarly, CRP would appear to be useful in the diagnostic and prognostic management of peripheral vascular disease (Ridker et al., 1998). Various analytical methods are available for CRP determination, such as ELISA, immunoturbidimetry, rapid immunodiffusion, and visual agglutination.

1.3.3 Biomarkers of endothelial function

Vascular endothelial cells play a major role in maintaining cardiovascular homeostasis. In addition to providing a physical barrier between the vessel wall and lumen, the endothelium secretes a number of mediators that regulate platelet aggregation, coagulation, fibrinolysis, and vascular tone (Frick et al., 2007). Endothelial cells secrete several mediators that can alternatively mediate either vasoconstriction, such as endothelin-1 and thromboxane, or vasodilation, such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF).

Endothelial dysfunction refers to a condition in which the endothelium loses its physiological properties and in particular a decreased bioavailability of NO, a critical endothelium-derived vasoactive factor with vasodilatory and antiatherosclerotic properties (Tatematsu et al., 2007).

The first step in plaque progression occurs when endothelial cells express adhesion molecules upon injury, which facilitates the adhesion of monocytes onto endothelium (Onat et al., 2011). These adhesion molecules may include selectins, which mediate a loose rolling of monocytes on the activated endothelial cells, and more importantly ICAM-1 and VCAM-1, which mediate the firm adhesion (Onat et al., 2011; Li & Glass 2002). The monocytes can subsequently migrate through the endothelium to the intima, accumulate lipids and transform to lipid laden foam cells, which result in the earliest visible atherosclerotic lesions as fatty streaks (Moore & Tabas 2011; Li & Glass 2002). Risk factors, such as hypertension, hyperlipidemia, hyperglycemia and environmental exposure (i.e. smoking), could accelerate the progression of plaque, which involves the migration of not only the macrophage foam cells, but also smooth muscle cells and as well as increased death of foam cells to form the necrotic core (Valledor et al., 2011; Packard & Libby 2008; Li & Glass 2002). Furthermore, the foam cells may also contribute to the plaque progression by amplifying the inflammatory response and lipoprotein modifications (Packard & Libby 2008; Hansson & Libby 2006. All of these changes can lead to the formation of advanced vulnerable plaque, which may rupture, lead to thrombus formation and finally result in CVD (Moore & Tabas 2011; Packard & Libby 2008; Li & Glass 2002).

Thus, the central role of the endothelium in the development of vascular disease has led to the search for biologically relevant biochemical markers to estimate endothelial function and injury. This includes soluble biomarkers and physiological parameters such as measures of arterial stiffness and endothelial function.

Several studies have shown that surrogate markers of endothelial dysfunction such as NO, ICAM, VCAM, E-selectin, and flow mediated dilation (FMD) may serve as potential markers of endothelial damage (Suliman et al., 2006; Wang et al., 2005; Kock et al., 2005)

1.3.3.1 Nitric oxide

NO is one of the endothelial biomarkers which decreases in condition of endothelial dysfunction. NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin (Fostermann & Munzel 2006). This gas diffuses to the vascular smooth muscle cells and activates guanylate cyclase which leads to cGMP-mediated vasodilatation.

When NO forms, it has a very short half life (few seconds) and can react with superoxide to form peroxynitrite, which can generate damage to the cell membrane inducing lipid peroxidation. Superoxide can quench NO thus reducing its bioavailability and induce endothelial dysfunction. In normal vascular physiology, NO plays a key role to maintain the vascular wall in a quiescent state by inhibition of inflammation, cellular proliferation, and thrombosis. This is in part achieved by s-nitrosylation of cysteine residues in a wide range of proteins, which reduces their biological activity (Stamler et al., 2001). The target proteins include the transcription factor NF-κB, cell cycle–controlling proteins, and proteins involved in generation of tissue factor (Gosh et al., 2002).

At present, there are several reasons to believe that *in vivo* NO synthesis from L-arginine could indeed be impaired in atherosclerosis, hypertension, dyslipidemia, diabetes, obesity, insulin resistance, metabolic syndrome, as well as in ageing (Vickers et al., 2009; Park et al., 2009; Njajou et al., 2009; Huang, 2009; Park et al., 2010; Tabit et al., 2010).

Determination of the NO radical is difficult because of its radical nature and very short half-life; so measurements of plasma nitrite, nitrate and nitrosylated proteins are increasingly being mentioned as markers for determining NO bioavailability (Tsikas 2005; Bryan et al., 2004). It has been shown that up to 70–90% of plasma nitrite is derived from eNOS activity in fasted humans (Lundberg et al., 2006). However, circulating levels of nitrites and nitrosylated proteins are difficult to measure (Rassaf et al., 2004; Rassaf et al., 2005), and the values may be perplexed by other sources of NO formation and variation in dietary NO (Lundberg 2006).

1.3.3.2 Cell adhesion molecules

The role of cell adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1), vascular endothelial cell adhesion molecule-1 (VCAM-1) and E-selectin, has been studied extensively in the process of endothelial dysfunction (Ross 1999). The determination in blood is very easy since these molecules can be measured with commercial immunoassays.

Soluble ICAM-1 are expressed on the surface of endothelial cells, leukocytes and smooth muscle cells in reaction to stimuli such as shear stress, bacterial toxins, pro-inflammatory cytokines and oxidants. ICAM-1 can be induced by IL-1 and TNF-α and are expressed by the vascular endothelium, macrophages and lymphocytes. ICAM-1 mediate attachment of circulating leukocytes to the endothelium and their subsequent transmigration and accumulation in the arterial intima (Tailor et al., 2000), processes critical to the development and progression of atherosclerosis (Ross 1999). However, their role in initiation and formation of endothelial lesions is overshadowed by the contribution of VCAM-1 (Cybulsky et al., 2001).

VCAM-1 is expressed on both large and small vessels after the endothelial cells are stimulated by cytokines (Carlos et al., 1990). These molecules facilitate the rolling, adhesion and migration of leukocytes across the endothelial barrier.

In large prospective studies of healthy individuals, sICAM-l, but not sVCAM-1, appears consistently related to incident coronary artery disease (Blankenberg et al., 2003; de Lemos et al., 2000; Ridker et al., 1998). sICAM-l appear as a general marker of a pro-inflammatory status (Blankenberg et al., 2003). By contrast, VCAM-1 is not expressed in baseline conditions, but is rapidly induced by pro-atherosclerotic conditions (O'Brien et al., 1993). Therefore, it seems that sVCAM-1 does not appear as a risk factor in healthy individuals, but emerges as a strong risk predictor in patients suffering from pre-existing disease. Support for a different role of ICAM-1 and VCAM-1 according to the type of population comes from studies on peripheral arterial disease; in healthy individuals, higher levels of ICAM-1 have been shown to predict future cardiovascular events, symptomatic disease (Luc et al., 2003; Pradhan et al., 2002), whereas in patients with established disease, VCAM-1 is a better marker of the extent and severity of atherosclerosis (Jager et al., 2000; Peter et al., 1997).

1.3.3.3 Flow-mediated dilatation, blood pressure and arterial stiffness

Surrogate markers of endothelial dysfunction and atherosclerosis, including blood pressure, brachial artery flow-mediated dilatation (FMD), and arterial stiffness have been increasingly used.

Endothelial dependent vasodilation has been identified as the most common used clinical assessment of endothelial function. These tests evaluate pharmacological and/or physiological stimulation of endothelial release of NO and other vasoactive compounds, and often a comparison of vascular responses to endotheliumin dependent dilators such as nitroglycerine.

Several invasive and non-invasive techniques have been developed to evaluate endothelial function. Invasive techniques, which involve intracoronary or intrabrachial infusions of vasoactive agents, are still considered to be the gold standard for early detection of endothelial dysfunction (Kandhai-Ragunath et al., 2013). Invasive tecniques include intracoronary and intrabrachial infusions of vasoactive agents. Although intracoronary studies are considered to be the gold standard for early detection of endothelial dysfunction in the coronary arteries, they have the disadvantage of being invasive and expensive, with the risks of coronary catheterisation, and cannot be used as a screening test in the general population (Widlansky et al., 2003). For the assessment of pre-clinical disease, the ideal technique for measuring endothelial function must be noninvasive, reliable, reproducible, cheap and easy to perform. For that reason several non-invasive techniques have been developed, with comparable results and good reproducibility (Kandhai-Ragunath et al., 2013). The non-invasive testing are:

-*Ultrasound flow mediated dilatation (FMD) of the brachial artery.* It is the most widely used method for both small and large population studies of adults and children (Leeson et al., 2006). This testing involves measuring the diameter of an artery by noninvasive ultrasound before and after increasing shear stress (provided by reactive hyperemia), with the degree of dilatation reflecting (in large part) arterial endothelial NO release (Joannides et al., 1995). Measurement of ultrasound-based FMD in the brachial artery has intrinsic appeal: it is noninvasive, relatively repeatable and reproducible, reflects important biology, has some data to support its predictability, and is useful in serial studies of disease reversibility (Celermajer et al., 1992). For these reasons this test represents the gold standard for clinical research on conduit artery endothelial biology (Celermajer et al., 1992). Nevertheless, the measurement of FMD by ultrasound has also proven problematic for risk stratification in individuals. The method is technically demanding, requiring specific training and also the care required to minimize the effect of environmental or physiological influences, such as exercise, eating, caffeine ingestion, and important variations in temperature. FMD tests reflect in part the synthesis and bioavailability of NO. Brachial artery FMD has been shown to correlate with measures of coronary endothelial function (Anderson et al., 1995). This technique has been extensively used with very good reproducibility and low observer variability. This technique is excellent for acute interventions with repeated measurements.

-*Pulse amplitude tonometry or peripheral arterial tone (PAT).* Interest has recently grown particularly in testing endothelial vasomotor function after reactive hyperemia by PAT (RH-PAT), as measured in the fingertips (Hamburg et al., 2008). This non-invasive method assesses post-occlusive volume changes at the fingertip and has been shown to identify coronary artery dysfunction and to correlate with brachial artery reactivity testing in adults (Hamburg & Benjamin 2009). There more EndoPAT tests can be carried out in both the office and hospital settings, with patients positioned either sitting or supine. The test takes 15 minutes to complete, is very easy to perform, and is both operator and interpreter independent (Faizi et al., 2009).

Blood pressure (BP) has long been known to be an important risk factor for cardiovascular risk. Risk increases from BP levels 120/80 mm Hg, and there is no level at which the risk suddenly increases. An increase of 20 mm Hg in systolic BP is associated with a 2-fold increase in risk of death from stroke and ischemic heart disease (Lewington et al., 2002). Moreover, risk increases as the level and number of risk factors increases (Kannel 1996). This is especially evident in the case of the metabolic syndrome, which includes impaired glucose tolerance or type 2 diabetes mellitus, high BP (systolic BP >140 mm Hg), obesity, an increase in serum triglycerides and cholesterol (Cohon et al., 2004). While initial work concentrated on diastolic pressure, more recently, it has been recognized that systolic and pulse (difference between systolic and diastolic) pressures play a more important role in endothelial dysfunction (Cohon et al., 2004). Systolic pressure is influenced by arterial stiffness and increases continuously with age.

Measurements of arterial stiffness provide useful information regarding the health of the arterial vasculature. In fact, it provides assessment of both the structure and function of the artery. There are many methods that have been used to measure arterial stiffness in humans (Mackenzie et al., 2002). Large artery stiffness, as assessed by pulse pressure and pulse wave velocity (PWV), is age-dependent and reflects structural alterations in the conduit arteries that are accelerated by hypertension and atherosclerosis. PWV is considered the gold standard method and most consistently predictive of CVD risk (Mitchell et al., 2010). The augmentation index (Aix) is an indirect measure of arterial stiffness and increases with age. It is calculated as augmentation pressure divided by pulse pressure ×100 to give a percentage (Mackenzie et al., 2002). An increased Aix is associated with increased cardiovascular risk (Nürnberg et al., 2002).

1.4 Health effect of polyphenol bioactives: role of anthocyanins in the modulation of cardiovascular biomarkers

Fruit and vegetables are rich sources of numerous phytochemicals with powerful bioactive properties. The protective activity and mechanisms of action of several phytochemicals was evidenced in a large number of studies carried out in cell culture and animal models. However, it is not clear whether the effects in animals can be readily extrapolated to humans because of specie differences, employment of genetically susceptible animals and relative doses of bioactives highly above those typically consumed through the diet by human beings.

Therefore, experimental dietary studies in humans represent the link between nutritional epidemiological research and trials performed in cell-culture systems and animal models. Intervention studies relay on intermediate endpoints related to disease risk by using biological markers that could help in understanding the effect and mechanism of phytonutrients in humans.

In the last years, a great interest has been devoted to the role of a particular class of bioactive compounds, included in the class of polyphenols, named anthocyanins (ACNs).

ACNs provide blue and purple colour to vegetable and fruits, in particular berries, such as cranberries, blueberries, strawberries, blackcurrant and raspberries (Wallace 2011).

ACNs exist in plant predominantly as glycoside conjugates; however, they can be found in different structural arrangement depending on the number of glycosilating sugars, position in the aglycon, and the degree and nature esterification of the sugar with aliphatic or aromatic acids Fig 1.1 (Wang & Stoner 2008).

Fig. 1.1 *Chemical structures of anthocyanidins (Adopted from Wang & Stoner 2008)*

	R1	R2
Delphinidin	OH	OH
Petunidin	OCH_3	H
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
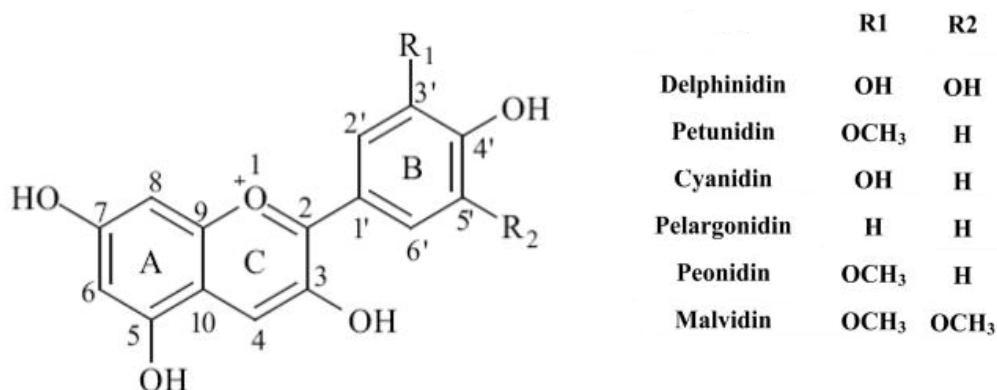
The six ACNs commonly found in plants are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Wallace et al., 2011). The daily intake of ACNs in humans is approximately 180–215 mg/d, with the major sources of ACNs being berries, purple grapes and red wine (Mink et al., 2007).

Their bioavailability is very low and only less than 0.1% of the amount introduced is detected in urine within 24 h of consumption (Croizier et al., 2010). Most of them reach the colon where are extensively metabolized by intestinal microbiota. Various metabolites are formed during the metabolism of ACNs and anthocyanidins (aglycon) and include glucuronides and methylated and sulphated derivatives of ACNs, but also phenolic acids (Del Rio et al., 2010a).

The relation between ACNs intake and CVD mortality was examined in several studies in which was observed a decrease in risk comparing higher with lower intakes (McCullough et al., 2012; Cassidy et al., 2013). In general, the beneficial effects of ACNs on the cardiovascular system have been attributed to their ability to reduce oxidative stress. In addition to the antioxidant effects, several *in vitro* and *in vivo* studies have demonstrated the anti-inflammatory activity and a protective effect on vascular function (Tsuda 2012; Wallace 2011).

1.4.1 Anthocyanins in the modulation of markers of oxidative stress

Antioxidant activity is one of the most important and recognized mechanism exerted by ACN compounds. In fact, several *in vitro* studies documented the capacity of ACNs to modulate markers of oxidative stress by acting as scanvenger of ROS (Zhu et al., 2012). For example, Youdin et al., (2000) found a significant increase of blood cell resistance to H_2O_2-induced ROS production after incubation with phenolic compounds. Acquaviva et al., (2003) documented that incubation with flavonoids, in particular ACNs (cyanidin and cyanidin 3-glucoside: 400µmol/L) inhibited DNA cleavage and was able to scavenge free radicals activity. A protective effect, against free radical species production and DNA damage induced by Ochratoxin A, was demonstrated in human fibroblasts cell culture after addition of cyanidin 3-glucoside (0.125, 0.250 mM) (Russo et al., 2005). Moreover, Lazzè et al., (2003) demonstrated that ACNs have the ability to protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells.
In addition, numerous *in vitro* studies reported the capacity of ACNs to prevent peroxinitrate-induced damage to endothelial cells (Paixão et al., 2012a; Paixão et al., 2012b; Paixão et al., 2011; Serraino et al., 2003), reduce oxidative damage to lipids and lipoproteins (Chen et al., 2010).

Apart from studies *in vitro,* others researchers have examined the impact of polyphenolic compounds on measures of oxidative stress *in vivo*. For example, supplementation of rats with blueberry ACNs reduced isolated red blood cell susceptibility to free radical damage and decreased liver DNA damage in rats, but unaffected the levels of urinary IsoPs (Dulebohn et al., 2008). Harris et al., (2001) reported that 2.5-10% black raspberry supplementation reduced DNA damage in rats treated with carcinogens. In a recent study, the supplementation with a mix of blueberry, strawberry and red raspberry, rich in ACNs, incorporated to the diet (5% w/w) for 12 weeks resulted in a moderate reduction in endogenous DNA adducts in female mice (Aiyer & Kichambare 2008). Ramirez-Tortosa et al., (2005) reported that rats fed a vitamin E–deficient diet for 12 wks and then a diet containing a highly purified ACN- rich extract (1 g/kg diet) for the same period, resulted in a significant reduction of the levels of liver DNA damage estimated as *8*-OHdG and lipid hydroperoxidation in rats. Moreover, the supplementation with lyophilized extract of *Vaccinium ashei* (10%w/w) for a period of 3 weeks, reduced isolated blood cell susceptibility to free radical damage and decreased liver DNA damage in rats (Dulebohn et al., 2008). Barros et al., (2006) demonstrated that the supplementation for 4 weeks with lyophilized extract of different berries (2.6–3.2 mg/kg/day) decreased neural DNA damage in mice. Also in our laboratories, we documented that 8-week supplementation with 8% w/w wild blueberry powder (providing about 24mg ACNs per day) was able to improved DNA resistance against oxidative damage in rat lymphocytes (Del Bo' et al.,2010a).

The effects of ACN-rich foods on oxidative stress biomarkers have been also investigated in different human intervention studies, but results are still inconclusive. Riso et al. (2005) documented that daily intake for 6 weeks of 600 mL of blood orange juice increased DNA resistance to H_2O_2 in a group of healthy female volunteers. Also Bub et al., (2003) documented that the daily dose of 300 mL ACN-rich and flavonol-rich juice for a period of 2 weeks was able to decrease endonuclease III (ENDOIII)-sensitive sites in healthy volunteers. Similar results were also reported by Weisel et al., (2006) that documented a reduction in oxidative cell damage in healthy

subjects after 3-week consumption of ACN and polyhenol-rich juice, and by Sporemann et al., (2008) that reported a significant reduction in the levels of DNA oxidation damage, as well as protein and lipid peroxidation after consuming 200 mL/day of red fruit juice for a period of 4 weeks.

A significant reduction in the levels of lipid peroxidation was observed after regular consumption of blueberries (Basu et al., 2010), strawberries (Basu et al., 2009), pigmented potatoes (Kaspar et al., 2011), lyophilized grape powder (Zern et al., 2005), and concentrated red grape juice (Castilla et al., 2006).

On the contrary, Duthie et al., (2006) documented that the intake of 750 mL/day for 2 weeks of cranberry juice (providing 2.2mg of ACNs and 852 mg of total phenols) had no effect on endogenous DNA damage, ENDOIII sites, and H_2O_2 sensitivity. In addition, urinary 8-oxodG decreased similarly in the test and control group during the intervention period, indicating no specific effect of the juice bioactives (Duthie et al., 2006).

A significant reduction in the levels of urinary 8-oxodG, but not of urinary excretion of IsoPs, was observed after 2 weeks of tart cherry juice (240 mL/day) in a group of older volunteers (Traustadóttir et al., 2009). Another study showed no effect in DNA strand breaks following 2-week consumption of berry dessert (200g/day) in a group of elderly volunteers consumed (Ramirez Tortosa et al., 2004). No effect on strand breaks, as well as ENDOIII and FPG sensitive sites, was also found by Moller et al., (2004) following 3-week supplementation with blackcurrant juice and ACN drink in a group of healthy volunteers.

1.4.2 Anthocyanins in the modulation of marker of inflammation

ACNs can positively modulate inflammatory status by influencing the expression of proinflammatory and anti-inflammatory cytokines, but also to down-regulate the pathways involved in the activation of inflammoty processes.

Direct inhibition of LPS-induced NF-κB transactivation by ACNs was observed in human monocytes (Karlsen et al., 2007). Similarly, red wine has been reported to inhibit NF-κB production of proinflammatory factors in endothelial cells and inflammatory cells (Blanco-Calio et al., 2000; Zern et al., 2003). ACNs may protect against TNFα–induced monocyte chemoattractant protein-1 secretion in human endothelial cells (Suganami et al., 2005). Treatment of endothelial cells with cyanidin 3-glucoside and pelargonidin 3-glucoside has been reported to inhibit the production of cytokines and matrix metalloproteinases (MMP), including MMP-1 and MMP-9 (Atalay et al., 2003). Also black currant extract and cyanidin-3-O-glucoside have shown to inhibit the LPS-induced secretion of IL-6 by human macrophages (Desjardins et al., 2012).

Purified ACN mixture, delphinidin-3-O-β-glucoside and cyanidin-3-O-β-glucoside were shown to inhibit IL-6 and IL-1β-induced CRP production in human hepatocellular liver carcinoma cell line (HepG2) in a dose-dependent manner (Zhu et al., 2013).

Decendit et al., (2013) reported that malvidin-3-O-β glucoside, the major grape ACN, decreased the transcription of genes encoding inflammatory mediators, by the inhibition of TNF-α, IL1, IL-6 expression in human PBMCs. Similar results were also observed in another study in which the anti-inflammatory effect of purple sweet potato leaf extract, rich in cyanidin-3-O-glucoside, was documented in human aortic endothelial cells (Chao et al., 2013).

The anti-inflammatory activity of ACNs and ACN-rich foods was also investigated in several animal models. Decreased TNF-α expression was reported in mice fed a high-fat diet and supplemented with ACN-rich extracts (Lefevre et al., 2008; Tsuda et al., 2003). Addition of 4% w/w whole blueberry powder for 8 weeks to a high fat diet of mice also resulted in reduced expression of TNF- α in adypocytes, although IL-6 expression did not change (DeFuria et al., 2009). Lim et al., (2013) reported that 12-weeks of mulberry fruit extract (rich in ACNs) were able to reduce the levels of CRP, TNF-α and IL-1 in adipose and liver tissue in obese mice. Very recently, Vendrame et al., (2013) documented that 8-week supplementation with 8% wild blueberry diet

(providing about 24mg/day ACNs) was able to decreased plasma concentration of CRP, TNF-α and IL-6 in obese Zucker rats. Moreover, the expression of the same inflammatory markers and NF-kB was down-regulated in both the liver and abdominal adipose tissue in the animal model (Vendrame et al., 2013).

Different studies reported an inverse association between ACN intake and inflammation (Chun et al., 2008). This observation was also confirmed after ACNs and ACN-rich food consumption in human intervention studies. Several NF-κB–related proinflammatory chemokines, cytokines, and mediators of inflammatory responses were shown to decrease in the plasma of healthy adult participants after supplementation with ACNs in parallel-designed, placebo-controlled, clinical trials, suggesting mediated inhibition of NF-κB activation by ACNs *in vivo* (Scarabelli et al., 2009; Karlsen et al., 2007; Kelley et al., 2006). In a recent randomized, double-blind trial, the consumption of a purified ACN mixture (320 mg/day) twice a day for 24 weeks, significantly decreased the levels of serum hs-CRP and plasma IL-1β in hypercolesterolimic subjects (Zhu et al., 2013).

Kaspar et al., documented that 6-week intake of 150 g of cooked pigmented potatoes (rich in ACN compounds) was able to reduce the circulating levels of CRP and IL-6 in free-living healthy subjects. Karlsen et al. (2007) showed significant improvement of plasma risk biomarkers after supplementation with bilberries and blackcurrant ACNs (300 mg/d) in a group of volunteers.

On the contrary, Basu et al., (2010c) reported that 4-week consumption of two cups of strawberry drink did not affect markers of inflammation including C-reactive protein and adiponectin in subjects with metabolic syndrome. Similar result was also observed by Stull et al., (2010), in which no effect of 6-week blueberry bioactives supplementation was observed, and by Hassellund et al., (2013) after 4-week supplementation with ACNs (640mg/day) in a group of pre-hypertensive men.

1.4.3 Anthocyanins in the modulation of markers of endothelial function

The beneficial effect of ACNs in the modulation of endothelial function has been extensively reported in *in vitro* studies.

ACNs treatment was shown to upregulate endothelial nitric oxide synthase (eNOS) in bovine artery endothelial cells, and increase protein level of eNOS in human umbilical vein endothelial cells (Xu et al., 2004; Lazzé et al., 2006). Treatment of rabbit aortic rings with a freeze-dried strawberry extract, rich in ACNs, produced a dose-dependent endothelial relaxation attributed to the phosphorylation of eNOS (Edirisinghe et al., 2008).

Mudnic et al., (2012) documented that the vasodilatory effects of blackberry wine, in addition to their flavonoid and total phenolic content, was most significantly associated with the content of ACNs.

Andriambeloson et al. (1997) clearly have shown the endothelial-NO dependent effect of ACN-enriched blueberry extracts on rat aortic rings, by determining that the delphinidin (but not malvidin or cyanidin), induced an endothelial-NO dependent vasorelaxation completely mediated by NO. Moreover, in another study, Andriambeloson et al. (1998) documented that red wine polyphenolic compounds induced endothelial-NO dependent vasorelaxation in rat aortic rings, by increasing the production and release of NO and cyclic-guanosine 3', 5-monophosphate.

Also Auger et al., (2011) showed that fruit juices and purées caused endothelium-dependent relaxations in the porcine coronary artery, with blackcurrant, aronia, cranberry, blueberry, lingonberry, and grape being the most effective fruits. This effect involved both endothelium-derived NO and EDHF, and appeared to be dependent on the polyphenolic composition rather than on the polyphenolic content.

However, the effects of ACN-rich extracts or single ACNs are not limited to the modulation of vascular reactivity, but also to several other functions and markers such as capillary permeability, platelet aggregation, thrombus formation, vascular cell adhesion molecules, that are directly or indirectly related to endothelial function (Youdim et al., 2000; Youdim et al., 2002; Fitzpatrick et al., 1993; Serraino et al., 2003, Rechner & Knoner 2005; Cimino et al., 2006; Oak et al., 2006 Youdin et al., 2008; Chao et al., 2013).

The effect of ACN-rich foods in the modulation of vascular tone in animal models has been deeply investigated in our laboratories. For example, we recently documented that 7-week wild blueberry supplementation (24 mg/day ACNs) was able to improve vasomotor tone in Sprague-Dawley rats (Del Bo' et al., 2010b). Similar effects were also observed after 8-week wild blueberry enriched diet supplementation in young and adults spontaneously hypertensive rats (Kristo et al., 2011; Kristo et al., 2010; Kalea et al., 2010). We also documented that wild blueberry supplementation influenced the structure and organization of the extracellular matrix that may have implications on the regulation of the vascular tone and LDL binding (Kalea et al., 2005) and may decrease COX-derived vasoconstrictors in spontaneously hypertensive rats (Kalea et al., 2006).
Our results were also corroborated by others studies reported in literature. For example, Mizutani et al. (1999) reported that hypertensive rats fed with red wine polyphenolic compounds for 8 weeks reduced blood pressure and improved arterial biomechanical properties. Benito et al., (2002) reported that flavonoid-rich diets (red wine phenolic compounds) induced endothelium-dependent vasorelaxation in rat aorta through increasing NO production and preserving NO bioavailability.

Unfortunately, very few studies investigated the effect of ACN-rich food consumption on the endothelial function in humans. Most recently, Rodriguez-Mateos et al., (2013) showed that blueberry intake acutely improved vascular function in healthy men in a time- and intake-dependent manner. Dohadwala et al., (2011) documented an improvement of vascular function after the intake of a portion of cranberry juice (480 ml, providing 835 mg total polyphenols, and 94 mg ACNs) in subjects with CVD, while no effect was observed in the same subjects after 4-week cranberry consumption.
Basu et al., (2009) showed that the consumption of 4 cups of freeze-dried strawberry beverage (50 g freeze-dried strawberries, approximately 3 cups fresh strawberries) daily for 8 weeks decreased circulating levels of sVCAM-1 in subjects with metabolic syndrome.
However, most of the beneficial effects on endothelial function derive from studies on polyphenol-rich foods. For example, Chavez et al., (2009) reported that acute consumption of grape polyphenols, equivalent to 1.25 cups of fresh grapes, caused significant improvement of endothelial function and prevented vascular endothelial dysfunction induced after consumption of a high fat meal. On the contrary, Van Mierlo et al., (2010) documented that 2-week intervention periods with grape polyphenols (about 800 mg/day) had no impact on vascular function in healthy men.
Recently, the effect of flavonoid-rich dark chocolate on endothelial function, aortic stiffness and wave reflections were investigated. The results of the study showed, for the first time, that consumption of dark chocolate acutely decreased wave reflection and increased flow mediated dilation after chocolate consumption in healthy adults (Loffredo et al., 2011). Similar results were also obtained in different acute studies after consumption of flavonol-rich cocoa in subjects with endothelial function and dysfunction (West et al., 2013; d'El-Rei et al., 2013)
Moreover, the beneficial effect on endothelial function has been also demonstrated after green tea consumption. Alexopoulos et al., (2008) showed that green tea consumption had an acute beneficial effect on endothelial function in healthy individuals. Oyama et al., (2010) found that green tea catechins improved dysfunctional vessels in smokers by increasing the level of nitric oxide.

1.5 Role of blueberries in the modulation of cardiovascular markers

The consumption of berries fruits (i.e. cranberry, strawberry, raspberry, elderberry, blackberry, black current, and blueberry) and their contribution to improving cardiovascular health is subject of considerable interest. Berries are low in calories and high in moisture and fiber; moreover they are a good source of polyphenols. Their content varies considerably among berries; blueberry and cranberry contain predominantly proanthocyanidin and ACNs, whereas blackberries, black raspberries and strawberries contain predominantly ellagitannins (Seeram 2008). Blueberries, in particular highbush (*Vaccinium corymbosum L.*) and lowbush blueberry or wild (*Vaccinium augustifolium*) are a good source of ACNs. Five major ACNs have been reported: delphinidin, malvidin, petunidin, cyanidin and peonidin found as 3-glucosides, 3-galactosides and 3-arabinosides (Basu et al., 2010b; Bushway et al., 1983).

Therefore, the class of compound present in a particular berry type may contribute significantly to its unique biological properties. In this regard, more research is needed to understand which molecules are effective and what mechanisms are involved in their action (Del Rio et al., 2013; Del Rio et al., 2010b).

Very few studies evaluated the effect of the consumption of blueberry in the cardiovascular area. As of today, the research carried out seems to support that such food is potentially able to improve several functions related to antioxidant status and oxidative stress in humans (Table 1.1), while the results on inflammation and endothelial function are poor and controversial.

This suggests a need to investigate the effect of blueberry on such markers. Several factors such as the amount consumed, time of administration, the type of subjects, and the selection of biomarker could be important determinants that may influence the demonstration that regular consumption of blueberry can lead to improvement of one or more functions of the organism.

Table 1.1 *Summary of blueberry intervention trials (adopted from Basu et al., 2010b)*

Reference	Duration	Study design	Study subjects	Control	Intervention	Significant findings
Pederson et al., 2000	Postprandial	Randomized controlled trial	Nine healthy female volunteers (mean age, 31 ± 2 years)	9% (w/v) sucrose in water (500ml)	500 ml blueberry juice or cranberry juice	Increase in plasma antioxidant capacity, vitamin C and phenols with cranberry juice ($P<0.05$), while no effect with blueberry juice
Van den Berg et al., 2001	Three weeks	Randomized controlled trial	Twenty-two male smokers (mean age, 33 ± 11 years)	Control drink (330ml)	330 ml fruit drink containing 30% clarified blueberry juice concentrate	Increase in vitamin C, and plasma antioxidant capacity ($P<0.05$)
Kay & Holub 2002	Postprandial	Single-blind crossover study	Eight middle-aged male subjects (mean age, 47 ± 2 years)	High-fat meal	High-fat meal supplemented with 100g freeze-dried wild blueberry powder	Increase in serum antioxidant status ($P<0.05$)
Mazza et al., 2002	Postprandial	Single-blind crossover study	Five male subjects (mean age, 47 ± 2 years)	High-fat meal	High-fat meal supplemented with 100g freeze-dried wild blueberry powder	Increase in serum antioxidant status ($P<0.05$)
Bub et al., 2003	Ten weeks	Randomized crossover study	Twenty-seven non-smoking men (mean age, 35 ± 4 years)	None	ACN-rich juice containing aronia, blueberries, and boysenberries in a mixture of apple, mango, and orange juice (76% w/w water); 300ml/day	Decrease in plasma thiobarbituric acid reactive substances; decrease in oxidative DNA damage in PBMC ($P<0.001$)
McAnulty et al., 2005	Three weeks of postprandial	Randomized controlled trial	Twenty smokers (mean age: blueberry group, 26 ± 3.3; control group, 29 ± 4.2 years)	Usual diet and lifestyle with restriction of large amounts of fruits, vegetables and supplements	Acute or daily consumption of 250 blueberries	Decrease in lipid hydroperoxides in blueberry group versus control at 3 weeks ($P<0.05$)
Jensen et al., 2008	Postprandial	Randomized double-blind, placebo-controlled, crossover trial	Twelve healthy subjects (mean age, 19-52 years)	Placebo vegetable capsules (0.5g each)	120ml juice containing acai berry, cranberry, blueberry, wolfberry and bilberry in addition to other fruit juices	Increase in serum antioxidant status and inhibition of lipid peroxidation versus placebo ($P<0.03$)
Stull et al., 2010	Six weeks	Randomized controlled trial	Thirty-two obese, nondiabetic and insulin-resistant subjects (mean age: blueberry group, 54 ± 3 years; control group, 49 ± 3 years)	Control smoothie	Smoothie supplemented with 22.5 g blueberry powder (731 mg total phenolics, 334 mg ACNs). Two portion per day.	Improve in insulin sensitivity in the blueberry group compared to control group ($P<0.05$). No effect on markers of inflammation
Basu et al., 2010b	8 weeks	Randomized controlled trial	Forty-eight obese subjects with metabolic syndrome (4 males and	Control water	Freeze-dried blueberry beverage (50 g freeze-dried blueberries, approximately	Decrease in systolic and diastolic blood pressures, plasma oxidized LDL, malondialdehyde and hydroxynonenal

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			44 females; mean age, 50.0 ± 3.0 y)		350 g fresh blueberries)	concentrations in the blueberry-supplemented group compared to control (P<0.05). No effect on serum glucose concentration and lipid profiles.
Rodriguez-Mateos et al., 2013	Postprandial	Randomized controlled trial	Ten healthy men (mean age, 27 ± 1.3 years)	Control drink	Different blueberry drinks providing (766, 1278 and 1791 mg total polyphenols, equivalent to 240, 400, and 560 g fresh blueberry, respectively).	Improve in endothelial function in the blueberry group compared to control (P<0.05) in a time dependent manner
	Postprandial	Randomized controlled trial	Eleven healthy men (mean age, 27 ± 1 years)	Control drink	Different blueberry drinks providing (319, 639, 766, 1278 or 1791 mg total polyphenols, equivalent to 100, 200, 240, 400, and 560 g fresh blueberry, respectively).	Improve in endothelial function in the blueberry group compared to control (P<0.05) in a dose-dependent manner

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2. EVALUATION OF THE REPRODUCIBILITY OF DNA DAMAGE MEASUREMENTS (BY COMET ASSAY), AS MARKER OF OXIDATIVE STRESS, IN FRESH AND CRIOPRESERVED PERIPHERAL BLOOD MONONUCLEAR CELLS

2.1 AIM OF THE STUDY

Despite the comet assay is widely recognized as a valid technique for analyzing and quantifying DNA damage at the level of the individual cell, only few information is available on the effect of freezing and freeze-thaw process on the levels of DNA damage in human cells before and after storage. This is particularly important in dietary intervention studies where, for logistic reasons, sometimes samples cannot be used immediately and need to be stored even for long time periods. The aim of this task was to verify the reproducibility of the DNA damage measurement (through the comet assay) in samples of peripheral blood mononuclear cells (PBMCs) stored in the freezer and obtained from an intervention study compared to the same marker analysed in fresh samples one year before.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Normal-melting-point (NMP) agarose, low-melting-point (LMP) agarose, ethylenediaminetetraacetic acid (EDTA), Na_2EDTA, TRIS acetate-EDTA-buffer, Dimethyl Sulfoxide (DMSO), Phosphate Saline Buffer (PBS), RPMI-1640 medium, hystopaque 1077, Fetal Bovine Serum (FBS), hydrogen peroxide (H_2O_2), ethidium bromide, N-lauroylsarcosine, triton X-100, trizma, sodium hydroxide, albumin from serum bovine, sodium chloride, were from Sigma (St. Louis, MO, USA). Formamildopyrimidine DNA glycosylase (FPG) enzyme was kindly provided by Prof. Andrew Collins from University of Oslo.

2.2.2 Isolation of peripheral blood mononuclear cells

Venous blood sample (1 x 10mL) was collected from individuals with risk factors for cardiovascular disease by venepuncture. The whole blood (100μL) was gently mixed in micro tubes with 900uL cold RPMI-1640 medium. Then 100μL Histopaque-1077 was carefully added to the bottom of the tube and centrifuged at 200 xg for 4 min. at room temperature. Cells were collected and 1mL PBS solution was used to wash the cells. The samples were then centrifuged for 10 sec at 5000 xg at room temperature to pellet the cells. The supernatants were poured off and pellets resuspended in PBS and immediately used, or resuspended in freezing mix composed of 50% FBS, 40% RPMI, supplemented with 10% DMSO as cryoprotectant, and stored at -80°C for 12 months.

2.2.3 Experimental design

Samples under analysis derived from a previous dietary intervention study in which 20 male volunteers received, for a period of 6 weeks, a wild blueberry or a placebo drink. PBMCs were obtained from blood collected at the beginning and at the end of each experimental period (before/after wild blueberry drink and before/after placebo drink; n= 80 samples) (Riso et al., 2012). The analysis of the levels of DNA damage (endogenously oxidized DNA bases, and the levels of H_2O_2 -induced DNA damage) was performed on fresh PBMCs or cryopreserved cells samples. The latter, an aliquot of cells was gently thawed in a water bath at 37°C and centrifuged in order to remove the media. Cells were put on ice and washed twice; first with PRMI-1640 media and then with PBS. Finally, cells were suspended in 50μl of PBS.

2.2.4 Evaluation of DNA damage through Comet assay

The alkaline comet assay was performed as previously described by Riso et al., (2010) and Guarnieri et al., (2007). Cell suspension was supplemented with low melting point agarose (1.5% w/v) in Tris-acetate EDTA buffer, pH 7.4, at 37°C and immediately pipetted onto 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. The level of oxidized bases in PBMCs was determined as FPG-sensitive sites; the protein detects 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. The cell resistance against oxidatively-generated DNA damage (i.e. strand breaks and alkali labile sites) was measured by treating cells with H_2O_2. Two slides with two gels each and for both the comet protocols were prepared for all the samples. One slide was treated with H_2O_2 (500μmol L^{-1} in PBS, for 5 min), while the other one with FPG enzyme (100ng mL^{-1}, for 45 min at 37°C). The others two slides acted as controls. Figure 2.1.1 summarized the steps of the comet assay for the evaluation of endogenous and H_2O_2-induced DNA damage.

Figure 2.1.1 *Evaluation of endogenous and oxidatively-induced DNA damage (cell resistance against H_2O_2 insult) in PBMCs evaluated by comet assay*
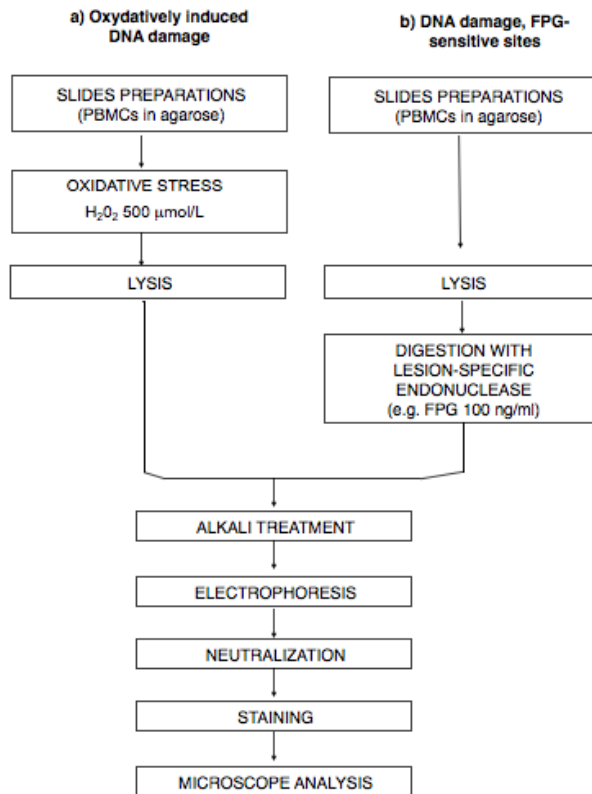
Slides were placed in lysis buffer (2.5M NaCl, 0.1M Na_2EDTA, 10mM tris 1% TRITON x-100, 1% dimethyl sulfoxide, 1% N-Lauroylsarcosine sodium salt, pH10) for 1 h at 4°C in the dark, and then were left for 40 min in the electrophoresis buffer (0.3M NaOH, 1mM Na_2EDTA, 40min at 4°C in the dark). Electrophoresis was performed at 1.1 V/cm^2 for 20 min. Slides were successively neutralized (0.4M tris-HCl, pH 7.5) for 15min at 4°C in the dark, stained with ethidium bromide (2μg mL^{-1}), washed in PBS, drained, and covered with cover slips. One hundred cells 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 (not treated with H_2O_2 or not incubated with FPG) was subtracted from the percentage DNA in tail of H_2O_2-treated or FPG incubated cells, respectively.

2.2.4 Statistical analysis

Statistical analysis was performed using STATISTICA software (Statsoft Inc, Tulsa, OK, USA). Two-way ANOVA was used to verify the effect of protocol (independent factor) on FPG-sensitive sites and oxidatively induced DNA damage in PBMCs before and after wild blueberry or control treatment (dependent factor). Differences between means were evaluated by the least significant difference (LSD) test. Differences were considered significant at $P \leq 0.05$.

2.3 RESULTS AND DISCUSSION

Oxidatively damaged DNA is one of the most widely measured biomarkers of oxidative stress. The comet assay offers a number of advantages for the detection of DNA damage. In fact, it is rapid, simple, and relatively inexpensive to perform (McKelvej-Martin et al., 1993); it allows the collection of data at the individual cell level and requires a relatively small number of cells (< 10.000) in each sample (Fairbairn et al., 1995). Moreover, comet assay can be used for analysis of all types of eukaryotic cells, both *in vitro* and *ex vivo* (McKelvej-Martin et al., 1993). In addition, comet assay shows a high sensitivity for the detection of DNA damage (Fairbairn et al., 1995) and for these reasons is widely used not only in human biomonitoring studies, but also to evaluate the detrimental effects of pollutants and environmental contaminants (Kassie et al., 2000; Moller 2005; Valverde M & Rojas 2009; Azqueta et al., 2009). In the last years, a great interest has been turned to the role of the comet assay as tool to study the influence of diet on DNA stability and human health (Riso et al., 2012; Riso et al., 2010; Lampe et al., 2009; Brivida et al., 2008; Brivida et al., 2007; Moller et al., 2004; Collins et al., 2001).

Two approaches of the assay are commonly used in dietary intervention studies. One it is the measurement of oxidized bases generated by the use of selected enzymes able to detect oxidized pyrimidine and purine bases. The FPG protein detects 8-oxodG and ring-opened formamidopyrimidine nucleobases. The analysis of oxidatively damaged DNA has been thoroughly validated in inter-laboratory trials and showed generally good dose-response relationship with agents that preferentially generate 8-oxodG (Ersson et al., 2013). The other approach is to study DNA *ex vivo* resistance to oxidative stress by incubating the cells with hydrogen peroxide (Duthie & Collins 1997).

One of the most problematic issues of comet assay in human trials concerns the adequate experimental design of the studies. Factors such as age, gender, ethnicity, lifestyles of the participants should be standardized since they are potential confounding factors (Moller et al., 2000). However, also the type of cells and the operational aspects may increase the variability. PBMCs provide a convenient and readily available source of human material and are routinely used experimentally in the comet assay (Duthie et al., 2002). Cryopreserved PBMCs seem a good alternative to fresh sample in clinical trials, especially when samples are collected at multiple steps. However, the isolation, freezing and thawing of samples seems to be potential bias since can affect the antioxidant capacity of cells.

In this regard, no information is reported on the variability of the levels of DNA damage in cells when comparing fresh versus cryopreserved PBMCs, in particular after a dietary intervention trial.

In present study, it has been evaluated the reproducibility of the levels of DNA damage, evaluated as endogenous and oxidatively induced strand breaks in PBMCs, following 12 months of storage period at -80°C.

Samples were obtained from a previous trial in which volunteers received, for a period of 6 weeks, a wild blueberry or a placebo drink (Riso et al., 2012). We documented that 6-week WB drink consumption reduced the level of oxidized DNA bases and increased the protection from the *ex vivo* H_2O_2 induced DNA damage in fresh PMBCs. In particular, estimation of oxidized purines in PBMC DNA through quantification with the formamidopyrimidine DNA glycosylase (FPG)-sensitive sites indicated a statistically significant decrease following the WB drink (-23.2%, p≤0.01) with respect to the PL drink (-0.83%, p=0.89). Moreover, the intake of the WB drink significantly decreased the levels of H_2O_2-induced DNA damage (-18.8%, p≤0.01), while no effect was observed after the PL drink (-1.11%, p=0.84).

In Tables 2.1.1 and 2.1.2 are reported the results on the levels of FPG sensitive sites and H_2O_2-induced DNA strand breaks obtained in stored and fresh cells. In general, a small but significant effect of cryopreservation on DNA integrity has been observed. In particular, cryopreservation increased the levels of DNA damage in untreated cells (control cells: without

Table 2.1.2 *Effect of fresh versus cryopreserved on background and H$_2$O$_2$-induced strand breaks in PBMCs evaluated trough the comet assay (N=16).[1]*

DNA damage	Before WB	After WB	Before PL	After PL
Fresh samples (N= 16 subjects)				
Background SBs (% DNA in tail, PBS)	$6.1 \pm 1.1^{a,C}$	$6.4 \pm 1.1^{a,C}$	$6.1 \pm 1.3^{a,C}$	$6.5 \pm 1.4^{a,C}$
Net H$_2$O$_2$-induced DNA damage (% DNA in tail)	$44.7 \pm 7.5^{a,E}$	$36.3 \pm 9.3^{b,E}$	$46.1 \pm 11.5^{a,E}$	$45.9 \pm 7.1^{a,E}$
Cryopreserved samples (N= 16 subjects)				
Background SBs (% DNA in tail, PBS)	$10.5 \pm 5.0^{a,D}$	$9.7 \pm 4.4^{a,D}$	$11.8 \pm 6.0^{a,D}$	$10.1 \pm 4.2^{a,D}$
Net H$_2$O$_2$-induced DNA damage (% DNA in tail)	$22.7 \pm 6.1^{a,F}$	$19.1 \pm 7.0^{b,F}$	$21.3 \pm 6.4^{a,F}$	$20.6 \pm 6.8^{a,F}$

[1]*Data are expressed ad mean $\pm$ SD. WB, wild blueberry; PL, placebo; SBs, strand breaks; PBS, phosphate buffer saline; EB, endonuclease buffer.*
[a,b]*Data with different letters within the same row are significantly different (P$\leq$0.05)*
[(C,D);(E,F)]*Data with different letters between the rows are significantly different (P$\leq$0.05)*

2.4 CONCLUSIONS

In conclusion, this study documented that the cryopreservation protocol used is associated with DNA damage in PBMCs. The response of the dietary treatment to levels of oxidized bases, measured as FPG-sensitive sites, seem comparable in fresh and frozen PBMCs even after 1 year storage. On the contrary, cell protection against oxidative insult is consistently different between fresh and frozen cells; thus stored cells can be successfully used for the detection of FPG-sensitive sites, that but not for the resistance against oxidative insult. By considering the results obtained, fresh cells represent the best samples to use for studying the effect of dietary intervention through comet assay.

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3. EVALUATION OF THE INTER-DAY REPRODUCIBILITY OF PERIPHERAL ARTERIAL FUNCTION MEASURED THROUGH ENDO-PAT2000 TECHNOLOGY

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3.1 AIM OF THE STUDY

To assess the effect of an intervention on endothelial function, reliable methods are needed. In this regard, several invasive and non-invasive techniques have been developed during the last years to evaluate endothelial function. Reactive hyperaemia peripheral arterial tonometry (RHI-PAT) may be considered a valid non invasive approach to measure endothelial function, since it is reported to be correlated significantly with the most used frequently method called flow mediated dilation (FMD) assessed by brachial artery ultrasound scanning (Flammer et al., 2012; Santos-Garcia et al., 2011; Stout 2009; Bonetti et al., 2004). However, information on the between-day and within-day variability of PAT technique should be evaluated and these studies are lacking and conflicting for this technology. The aim of the present study was to determine the inter-day variability of the PAT measure.

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3.2 MATERIALS AND METHODS

3.2.1 Subjects recruitment

A homogeneous group of thirteen subjects, recruited from the student population of the University of Milan, were selected according to the following criteria: 20-30 years of age, moderate smoking (about 15 cigarette/day; smoking from at least 5 years), moderate physical activity (25-30 min per day of brisk walk or jog) and moderate alcohol consumption (up to 10-14 drinks per week). Subjects were selected on the basis of an interview by a dietitian to evaluate their dietary habits. Subjects declared no history of cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases, anemia, chronic asthma, allergy, traumas of the arms or hand, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations and chronic backache. Moreover, subjects were supplement/drug/ medication free for at least one month before the beginning of the study.

3.2.2 Experimental design

Subjects came in the laboratory in the morning, and the RHI was tested on two consecutive days. Following overnight fasting, the subjects were allowed to have a light breakfast before 7 am on each of the study days. Tests were performed in a dark, noise-and climate-controlled (22-24°C) room, at the same hour.
Moreover, on a subgroup of 5 people, RHI measurement was repeated, on the same day, for three times. The analysis was performed at baseline and after 1h, 2h and 3h.

3.2.3 Evaluation of peripheral arterial function

Endothelial-dependent vasodilation in the small finger arteries was assessed by Endo-PAT2000 (Itamar Medical Ltd., Caesarea, Israel).
The Endo-PAT equipment consists of two finger-mounted probes, which include a system of inflatable latex air-cushions within a rigid external case; pulsatile volume changes of the fingertip are sensed by a pressure transducer, located at the end of each probe, and transferred to a personal computer where the signal is band pass-filtered (0.3 to 30 Hz), amplified, displayed, and stored. For the evaluation, subjects are in the supine position and both hands on the same level in a comfortable, thermoneutral environment. Arterial systolic and diastolic blood pressure and heart rate frequency are measured before starting the test. A blood pressure cuff is placed on one upper arm (study arm), while the contralateral arm served as a control (control arm). After a 10-min equilibration period, the blood pressure cuff on the study arm is inflated to 60 mmHg above systolic pressure for 5 min. The cuff is then deflated to induce reactive hyperemia (RH) while the signals from both PAT channels (Probe 1 and Probe 2) are recorded by a computer. The technique provides values for the calculation of Reactive Hyperemia Index (RHI), which gives an indication of the endothelial vasodilator function. The Reactive Hyperemia Index (RHI) is a ratio of the post-to-pre occlusion PAT amplitude of the tested arm, divided by the post –to-pre occlusion ratio of the control arm.

3.2.4 Statistical analysis

Statistical analysis was performed using STATISTICA software (Statsoft Inc, Tulsa, OK, USA). Distribution of data was checked for normality by Shapiro-Wilk test. Analysis of variance was used to identify differences between-day (day 1 vs day 2) of RHI. Individual subject's CV (%) of RHI measured inter-day was used to determine the variability of the measure. Moreover, one way ANOVA, with time as dependent variable, was used to ascertain the difference in RHI values within the same day.

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3.3 RESULTS AND DISCUSSION

Endothelial nutrition is a new and innovative concept that involves the study of the role of dietary compound on endothelial function. Preventing the endothelium from becoming dysfunctional with nutrients or non-nutrients that modulate vascular tone and maintain homeostasis of the endothelium is of importance to human health. Invasive techniques, which involve intracoronary or intrabrachial infusions of vasoactive agents, are still considered to be the gold standard for early detection of endothelial dysfunction (Higashi & Yoshizumi 2003). However, when studying the effect of a dietary intervention on endothelial function, the ideal technique for measuring must be non-invasive, reliable, reproducible, cheap and easy to perform.

The most popular clinical method to assess vascular endothelial function is FMD by means of brachial artery ultrasound scanning (BAUS) (Onkelinx et al., 2012; Corretti et al., 2002). This technique, recommended by the international Brachial Artery Reactivity Task Force, is based on the percent change of the brachial artery diameter (%FMD) caused by reactive hyperaemia (Corretti et al., 2002).

Recently, an alternative method named Endo-PAT (peripheral arterial tone), has been developed and widely used to identify patients with coronary endothelial dysfunction. Endo-PAT showed a significant correlation with FMD ($r = 0.55$, $p < 0.0001$) and has the advantage of being operator independent (Patvardhan et al., 2010; Hamburg & Benjamin 2009).

When studying the effect of an intervention on endothelial function, the between-day variability of this technique should be evaluated and studies are lacking for the PAT method.

The main finding of the present study was the inter-day reproducibility of the RHI measure. In Figure 3.3.1A are reported the values of RHI obtained in two consecutive days in each subject. The average data obtained from the two PAT measures (2.03 ± 0.30 at day 1 and 2.03 ± 0.29 at day 2) did not demonstrate ($p=0.94$) a difference on vascular function. Intra-subject variability of RHI presented as CV (%) was very low and around 4% (Figure 3.3.1B).

Fig. 3.3.1 *(A) Individual values of RHI measured in two consecutive days; (B) intra-subject variability of RHI*
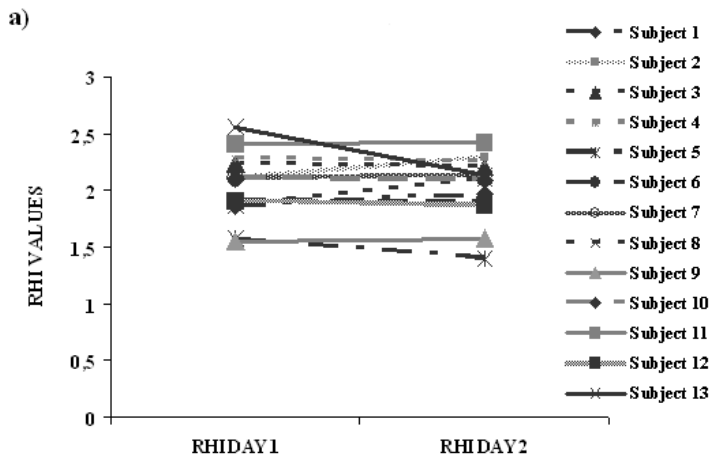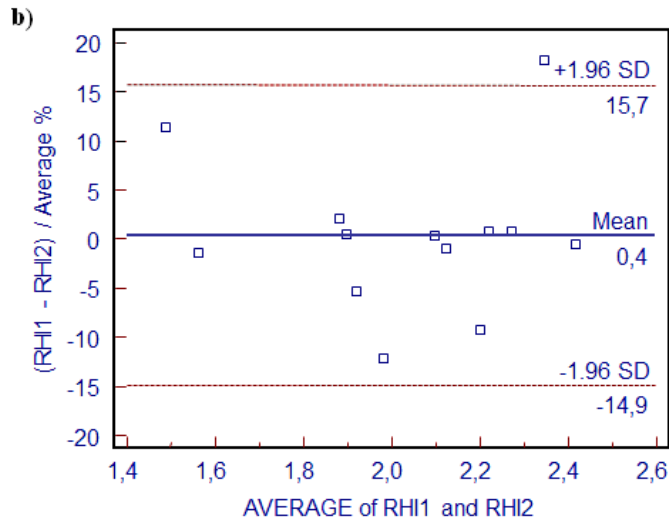
RHI, reactive hypermia index

      Missing information regards also within-day variability of RHI following a multiple measurement. In this regard, the International Brachial Artery Reactivity Task Force suggested that intra-day multiple measurements may promote vasodilation through NO production (Corretti et al., 2002). This effect may eventually mask improvement of vascular function due to the treatment under study (i.e. overestimation).

In the present study, we determined the within-day variability of PAT by repeating the measurements after 1h on the same day. Multiple measurements seemed to increase RHI for the tests at 1, 2, and 3 h with respect to baseline in all the subjects (Figure 3.3.2A). The raise was significantly different as documented by one-way ANOVA ($p = 0.002$; Figure 3.3.2B).

Fig. 3.3.2 *Mean (A) and individual values (B) of the reactive hyperimia index (RHI) following multiple measurements within-day*
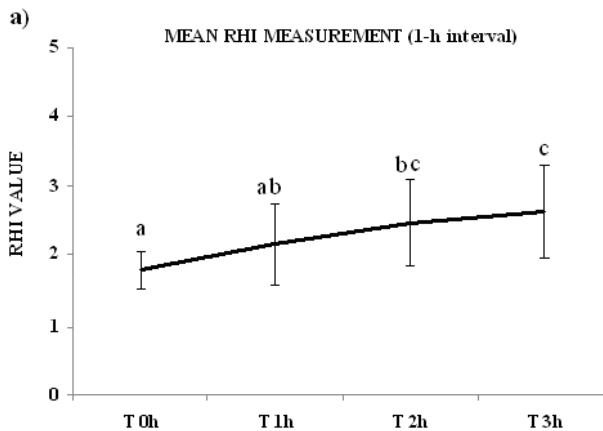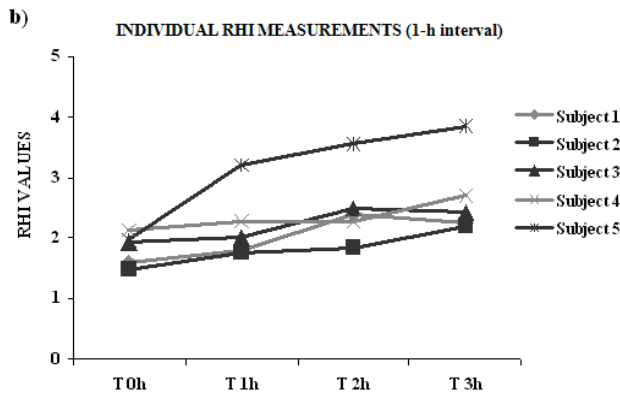
RHI, reactive hypermia index
[a,b,c]Data with different letters are significantly different (P<0.05)

In particular, RHI increased significantly after 2 h (+38.2%; p=0.002) and 3h (+47.5%; p=0.0005) with respect to time zero, and for time 3h also significantly different compared to 1h (+22%; p=0.02). An increase, even if not significant, was also observed at time 1h with respect to baseline (+21%; p=0.057). Thus, it seems that the time-period among subsequent measurements is an important variable in this type of assessment and it should be seriously considered to avoid cross-over effects.

These data seem in accordance with the observation of Liu et al., (2009) which documented a significant increase in the RHI when the PAT was measured at 0.5-hour intervals (for 2.5 h) indicating a crossover effect, but not at 1 h intervals (for 4 h) and 2 h intervals (for 12 h) in healthy male subjects. In addition, Forchhammer et al., (2012) demonstrated intra-day reproducibility in a group of healthy subjects whose vascular function was measured in four different occasions (in the morning, before and after lunch, in the afternoon) within the same day.

3.4 CONCLUSIONS

In conclusion, the results of the research documented a high inter-day reproducibility of RHI values measured through PAT technology. These results are important since they confirm the reliability of the instrument and of the outcome obtained in different days. On the contrary, the variability of the repeated measurement of endothelial function at intervals of 1h was very high. This could prejudge the PAT system for repeated measurements in a relatively short time frame. This information is extremely important in many clinical, pharmacological or dietary intervention studies where the repeated measures are performed. Thus, further investigations are needed in order to clarify the intra-day reproducibility of the PAT technology to prevent a crossover effect.

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4. STUDY OF THE BIOAVAILABILITY OF ANTHOCYANINS FROM TWO DIFFERENT BLUEBERRY PRODUCTS (UNBLANCHED VS BLANCHED BLEUEBRRY PURÉE) IN HUMAN VOLUNTEERS

4.1 AIM OF THE STUDY

Blueberries contain several phenolic bioactive compounds, such as ACNs, that play a potential beneficial role in human health (Del Rio et al., 2010; Chong et al., 2010; Wang & Stoner 2008). The health benefits of ACNs are strictly dependent on their absorption, metabolism, distribution and excretion. Studies focusing on ACN absorption have shown that their bioavailability is very low, and less than 1 % of consumed amounts of ACNs (180-215 mg/day) is generally absorbed, and their maximum concentration in the blood is in the order of nanomolar levels (Yang et al., 2011; Milbury et al., 2010).

Some studies have reported that the inclusion of a blanching preconditioning step, applied to inactivate oxidative enzymes before tissue mechanical processing, may improve ACN recovery in berry products, such as blueberries (Syamaladevi et al., 2012; Brambilla et al., 2008; Rossi et al., 2003; Skrede et al., 2000). Whole fruit thermal processing may influence not only phenolic abundance but also food matrix environment and microstructure with possible implications on the bioaccessibility of phenolic compounds (Brambilla et al., 2011; Yang et al., 2011; Parada & Aguilera 2007). Since the influence of food processing on ACN absorption rate has been poorly investigated, the aim of this study was to examine whether blanching whole berries could affect the absorption of ACNs from two blueberry purées in healthy human volunteers.

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4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Standards of cyanidin (Cy)-, delphinidin (Dp)-, peonidin (Pn)-, petunidin (Pt)- and malvidin (Mv)-3-O-glucoside (glc), -3-O-galactoside (gal), and Cy- and Mv-3-O-arabinoside (Ara) were purchased from Polyphenols Laboratory (Sandnes, Norway). Folin-Ciocalteau reagent and standards of gallic acid, chlorogenic acid and cyanidin-3,5-diglucoside (CydG), as ACN internal standard (IS) were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau was from Fluka Biochemicals. Sodium carbonate, sodium acetate, metaphosphoric acid (MPA), potassium chloride, potassium metabisulfite, hydrochloric acid, methanol, acetonitrile, acetone, formic acid and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA).

4.2.2 Blueberry products

Thirty kilograms of blueberry fruit (Vaccinium corymbosum L., cv. Brigitta) were cleaned, individually quick frozen (IQF) in a tunnel (Thermolab, Codogno, Italy) operating at −50 °C air temperature and 4.5 m/s air speed, divided into three lots (L1, L2, L3) of 10 kg each and stored at −20 °C for 1 month until analysis (L1) and processing (L2, L3). Each lot of berries was divided into three aliquots (3.3 kg/aliquot) and processed into three trials (one trial/day). L2 was processed into purée (P) after partial thawing at 20°C for 1 h (NB) while L3 was immediately processed after steam-blanching for 3 min and tap water-cooling in a pilot steam blanching tunnel (Ghizzoni Dante and Figlio, Felino, Parma, Italia).12 Purées were prepared by homogenizing berries for 1 min in a commercial food processor (Moulinex, Paris, France). Then, 300 g aliquots of purées were packed in 400-mL plastic vessels, sealed under partial vacuum using a 25 ìm thick polypropylene film and frozen within 20 minutes at −20 °C. From each lot of processed berries, thirty purée servings were obtained, randomly divided into two groups and assigned to biochemical analysis (10 servings) and absorption trials (20 servings).

4.2.3 Subject recruitment

Ten healthy male subjects, aged 20.8 ± 1.6 y with body mass index (BMI) 22.5 ± 2.1 kg/m^2, were recruited from the student population of the University of Milan according to the following inclusion criteria: no smoking; no history of cardiovascular, diabetes, hepatic, renal, or gastrointestinal diseases; and not having taken any supplement, drug, or medication for at least one month before the beginning of the study. Subjects were selected on the basis of an interview to evaluate their dietary habits and ensure that they were as homogeneous as possible, in particular for fruit and vegetable consumption. This was obtained by means of a food frequency questionnaire previously published and specifically revised to focus on food sources rich in antioxidants (Porrini et al., 1995). Exclusion criteria were: high (> 5 portions/day) or low (<2 portions/day) intake of fruit and vegetables, habitual alcohol consumption (< 3 drinks per week were tolerated). Volunteers who followed a specific diet (e.g., vegetarian, vegan, or macrobiotic) and those who had a specific aversion for blueberry consumption were excluded. The inclusion criteria were: sex (male), ethnicity (Western European), BMI within normal range (from 19 to 25 kg/m^2), age (from 19 to 30 years), physical activity (more than 30 minutes/day and from 2 till 4 days per week) and dietary habits as homogeneous as possible. On the basis of the questionnaire, ten volunteers were selected. All participants gave informed consent and the study was approved by the Ethics Committee of the University of Milan.

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4.2.4 Experimental design

Subjects were deprived of ACN-rich food sources 10 days before experimentation. Each volunteer received a complete list of foods to be avoided; the list included ACN-rich foods such as berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants, elderberries) red wine and red/violet fruits (i.e. grapes, cherries, pomegranates, red apples, plums, eggplants, tomatoes, peppers), and other colored products (i.e. marmalade, jams, juices containing berries). The repeated measure crossover design was chosen to limit inter-individual variability. Subjects were randomly divided into two groups of five subjects each and consumed a portion of blanched-purée (BL-P) or unblanched-purée (NB-P) in the following order: group 1 was assigned to the sequence BL-P/wash-out/NB-P, whereas group 2 followed the sequence NB-P/wash-out/BL-P. The two experimental treatments were separated by a 10 day wash-out period. The breakfast, lunch and dinner was standardized one day before the experiment and up to 48 days after purées consumption. Breakfast consisted of milk and biscuits (i.e. shortbread) while lunch consisted of two sandwiches (one with cooked ham and cheese and one with raw ham), For the dinner subjects could eat as first dish (pasta or rice with butter and cheese) and a steak with potatoes and two slices of white bread. Coffee, tea, wine, beer and chocolate were not allowed. Moreover, subjects were asked to exclude all ACN-containing foods as previously reported. On the scheduled day, after an overnight fast, subjects consumed a single dose of thawed NB-P or BL-P, providing approximately 300 mg of ACNs. The purées were consumed early in the morning and blood was collected by a phlebotomist at time 0 (before the consumption of blueberry purées) and after 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h and 24 h after BL-P or NB-P consumption. Blood samples were drawn into evacuated tubes with heparin as anticoagulant. Urines were collected 24 h before the experiment and then 24 and 48 h after BL-P or NB-P intake. To check for compliance to the dietary instructions, one day food record was kept by subjects 2 days before and 1 day after the intake of the blueberry purées. Moreover, a direct interview was scheduled with a dietitian.

4.2.5 Phenolic analysis of the blueberry purées

For each NB and BL purée samples, two vessels (2x300g) were thawed and analysis was conducted in duplicate on the product. Phenolic compounds were extracted in duplicate with a formic acid–water (5:95 v/v) solution according to Brambilla et al., (2008). A subsample (15 g) of each blueberry product was homogenized with the extraction solution (25 mL) and left at room temperature for 20 min in the dark before centrifuging at 6000 rpm for 5 min (PK 130 Centrifuge, ALC International, Milan, Italy). The pellet following centrifugation was washed with 25 mL of the extraction solution and centrifuged at 6000 rpm for 5 min and the resulting supernatant was combined with the initial one.

Spectrophotometric analysis of total phenolic content (TPC), monomeric anthocyanin pigment (MAP) and polymeric color (PC %) was carried out on the extracts with a UV4 UV/vis spectrometer (Unicam, Cambridge, U.K.). Total phenolic content, expressed as gallic acid equivalents (GAE), was measured by Folin-Ciocalteau assay; MAP, expressed as cyanidin 3-glucoside equivalents (C3GE), was measured by a pH-differential method and PC % was measured by a bisulfite bleaching method (Singleton & Rossi 1965; Giusti & Wrolstad 1991). Chlorogenic acid and individual ACN compounds were analyzed by gradient-RP-HPLC and diode array detection according to Brambilla et al., (2008). The chromatographic system consisted of a PU 1580 pump (Jasco Co., Tokyo, Japan), a 250×4.6 mm i.d., 5 µm, Inertsil ODS-3 column, heated at 40°C, and a Jasco MD 2010 Plus photodiode array detector. The mobile phases consisted of acetonitrile and water/formic acid (90:10, v/v) and elution was performed at a flow rate of 0.5 mL min^{-1} by linear gradient steps. All the ACN monoglycosides

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were expressed as C3GE and chlorogenic acid was expressed as mg 100g^{-1} FW by measuring detector response to the commercial standard.

4.2.6 Vitamin C (ascorbic acid) analysis of the blueberry purées

The ascorbic acid was extracted and determined by HPLC analysis according to Riso et al., (2005). Briefly, a sample (5 g), in duplicate, of blueberry purées (BL-P and NB-P) was homogenized and suspended in 10 mL of MPA (10 %), centrifuged at 3000 × g for 1 minute, filtered and injected for HPLC analysis. Ascorbic acid was determined by means of a chromatographic system consisting of a model 510 system pump (Waters), a 5 µm Atlantis C18 column (250 x 4.6 mm i.d.; Waters, Ireland) and an UV-Vis detector (Varian 9050). Samples were eluted with a mobile phase of 0.1% formic acid (1.4 mL/min) and the detection was achieved at 245 nm. The volume of injection was 50 µL. Chromatographic data were acquired by a Millenium 4.0 Workstation (Waters).

4.2.7 Plasma separation

Plasma was separated within 30 minutes after collection by centrifugation for 15 min at 2300 × g at 4°C. Two aliquots (1 mL) were acidified with TFA (1 %) to preserve ACN stability, centrifuged at 4500 × g for 1 minute and the supernatants were stored at −80 °C for no longer than 2 months.

4.2.8 Anthocyanin extraction and analysis in plasma

Anthocyanins were extracted from plasma using a Micro-Plate solid phase extraction (SPE) HLB Oasys Cartridge preactivated with methanol (500 µL) and washed with 500 µL acidified water (1% TFA). Plasma (400 µL) was diluted with 140 µL of acidified water (1% TFA) and 60 µL of water containing Internal Standard (50 ng mL^{-1} of CydG) used to correct the loss of ACNs during sample preparation. Samples were vortexed, centrifuged and loaded onto the cartridge. Samples were drained under gravity and the cartridge was washed with acidified water (100 µL; 1% TFA) and 100 µL of water-MethOH (80:20 v/v) acidified (TFA 0.1%). The ACNs were eluted from the cartridge using 50 µL of methanol containing TFA (0.1%) in order to concentrate 8 times the amount of ACNs in plasma. The filtered sample was collected and 20 µL were injected into an UHPLC MS/MS system for the analysis of individual ACNs, according to a method previously published Del Bo' et al., (2010). The lower limit of detection was 1 ng mL^{-1} for Mv-glc, 4 ng/mL for D-glc, and about 2 ng mL^{-1} for the other ACNs. For detailed information see Del Bo' et al., (2010).

4.2.9 Hippuric acid extraction and analysis in urine

Urine (0.2 mL) was diluted with 1.8 mL of acidified water (0.1 % formic acid) and centrifuged at 900 × g for 10 min. The supernatant was collected and 20 µL were injected into the UHPLC system for the analysis according to a method previously published (Del Bo' et al., 2010).

4.2.10 Statistical analysis

Statistical analysis on plasma samples was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US), whereas that of the biochemical data on blueberry purées was carried out using Statgraphics version 7 (Manugistic Inc., Rockville, MD, USA) software package. Data obtained on plasma levels of ACNs were analyzed by a two-way ANOVA for repeated measures design, using the type of product and time as dependent factors. Differences

between means were further analyzed by the Least Significant Difference (LSD) test. Differences were considered significant at P ≤ 0.05. Data of physicochemical parameters of berries and blueberry purées, ACNs and phenolic composition were analyzed by one way analysis of variance (ANOVA procedure) and means were compared by Tukey's test (P ≤ 0.05).

4.3 RESULTS AND DISCUSSION

4.3.1 Composition and characteristic of the blueberry purées
The ACN and phenolic profiles of the purée are reported in Table 4.3.1.

Table 4.3.1 - *Composition of the unblanched (NB-P) and blanched (BL-P) blueberry purées. Data are expressed as mean±SD.*

	NB-P	BL-P
TPC (mg GAE/100g FW)	242.43 ± 23.91^{b}	285.17 ± 11.17^{a}
MAP (mg C3GE/100g FW)	88.78 ± 2.59^{a}	92.73 ± 2.84^{a}
PC (%)	7.52 ± 1.61^{a}	2.51 ± 0.47^{b}
Chlorogenic acid (mg/100g FW)	30.09 ± 1.24^{b}	40.51 ± 3.21^{a}
Total anthocyanin (mg C3GE/100g FW)	116.12 ± 6.98^{a}	119.48 ± 6.51^{a}
Dp-3-gal	19.04 ± 2.04^{b}	26.76 ± 2.83^{a}
Dp-3-glc	0.57 ± 0.11^{a}	0.41 ± 0.03^{b}
Cy-3-gal + Dp-3-ara	15.45 ± 1.27^{b}	19.37 ± 1.39^{a}
Cy-3-glc	0.51 ± 0.02^{a}	0.11 ± 0.05^{b}
Pt-3-gal	12.31 ± 1.44^{a}	14.18 ± 1.39^{a}
Cy-3-ara	1.77 ± 0.06^{a}	1.30 ± 0.10^{b}
Pt-3-glc	2.36 ± 0.10^{a}	2.18 ± 0.79^{a}
Peo-3-gal + Pt-3-ara	8.07 ± 0.31^{a}	8.60 ± 0.50^{a}
Peo-3-glc	1.26 ± 0.04^{a}	0.32 ± 0.08^{b}
Mv-3-gal	31.18 ± 1.54^{a}	26.85 ± 1.70^{b}
Mv-3-glc	2.72 ± 0.08^{a}	1.14 ± 0.19^{b}
Mv-3-ara	16.71 ± 0.82^{a}	15.07 ± 1.11^{a}

TPC, total phenolic content; MAP, monomeric anthocyanin pigment, PC, polymeric color; Dp-3-gal, delphinidin-3-galactoside; Dp-3-glc, delphidin-3-glucoside; Cy-3-gal + Dp-3-ara, cyanidin-3-galactoside + delphinidin-3-arabinoside; Cy-3-glc, cyanidin-3-glucoside; Pt-3-gal, petunidin-3-galactoside; Cy-3-ara, cyanidin-3-arabinoside; Pt-3-glc, petunidin-3-glucoside; Peo-3-gal + Pt-3-ara, peonidin-3-galactoside + petunidin-3-arabinoside; Peo-3-glc, peonidin-3-glucoside; Mv-3-gal, malvidin-3-galactoside; Mv-3-glc, malvidin-3-glucoside; Mv-3-ara, malvidin-3-arabinoside.
Data with different letters in the same row are statistically different at $P \leq 0.05$ (Tukey's test).

Total phenolic content (TPC) and chlorogenic acid content was higher in BL-P compared to NB-P while PC%, index of phenolic degradation products, was higher in NB-P. Total anthocyanin content, computed either by the pH differential method or the RP_HPLC detection, was not significantly different between the BL and NB samples. Galactoside and arabinoside forms were

the prominent compounds detected in the blueberry products with Mv-3-gal, and Dp-3-gal as the dominant ACNs, followed by Mv-3-ara, Cy-3-gal + Dp-3-ara and Pt-3-glc (14.4%, 13.3% and 10.6%, respectively) in NB samples and by Cy-3-gal + Dp-3, Mv-3-ara and Pt-3-glc (16.7%, 13% and 12.2%, respectively) in the BL samples. On the contrary, glucoside forms were detected at very low concentrations. Blanching had an influence on individual ACN amounts found in purées: the main differences concerning Dp-3-gal and Cy-3-gal + Dp-3-ara were higher in BL-P (+40. % and +25.3 %, respectively) and Mv-3-gal was higher in NB-P (+16.1%). The content in ACN-glucoside derivatives was relatively low in all the samples, but significantly higher in NB-P with respect to BL-P (7.42 vs 4.16 mg C3GE/100g FW respectively), especially for Dp-3-glc, Cy-3-glc and Mv-3-glc. The ascorbic acid content of berries was relatively low (0.8 mg/100g FW) and did not significantly change following purée processing.

4.3.2 Plasma anthocyanins concentration after the consumption of unblanched and blanched blueberries purées.

In Figure 4.3.2 are reported the plasma levels of ACN following the consumption of NB and BL blueberry purées. Only 3 of the 12 ACNs identified in the blueberry purées were detected in plasma. These compounds (Cy-3-glc, Mv-3-glc and Dp-3-glc) were present at low concentration in both purées. ACNs were rapidly absorbed and their plasma concentration increased 1 h after consumption, achieving their maximum concentration at 1.5 h. These levels decreased after 2 and 3 h reaching baseline values after 4 h (Figure 4.3.2). Two-way ANOVA did not show on the whole a significant effect of processing on ACN-absorption, but a significant effect of time ($P \leq 0.0001$). However, post hoc comparisons demonstrated that plasma ACN concentrations were different at specific time–points; ACN absorption was higher (+25 %; $P \leq 0.05$) after 1.5 h following BL-P intake (31.1 ± 11.4 nmol L^{-1}) with respect to NB-P (24.9 ± 14.0 nmol L^{-1}). Moreover, plasma ACN concentration tended to decrease after 2 h with respect to 1.5 h for both the purées, but remained significantly higher up to 2 h following only the intake of BL-P (24.9 ± 10.1 nmol L^{-1} *vs* 14.3 ± 9.6 nmol L^{-1}; $P \leq 0.001$) (Figure 4.3.2a).

Considering the absorption of single ACNs it has been observed a similar trend for malvidin and cyanidin. In particular, it has been documented a significant ($P < 0.001$) increase of Mv-3-glc after the intake of BL-P and NB-P. The concentration remained significantly higher up to 2 h after consumption with respect to 1 h following the intake of BL-P (15.9 ± 7.5 nmol L^{-1} vs 9.8 ± 7.3 nmol L^{-1}; $P \leq 0.01$). Mv-3-glc was the only ACN detected after 3 h from the intake of both purées (1.7 ± 1.7 nmol L^{-1} in BL-P and NB-P) (Figure 4.3.2b). The same trend was observed for Cy-3-glc even if plasma concentrations were lower with respect to Mv-3-glc. Plasma Cy-3-glc concentrations were higher after BL-P intake with respect to NB-P both at 1.5 h (11.7 ± 4.3 nmol L^{-1} for BL-P and 9.5 ± 5.2 nmol L^{-1} for NB-P; $P \leq 0.05$) and at 2 h (9.0 ± 3.2 nmol L^{-1} for BL-P and 6.2 ± 3.2 nmol L^{-1} for NB-P; $P \leq 0.05$) (Figure 4.3.2c). ACN-concentration remained significantly higher up to 2 h with respect to 1 h following the intake of BL-P (9.0 ± 3.2 nmol L^{-1} vs 4.5 ± 2.6 nmol L^{-1}; $P \leq 0.01$). Additionally, Dp-3-glc exhibited similar time patterns as the other ACNs; however this compound was detected at low concentrations only in the plasma of few volunteers (4 out of 10; data not shown) who were also those having higher absorption of Mv and Cy- 3-glc, suggesting subject-specific absorption.

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Figure 4.3.2- *(A) Total plasma anthocyanin (ACNs), (B) Mv-3-glc and Cy-3-glc (C) concentration after the consumption of 300 g of unblanched (NB-P) and blanched (BL-P) blueberries purées.*

4.3.3 Total hippuric acid concentration in urine before after the consumption of unblanched and blanched blueberry purées.

The levels of hippuric acid (expressed as total mg) detected in the urine collected 24 h before and 24 and 48 h after the consumption of BL-P and NB-P purée are reported in Figure 4.3.3. Statistical analysis did not show a significant effect of the type of purée, but confirmed a significant effect of time ($P < 0.05$). The intake of both purées significantly increased the excretion of hippuric acid after 24 h of blueberry consumption in both groups: BL-P (223 ± 124 mg total *vs* 469 ± 282 mg total, $P < 0.05$) and NB-P (210 ± 101 mg total *vs* 544 ± 311 mg total, $P < 0.05$). The levels of hippuric acid decreased after 48 h from NB-P intake (130 ± 102 mg total) while they remained higher, even if not significantly, after the BL-P intake (367 ± 283 mg total) with respect to 24 h pre-intervention. These results suggest that ACNs and other phenolic compounds were absorbed, metabolized and excreted as hippuric acid within 24 until 48 h after the consumption of both purées.

Figure 4.3.3 - *Total hippuric acid concentration in urine before and 24 and 48 h after the consumption of 300 g of unblanched (NB-P) and blanched (BL-P) blueberry purées.*
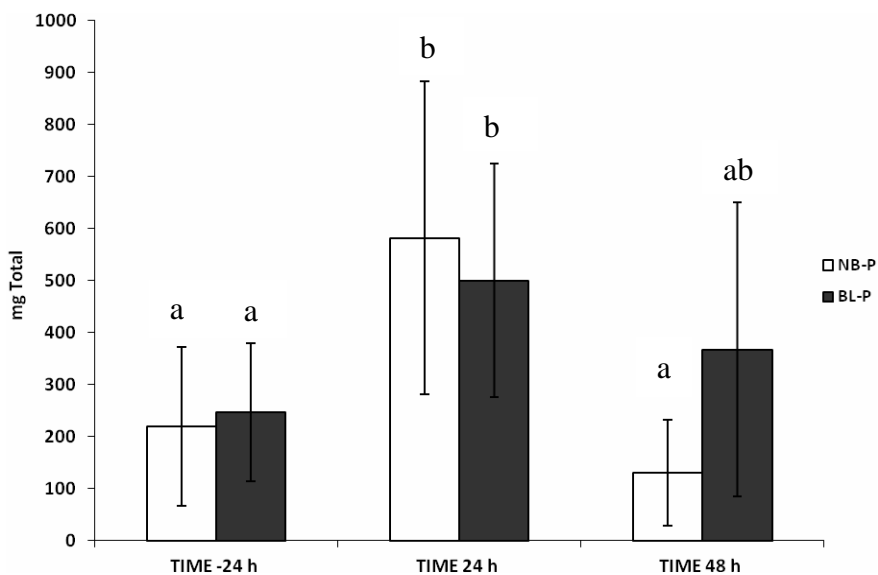
NB-P, unblanched blueberry purée;
BL-P, blanched blueberry purée;
Data are present as mean ± SD.
Data with different letters are significantly different P ≤ 0.05

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4.4 CONCLUSIONS

The impact of food processing on ACN-rich products has not been thoroughly investigated but, it is of crucial importance for the food industry since it is recognized that thermal treatment is detrimental for nutrients and bioactive components of the food (Tadapaneni et al., 2012). Thus, food processing based on mild technologies has been developed to preserve or enhance nutritive value of foods. In the present study it has been investigated whether a blanching treatment could enhance ACN and phenolic content in a blueberry purée compared to a purée that did not undergo the blanching treatment.

The results of the research documented that, even though the mild blanching process applied had no significant effect on total ACN content (possibly due to the low degree of exposure to native enzymes responsible for ACN degradation typical of the juice-making process), berry blanching enhanced ACN absorption from minimally processed blueberry purées 1.5 and 2 h after consumption. This is particularly important for food industry that wants to put on the market food products rich in bioactive compounds with potential beneficial effects on human health.

In addition, these results confirmed that ACNs from foods are poorly absorbed and their presence in the blood is limited at few hours as also supported by several studies (Yang et al., 2011; Mazza et al., 2002). The influence of processing on ACN metabolism and health effects *in vivo* should be the focus of future research.

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5. DEVELOPMENT OF ACUTE DIETARY INTERVENTION STUDY TO EVALUATE THE EFFECT OF A SINGLE PORTION OF BLUEBERRY IN THE MODULATION OF MARKERS OF OXIDATIVE STRESS AND ENDOTHELIAL FUNCTION IN YOUNG NONSMOKER VOLUNTEERS

5.1 AIM OF THE STUDY

It has been previously documented that blueberries are rich sources of phenolic compounds such as ACNs. Despite their low bioavailability, ACNs play several beneficial effects on human health and, in this regard, it has been hypothesized that their intake could improve cell protection against oxidative stress and affect endothelial function in humans. The aim of the study was to investigate the effect of one portion (300g) of blueberries on selected biomarkers of oxidative stress and antioxidant protection (endogenous and oxidatively-induced DNA damage) and of vascular function (changes in peripheral arterial tone and plasma nitric oxide levels) in a group of healthy male subjects.

5.2 MATERIALS AND METHODS

5.2.1 Blueberry and placebo preparation

Blueberries (*Vaccinium corymbosum* L. "Brigitta") from a single batch were purchased, sorted and immediately frozen by Individually Quick Freezing technique in a tunnel (Thermolab, Codogno, Italy) and stored at −20°C until use. For the study, BB was partially thawed (3 h at 20°C) and homogenized in a commercial food processor (Moulinex, Paris, France). They were packed in portions of 300 g, thermally sealed under partial vacuum (Minipack-Torre S.P.A., Dalmine, Bergamo, Italy) and stored at −20°C for few days. The evening before the experiment, the BB portions were placed at + 4°C for defrosting. The BB was gelatinous in texture; for this reason, a control jelly (CJ) was utilized as placebo. The CJ was prepared by suspending 20g of food grade gelatin (Universal, Peru) and adding the same amount of BB sugars (about 27.1g total, 16.4g fructose and 10.7 g glucose) in 200mL of hot water. The CJ containing a food colorant was prepared the day before the experiment and stored at +4°C to solidify.

5.2.2 Study subjects

Ten healthy male subjects, ages 20.8 ± 1.6 y with body mass index (BMI) 22.5 ± 2.1 kg/m^2, were recruited from the student population of the University of Milan according to the following inclusion criteria: no smokers; no history of cardiovascular, diabetes, hepatic, renal, or gastrointestinal diseases; not consuming any dietary supplement, drug, or medication for at least one month before the beginning of the study. Subjects were selected on the basis of an interview to evaluate their dietary habits and ensure that they were as homogeneous as possible, in particular for fruit and vegetable consumption. This was obtained by means of a food frequency questionnaire previously published and specifically revised to focus on food sources rich in antioxidants (Porrini et al., 1995). Exclusion criteria were: hypertension (systolic blood pressure > 140 mm Hg and/or diastolic blood pressure > 90 mm Hg), high total serum cholesterol (TSC) ($\geq$5.17 mmol L^{-1}), low high-density lipoprotein (HDL)-cholesterol (<1.03 mmol L^{-1}), high low density lipoprotein (LDL)-cholesterol ($\geq$3.36 mmol L^{-1}), high triglycerides (TG) ($\geq$1.69 mmol L^{-1}), overweight (BMI $\geq$ 25 kg/m^2). Other exclusion criteria were as follows: high (> 5 portions/day) or low (< 2 portions/day) intake of fruit and vegetables and alcohol consumption (< 3 drinks per week were acceptable). Volunteers who followed a specific diet (e.g. vegetarian, vegan, or macrobiotic) and those who had a specific aversion for blueberry consumption were excluded. All participants gave informed consent and the study was approved by the Ethics Committee of the University of Milan.

5.2.3 Experimental design

Subjects were deprived of ACN-food sources 10 days before experimentation. Volunteers received a complete list of ACN-rich foods to be avoided; the list included ACN-rich foods such as berry fruits, red wine and red/purple fruits and other colored products. Subjects were randomly divided into two groups of 5 subjects each: group 1 was assigned to the sequence BB/wash-out/CJ, whereas group 2 followed the sequence CJ/wash-out/BB. The study was scheduled at different days to avoid interference between withdrawal times and the study of vascular function. Each analysis was separated by 10 days of wash-out period. Lunch and dinner was standardized and subjects were asked to exclude all ACN-containing foods and maintain their regular lifestyle. For the present study, peripheral arterial function was measured in two consecutive days since no inter-day effect on vascular function was observed as previously reported. Baseline levels were assessed the first day, while the second day peripheral arterial function was evaluated 1h after the intake of BB or CJ.

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The subjects fasted overnight before the ingestion of one portion of thawed BB (providing 348 mg of ACNs) or CJ (without ACNs). The products were consumed early in the morning and blood was collected by a phlebotomist at time 0 (before the consumption of the products) and 1 h, 2 h and 24 h after BB or CJ consumption. Samples were drawn into evacuated tubes with heparin as anticoagulant. One day-food records were kept by subjects in each experimental session, 2 days before and 1 day after the intake of the BB product to check compliance to the dietary instructions. Moreover, a direct interview by a registered dietitian was scheduled.

5.2.4 Sugars, total phenolics and vitamin C determination in blueberry and control jelly

A duplicate sample (50 g) of BB was homogenized and suspended in water, centrifuged at 3000 x g for 1 minute, filtered and injected for the analysis. Glucose and fructose were quantified by ultra performance liquid chromatography (UPLC). The LC consisted of an Alliance model 2695 (Waters, Milford, MA) equipped with a model 2996 photodiode array detector (Waters), coupled with mass spectrometry (Micromass, Beverly, MA). The separation was carried out on BEH Amide column (150 x 2.1 mm, 1.7 µm, Waters) at 35°C. Solvents were triethanolamine 0.2% and acetonitrile: triethanolamine at a ratio of 74:26 (v/v). The elution gradient was linear and the amount of triethanolamine was increased from 0% to 35% in 11 minutes at set up flow rate of 0.45 mL min^{-1}. The calibration curve was obtained from 5 mg L^{-1} to 100 mg L^{-1} for both sugars. The percentage relative standard deviation was calculated after injecting standard solutions of glucose and fructose at increasing concentration (2 mg L^{-1}, 10 mg L^{-1} and 50 mg L^{-1}) in quintuplicates. Phenolic compounds were extracted in duplicate from BB by applying a formic acid-water (5:95 v/v) extracting media, according to Brambilla et al., (2008). Total phenolic compounds of the extracts were analyzed by Folin-Ciocalteau assay (Giusti & Wrolstad 2001) and expressed as gallic acid equivalents (GAE mg/100g) while chlorogenic acid and individual anthocyanin compounds were analyzed by gradient reverse phase-high-performance liquid chromatography (RP-HPLC) and diode array detection and were quantified by measuring detector response to the commercial standards (Polyphenols Laboratory Sandes, Norway) (Brambilla et al., 2008). All ACN monoglycosides were expressed as cyanidin 3-glucoside (CydG) equivalents. Vitamin C was extracted and determined by HPLC analysis as previously described by Riso et al., (2005).

5.2.5 Analysis of biochemical parameters

Blood samples were drawn and immediately centrifuged at 1000 x g for 15 minutes for plasma and serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was performed in serum including evaluation of lipid profile (TG, TSC, LDL-C and HDL-C) and glucose. All these parameters were determined using a cobas® 6000 analyzer series (Roche Diagnostics, North America). Plasma concentration of total NO was calculated by measuring the products of oxidation (nitrate and nitrite) by a Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI).

5.2.6 Anthocyanin extraction and analysis in plasma

Two aliquots of plasma (1 mL) were acidified with trifluoroacetic acid (TFA, 1%), vortexed, and centrifuged for 1 min at 4500 x g and the supernatant was stored at -80°C until analysis. Anthocyanins were extracted from plasma and analyzed as previously described and reported in Del Bo' et al., (2012, 2010).

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5.2.7 Evaluation of endogenous DNA damage and cell resistance against H₂O₂-induced DNA damage

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation as previously described (see **study1**) and reported in Collins et al., (1996). The FPG-sensitive sites (oxidized purines) and cell resistance against H_2O_2 (500 µmol L^{-1}, 5 min) induced DNA damage were evaluated by the comet assay as previously described in detail (see **study 1**) and reported in previous studies (Collins et al., 1996; Riso et al., 1995).

5.2.8 Evaluation of peripheral arterial function

Endothelial-dependent vasodilation in the small finger arteries was assessed by a non-invasive plethysmographic method (Endo-PAT 2000, Itamar Medical Ltd., Caesarea, Israel) based on the registration of pulsatile blood volume in the fingertips of both hands. Detailed information is previously described (see **study 2**) and reported in a previous study by Bonetti (2005).

5.2.9 Statistical analyses

Sample size has been calculated taking into account the expected variation in the DNA damage as primary endpoint. In particular, ten subjects were calculated to be more than sufficient to evaluate a difference of DNA damage after the wild blueberry drink of 8.6 (standard deviation 0.9), with alpha=0.05 and a statistical power of 80%. This number of subjects is comparable to those used in previous acute studies (Schroeter et al., 2006; Heiss et al., 2005; Grassi et al., 2005). For the evaluation of vascular function modulation (secondary endpoint). Moreover, the "repeated measure" experimental design used, in which each subject acts as its own control, reduces the error variance, thus increasing statistical power.

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Data were analyzed by ANOVA for repeated measures design. ANOVA with treatment (BB vs CJ) and time (before and after each treatment) as dependent factors was applied to evaluate the effect of BB on the variables under study. Differences were considered significant at $P \leq 0.05$; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $P \leq 0.05$ as level of statistical significance. Data are presented as means ± standard deviation (SD).

5.3 RESULTS AND DISCUSSION

5.3.1 Composition and characteristic of the blueberry portion

The nutritional composition of BB is reported in Table 5.3.1. One portion (300 g) of the BB provided about 27 g of sugars (fructose and glucose), 348 mg of ACNs (malvidin-galactoside, delphinidin-galactoside and malvidin-arabinoside making up more than 50% of the total ACN content), 727 mg of total phenolic acids, 90 mg of chlorogenic acid and 2.4 mg of vitamin C. The CJ provided the same amount and type of sugars but not of bioactive compounds.

Table 5.3.1-*Nutritional composition of Blueberry (BB) and Control jelly (CJ)*

	BB	CJ
Sugars (g/100g)		
Fructose	5.46 ± 0.10	5.46
Glucose	3.57 ± 0.18	3.57
Total phenolic compounds (mg/100g)	242.4 ± 23.9	-
Chlorogenic acid (mg/100g)	30.1 ± 1.2	-
Total anthocyanins (mg/100g)	116.1 ± 6.9	-
Vitamin C (mg/100g)	0.8 ± 0.1	-

Data are expressed as means ± SD.

5.3.2 Baseline characteristics of the subjects

Baseline anthropometric and clinical characteristics of the subjects are reported in Table 5.3.2. All data were within the range of normality.

Table 5.3.2- *Subject characteristics*

	Value
Age (y)	20.8 ± 1.6
Body weight (kg)	72.4 ± 7.9
BMI (kg/m^2)	22.5 ± 2.1
Systolic pressure (mmHg)	119.5 ± 8.8
Diastolic pressure (mmHg)	76.5 ± 6.2
Heart rate (beat/min)	62.2 ± 15.3
Glucose (mmol L^{-1})	3.92 ± 0.26
TG (mmol L^{-1})	1.69 ± 0.49
TSC (mmol L^{-1})	4.4 ± 0.64
HDL-C (mmol L^{-1})	1.44 ± 0.33
LDL-C (mmol L^{-1})	2.43 ± 0.37

Data (n=10) are expressed as means ± SD.

BMI, body mass index; TG, triglycerides; TSC, total serum cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

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5.3.4 Plasma concentration of anthocyanins following blueberry and control jelly intake

Anthocyanins were not detectable in plasma at baseline while a significant (P<0.001) increase was observed 1 h (13.7 $\pm$ 10.7 nmol L^{-1}) and 2 h (18.7 $\pm$ 6.4 nmol L^{-1}) after BB intake. Twenty four hours after BB intake, ACNs were not detected in plasma; CJ intake resulted in undetectable plasma ACNs.

5.3.5 Effect of blueberry and control jelly intake on the levels of DNA damage in peripheral blood mononuclear cells

Results on DNA damage in PBMCs are reported in Table 5.3.3. Oxidized purines evaluated through quantification of FPG-sensitive sites were not significantly different following BB or CJ intake. This result is not surprising, since the levels of FPG-sensitive sites measured, represent the steady-state levels of oxidatively damaged DNA. In fact, in cultured cells, the repair of FPG-sensitive sites has a half-life of 1-5 hours (Danielsen et al., 2009; Collins & Harrington 2002; Will et al., 1999). Since the removal of DNA damage is not instantaneous, a long-term supplementation may be required to establish the possibility of *in vivo* efficacy of BB intake on endogenous levels of oxidatively damaged DNA (Collins et al., 2001).
Concerning the levels of H_2O_2-induced DNA damage, a significant reduction was observed 1 h after the BB intake (from 51.7 $\pm$ 4.9% to 42.7 $\pm$ 8.7%, P $\leq$ 0.01), while no effect was observed after CJ (from 53.2 $\pm$ 2.8% to 52.0 $\pm$ 7.6%, P = 0.84). These results are in accordance with several human studies that demonstrated a greater protection against oxidatively induced DNA damage after the intake of single portions of fruits such as kiwifruits, apples and orange juice (Collins et al., 2001; Brivida et al., 2007; Guarnieri et al., 2007; Nguyen et al., 2003). However, the protective effect was transient and the level of H_2O_2-induced DNA damage returned to baseline 2 h after BB consumption when the maximum peak of plasma ACNs absorption was observed. Moreover, no correlation between the decrease in H_2O_2-induced DNA damage and the increase in ACNs was observed at 1 h. Thus, the protection against oxidative stress may be related to other bioactives absorbed, apart from ACNs (e.g. phenolic acids, vitamin C), acting alone or synergistically. Moreover, these compounds could have indirectly activated signaling mechanisms of defense (e.g. antioxidant enzymes through gene expression modulation) (Nguyen et al., 2003) even though the effect is not maintained at 2 h.

5.3.6 Effect of blueberry and control jelly intake on peripheral arterial function and plasma nitric oxide levels

The results on peripheral arterial function (RHI), blood pressure, heart rate and plasma NO levels, before and after BB and CJ consumption, are reported in Table 5.3.4. According to the repeated measures ANOVA, after either the WB or the CJ intake no significant changes were observed for RHI. The mean percent change in RHI index between the pre-to-post intervention was +0.5% (95% CI: -7.3%, +8.4%) after the BB and -4.5% (95% CI: -13.9%, +6.4%) after the CJ intake. On the whole, a high inter-individual variability was observed in the percent changes of RHI index (**Figure 1**). The lack of the effect in the modulation of peripheral vascular function could be dependent to the fact that subjects enrolled were healthy, and most of them had RHIs in the normal range (RHI $\geq$ 1.67). It seems plausible that improvements may be easier demonstrated in subjects with reduced vascular function (e.g. elderly or subjects who are at risk of developing cardiovascular diseases) or after vascular function challenges (e.g. following smoking or a meal rich in saturated fats). Another reason of the lack of modulation may be attributed to the length of time between the BB intake and the measurement of peripheral arterial function (1 h). In fact, more time may be necessary to detect an effect on endothelial function following the exposure to BB and their bioactives. In this regard, Dohadwala et al., (2011)

documented an improvement of vascular function at 2 h and 4 h after the intake of a single portion of cranberry juice.

The observations on vascular function are consistent with the non-significant changes in plasma total NO, indicating that the short-term consumption of BB did not exert any changes on this marker. Plasma NO concentration is mainly related to systemic inflammation, whereas the endothelium-derived nitric oxide synthase (eNOS) production of NO is a minor contributor to alterations in its plasma concentration. Nevertheless, it cannot exclude that a modulation of NO occurred at the endothelial level without influencing total plasma levels. In fact, some authors reported that the consumption of red wine polyphenols and flavonoids may affect vascular function by increasing the half-life of endothelial NO (Dell'Agli et al., 2004). Future studies with larger numbers of subjects or with established vascular dysfunction may contribute to our understanding of the beneficial effects of BB consumption on vascular function and modulation of plasma NO levels.

Table 5.3.3- *Effect of one portion of Blueberry (BB) or control jelly (CJ) on background, FPG sensitive sites and H_2O_2-induced strand breaks.*

	T 0h	T 1h	T 2h	T 24h
BB consumption				
Background SBs (% DNA in tail, EB)	5.9 ± 0.6	5.9 ± 0.6	5.9 ± 0.7	6.0 ± 0.4
Net FPG-sensitive sites (% DNA in tail)	12.9 ± 2.1	13.1 ± 1.4	12.4 ± 1.4	13.1 ± 1.4
Background SBs (% DNA in tail, PBS)	8.1 ± 1.0	7.9 ± 1.5	8.00 ± 1.2	8.3 ± 0.8
Net H_2O_2-induced DNA damage % (DNA in tail)	51.7 ± 4.9	42.7 ± 8.7*	50.1 ± 9.1	51.8 ± 6.1
CJ consumption				
Background SBs (% DNA in tail, EB)	6.1 ± 0.6	5.9 ± 0.6	5.9 ± 0.3	5.8 ± 0.2
Net FPG-sensitive sites (% DNA in tail)	14.5 ± 3.6	14.1 ± 2.7	13.5 ± 1.4	14.8 ± 0.8
Background SBs (% DNA in tail, PBS)	8.5 ± 0.7	8.6 ± 0.9	8.7 ± 0.1	8.4 ± 0.3
Net H_2O_2-induced DNA damage (% DNA in tail)	53.2 ± 2.8	52.0 ± 7.6	54.0 ± 4.3	49.3 ± 3.3

Data (n=10) are expressed as means $\pm$ SD.

SBs, strand breaks; PBS, phosphate-buffered saline; EB, endonuclease buffer; FPG,formamidopyrimidine DNA glycosylase.

*Significantly different from each other time point in the same row and different with respect to each other time point for the CJ group; p≤0.01.

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Table 5.3.4- *Effect of one portion of Blueberry (BB) or Control jelly (CJ) intake on peripheral arterial function and total plasma nitric oxide (NO).*

Variables	Before BB	After BB	Before CJ	After CJ
Systolic blood pressure (mmHg)	119.5 ± 8.8	118.6 ± 8.7	122.5 ± 10.4	121.3 ± 8.5
Diastolic blood pressure (mmHg)	76.5 ± 6.2	73.9 ± 6.1	76.3 ± 4.8	76.3 ± 10.3
Heart rate (beat min^{-1})	62.2 ± 15.3	61.6 ± 17.1	63.5 ± 16.4	64.0 ± 19.8
RHI	1.96 ± 0.39	1.95 ± 0.30	1.94 ± 0.30	1.82 ± 0.16
Total NO ($\mu mol\ L^{-1}$)	64.6 ± 22.5	64.7 ± 18.6	72.2 ± 21.8	72.5 ± 18.7

Data (n=10) are expressed as means $\pm$ SD.

RHI; reactive hyperemia index; NO, nitric oxide.

5.4 CONCLUSIONS

This study documented that blueberries can improve cell resistance against H_2O_2-induced DNA damage, and this is in accordance with previous observations with other fruits provided in single portions, thus supporting the importance of consuming vegetable foods regularly.

Concerning vascular function, no effect was observed after acute consumption of blueberry. These results are in contrast with what found by other authors following consumption of red wine polyphenols, flavonoids, cocoa and chocolate (Hollenberg & Fisher 2007; Vlachopoulos et al., 2005; Fisher et al., 2006; Alexopoulos et al., 2008; Oyama et al., 2010; Vlachopoulos et al., 2003).

Possible study limitations are the small sample size of healthy subjects considered for the demonstration of an effect on vascular function at one time-point after the ingestion of BB.

Future studies with larger numbers of subjects or with established vascular dysfunction may contribute to our understanding of the beneficial effects of BB consumption on vascular function and modulation of plasma NO levels.

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6. DEVELOPMENT OF A POTENTIAL HUMAN MODEL OF CHRONIC TOBACCO SMOKERS FOR STUDYING VASOACTIVE PROPERTIES OF FOOD BIOACTIVES

6.1 AIM OF THE STUDY

Based on results obtained in a previous study where we failed to demonstrate a significant modulation of endothelial function following the consumption of a single blueberry portion in healthy subjects with normal endothelial function, in this study we used smoking as a stressor to develop a model that may better demonstrate the effect of dietary vasoactive compounds. In fact, cigarette smoking is one condition that increases oxidative stress but also impairs endothelial function (Sanada et al., 2012; Giudice et al., 2012). The mechanism of endothelial dysfunction through smoking seems to be attributed to the known/unknown substances (i.e. reactive oxygen and nitrogen species, nicotine, benzopyrene and acrolein) that constitute the particulate (tar) and gaseous phase of the cigarette (Yamaguchi et al., 2007). These components may induce oxidative stress and inflammation with detrimental consequences on endothelial function and arterial stiffness. Endo-PAT device has been proposed as a tool to evaluate the effect of a single or long term exposure to foods/bioactive compounds on arterial function with conflicting results. This may depend on subject characteristics such as age, sex, dietary and life-style habits, physical activity, but also on the specific experimental protocol used.

The aim of the present study is to evaluate whether peripheral arterial tone (PAT) technology is able to detect the acute effects of a single cigarette smoking on RHI and arterial stiffness in a homogeneous group of young healthy male smokers. If PAT technology will be able to detect the acute effects of smoking a single cigarette on peripheral arterial function, then this model could be applied for studying the impact of dietary bioactive compounds in the modulation of endothelial function administered before smoking.

6.2 MATERIALS AND METHODS

6.2.1 Subject recruitment

In order to select a homogeneous group of healthy smokers volunteers, twenty males, 23.6 ± 2.9 average age with body mass index (BMI) of 22.4 ± 2.2 kg/m^2, were recruited from the student population of the University of Milan according to the following criteria: 20-30 years of age, moderate smoking (about 15 cigarette/day; smoking from at least 5 years), moderate physical activity (25-30 min per day of brisk walk or jog) and moderate alcohol consumption (up to 10-14 drinks per week). Subjects were selected on the basis of an interview by a dietitian to evaluate their dietary habits. Exclusion criteria were as follows: hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90mmHg), hyperglycemia (> 10 mmol L^{-1}), hypertriglyceridemia (TG$\geq$1.69 mmol L^{-1}) and hypercholesterolemia [total serum cholesterol (TSC) $\geq$5.17 mmol L^{-1}, low high-density lipoprotein (HDL) cholesterol (C) <1.03 mmol L^{-1}, high low density lipoprotein (LDL) cholesterol (C) $\geq$3.36 mmol L^{-1}], and overweight (BMI $\geq$ 25 kg/m^2). Endothelial dysfunction (RHI <1.67) was another important exclusion criterion. Subjects declared no history of cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases, anemia, chronic asthma, allergy, traumas of the arms or hand, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations and chronic backache. Moreover, subjects were excluded if they were taking any supplement, drug, or medication for at least one month before the beginning of the study. The study was approved by the Ethics Committee of the University of Milan. All participants gave informed consent and the study was in accordance with the Declaration of Helsinki.

6.2.2 Experimental design

Subjects were asked to avoid foods containing substances with possible vasoactive properties 10 days before experimentation. Specific attention was devoted to foods such as chocolate, berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants and elderberries), red wine and red/violet fruits, green tea. All participants were asked to refrain from physical activity from the day before the experiment and to smoke the number of cigarettes/day declared in the questionnaire (i.e. about 15 cigarettes per day). The day before the experiments and during the trial, breakfast, lunch and dinner were standardized. The standardized dinner was consumed by 9.00 pm and only one coffee was permitted with a maximum of two coffees during the day. Moreover alcoholic drinks or soft drinks were not allowed.

With regard to the establishment of the peripheral arterial function evaluation protocol, we chose to avoid multiple measurements (involving 5 min arterial occlusion through cuff inflation) in a short time-period, because it could promote vasodilatation through NO production between test and re-test measurements (Onkelink et al., 2012). Thus, peripheral arterial function was measured in two consecutive days. Moreover, we pre-tested the protocol to exclude an inter-day variability and to assure a within-subject repeatability of basal levels of RHI as also reported by other authors (Munir et al., 2008; Bonetti 2005). For the present study, overnight fasted volunteers came to our laboratory early in the morning; baseline levels were assessed the first day, while the second day RHI was evaluated 20 minutes after smoking a single cigarette (containing approximately 8 mg of Tar by volume, 0.6 mg of nicotine and 9 mg of carbon monoxide) in a smoking room close to the test room. Systolic blood pressure (SyBP), diastolic blood pressure (DiPB) and HR were measured before and 5 min after smoking and at the end of the evaluation of peripheral vasoreactivity (30 min after smoking). The study design is reported in Figure 6.2.1.

Figure 6.2.1- *Experimental design*
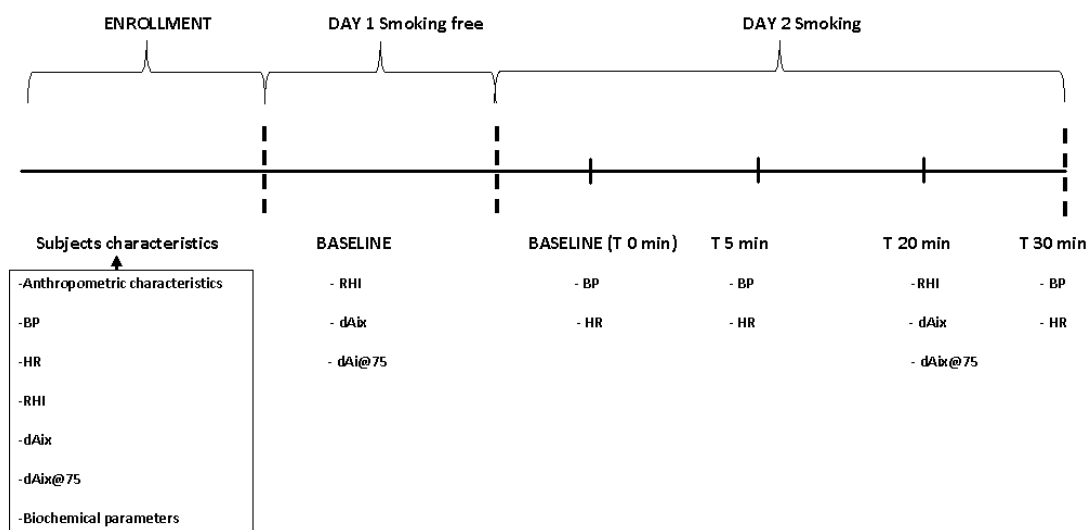
BP, blood pressure; HR, heart rate; RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm

6.2.3 Determination of peripheral arterial function and arterial stiffness

Endothelial-dependent vasodilation in the small finger arteries was assessed by Endo-PAT2000 as previously reported (see **study 2**).
The EndoPAT device also generates dAix, strongly correlated to aortic Aix, calculated from the shape of the pulse wave recorded by the probes during baseline (Reisner et al., 2007). Because dAix is influenced in an inverse and linear manner by heart rate, the dAix was automatically normalized by the tool for a heart rate of 75 bpm (dAix@75).

6.2.4 Analysis of biochemical parameters

Blood samples were drawn and immediately centrifuged at 1000 x g for 15 minutes for plasma and serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was performed in serum including evaluation of lipid profile (TG, TSC, LDL-C and HDL-C) and glucose. All these parameters were determined using a cobas® 6000 analyzer series (Roche Diagnostics, North America).

6.2.5 Statistical analysis

Sample size was calculated according to the expected variation in the primary endpoint considered (RHI). In particular, twenty subjects was calculated to be sufficient to evaluate a difference of RHI after smoking of 0.28 (standard deviation 0.40), with alpha=0.05 and a statistical power of 80%. Statistical analysis was carried out with the Statistical Package for the Social Sciences (SPSS inc., Chicago, Ill., U.S.) release 17.0 for Windows. Pre and post-smoking differences were tested by paired Student's t-test while ANOVA with repeated measures, followed by the Bonferroni test for multiple comparisons, was used to assess the variation over time of SyBP, DiBPand HR. The relationship among different variables was determined using

the Pearson correlation test. The level of significance was set at p≤0.05. Data are presented as mean and standard deviation.

6.3 RESULTS AND DISCUSSION

6.3.1 Baseline characteristics of the study population

The baseline clinical and anthropometric characteristics of the enrolled volunteers are reported in Table 6.3.1. Subjects presented normal vital signs (SyBP/DiBP<140/90 mmHg) and endothelial function (RHI >1.67) consistent with their young age.

Table 6.3.1- *Antropometric and clinical characteristics of the subjects[1]*

Variables	Value
Age (years)	23.6 ± 2.9
Hight (cm)	177.2 ± 6.7
Weight (kg)	70.6 ± 9.9
BMI (kg/m^2)	22.4 ± 2.2
Neck circumference (cm)	36.2 ± 1.4
Shoulder circumference (cm)	109.5 ± 5.1
Chest circumference (cm)	94.2 ± 6.3
Waistline circumference (cm)	78.2 ± 6.2
Paunch circumference (cm)	83.0 ± 5.9
Sides circumference (cm)	95.5 ± 6.3
Arm circumference (cm)	27.6 ± 2.4
Forearm circumference (cm)	25.7 ± 1.8
Wrist circumference (cm)	17.0 ± 0.6
Smoking (cigarettes/day)	15 ± 2
SyBP (mm Hg)	114.4 ± 7.9
DiBP (mm Hg)	72.2 ± 6.3
HR (beat min^{-1})	57.9 ± 7.6
RHI	2.23 ± 0.28
dAix (%)	-8.6 ± 8.0
dAix@75 (%)	-18.4 ± 8.9

[1]*Data are expressed as means ± SD (standard deviation). BMI, body mass index; SyBP, systolic blood pressure; DiBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.*

6.3.2 Effect of smoking on arterial function and digital augmentation index

In Table 6.3.2 are reported the values of RHI, dAix and dAix@75 before and after smoking. Peripheral arterial function was impaired 20 min after cigarette smoking. The mean percentage change pre-to post smoking was –27.6% (95%CI: -35.2%, -20.1%). The RHI reduction was observed in all subjects and was particularly high insomuch as to induce endothelial dysfunction (RHI<1.67) in more than a half of the subjects (13 out of 20).

Table 6.3.2 - Arterial function and arterial stiffness before and 20 min after smoking a cigarette in the overall population[1]

	Before smoking	20 min After smoking	p value[2]
RHI	2.23 ± 0.28	1.59 ± 0.27	0.0001
dAix (%)	-8.6 ± 8.0	-13.1 ± 7.5	0.007
dAix@75 (%)	-18.4 ±8.9	-18.0 ± 8.9	0.785

[1]*N =20. Data are expressed as means ± SD. RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.*
[2]*Overall P value for t-TEST with Statistical Package for the Social Sciences (SPSS inc., Chicago, Ill., U.S.)*

These results are in accordance with Xue et al. (2011) that found a significant reduction in peripheral arterial function, 20 min after smoking, but in contrast with the study of Moerland et al., (2012) in which no effect was observed at 30 and 90 min after smoking.

The divergence between these results may dependent from the protocol used and subject population. It is widely recognized that gender, age and physical activity may strongly influence endothelial function (Black et al., 2008). In present study, the protocol has been standardized through a selection of a homogeneous population for gender, age, smoking habits, alcohol consumption, dietary habits, physical activity and anthropometric characteristics, and thus, it has been possible to document that acute smoking induces peripheral arterial dysfunction in young male smokers.

Regarding dAix, in contrast from data previously reported (Doonan et al., 2010), a significant reduction was observed after smoking (Table 6.3.2). However, because heart rate has been shown to be a possible confounder in the determination of dAix, no effect was observed after normalization for heart rate (dAix@75) (Weber et al., 2004). This could be attributed to the young age of smokers having a better compensatory response to an injury (Seet et al., 2012; Nishiyama et al., 2008).

6.3.3 Effect of smoking on blood pressure and heart rate

Several studies suggest that impaired microvascular function contributes to an increase in blood pressure and heart rate. Figure 6.3.1 shows the values of blood pressure and heart rate before and after smoking. Repeated measures ANOVA analysis, revealed a significant variation over time ($P<0.001$) of SyBP, DiBP and HR; in particular all parameters assessed 5 min after smoking were significantly higher with respect to baseline and after RHI. The mean percent change pre-to post smoking was +13.8% (95%CI: +11.4%, +16.2%) for SyBP, +10.2% (95%CI: +6.4%, +14%) for DiBP and +12.8% (95% CI: +6.2%, +19.4%) for HR. This effect was transient and the values fell to baseline after 30 min.

Figure 6.3.1- *Variation of systolic and diastolic blood pressure (A) and heart rate (B) in the overall population over time.*
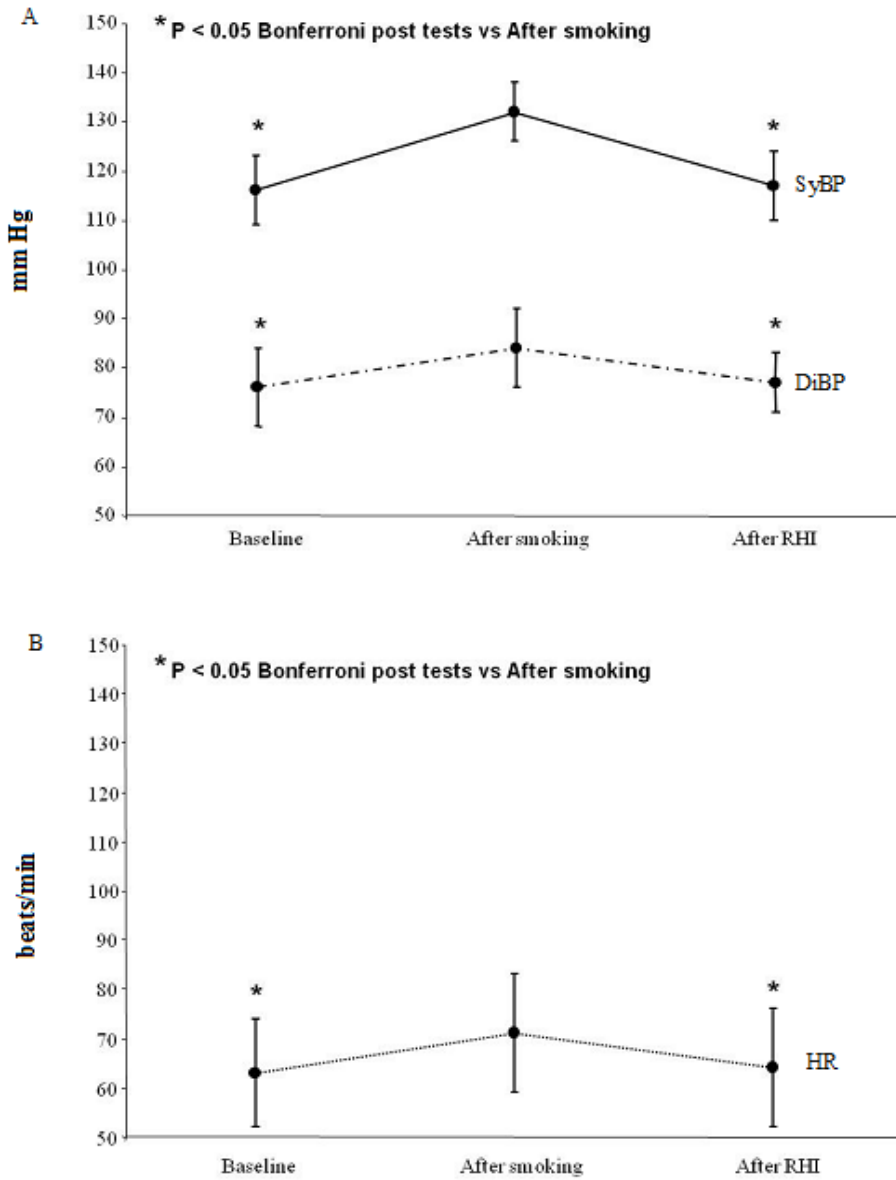
SyBP, systolic blood pressure; DiBP, diastolic blood pressure; HR, heart rate
*Data not significantly different

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6.3.4 Correlation among endothelial function, arterial stiffness and vital signs

The correlation among endothelial function variables and vital signs in the overall population is presented in Table 6.3.3. As shown, endothelial function was not correlated with blood pressure values, HR and dAix and dAix@75. Only baseline dAix@75 and post-smoking dAix@75 variables correlated with pre-smoking HR (R = 0.503; P = 0.024) and with both pre-smoking (R = 0.593; P = 0.006) and post-smoking (R = 0.496; P = 0.026) HR levels, respectively.

Table 6.3.3 - *Correlation coefficients among endothelial function variables and vital signs in the overall population.*

N = 20		SyBP_B	DiBP_B	HR_B	SyBP_S	DiBP_S	HR_S
RHI_B	R	.255	-.033	.315	.063	-.259	.411
	P value	.278	.889	.176	.792	.270	.072
RHI_S	R	.012	.007	-.156	.079	.309	.127
	P value	.960	.976	.513	.742	.185	.592
dAix_B	R	-.349	-.094	.156	-.110	-.174	-.149
	P value	.132	.693	.513	.643	.463	.531
dAix_S	R	-.370	-.065	-.019	.045	-.066	-.274
	P value	.108	.785	.935	.850	.784	.242
dAix@75_B	R	-.072	.259	**.503**	.088	.092	.230
	P value	.762	.270	**.024**[*]	.712	.699	.329
dAix@75_S	R	-.146	.326	**.593**	.125	.183	**.496**
	P value	.539	.161	**.006**[*]	.600	.440	**.026**[*]

B, baseline; S, after smoking; RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; SyBP, systolic blood pressure; DiBP, diastolic blood pressure; HR, heart rate. R, correlation coefficient

6.3.5 Correlation among endothelial function, arterial stiffness and biochemical parameters

Diet and nutritional status can affect endothelial function and arterial stiffness. It is widely recognized that high circulating levels of triglycerides and cholesterol are associated with endothelial dysfunction and impairment of arterial stiffness in the brachial artery (Wilkinson et al., 2002). However, the association in the small and distal arteries is not well understood. Twelve out of the twenty volunteers recruited, gave their consent to provide a blood sample for biochemical analysis. Data obtained were used to test the relationship between parameters of nutritional status and arterial function measurements. Data on lipid profile (total, HDL and LDL cholesterol, and triglycerides), glycaemia (fasting blood glucose) and vitamin status (folate and vitamin B_{12}) were within the range of normality (Table 6.3.4).
No correlation was observed between serum cholesterol and RHI, and dAix@75 at baseline, while a positive and interesting correlation was observed between total serum cholesterol levels at baseline and the levels of dAix@75 after smoking (Table 6.3.5). This may suggest that people with high serum cholesterol levels exhibit a higher vessel stiffness following smoking. This is an important finding not only in underlying the role of cholesterol for arterial function even in young individuals, but also suggesting that lipid profile characterization may be useful when considering subjects enrolled in studies on endothelial function.

Table 6.3.4 - *Baseline biochemical parameters in a sub-sample.[1]*

Variables	Value
TSC, (mmol L^{-1})	4.16 ± 0.54
LDL-cholesterol, (mmol L^{-1})	1.5 ± 0.34
HDL-cholesterol, (mmol L^{-1})	2.17 ± 0.41
TG, (mmol L^{-1})	1.04 ± 0.42
Fasting glucose, (mmol L^{-1})	3.89 ± 0.67
Folate, (ng mL^{-1})	6.4 ± 1.9
Vitamin B$_{12}$, (pg mL^{-1})	403 ± 105

[1]N=12. Data are expressed as means $\pm$ SD.
LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; TSC, total serum cholesterol.

Table 6.3.5-derived*Correlation coefficients among endothelial function variables and baseline biochemical parameters in a sub-sample.*

N=12		TSC_B	HDL-C_B	LDL-C_B	TG_B	GLC_B	VitB$_{12}$_B	Folate_B
RHI_B	R	.125	-.532	.397	.391	-.208	-.074	-.145
	P value	.714	.092	.226	.235	.540	.830	.672
RHI_S	R	-.213	-.037	-.222	-.074	-.483	-.225	-.328
	P value	.529	.914	.513	.829	.132	.506	.324
dAix_B	R	.190	.408	.020	-.200	-.149	.203	.406
	P value	.577	.213	.953	.555	.662	.549	.215
dAix_S	R	.307	.521	-.022	.033	-.391	-.093	.058
	P value	.359	.101	.949	.924	.234	.786	.865
dAix@75_B	R	.488	.332	.341	.071	-.260	.398	.409
	P value	.128	.319	.305	.836	.440	.225	.211
dAix@75_S	R	**.681**	.360	.466	.290	-.063	.429	.394
	P value	**.021**[*]	.276	.148	.388	.855	.188	.230

B, baseline; S, after smoking; RHI, reactive hyperemia index; Aix, augmentation index, Aix@75 standardized for heart rate of 75 bpm; TSC, total serum cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; GLC, glycaemia; R, correlation coefficient.

6.4 CONCLUSIONS

In conclusion, acute cigarette smoking increased arterial blood pressure and heart rate and reduced peripheral arterial function, measured by peripheral arterial tonometry, in young moderate smokers. The experimental model described here, where a decrease of RHI is easily induced by cigarette smoking could be useful in studying the impact of dietary vasoactive compounds in modulating peripheral arterial function.

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7. DEVELOPEMT OF ACUTE DIETARY INTERVENTION STUDY TO EVALUATE THE EFFECT OF A SINGLE PORTION OF BLUEBERRY IN THE MODULATION OF MARKERS OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL FUNCTION IN A HOMOGENEOUS GROUP OF YOUNG SMOKER VOLUNTEERS

7.1 AIM OF THE STUDY

It has been previously "set up" a potential human model for studying the effects of smoking on peripheral arterial function by Endo-PAT2000. In that study, we demonstrated that PAT technology was able to detect a peripheral arterial dysfunction following a single cigarette smoking in a homogeneous population of smoker volunteers. Thus, in the present study, we proposed to use the same human model for studying the impact of blueberry consumption in the modulation of RHI and markers of oxidative stress, endothelial function and inflammation.

7.2 MATERIALS AND METHODS

7.2.1 Subject recruitment

Sixteen healthy male smokers, 23.6 ± 2.9 average age and BMI of 23.0 ± 1.9 kg/m^2, were recruited from the student population of the University of Milan according to the following criteria: 20-30 years of age, moderate smoking (about 15 cigarette/day), moderate physical activity (25-30 min per day of brisk walk or jog) and moderate alcohol consumption (up to 10-14 drinks per week). Subjects were recruited on the basis of an interview by a dietitian to evaluate their dietary habits. 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/m^2). 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. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. All participants signed informed consent form.

7.2.2 Experimental design

Volunteers were selected for a repeated measures crossover study (Figure 7.2.1) 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).

Figure 7.2.1- *Randomized cross-over experimental design.*
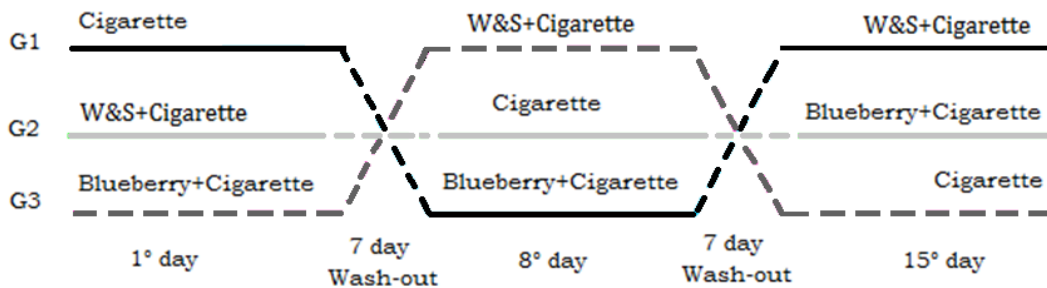
G, group; W&S, water + sugars

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. Subjects were deprived of polyphenol vasoactive substances 10 days before experimentation. Specific attention was devoted to food such as chocolate, berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine and red/violet fruits, and green tea. Volunteers abstained also from drinking caffeine-rich beverages and limited coffee to three per

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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 permitted at the end of the dinner. No alcoholic drinks or soft drinks were permitted. Moreover, all participants were asked to refrain from physical activity from the day before the experiment and to continue smoking the number of cigarettes/day as declared in the questionnaire.

Also for the present study, peripheral arterial function was measured in two consecutive days. Therefore, overnight fasted volunteers came in our laboratory early in the morning; baseline levels were assessed the first day. The second day, subjects consumed a portion of blueberry or a control drink or nothing and smoked one cigarette. The protocol was set to measure peripheral arterial function 2 h after blueberry intake; these conditions were chosen by considering previous observations on the beneficial effect on endothelial function observed at this specific time-point following the intake of a polyphenol-rich food (Dohadwala et al., 2011; Loffredo et al., 2011). The cigarette, containing approximately 6 mg of Tar by volume, 0.5 mg of nicotine and 0.9 mg of carbon monoxide, was smoked 1 h and 40 min after blueberry or control drink consumption. Reactive hyperemia index (RHI), and digital augmentation index (dAix) were tested 20 min after smoking. Systolic (Sy), and diastolic (Di) blood pressure (BP), and heart rate (HR) were measured before and 5 min after smoking a cigarette and at the end of the endothelial function measurement. Blood pressure and heart rate were measured before and 5 min after smoking and at the end of the evaluation of peripheral vasoreactivity (30 min after smoking).

7.2.3 Determination of peripheral arterial function and arterial stiffness

Endothelial-dependent vasodilation in the small finger arteries was assessed by Endo-PAT2000. In addition to the RHI it has been also reported the Framingham RHI (F-RHI), which was automatically calculated using, however, a different post-occlusion hyperaemia period (90 to 120 s) without baseline correction factor. The F-RHI, that has been shown to correlate with other CVD risk markers (Hamburg et al., 2008), was expressed as natural log of the resulting ratio. The EndoPAT device also generates dAix, strongly correlated to aortic Aix, calculated from the shape of the pulse wave recorded by the probes during baseline (Munir et al., 2008). Because Aix is influenced in an inverse and linear manner by heart rate, the dAix was automatically normalized by the tool for a heart rate of 75 bpm (dAix@75).

7.2.4 Biochemical Measurements

Blood samples were drawn and immediately centrifuged at 1000 x g for 15 min. for serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was performed in serum, including evaluation of lipid profile (TG, TSC, LDL-C and HDL-C), and glucose. All these parameters were determined using standard laboratory methods (Del Bo' et al., 2013a).

7.2.5 Evaluation of endogenous DNA damage and cell resistance against H_2O_2-induced DNA damage

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation as previously reported (**Study 1**). The FPG-sensitive sites (oxidized purines) and cell resistance against H_2O_2 (500 µmol L^{-1}, 5 min) induced DNA damage were evaluated by the comet assay as previously described in detail (**Study 1**).

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7.2.6 Evaluation of marker of inflammation and endothelial function

Serum was obtained within 1 h, by centrifugation for 15 min at 2300 x g at 4°C. Markers of inflammation and endothelial function such as: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), were measured by Bio" plex Pro TM Array.

7.2.7 Preparation of blueberry portion and control-drink

Blueberries (*Vaccinium corymbosum L.* "Brigitta") from a single batch were purchased, sorted and immediately frozen by Individually Quick Freezing technique in a tunnel (Thermolab, Codogno, Italy) and stored at −20°C until use. For the study, 300g of blueberry was thawed at + 4°C overnight. The control-drink was prepared daily, by suspending in 300 mL of water the same amount of sugars found in the blueberry. No bioactive compounds were added in the control drink.

7.2.8 Sugars, anthocyanins, total phenolics and vitamin C determination in blueberry portion

Sugar (glucose and fructose) content was quantified by ultra high pressure liquid chromatography-mass spectrometry as previously described (Del Bo' et al., 2013a). Individual ACNs were analyzed by high performance liquid chromatography (HPLC) analysis (Del Bo' et al., 2010), while total phenolic compounds were analyzed by Folin-Ciocalteau assay (Singleton & Rossi 1965) and expressed as gallic acid equivalents (mg/100g). Vitamin C (ascorbic acid) was extracted and determined by HPLC analysis as previously described by Riso et al. (2005).

7.2.9 Statistical analysis

Sample size has been calculated taking into account the expected variation of RHI as primary endpoint considered. In particular, by considering our previous observations (Del Bo' et al., 2013), sixteen subjects were calculated to be sufficient to evaluate a difference of RHI after blueberry intake of 0.30 (standard deviation 0.40), with alpha=0.05 and a statistical power of 80%. Moreover, the "repeated measure" experimental design in which each subject acts as its own control, allows reduction of the error variance.
Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). The Shapiro-Wilk test was applied to verify the normal distribution of the variables. Data of the variables under study were analyzed by one way ANOVA with time (before and after smoking) or treatment (smoking *vs* consuming a portion of blueberry + smoking *vs* consuming a control drink + smoking) as dependent factors. The variables of the treatment were reported as the percentage change (i.e. [after treatment-before treatment]/ before treatment *100). The mean changes are described as mean with 95% confidence interval (CI). Differences are considered significant at $p \leq 0.05$; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as level of statistical significance. Data presented as mean values standard error of the mean (SEM).

7.3 RESULTS AND DISCUSSION

7.3.1 Baseline characteristics of the study population
The baseline clinical and anthropometric characteristics of the enrolled volunteers are reported in Table 7.3.1. Subjects presented normal vital signs (SyBP/DiBP<140/90 mmHg), endothelial function (RHI >1.67), serum lipid profile (TG, TSC, LDL-C and HDL-C), glucose, consistent with their young age.

Table 7.3.1- *Anthropometric and clinical characteristics of the subjects at baseline (n=16)[1]*

Variables	Value
Age (years)	23.6 ± 0.7
Height (cm)	178.1 ± 1.7
Weight (kg)	73.1 ± 2.3
BMI (kg/m^2)	23.0 ± 0.5
Neck circumference (cm)	36.6 ± 0.3
Shoulder circumference (cm)	110.3 ± 1.3
Chest circumference (cm)	94.8 ± 1.7
Waistline circumference (cm)	79.5 ± 1.7
Abdominal circumference (cm)	84.2 ± 1.5
Hip circumference (cm)	97.5 ± 1.2
Arm circumference (cm)	28.2 ± 0.5
Forearm circumference (cm)	26.1 ± 0.4
Wrist circumference (cm)	17.2 ± 0.1
Smoke (cigarettes/day)	15 ± 1
SyBP (mm Hg)	116.0 ± 1.7
DiBP (mm Hg)	76.1 ± 2.1
HR (beat min^{-1})	63.3 ± 2.9
RHI	2.23 ± 0.07
F-RHI	0.65 ± 0.07
dAI(%)	-8.6 ± 2.0
dAI@75 (%)	-18.4 ± 2.2
TSC (mmol L^{-1})	4.13 ± 0.08
HDL-C (mmol L^{-1})	1.43 ± 0.10
LDL-C (mmol L^{-1})	2.20 ± 0.10
Triglycerides (mmol L^{-1})	1.01 ± 0.08
Glucose (mmol L^{-1})	4.34 ± 0.17

[1]Data are expressed as mean ± SEM. SyBP, systolic blood pressure; DiBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAI, digital augmentation index; dAI@75, digital augmentation index standardized for heart rate of 75 bpm; TSC, total serum cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

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7.3.2 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 309mg of ACNs (malvidin-galactoside, delphinidin-galactoside, petunidin-galactoside and malvidin-arabinoside were the dominant compounds), 856mg of total phenolic acids and 2.4mg of ascorbic acid. The control drink provided the same amount and type of sugars but no bioactive compounds.

7.3.3 Effect of smoking on reactive hyperemia index and arterial stiffness

In Table 7.3.2 are reported the values of RHI, F-RHI, dAix and dAix@75 before and after smoking. Peripheral arterial function, measured through the digital hyperemic response by the RHI, was impaired after 20 min of smoking.

Table 7.3.2- *Arterial function and arterial stiffness measure before and 20 min after smoking a cigarette (n=16)[1]*

	Before smoking	20 min After smoking	p value[2]
RHI	2.23 ± 0.08	1.64 ± 0.07	0.0001
F-RHI	0.65 ± 0. 08	0.31 ± 0.07	0.002
dAix (%)	-7.8 ± 2.1	-14.1 ± 1.8	0.003
dAix@75 (%)	-18.8 ±2.2	-19.1 ± 2.2	0.819

[1]*Data are expressed as mean ± SEM. RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.*

[2]*Overall P value for one-way ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).*

The RHI reduction was particularly high insomuch as to induce an endothelial dysfunction (RHI<1.67) in more than a half of the subjects (9 out of 16) and a reduction in the others (Figure 7.2.2a). A significant impairment was also observed for F-RHI (Table 7.3.2). The F-RHI reduction occurred in 13 out of 16 subjects, while a small increase with respect to baseline value was observed in 3 subjects (Figure 7.2.2b). The deleterious effects observed are in accordance with several studies (Gül et al., 2011; Bard et al., 2010; Lekakis et al., 1997) and with ther previous observation in which it has been reported that smoking induced a significant reduction (-27.6%) of RHI in young healthy smokers (Del Bo' et al., 2013b).

Regarding dAix, a significant (p=0.003) reduction was also observed (Table 7.3.2), while no significant (p=0.819) effect was detected after normalization for heart rate (dAix@75), as also confirmed in the study previously reported. These results are in agreement with those studies in which no effect was observed on arterial stiffness after acute smoking in young smokers (Seet et al., 2012), but in contrast with those that showed increase in arterial stiffness in older smokers (Rehill et al., 2006). Thus, the lack of an effect or the apparent improvement for dAix could be dependent of the age of volunteers. Young people have more elastic walls able to counteract the vasoconstriction induced by smoking (Rehill et al., 2006).

Figure 7.2.2-*Individual values of reactive hyperemia index (A) and Framingham reactive hyperemia index (B) measured by before and 20 minutes after smoking a single cigarette in a group of smokers (n = 16).*
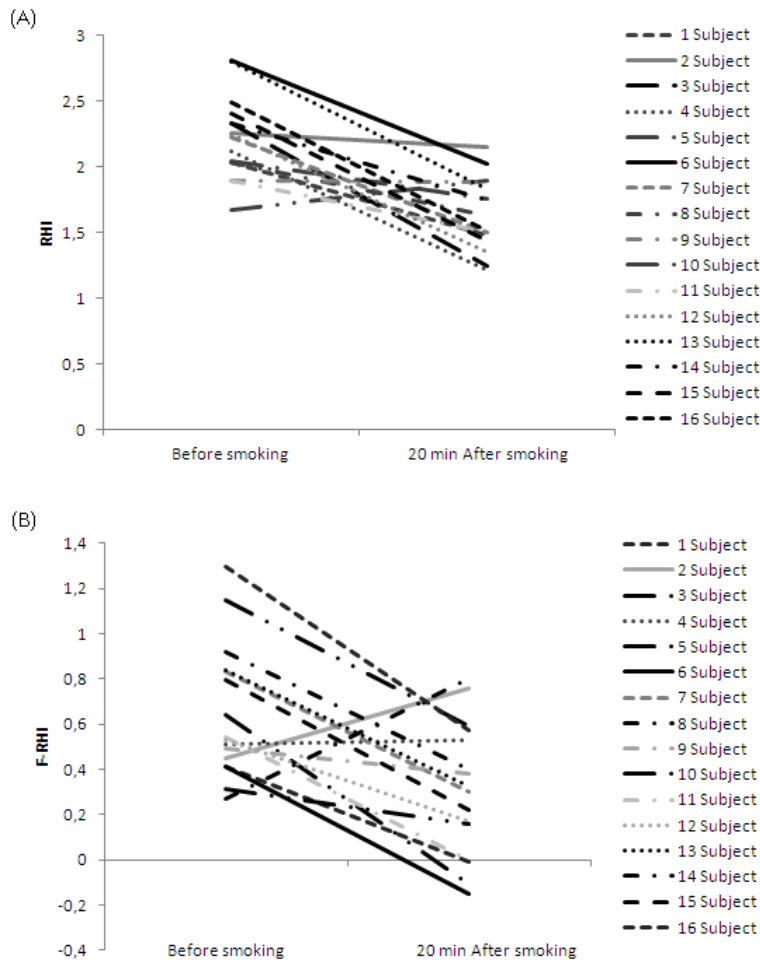
RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index

7.3.4 Effect of smoking on blood pressure and heart rate

Short-term smoking has also been shown to increase blood pressure and heart rate. In the present study, it has been documented that acute cigarette smoking impaired blood pressure and heart rate. Smoking a single cigarette significantly increased the levels of SyBP (+13%), DiBP (+10%), and HR (+15%). This effect was transitional and the values dropped to baseline 30 min after smoking (Table 7.3.3). These results are in accordance with data reported by Lekakis et al. (1997) and Stefanadis et al. (1997), who documented a prompt increment in heart rate and blood pressure during the first 5 min after smoking probably attributed to an increase in circulating levels of catecholamines that seem to reach a maximum concentration after 5-10 min. from smoking and to return to baseline levels after 30 min (Lekakis et al., 1997).

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Table 7.3.3- *Blood pressure and heart rate, before, 5 and 30 min after smoking a cigarette (n =16)[1]*

	Before smoking	5 min After smoking	30 min After smoking	P value[2]
SyBP (mmHg)	116.0 ± 1.7^a	131.7 ± 1.6^b	117.0 ± 1.9^a	0.0001
DiBP (mmHg)	76.1 ± 2.1^a	83.5 ± 1.9^b	76.8 ± 1.7^a	0.005
HR (beat min^{-1})	63.3 ± 2.9^a	70.7 ± 2.9^b	64.0 ± 2.9^a	0.047

[1]*Data are expressed as mean ± SEM. SyBP, systolic blood pressure; DiBP, diastolic blood pressure; HR, heart rate*
[2]*Overall P value for one-way ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).*

7.3.5 Effect of smoking on markers of oxidative stress, inflammation and endothelial function

The impairment of vascular function and blood pressure may be related to an increase of oxidative stress or inflammatory status. In fact, smoking elevates the circulating levels of potential blood vessel vasoconstrictor agents, pro-inflammatory cytokines and the damage of several macromolecules such as lipids and DNA due to a rapid decrease of antioxidant status. This condition increases immediately after smoking by inducing hypertension and endothelial dysfunction (Bard et al., 2010; Csiszar et al., 2009). In Table 7.3.4 are reported the effect of cigarette smoking on the levels of DNA damage in a subgroup of 6 subjects. No significant effect was also observed for the levels of DNA damage measured as FPG-sensitive sites and DNA strand breaks, before and after smoking in the group of subjects (Table 7.3.4).

Table 7.3.4- *Effect of cigarette smoking on background FPG sensitive sites, and H_2O_2 induced strand breaks. (n=6)[1]*

Variables	Time 0 min	Time 20 min	Time 24 h	P value[2]
Background SBs (%DNA in tail, EB)	6.3 ± 1.0	6.7 ± 0.8	6.6 ± 0.3	0.201
Net FPG-sensitive sites (% DNA in tail)	14.3 ± 2.3	16.3 ± 4.1	14.3 ± 2.9	0.390
Background SBs (% DNA in tail, PBS)	7.2 ± 1.2	6.8 ± 1.1	7.2 ± 1.3	0.594
Net H_2O_2- induced DNA damage (% DNA in tail)	46.6 ± 5.1	47.3 ± 3.9	47.3 ± 4.3	0.370

[1]*Data are expressed as mean±SD. SBs, strand breaks; PBS, phosphate buffer saline; EB, endonuclease buffer; FPG, formamidopyrimidine DNA glycosilase.*
[2]*Overall P value for repeated-measured ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).*

In Figure 7.3.1 are reported the effect of cigarette smoking in the serum circulating levels of VEGF, ICAM-1, sVCAM-1, IL-8. ANOVA did not show any significant effect on these markers, even if serum concentrations tended to increase after 20 min from cigarette smoking, while a decrease seemed occur for IL-8. If these results will be confirmed on the whole group of subjects, they could explain the reasons of this impairment in the endothelial function.

Figure 7.3.1-*Effect of cigarette smoking on serum circulating levels of VEGF (A), VCAM (B), ICAM (C) and IL-8 (D), (n=6)*[1]
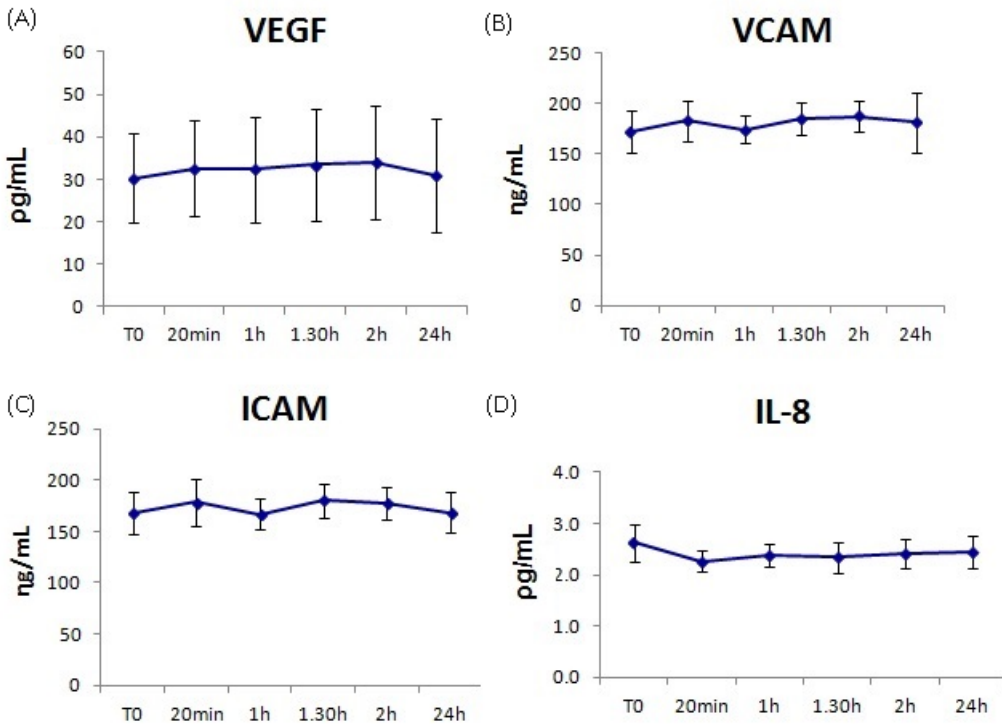
[1]*Data are reported as mean±SD*

7.3.6 Effect of a single blueberry portion and control drink on reactive hyperemia index and arterial stiffness

The role of bioactive rich-food in the modulation of vascular function has been investigated in several studies but controversial results have been observed. Short term supplementation with vitamin C and E has shown to improve endothelial function in healthy young smokers, while no beneficial effect has been observed in the long term (Raitakari et al., 2000; Neunteufl 2000). Similar results have been also observed after the consumption of a single portion of polyphenol-rich food such as red wine (Karatzi et al., 2007), dark chocolate (Loffredo et al., 2011) and cranberries (Dohadwala et al., 2011). In the present study, the consumption of a portion of blueberry was able to counteract the endothelial dysfunction induced by smoking, 2 hours after blueberry consumption. On the contrary, no effect was observed following consumption of the control drink.

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The mean percentage variation values of RHI (A), F-RHI (B), for each treatment are reported in Figure 7.3.2. Repeated measures ANOVA revealed a significant effect of treatment for the variable RHI (p=0.0006), and F-RHI (p=0.003). The mean percentage change pre to post treatment for RHI was -25.2% (95%CI: -34%, -16.2%) after S treatment, -17.5% (95%CI: -26%, -8.9%) after CS treatment and -6.6% (95%CI: -13%, -0.5%) after BS treatment (Figure 7.3.2a). The mean percentage change pre to post treatment for F-RHI was -42.7% (95%CI: -85.4%, -0.15%) after S treatment, -8.1 % (95%CI: -36.5%, +20.3%) after CS treatment and +28.3% (95%CI: -12.6%, +69.2%) after BS treatment (Figure 7.3.2b). Post-hoc analysis (LSD test) revealed that the consumption of a single blueberry portion counteracted significantly the reduction of RHI and F-RHI after S treatment (BS *vs* S, p=0.0001 and p=0.0008, respectively). However, the reduction was significantly different with respect to CS treatment (BS *vs* CS, p= 0.01) for RHI, but not for F-RHI (BS *vs* CS, p= 0.006). No effect was observed between S *vs* CS treatment for both the variables (RHI, p=0.09 and F-RHI, p=0.08). These results are in accordance with the previous observations in which polyphenol-rich foods affect vascular function 2 hours after their intake (Karatzi et al., 2007; Loffredo et al., 2011; Dohadwala et al., 2011).

Figure 7.3.2- *Mean percent variation of RHI (A), F-RHI (B) measured during each treatment (n=16)[1].*
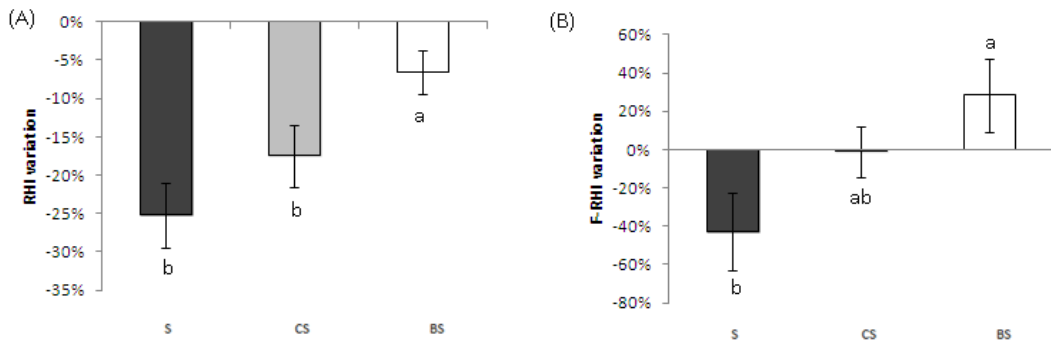
[1]*Data are expressed as mean ± SEM. S, smoking treatment; CS, control-drink + smoking treatment; BS, blueberry intake + smoking treatment; RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.*
[a,b] *Graphs with different letters are significantly different from other treatments (p≤0.01).*

Endothelial dysfunction is frequently associated with high levels of arterial stiffness. Some studies documented that smoking and unbalanced dietary habits (i.e. meals rich in saturated fatty acid, refined sugars and salt), may have detrimental effect on arterial stiffness (Hall 2009; Mahmud & Feely 2003). On the contrary, the consumption of polyphenol-rich food seems to reduce and improve the arterial stiffness (Ruel et al., 2013; Karatzi et al., 2013).
In the present study, no significant effect was also observed for dAix and dAix@75 (p=0.20 and p=0.79, respectively) following the consumption of blueberry and control drink (Figure 7.3.3). These results are in accordance with Mathew et al., (2012) in which no effect on arterial stiffness was observed following consumption of a high fat meal and pomegranate juice extract, but in contrast with Karatzi et al., (2012) that found modulation of arterial stiffness following an acute consumption of beer.

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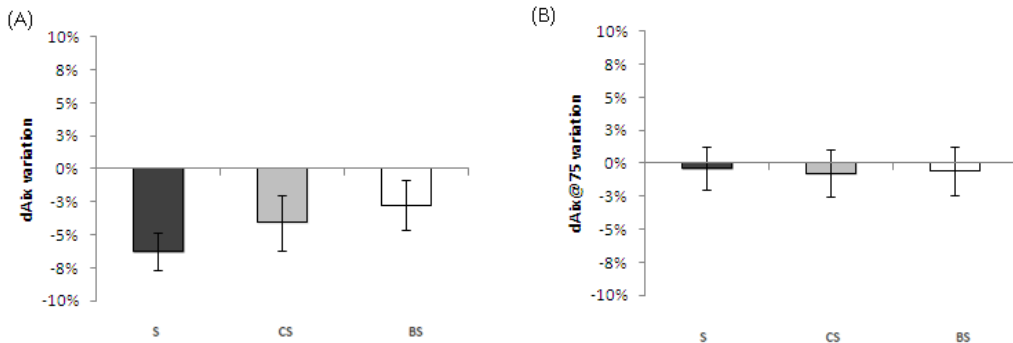**Figure 7.3.3-** *Mean percent variation of dAix (A), dAix@75 (B) measured during each treatment (n=16)[1].*

[1]*Data are expressed as mean ± SEM. S, smoking treatment; CS, control-drink + smoking treatment; BS, blueberry intake + smoking treatment; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.*

7.3.7 Effect of a single blueberry portion and control drink on systolic and diastolic blood pressure, and heart rate

Diets rich in antioxidant compounds seem to modulate blood pressure levels. A recent meta-analysis has reported for the first time, that the intake of polyphenol and ACN-rich foods is associated with low levels of blood pressure (Jennings et al., 2012). Similar results were also observed by Mathew et al., (2012) in which the consumption of pomegranate extract has resulted in suppression of the postprandial increase in SyBP following a high-fat meal. On the contrary, two recent dietary intervention studies reported that 4-week consumption of an ACN-extract did not reduce the levels of blood pressure in healthy and pre-hypertensive men (Hassellund et al., 2012; Hassellund et al., 2013).

In the present study, the effect of blueberry consumption on SyBP, DiBP and HR in each treatment is reported in Figure 7.3.4 (a-c). The mean percentage change between the pre to post treatment was +13.1% (95%CI: 10.5%, 15.7%) after S treatment, +12.7% (95%CI: 10.2%, 15.2%) after CS treatment, and +8.4% (95%CI: 5.4%, 11.4%) after BS treatment (Figure 7.3.4a). Post-hoc analysis (LSD test) showed that the consumption of a single blueberry portion counteracted significantly the increment of SyBP after S treatment (BS *vs* S, p=0.008). This effect was also significantly different with respect to CS treatment (BS *vs* CS, p= 0.01) while no difference was observed between S and CS (p=0.90). On the contrary, no effect was observed after blueberry intake for the variables DiBP and HR among the three treatments (p=0.71 and p=0.50, respectively).

These results seem to support the potential beneficial effect of polyphenol compounds in the modulation of blood pressure as also previously reported by other authors (Jennings et al., 2012; Mathew et al., 2012).

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Figure 7.3.4-*Mean percent variation of SyBP(A), DiBP (B) and HR (C) measured during each treatment (n=16)[1].*
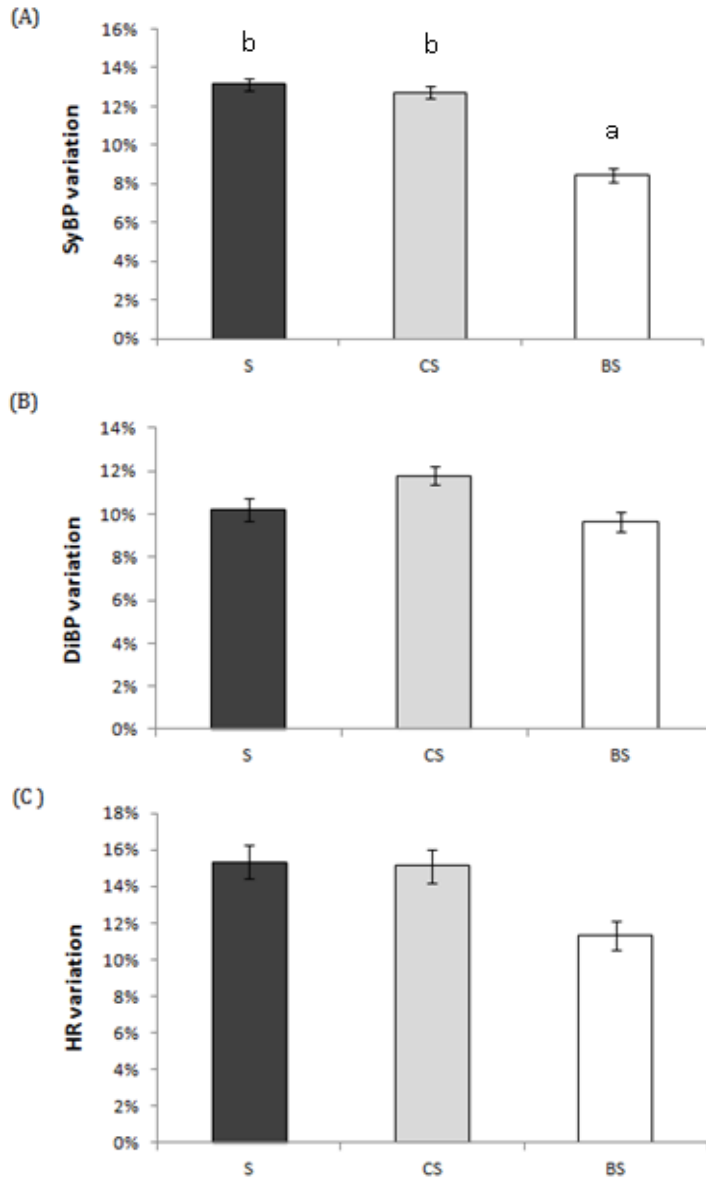
[1]*Data are expressed as mean ± SEM. S, smoking treatment; CS, control-drink + smoking treatment; BS, blueberry intake + smoking treatment; SyBP, systolic blood pressure; DiPB, diastolic blood pressure; HR, heart rate.*
[a,b] *Graphs with different letters are significantly different from other treatments (p≤0.01).*

7.4 CONCLUSIONS

In the present study we documented that acute smoking significantly reduced peripheral arterial function and increased blood pressure and heart rate in healthy male smoker volunteers, while the consumption of blueberries prevented peripheral arterial dysfunction. These results are important and confirm the protective role of blueberry in the modulation of vascular function, emphasizing the importance of regular berry fruit consumption especially in people exposed to oxidative stress such as smokers. The analysis of data on the whole group of subjects will clarify the effect of smoking and blueberry in the modulation of biomarkers of oxidative stress, inflammation and endothelial function understudy. Prospective short-term studies in healthy subjects and in patients with cardiovascular risk factors (i.e. endothelial dysfunction) are needed to confirm the beneficial effects on vascular function, focusing also on the mechanisms involved in their modulation.

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8. DEVELOPMENT OF A DIETARY INTERVENTION STUDY TO EVALUATE THE EFFECT OF 6-WEEK BLUEBERRY CONSUMPTION ON MARKERS OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL FUNCTION, IN SUBJECTS WITH RISK FACTORS FOR CARDIOVASCULAR DISEASE

8.1 AIMS OF THE STUDY

The protective effect of wild blueberry intake has been studied in several animal models in our laboratories (Del Bo' et al., 2012; Del Bo' et al., 2010a; Del Bo' et al., 2010b; Kalea et al., 2010; Kristo et al., 2010; Kalea et al., 2009; Norton et al., 2005). In particular, we demonstrated that the intake of wild blueberries (24 mg ACNs per day) can improve vasomotor tone in the rat aorta and lymphocyte resistance against oxidatively induced DNA damage (Del Bo' et al., 2012; Del Bo' et al., 2010b). However, such observations in animal experimental models need to be confirmed in controlled human intervention studies where a wide range of biomarkers are used and specific targets of subjects at risk are considered. To our knowledge there is a paucity of studies that have evaluated the *in vivo* effect of blueberry consumption in humans with risk factors for CVD (Basu et al., 2010; Stull et al., 2010).

We have previously reported that blueberry was able to reverse endothelial dysfunction induced by acute cigarette smoking in a group of young healthy smokers. In the present study, we investigated the effect of 6-week consumption of blueberry on markers of oxidative stress, inflammation and endothelial function in subjects with risk factors for CVD.

8.2 MATERIALS AND METHODS

8.2.1 Recruitment of subjects

Subjects involved in the study were recruited from the staff of the University of Milan and other Institutes through advertisement on bulletin boards. Inclusion criteria were: healthy subjects with at least one risk factor for CVD such as: pre-hypertension (systolic pressure 120-139 mm Hg and diastolic pressure between 80-89 mm Hg), high serum cholesterol ($\geq$5.17 mmol L^{-1}), low high density lipoprotein (HDL)-cholesterol (<1.03 mmol L^{-1}), high low density lipoprotein (LDL)-cholesterol ($\geq$3.36 mmol L^{-1}), high triglycerides ($\geq$1.69 mmol L^{-1}) overweight (BMI $\geq$25 kg/m^2) and smoking (>10 cigarettes/day). Individuals with secondary hypertension or obesity (BMI $\geq$30) were excluded. A medical history questionnaire was used to exclude subjects with clinical diseases such as diabetes, renal insufficiency, known food allergic reactions, chronic constipation, diarrhea or any other gastrointestinal problem or disease. Subjects were specifically excluded from the study if they were taking drugs, supplements, specific probiotics or medications during the last month before the beginning of the experiment. Weight and height were assessed and subjects were selected on the basis of an interview to evaluate their dietary habits and ensure that they were as homogeneous as possible, in particular for fruit and vegetables consumption. This was obtained by means of a food frequency questionnaire previously published and specifically revised to focus on food sources rich in antioxidants (Porrini et al., 1995). Other exclusion criteria were: high (> 5 portions/day) or low (<2 portions/day) intake of fruit and vegetables and habitual alcohol consumption (< 3 drinks per week were tolerated). Volunteers who followed a specific diet such as vegetarian, vegan or macrobiotic, and those who had a specific aversion to blueberries or their products were excluded. Twenty male subjects, ages 47.8 $\pm$ 9.7 years with body mass index (BMI) 24.8 $\pm$ 2.6 kg/m^2 were selected. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. All participants signed informed consent form.

8.2.2 Blueberry and placebo drink preparation

The wild blueberry (WB) drink was prepared by suspending 25 g of WB freeze-dried powder (i.e. a composite from Wayman's (Cherryfield, ME), standardized at 1.5% total ACNs by FutureCeuticals, (Momence, IL, USA)) in 250 mL of water to give an amount of WB equivalent to 1 cup of raw fruits (148 g, providing approximately 375 mg of ACNs). The nutritional composition of the soluble WB powder used in the study is reported in Table 8.2.1. The placebo (PL) drink was prepared to have sensory characteristics similar to the WB drink but it did not contain polyphenols. The PL drink consisted of 250 mL water, 7.5 g fructose, 7 g glucose, 0.5 g citric acid, and 0.03 g blueberry flavor (Kerry Ingredients & Flavours Italia S.p.A., Bergamo, Italy). In order to reach the same color, small amounts of food colors typically used by food industry for the production of sweetened soft drinks were added to the PL drink.

8.2.3 Experimental design

A randomized, repeated measures crossover design was utilized. Subjects were randomly divided (by using a computer random number generator) into two groups of ten subjects each: group 1 was assigned to the sequence WB drink/wash-out/PL drink, whereas group 2 followed the sequence PL drink/wash-out/WB drink. WB and PL drinks were consumed daily for 6 weeks; the two treatments were separated by a 6 week wash-out period. Subjects received each morning the freshly prepared WB or PL drink in appropriate iceboxes. Participants were instructed to keep the drink under refrigeration and to avoid exposing it to a heat source or light

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and consume the drink within the morning. Additionally participants were asked to mix the beverage very well before drinking, rinse out each bottle with water and drink it to ensure complete intake of the WB powder. Every Friday, subjects received the drinks for the week-end and kept them refrigerated. Subjects were instructed to maintain their normal dietary and lifestyle habits (as declared before enrollment) but to abstain from consuming berries and other ACN-rich food sources. For this reason, during the experimental period, each subject received a complete list of foods high in ACNs to be avoided. A 24 h record of food consumption was kept by each volunteer one day before blood collection to check compliance to the dietary instructions. Moreover a 3-day food record and a weekly direct interview were scheduled randomly during the two experimental periods. At the beginning and at the end of each treatment period, fasting venous blood samples were collected early in the morning after an overnight fast.

8.2.4 Variables

The improvement of endothelium-dependent vasodilation (measured by a non-invasive plethysmographic method), the reduction of oxidized purines and the improvement of cell resistance to H_2O_2-induced DNA damage (evaluated in mononuclear blood cells by the comet assay) were considered as the primary endpoints. The other variables under study were: nutritional biomarkers (ACNs, vitamin C, folate, vitamin B_{12}, reduced glutathione (GSH), oxidized glutathione (GSSG)), lipid profile (triglycerides, total cholesterol, LDL and HDL-cholesterol), glucose, markers of inflammation (interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), soluble vascular adhesion molecule (s-VCAM-1), total nitric oxide (NO), augmentation index (AI), augmentation index standardized for heart rate of 75 bpm (AI@75), blood pressure, DNA repair activity, enzymes activity (glutathione S-transferase activity (GST), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), serum creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT).

8.2.5 Anthocyanin and phenolic acids extraction and analysis in the wild blueberry powder by LC-DAD-MS/MS

The Liquid Chromatography-diode array detector-mass spectrometry (LC-DAD-MS/MS) analysis of ACNs in the WB powder was performed in accordance to a procedure previously published (Del Bo' et al., 2010).

8.2.6 Soluble and insoluble fiber analysis in the wild blueberry powder

Soluble and insoluble dietary fiber in the WB powder was determined by the AOAC (Association of Official Analytical Chemists) International method 991.43; an AACC (American Association for Clinical Chemistry) 46.13 for the protein in the residue was used (AOAC method 1991).

8.2.7 Sample collection and separation

Blood was collected early in the morning by a phlebotomist. Samples were drawn into evacuated tubes with or without heparin. Plasma was separated within 30 min after collection while serum within 1 h, by centrifugation for 15 min at 2300 x g at 4°C. Peripheral blood mononuclear ells (PBMCs) were obtained from whole blood by density gradient centrifugation with Histopaque 1077 (Sigma Chemicals Co., St. Louis, MO). Mononuclear blood cells were removed from the gradient, washed with Phosphate Buffer Saline (PBS) and used for the determination of endogenous and cell resistance to H_2O_2-induced DNA damage. An aliquot of PBMC was

isolated and dissolved into an appropriate medium (90% RPMI media, 10% Dimethyl sulphoxide, Sigma) and stored at -80°C until evaluation of repair activity. Red blood cells were obtained from whole blood by centrifugation at 2300 x g for 10 min and were washed with an equal volume of PBS solution. Red blood cells (1 mL) were diluted with iced water (1:4) and centrifuged at 10000 x g for 10 min. All samples were then aliquoted, stored at -80°C until determination of hemoglobin and evaluation of SOD and GSH-Px activities. Red blood cells (500 μL) used for glutathione analysis were acidified with an equal solution of metaphosphoric acid (10%) before storage.

8.2.8 Analysis of biochemical parameters

A general laboratory biochemical assessment was performed including evaluation of hepatic and renal function (serum creatinine, AST, ALT and GGT), lipid profile (triglycerides, total cholesterol, and HDL-cholesterol) and glucose (Campolo et al., 2006). All these parameters were determined using standard laboratory methods. LDL cholesterol was calculated using the Friedewald's method. Plasma IL-6, TNF-α, CRP and s-VCAM 1 concentrations were measured by Quantikine human colorimetric sandwich ELISA immunoassay kits (R&D Systems, Inc. Minneapolis, MN). Plasma concentration of total NO was calculated by measuring the products of oxidation (nitrate and nitrite) by a Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI). Lymphocyte GST activity analysis was performed in accordance to the procedure described previously (Riso et al., 2009). GST activity was standardized to milligrams of protein and determined using a BCATM Protein Assay Kit (Pierce, Rockford, IL, USA). SOD and GSH-Px activities were measured in erythrocytes using commercial kits (Cayman chemical, Ann Arbor, USA). Enzyme activities were standardized to milligrams of hemoglobin determined by the use of the Drabkins reagent (Sigma Diagnostic, Co., St. Louis, MO). Vitamin C was determined in plasma by HPLC analysis as previously published (Riso et al., 2005). Vitamin B_{12} and total folate were measured by competitive immunoassay using direct chemiluminescence (Campolo et al., 2006). Analysis of GSH and GSSG in the erythrocyte lysate was performed following the instructions reported in a commercially available kit (Cayman chemical, Ann Arbor, USA).

8.2.9 Anthocyanin extraction and analysis in plasma

Two aliquots of plasma (1 mL) were acidified with TFA (1%), vortexed, and centrifuged for 1 min at 4500 x g and the supernatant was stored at -80°C until analysis. Anthocyanins were extracted from plasma and analyzed as previously reported (see **Study 3**).

8.2.10 Determination of peripheral arterial function

Evaluation of peripheral vasoreactivity was performed before blood drawing to avoid possible side effects on blood pressure and vasoactivity. Endothelial-dependent vasodilation and arterial stiffness in the small finger arteries was assessed by Endo-PAT2000 as previously reported (see **Study 2**).

8.2.11 Evaluation of DNA damage, repair activity and cell resistance against H_2O_2-induced DNA damage

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation as previously reported (see **Study 1**). The FPG-sensitive sites (oxidized purines) and cell resistance against H_2O_2 (500 μmol/L, 5 min) induced DNA damage were evaluated by the comet assay as previously described in detail (see **Study 1**).

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The DNA repair activity was determined in cryopreserved PBMCs. Assessment of DNA repair incisions was analysed by the Comet assay as previously described by Guarnieri et al. (2008). The repair activity was measured as the incision activity of substrate DNA treated with Ro19-8022/white light, which generates 8-oxo-7,8-dihydroguanine. Oxidised bases were introduced into A549 lung epithelial cells substrate nuclei by irradiating cells with white light in PBS with 1 lM Ro 19-8022 (the photosensitiser was a gift from F. Hoffmann-LaRoche, Basel, Switzerland) at 0°C. The cells were washed and resuspended in freezing medium (50% FBS, 40% RPMI and 10% DMSO) to a concentration of 3×10^6 cells/ml and frozen at –80°C. For the preparation of human PBMCs extracts, the cryo-preserved cells were centrifuged (300 g, 5 min and 4°C), and the pellet was resuspended in buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol; pH 7.8) at a volume of 20µL per 10^6 cells. The resuspended cells were divided in aliquots of 50 µL to which 12 µL 1% Triton X-100 was added. The lysate was centrifuged (700 g, 5 min and 4.C) and the supernatant was mixed with 200 µL buffer B (40 mM HEPES, 0.1 M KCl, 0.5 mM Na 2 EDTA and 0.2 mM BSA; pH 8). Approximately 3×10^4 substrate cells were embedded in agarose and applied on 85-100 mm GelBond. films and lysed as described for the Comet assay. After three times washing (5 min each) in buffer B, repair incisions were detected by incubation of the agarose-embedded nuclei with 60 µL PBMCs extract or buffer B for 20 min at 37°C. The subsequent alkaline treatment and electrophoresis were identical to the conditions used to determine DNA damage using the Comet assay. An assay control (a sample for FPGsensitive sites evaluation) was included in each electrophoresis run. After neutralisation with 0.4 M Tris–HCl (pH 7.5), cells were placed in 96% ethanol for 1.5 h or overnight. Nuclei were visualised as previously described for strand breaks and FPG-sensitive sites estimation after staining with 40 µL ethidium bromide (4 µg mL^{-1}) in PBS solution. The repair activity of the PBMCs extract was determined as the difference in % DNA in tail between parallel gels incubated with extract and control solution.

8.2.12 Statistical analysis

Sample size has been calculated taking into account the expected variation in the primary endpoints considered. In particular twenty subjects were calculated to be sufficient to evaluate a difference of RHI after WB drink of 0.28 (standard deviation 0.40), with alpha=0.05 and a statistical power of 80%. Moreover, the "repeated measure" experimental design in which each subject acts as its own control, allows conducting experiment more efficiently reducing the error variance. Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). The Shapiro-Wilk test was applied to verify the normal distribution of the variables. Data obtained were examined by analysis of variance (ANOVA) with the treatment sequence (WB drink vs PL drink or vice versa) as the independent factor in order to exclude the presence of carry-over effects. As none was detected, data were analyzed by ANOVA for repeated measures design. ANOVA with treatment (WB drink vs PL drink) and time (before and after each treatment) as dependent factors was applied to evaluate the effect of the WB drink on the variables under study. The difference in responses to the WB and PL drink periods was evaluated by statistically significant P-values for the interaction between treatment and time in the overall repeated ANOVA. Moreover, ANOVA with type of treatment as the dependent factor was used to evaluate percentage change (i.e. [after treatment-before treatment]/ before treatment *100) at the different endpoints of peripheral arterial function. The mean changes are described as mean with 95% confidence interval (CI). Differences are considered significant at p ≤ 0.05; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with p ≤ 0.05 as level of statistical significance.

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8.3 RESULTS AND DISCUSSION

8.3.1 Composition and characteristics of the wild blueberry powder

One portion of the WB powder provided about 102 kcal, 1.5 g of lipids, 1 g of proteins, 17 g of sugars (glucose and fructose) and 4.2 g of total fiber. The soluble and insoluble fiber was 0.6 g and 3.6 g, respectively. Chlorogenic acid (127.5 mg / 25 g) was the main phenolic acid detected in the WB powder. The total ACN concentration was 375 mg / 25 g with peonidin-3-glucoside, malvidin-3-galactoside, delphinidin-3-glucoside and delphinidin-3-galactoside as the dominant ACN compounds which represented about 35% of the total amount of ACNs.

8.3.2 Baseline characteristics of the subjects

Twenty people began the study and eighteen completed the whole protocol. Two subjects withdrew from the study due to personal reasons. Baseline anthropometric and clinical characteristics of the remaining 18 subjects are available in Table 8.3.1. Blood pressure, lipid profile and inflammatory markers were within the range of normality. According to the guidelines of the American Heart Association (AHA 2011), 8 subjects were classified as overweight (BMI > 25 kg/m^2), 7 had high levels of total cholesterol (≥ 6.2 mmol L^{-1}) and 7 borderline high (5.17-6.18 mmol L^{-1}). Six subjects had high levels of LDL-cholesterol (≥ 4.13 mmol L^{-1}) and 7 were borderline high (3.36-4.11 mmol L^{-1}). Ten subjects were pre-hypertensive (systolic pressure 120-139 mm Hg and diastolic pressure between 80-89 mm Hg). Nine subjects were smokers/ex-smokers. Finally eight subjects had below normal endothelium-dependent vasodilation with a RHI value ≤ 1.67.

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TABLE 8.3.1- *Subject characteristics at the beginning of the study (n=18)[1]*

Variables	Value	Variables	Value
Age (y)	47.8 ± 9.7	NO (μmol L^{-1})	45.3 ± 7.3
Body weight (kg)	75.7 ± 8.7	sVCAM-1 (ng mL^{-1})	402 ± 116
BMI (kg/m^2)	24.8 ± 2.6	CRP (mg dL^{-1})	0.19 ± 0.22
Glucose (mmol L^{-1})	4.9 ± 0.5	IL-6 (pg mL^{-1})	2.1 ± 1.1
TG (mmol/L^{-1})	1.2 ± 0.5	TNF-α (pg mL^{-1})	3.4 ± 1.5
TSC (mmol/L^{-1})	5.8 ± 1.2	Diastolic pressure (mm Hg)	79.4 ± 8.7
LDL-cholesterol (mmol/L^{-1})	3.8 ± 1.0	Systolic pressure (mm Hg)	121 ± 16
HDL-cholesterol (mmol/L^{-1})	1.4 ± 0.3	RHI	1.84 ± 0.46
AST (U L^{-1})	22.8 ± 4.8	FRHI	0.32 ± 0.27
ALT (U L^{-1})	26.7 ± 1.5	dAix	5.22 ± 18.5
GGT (U L^{-1})	26.8 ± 10.7	dAix@75	0.0 ± 17.4
Creatinine (mmol L^{-1})	79.6 ± 8.8	H_2O_2-induced DNA damage (% DNA in tail)	45.6 ± 10.1
Anthocyanins (μmol L^{-1})	nd	FPG sensitive sites (% DNA in tail)	13.4 ± 5.6
Vitamin C (μmol L^{-1})	63.1 ± 17.3	DNA repair activity (%DNA in tail)	6.5 ± 1.7
Folate (nmol L^{-1})	16.5 ± 4.5		
Vitamin B_{12} (pmol L)	312 ± 110		
GSH (μmol g^{-1} Hb)	5.8 ± 1.0		
GSSG (μmol g^{-1} Hb)	0.65 ± 0.42		
GSH/GSSG ratio	11.2 ± 4.53		
SOD activity (U mg^{-1} Hb)	4.7 ± 0.5		
GST activity (nmol min^{-1} mg^{-1} proteins)	35.1 ± 17.4		
GSH-Px activity (μmol min^{-1} mg^{-1} proteins)	17.4 ± 7.3		

[1]*Data are expressed as mean $\pm$ SD. BMI, body mass index; nd, not detectable; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; GST, glutathione S-transferase; GSH-Px, glutathione peroxidase; NO, nitric oxide; sVCAM-1, vascular cell adhesion molecule-1; CRP, C-reactive protein; IL-6, interleukin-6; TG, triglycerides; TNF-α, tumor necrosis factor alpha; TSC, total serum cholesterol; 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.*

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8.3.3 Effect of intervention on dietary markers

The effect of the 6 week intervention with the WB vs the PL drink on dietary markers evaluated in plasma is reported in Table 8.3.2. The intervention resulted in no significant effect on vitamin C, vitamin B_{12}, folate, GSH and GSSG concentrations and GSH/GSSG ratio. No ACNs were detected in the plasma after the WB or PL drink treatment after 6 weeks of intervention.

TABLE 8.3.2- *Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on nutritional biomarkers (n=18)[1]*

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Anthocyanins (μmol L^{-1})	nd	nd	nd	nd	-
Vitamin C (μmol L^{-1})	61.5 ± 16.8	61.7 ± 20.2	64.1 ± 15.8	67.5 ± 16.5	0.488
Folate (nmol L^{-1})	16.3 ± 5.0	15.0 ± 4.8	17.2 ± 4.8	15.9 ± 4.1	0.930
Vitamin B_{12} (pmol L^{-1})	308 ± 92	298 ± 99	314 ± 107	322 ± 105	0.392
GSH (μmol g^{-1} Hb)	6.8 ± 2.5	6.0 ± 0.9	6.2 ± 1.2	6.6 ± 1.5	0.140
GSSG (μmol g^{-1} Hb)	0.77 ± 0.44	0.74 ± 0.27	0.74 ± 0.40	0.83 ± 0.42	0.574
GSH/GSSG ratio	11.3 ± 6.6	8.99 ± 2.98	9.03 ± 3.74	9.45 ± 4.62	0.216

[1] *Data are expressed as mean $\pm$ SD. WB, wild blueberry; PL, placebo; nd, not detectable; GSH, reduced glutathione; GSSG, oxidized glutathione*
[2] *P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).*

8.3.4 Effect of intervention on vascular function and blood pressure

The values of RHI, FRHI, dAix, dAix@75, NO, sVCAM-1 and blood pressure are reported in Table 8.3.3. One subject was omitted from the analysis due to abnormal values registered in 2 different time points. Vascular function of the subjects measured by Endo-PAT2000 did not improve significantly, according to the repeated measures ANOVA, after the WB and PL drinks (P=0.452 for the interaction between treatment and time). However, an apparent decrease in the RHI was observed during consumption of the PL drink with respect to WB drink. The mean percent change between the pre-to-post intervention was +4.9% (95%CI: -11%, +20.8%) after the WB drink and -4.9% (95%CI: -12.6%, +3.9%) after the PL drink.
On the whole a high inter-individual variability was observed in the percent change of RHI (Figure 8.3.1) with about half of the subjects (53%; 9 out of 17) having an improvement following the intervention with the WB drink while the other half experienced (47%; 8 out of 17) a decrease. In the placebo group we observed that few subjects (23%; 4 out of 17) had an improvement following the intervention with the PL drink, while more than a half of the subjects (59%; 10 out of 17) showed a decrease. The remaining subjects (18%; 3 out of 17) did not show any effect. No significant changes in FRHI, dAix, dAix@75, blood pressure (systolic and diastolic), total plasma NO and sVCAM-1 were documented after the 6-week intervention with the WB drink or the PL drink.

TABLE 8.3.3- *Effect of a 6-week intervention with wild blueberry (WB) drink or placebo (PL) drink on vascular function, blood pressure, plasma total nitric oxide and soluble vascular cell adhesion molecule-1 (n=18)[1]*

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
RHI	1.83 ± 0.43	1.86 ± 0.55	1.87 ± 0.48	1.76 ± 0.41	0.452
FRHI	0.35 ± 0.28	0.29 ± 0.33	0.34 ± 0.31	0.30 ± 0.25	0.838
dAix	3.57 ± 15.6	3.18 ± 14.9	6.94 ± 17.5	4.71 ± 16.5	0.407
dAix@75	-1.71 ± 15.6	-3.76 ± 14.1	0.00 ± 17.9	0.47 ± 16.3	0.458
Diastolic pressure (mm Hg)	81.6 ± 8.8	81.4 ± 7.2	78.3 ± 8.2	78.8 ± 8.5	0.740
Systolic pressure (mm Hg)	123 ± 16	122 ± 16	122 ± 16	120 ± 16	0.878
Total NO (μmol L^{-1})	48.5 ± 24.1	49.8 ± 23.6	45.9 ± 16.9	43.7 ± 20.2	0.666
sVCAM-1 (ng mL^{-1})	410 ± 103	576 ± 364	417 ± 129	540 ± 232	0.762

[1]*Data are expressed as mean±SD; (n=17 vascular function). WB, wild blueberry; PL, placebo; RHI, reactive hyperemia index; FRHI, Framingham reactive hyperemia index; AI, augmentation index; AI@75, augmentation index standardized for heart rate of 75 bpm; Total NO, total nitric oxide; sVCAM-1, vascular cell adhesion molecules-1.*
[2]*P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).*

These results are in accordance with Dohadwala et al. (2011) who demonstrated that 4 weeks of supplementation with cranberry juice (480 ml/day, providing 94 mg ACNs and 835 mg total polyphenols) had no effect on vascular function (measured by flow-mediated dilation in the brachial artery) in subjects with coronary artery disease. However, many other factors may have affected these results such as: the large inter individual variation, individual differences in CVD risk factors, the duration of the intervention, and the amount and form of the WB product. We provided our subjects with a dietary daily amount of ACNs through the WB drink since it was interested in studying a realistic condition. Future studies with larger numbers of subjects with homogenous and/or more pronounced CVD risk factors or established endothelial dysfunction may show beneficial effect of blueberry intervention.

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Figure 8.3.1- *Individual values of reactive hyperemia index (RHI) measured by Endo-PAT2000 and registered before and after a six weeks WB drink and PL drink intake in the group of volunteers (n = 17).*
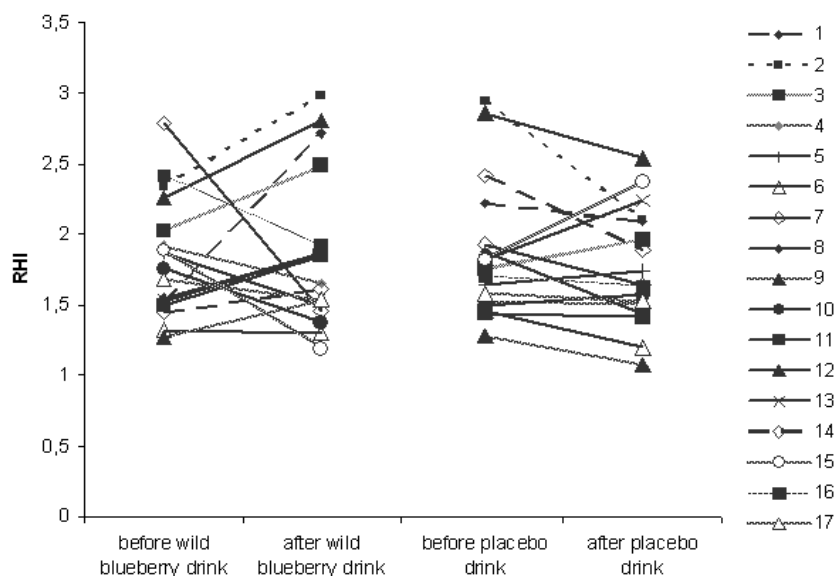
RHI, reactive hyperemia index; WB, wild blueberry drink; PL, placebo drink

8.3.5 Effect of intervention on DNA damage and repair activity in peripheral blood mononuclear cells

Results from intervention studies with berries (single berry items or mixture of different berries) have shown a reduction of the levels of oxidatively damaged DNA evaluated by means of the comet assay (Wilms et al., 2007; Freese 2006), whereas there are studies showing no effect after ingestion of ACN-rich blackcurrant juice or an ACN drink (Møller et al., 2004). Results on the levels of DNA damage and repair activity are shown in Table 8.3.4. There was a significant different treatment effect between the WB and PL drink periods (P=0.039 for the interaction between treatment and time). Estimation of oxidized purines in PBMC DNA through quantification with the formamidopyrimidine DNA glycosylase (FPG)-sensitive sites indicated a statistically significant decrease following the WB drink (from $12.5 \pm 5.6\%$ to $9.6 \pm 3.5\%$, $p \leq 0.01$) with respect to the PL drink (from $12.0 \pm 4.5\%$ to $11.9 \pm 4.4\%$, p=0.89). Moreover, there was also a different treatment effect between the WB and PL drink periods (P=0.037 for the interaction between treatment and time). The intake of the WB drink significantly decreased the levels of H_2O_2-induced DNA damage (from $45.8 \pm 7.9\%$ to $37.2 \pm 9.1\%$, $p \leq 0.01$), while no effect was observed after the PL drink (from $44.9 \pm 12.2\%$ to $44.4 \pm 8.4\%$, p=0.84).

The protection against DNA damage was particularly evident in the group of smokers/ex-smoker subjects with respect to nonsmokers. In fact, it was observed that the reduction in the levels of H_2O_2-induced DNA damage after the WB intervention was higher: smokers/ex-smokers ($44.4 \pm 7.7\%$ vs $33.7 \pm 5.9\%$, $p \leq 0.01$) with respect to the nonsmoker group ($47.2 \pm 8.3\%$ vs $40.7 \pm 10.7\%$, p=0.08) as indicated by the LSD test. This effect on DNA damage may be dependent on phytochemicals present in WB drink that can exert a direct protective effect against oxidative insult by scavenging reactive oxygen species in blood. Alternatively, ACNs may act through upregulation of the expression of different antioxidant genes involved in the detoxification of molecules such as hydrogen peroxide (Shih et al., 2007). Other processes that may have been

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modulated by the WB drink could be: alkylation or formation of ACN-DNA complexes, and/or modulation of DNA repair genes (Traustadóttir et al., 2009; Guarrera et al., 2007). In this regard, it has been reported that a flavonoid-rich diet could affect the expression of DNA repair genes belonging to different repair pathways like nucleotide excision repair, base excision repair and double strand breaks repair (Guarrera et al., 2007). In the present study, 6 weeks of WB drink consumption did not show an increase in DNA repair activity (Table..).

Table 8.3.4- *Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on background and H_2O_2 induced strand breaks, FPG sensitive sites, DNA repair activity (n=18)[1]*

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Background SBs (% DNA in tail, EB)	6.3 ± 1.6	6.7 ± 1.2	6.2 ± 1.4	6.2 ± 1.5	0.201
Net FPG-sensitive sites (% DNA in tail)	12.5 ± 5.6	9.6 ± 3.5*	12.0 ± 4.5	11.9 ± 4.4	0.039
Background SBs (% DNA in tail, PBS)	5.9 ± 1.1	6.5 ± 1.2	6.2 ± 1.4	6.8 ± 2.0	0.594
Net H_2O_2-induced DNA damage (%DNA in tail)	45.8 ± 7.9	37.2 ± 9.1*	44.9 ± 12.2	44.4 ± 8.4	0.037
DNA repair activity (% DNA in tail)	6.4 ± 1.7	6.7 ± 1.9	6.6 ± 1.5	6.4 ± 1.8	0.425

[1]*Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; SBs, strand breaks; PBS, phosphate buffer saline; EB, endonuclease buffer. *Significantly different from each other point, P≤0.05*
[2]*P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).*

8.3.6 Effect of intervention on anthropometric measures and biochemical parameters

Body weight, BMI and biochemical markers before and after the WB or PL drink are presented in Table 8.3.5. The intervention had no significant effect on body weight and BMI, glucose, creatinine and enzyme activity (AST, ALT and GGT) in liver. In addition, no effect on SOD, GSH-Px or GST activity was observed suggesting that upregulation of antioxidant enzymes is not the mechanism for the increased resistance toward H_2O_2-induced DNA damage.

Studies on ingestion of polyphenol bioactive compounds have shown results on lipid profile data that are not univocal. Bilberries, blackcurrants, cranberry juice or extracts have shown a favorable effect on the lipid profile in dyslipidemic, hypercholesterolemic or metabolic syndrome subjects from improving HDL-cholesterol to lowering LDL-cholesterol and total triglycerides (Basu et al., 2009; Erlund et al., 2008; Curtis et al., 2009; Ruel et al., 2008). On the contrary Basu et al. (2010) reported that 8 weeks of supplementation with a freeze dried blueberry beverage (50 g of powder, providing 742 mg of ACNs per day) did not change the plasma lipid profile in subjects with the metabolic syndrome. Similarly Stull et al. (2010) found that the consumption of two blueberry smoothies per day (45 g of freeze dried blueberry powder, providing 668 mg of ACNs) did not affect the lipid profile in obese subjects after 6 weeks of intervention. The above studies are in agreement with the present study, since the WB drink intervention did not alter the plasma lipid profile in subjects with risk factors for CVDs.

In the present study, no significant differences in lipid profile (triglycerides, total cholesterol, LDL and HDL cholesterol) were observed following WB drink consumption.

TABLE 8.3.5- *Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on anthropometric and biochemical characteristics of volunteers (n=18)[1]*

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Body weight (kg)	75.9 ± 8.4	75.8 ± 8.7	76.2 ± 9.3	76.1 ± 9.3	0.977
BMI (kg/m^2)	24.9 ± 2.5	24.8 ± 2.6	24.9 ± 2.7	24.9 ± 2.6	0.977
Glucose (mmol L^{-1})	5.0 ± 0.5	5.0 ± 0.3	4.9 ± 0.6	5.0 ± 0.6	0.357
TG (mmol L^{-1})	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.6	1.4 ± 0.6	0.279
TSC (mmol L^{-1})	5.9 ± 1.2	5.7 ± 1.3	5.9 ± 1.2	5.7 ± 1.3	0.774
LDL-cholesterol (mmol L^{-1})	3.9 ± 0.9	3.7 ± 1.0	3.8 ± 1.2	3.6 ± 1.0	0.726
HDL- cholesterol (mmol L^{-1})	1.4 ± 0.3	1.3 ± 0.2	1.4 ± 0.3	1.3 ± 0.3	0.241
AST (U L^{-1})	23.8 ± 8.1	23.3 ± 6.2	23.1 ± 4.8	23.8 ± 6.9	0.644
ALT (U L^{-1})	26.4 ± 10.7	27.5 ± 12.8	27.2 ± 12.1	27.5 ± 10.8	0.679
GGT (U L^{-1})	27.2 ± 11.0	26.5 ± 11.4	27.0 ± 10.7	25.3 ± 11.0	0.217
Creatinine (mmol L^{-1})	81.3 ± 11.5	82.2 ± 13.3	80.4 ± 10.7	80.4 ± 13.3	0.734
SOD activity (U mg^{-1} Hb)	4.7 ± 1.3	4.4 ± 1.3	4.8 ± 1.3	4.5 ± 1.4	0.870
GSH-Px activity (μmol min^{-1} mL^{-1} g Hb)	18.6 ± 9.5	18.0 ± 5.6	16.5 ± 5.6	19.4 ± 7.0	0.121
GST activity (nmol min^{-1} mg^{-1} proteins)	25.9 ± 13.3	25.8 ± 9.6	31.4 ± 17.9	28.5 ± 11.0	0.672

[1]Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidise; GST, glutathione S-transferase; TG, triglycerides; TSC, total serum cholesterol.
[1]Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; BMI, body mass index;
[2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).

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8.3.7 Effect of intervention on biomarkers of inflammation

The role of berries in decreasing markers of inflammation has not been well investigated and very few observations have shown an antinflammatory effect (Elliss et al., 2011; Stull et al., 2010). The plasma levels of IL-6, TNF-α and CRP of the subjects before and after the WB and PL drink are reported in Table 8.3.6. No significant difference in inflammatory markers was detected as a result of the intervention.

TABLE 8.3.6- *Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on circulating levels of interleukin 6, Tumor Necrosis Factor-α and C-reactive protein (n=18)[1]*

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
IL-6 (pg mL^{-1})	2.1 ± 1.4	2.1 ± 0.9	2.4 ± 1.3	1.9 ± 1.0	0.303
TNF-α (pg mL^{-1})	3.6 ± 2.1	3.1 ± 1.0	3.4 ± 2.1	3.5 ± 1.9	0.438
CRP (mg dL^{-1})	0.18 ± 0.20	0.20 ± 0.15	0.16 ± 0.13	0.22 ± 0.21	0.717

[1]*Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; CRP, C-reactive protein.*
[2]*P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).*

These results are in accordance with other observations reported in literature (Elliss et al., 2011; Stull et al., 2010; Basu et al., 2010). It should be noted that the inflammatory markers in our volunteers were in the normal physiologic concentration ranges at the onset of the study, thus the potential of WB to affect such biomarkers maybe more appropriately evaluated on subjects with high initial levels.

8.4 CONCLUSIONS

In conclusion, the consumption of a WB drink, providing 375 mg of ACNs, for 6 weeks reduced the level of oxidized purines and improved the resistance against H_2O_2-induced DNA damage in PBMCs. This indicates that regular consumption of wild blueberries can reduce oxidative stress in subjects with risk factors for CVD, even though peripheral vascular function and the other variables studied here were not significantly affected.

Future, larger studies are necessary to understand the duration of exposure to ACNs, the dose and/or the form of the WB product that may be effective in modulating endothelial function and the other parameters studied.

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9. IN VITRO APPROACHES TO STUDY THE ANTIATHEROGENIC AND ANTIATHEROSCLEROTIC EFFECT OF DIFFERENT BLUEBERRY BIOACTIVES

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9.1 AIMS OF THE STUDY

Atherosclerosis is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, caused largely by the accumulation of macrophages and white blood cells and promoted by low-density lipoproteins (Packard & Libby 2008; Napoli et al., 2006).

Foods rich in bioactives such as polyphenols seem to prevent atherosclerosis, by reducing oxidative stress, inflammatory response, vasocostrictor agents and by increasing the production of vasodilators such as NO (Santakumar et al., 2013; Siaos et al., 2013; Tangney et al., 2013; Khurana et al., 2013; Quiñoes et al., 2013; Andriantsitohaina et al., 2012).

The objective of the present study task was to investigate the potential anti-atherogenic and anti-atherosclerotic effect of WB extracts, rich in different polyphenol compounds through two different cells models: the human monocytic (THP-1) cell line, and the human umbilical venous endothelial cells (HUVECs). The first model was used to evaluate the capacity of WB extracts to reduce lipid accumulation (antiatherogenic effect), while the second one was used to assess whether the addition of WB extracts was able to reduce the ability of THP-1 cells to adhere to HUVECs following stimulation with pro-inflammatory cytokines (antiatherosclerotic effect).

9.2 MATERIALS AND METHODS

9.2.1 Chemicals

Standard of cyanidin (Cy)-, delphinidin (Dp)-, petunidin (Pt)-, peonidin (Pe)-, and malvidin (Mv)-3-*O*-glucoside (glc), Cy- and Pt-3-*O*-arabinoside (ara), Cy-3-*O*-galactoside (gal), Dp, Cy, and Mv were purchased from Polyphenols Laboratory (Sandes, Norway). Standard of chlorogenic, caffeic, ferulic, gallic, protocatechuic and syringic acids, glucose, fructose, Bovine Serum Albumin (BSA), Palmitic acid (PA), oleic acid (OA), hydrogen peroxide (H_2O_2), Hanks balanced salt solution, Pluronic F127, Nile Red, Phorbol 12-myristate 13-acetate (PMA), were from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid, methanol, ethanol, acetonitrile, triethylamine, phosphoric acid, trifluoroacetic acid (TFA) and ethyl acetate were from Merck (Darmstadt, D). Water was obtained from Milli-Q apparatus (Millipore, Milford, MA). Hepes, Sodium Pyruvate, Gentamin, RPMI-1640, trypsin-EDTA were obtained from Life Technologies (Grand Island, NY, USA). Fetal Bovine Serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Endothelial cell basal medium and endothelial cell growth supplement were from Cells applications, INC (San Diego, CA, USA). Freeze-dried Wild Blueberry (WB) powder, standardized at 1.5% total ACNs, was provided by Future-Ceuticals Company (Momence, IL, USA).

9.2.2 Extraction of bioactives from the wild blueberry powder

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

9.2.3 Analysis of anthocyanin and phenolic fractions

The analysis of ACN and Phe compounds of the fractions was performed by HPLC, which consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod. 2998 photodiode array detector (Waters). The separation was carried out through a C_{18} Kinetex column (150 x 4.6 mm, 2.6 µm, Phenomenex, Torrence, CA) at 45°C and 1.7 mL min^{-1} as flow rate. The eluents were (A) 1% H_3PO_4 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 minutes. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 (ACNs) and 320 nm (phenolic acids). Calibration curves ranged from 2 to 50 µg mL^{-1} were obtained for cyanidin (Cy-), delphinidin (Dp-), petunidin (Pt-), peonidin (Pe-) and malvidin (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

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from the stock solution with methanol acidified with 0.1% TFA. Each analysis was carried out in duplicate (Del Bo' et al., 2010).

9.2.4 Analysis of sugars

Glucose and fructose were quantified in the ACN and Phe fractions by UPLC (Acquity, Waters) coupled with a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA). The separation was carried out on BEH (Ethylene Bridged Hybrid) Amide column (150 x 2.1 mm, 1.7 μm, Waters) at 35°C. Solvents were (A) triethylamine 0.2% and (B) triethylamine 0.2% in acetonitrile. Flow-rate was 0.40 mL min^{-1}. The calibration curve was obtained from 1 to 50 μg mL^{-1} for both sugars. The mass spectrometer operated in ESI negative mode monitoring the ions with m/z 179 (glucose, fructose). The capillary voltage was set to 3.0 kV, the cone voltage to 20 V, the source temperature to 120 °C, and the desolvating temperature to 250°C.

9.2.5 Preparation of fatty acids and control solution

The stock solution of FA (0.2M OA/PA; 2:1 ratio) was prepared in EtOH and stored at -20°C. On the day of use, 5mM of FA water soluble solution (FA/BSA solution) was generated by incubating the FA in Hanks solution containing 10% BSA at 37°C for about 30 min with occasional vortex. The FA/BSA solution was added to the media to obtain final 500μM FA concentration for the exposure. Equal volumes of the Hanks solution/EtOH/fatty acid-free BSA were applied to control cells. The final concentrations of EtOH and BSA in exposure media were 0.05% and 0.2%, respectively.

9.2.6 Preparation of single anthocyanins and their metabolites

A stock solution of standards of Mv, Cy and Dp 3-*O*-glc, as well as their correspondent metabolic products as syringic, protocatechuic and gallic acid respectively, was prepared. We have chosen these ACNs because they were the compounds absorbed and detectable in plasma after consumption of a single portion of blueberry as recently reported (Del Bo' et al., 2012). Lyophilized standards (10 mg) were dissolved in 10 mL of acidified methanol (HCl 0.05 mM). Aliquots (1 mL) of standard were concentrated under nitrogen and subsequently quantified by spectrophotometric analysis. Standards were then stored at -20°C until use.

9.2.7 THP-1 cell culture

The monocytic THP-1 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were originally cultured from the peripheral blood of 1 year kid with acute monocytic leukemia from 1980s (Tsuchiya et al., 1980). According to the authors the cells maintained their monocytic characteristics for over 14 months (Tsuchiya et al., 1980), but in the study the cells were maintained for up to 3 months to keep their monocytic characteristics. The THP-1 cells are non-adherent cells, which can be differentiated into macrophage-like cells using PMA through a protein kinase C mediated ROS dependent signaling pathway (Traore et al., 2005). Treatment of THP-1 with PMA leads to a decrease in the expression of LDL receptor and an increase in the expression of scavenger receptors (Johnson et al., 2003). These alterations allow the cells to take up modified lipoproteins and to become foam cells with excessive accumulation of lipid, primarily cholesteryl ester, that make THP-1 a reasonable model to mimic monocyte-macrophage behaviour during the atherogenesis process.

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9.2.8 HUVEC cell culture

The human umbilical vein endothelial cells (HUVECs) were purchased from Cell Application (San Diego, CA, USA).These are primary cells that are derived from the endothelium of veins from the umbilical cord. When cultured *in vitro*, the cells form a monolayer which is similar to the endothelial cells in vivo, therefore they are commonly used as the *in vitro* model for the study of the function of endothelial cells (Park et al., 2006).

9.2.9 Study of the effect of the wild blueberry fractions in the modulation of lipid accumulation in THP-1 macrophages

Cells were cultured in complete RPMI cell media (RPMI-1640 medium supplemented with 1% Hepes, 1% sodium pyruvate, 0.1% gentamicin, and fetal bovine serum to a final concentration of 10%) at 37 °C and $5 - 7$ % CO_2. After growth, cells were differentiated into macrophages by treating with 10 ng mL^{-1} PMA overnight. Macrophages become adherent to the surface of the culture flasks, thus cells were washed with Hanks Solution to remove all non-adherent cells. Subsequently, 3 mL trypsin (0.05%)–EDTA (0.53mM) was added and incubatedfor 2 min at 37 °C and $5 - 7$ % CO_2 in order to re-suspend cells adherent to the flask. After incubation, 2 mL of complete RPMI cell media was added to inactivate trypsin. Cells were collected in falcon tubes, quantified in a CASY1 cell counter (Roche Innovatis AG, Reutlingen, D), and then centrifuged (mod. Sigma compact centrifuge 2-5, C&M Scientific, Livingston,West Lothian, UK) for 5 min at 250x g.

After centrifugation, pellet of cells was re-suspended in new complete RPMI media in order to reach a final concentration of approximately 2.5×10^5 cells / volume. Two hundred microliters of cell suspension (containing approximately 5×10^4 cells) in octuplicate were aliquoted into the wells of a 96-well dark microtiter plate and incubated for 24 h at 37 °C and $5 - 7$ % CO_2, in order to induce the cell adhesion to the surface of microtiter plate. The ACN (calculated considering the total ACNs concentration) and Phe fractions (calculated considering the chlorogenic acid concentration), as well as the single ACNs (Mv, Cy and Dp 3-glucoside) and their correspondent metabolites (syringic, protocatechuic and gallic acid, respectively) were tested. Media was removed and 200 µL of new complete RPMI media (containing 500 µM FA and different concentrations of bioactive compounds) was added. Concentrations of ACN and Phe fractions, of the single ACN and metabolite standards used were 0.05; 0.1; 0.3; 0.6; 1.25; 2.5; 5 and 10 µg mL^{-1}. Cells were incubated for 24 h at 37 °C and $5 - 7$ % CO_2. The lipid accumulation was measured by using the fluorescent dye Nile red. Nile red is not fluorescent in most polar solvents, but can be extensively fluorescent in lipid rich environments. Nile red is thus suitable for measuring lipid accumulation both in cells and in tissues. To measure the lipid accumulation in macrophages, after treatment, the cells were washed with Hanks solution and then stained in Hanks solution containing 0.5 µg mL^{-1} Nile red and 0.01% Pluronic F127 for 15 min at 37 °C and $5 - 7$ % CO_2. Later, cells were washed twice with 200 µL of Hanks solution and then exposed to 100 µL Hanks solution. The fluorescence (excitation: 544 nm, emission: 590 nm) was measured in a fluorescence spectrophotometer (mod. Fluoroskan Ascent FL, Thermo Scientific, USA) and the number of fold increase respect to the control (without FA) was calculated.

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9.2.10 Study of the effect of wild blueberry fractions in the modulation of THP-1 attachment to HUVEC cells

During atherosclerotic plaque formation, endothelial cells attract and bind monocytes to the inflammatory area in the intima of the endothelium. The inflammatory response may be caused by several pro-inflammatory citokynes. Co-cultures between THP-1 and human umbilical vein endothelial cells (HUVECs) have been used to study this process. In a typical experiment designed to detect the interaction, THP-1 cells are labelled with a fluorescent dye. The binding ability of THP-1 is determined by the fluorescent intensity of the adhered versus total cells, which is measured by a fluorescence plate reader.

In the present study, HUVECs were cultured in endothelial cell growth medium kit which contains 2% serum. After growth, cells were washed once with Hanks Solution to remove all non-adherent cells. Subsequently, 3 mL trypsin (0.05%)–EDTA (0.53mM) was added and incubated for 2 min at 37°C and 5 – 7 % CO_2 in order to re-suspend cells adherent to the flask. After incubation, 2 mL of media was added to inactivate trypsin. Cells were collected in falcon tubes, quantified and then centrifuged for 5 min at 250 x g. The pellet of cells was re-suspended in new media in order to reach a final concentration of approximately $1x10^6$ cells / volume. Two hundred microliters of cell suspension containing approximately $2x10^4$ cells in quintuplicate were aliquoted on 0.1% gelatin pre-coated 96-well black plate and growth for 24 h at 37°C and 5 – 7 % CO_2. After 24h, media was removed and 200 µL of new media, containing different concentrations of ACN and Phe fractions, was added. Concentrations of ACN and Phe fraction used were: 0.05; 0.1; 0.3; 0.6; 1.25; 2.5; 5 and 10 µg mL^{-1}; cells were incubated for 24 h at 37°C and 5 – 7 % CO_2. After incubation, $2x10^6$ THP-1 cells were spin-out, re-suspended in 1 ml serum free RPMI cell media (RPMI-1640 medium supplemented with 1% Hepes, 1% sodium pyruvate, 0.1% gentamicin) and labelled with CellTrackerTM Green CMFDA (5-Chloromethylfluorescein Diacetate, Invitrogen, USA) for 30 min at 37°C and 5 – 7 % CO_2. After staining, THP-1 cells were rinsed twice with complete RPMI cell media and re-suspended in HUVEC media at a density of $2x10^5$ cells/ml. One hundred microliter of THP-1 cells and 100 µl of TNF-α (1µg mL^{-1}) were added to HUVECs and incubated for 24 h at 37° C and 5 – 7 % CO_2. TNF-α induces a pro-inflammatory status and promote THP-1 adhesion as reported in several studies (Zhao et al., 2010; Sneddon et al., 2006).. After 24h cells were rinsed twice with Hank solution and the fluorescence (excitation: 485nm, emission: 538 nm) was measured in a fluorescence spectrophotometer. The increase of absorbance is dependent to the amount of labelled-THP-1 attached to HUVEC. So, data are presented as number of fold increase THP-1 attachment respect to the control (without stimulation with TNF-α).

9.2.11 Statistical analysis

The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Analysis of variance (ANOVA) was used to assess the effect of the different concentrations of ACN and phenolic compounds on lipid accumulation in THP-1 macrophages following stimulation with FA. Moreover, ANOVA was used to evaluate the capacity of ACN and phenolic fractions to reduce THP-1 attachment to HUVEC cells. Post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as level of statistical significance. Data are presented as mean±standard deviation (SD).

9.3 RESULTS AND DISCUSSION

9.3.1 Characterization of Wild blueberry fractions

The two extracts obtained from WB powder were analyzed in order to evaluate the content of sugars, Phe and ACNs. The ACN fraction composition is reported in Table 9.3.1. The total ACN content was 29.8 ± 5.17 mg mL^{-1}. Seventeen different ACNs were detected in the extract; the dominant compounds were: malvidin glycosides (about 14.4 mg mL^{-1}; 48% of the total ACN amount) and cyanidin glycosides (about 4.8mg mL^{-1}; 16%), followed by delphinidin glycosides (about 4.5mg mL^{-1}; 15%), while petunidin and peonidin glycosides represented about 13% and 7% of the total ACNs, respectively. In regard to conjugated sugars, the ranking order was glucoside > galactoside > arabinoside from the most to the least representative. No phenolic compounds, sugars were detected in the ACN fraction.

Chlorogenic acid was the main phenolic compound (13.1 ± 2.5 mg mL^{-1}) detected in the Phe fraction, while only traces of caffeic and ferulic acids were found. No sugar was detectable.

Table 9.3.1: *Characterization of the ACN fraction*

Compounds	Extracted mg mL^{-1}
malvidin 3-glc	7.89 ± 0.8
malvidin 3-gal	4.65 ± 0.7
malvidin 3-ara	1.84 ± 0.04
delphinidin 3-glc	2.18 ± 0.7
delphinidin 3-gal	1.55 ± 0.15
delphinidin 3-ara	0.75 ± 0.02
cyanidin 3-glc	2.19 ± 0.6
cyanidin 3-gal	1.31 ± 0.4
cyanidin 3-ara	1.26 ± 0.2
petunidin 3-glc	2.49 ± 0.8
petunidin 3-gal	0.67 ± 0.01
petunidin 3-ara	0.62 ± 0.03
peonidin 3-glc	1.61 ± 0.7
peonidin 3-gal	0.31 ± 0.04
peonidin 3-ara	0.11 ± 0.03
Total	29.9 ± 5.17

Data are reported as mean $\pm$ standard deviation.
glc, glucoside; gal, galactoside; ara, arabinoside

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9.3.2 Effect of anthocyanin fraction and single anthocyanin compounds on lipid accumulation in THP-1 macrophages

The effect of ACN fraction on lipid accumulation in TPH-1 macrophages is reported in Figure 9.3.1. ANOVA revealed a significant effect of the treatment (p<0.0001). In particular, the ACN fraction induced a reduction in the lipid accumulation at all the concentrations tested with respect to the control treatment with FA, but not with respect to the FA free control. The maximum reduction was observed for the highest concentration, i.e. 10 µg mL^{-1} (-27.4%; p≤0.0001).

Fig. 9.3.1 *Effect of ACNs fraction on lipid accumulation in THP-1 macrophages. 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.*
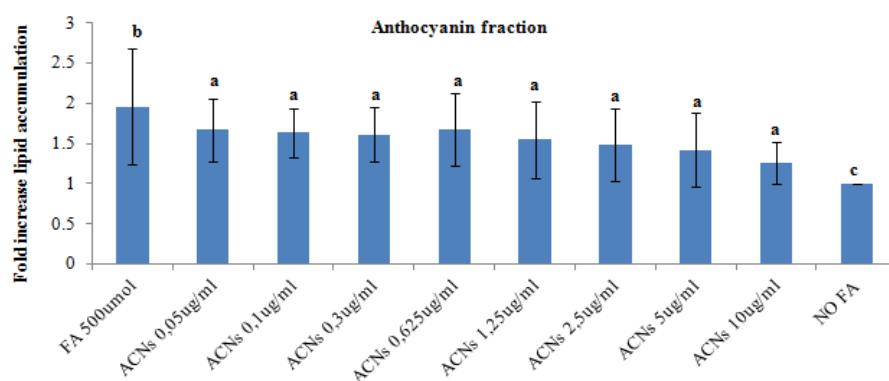
FA, fatty acids; ACNs, anthocyanins; NO FA, fatty acids free
[a,b,c] Data with different letters are significantly different (P≤0.05)

The modulation of lipid accumulation observed in our experiments seem in accordance with several mechanistic studies where the role of extracts of ACN-rich foods (e.g., sweet potato, berries, and oranges) was investigated (Chang et al., 2013; Hwang et al., 2011; Vendrame et al., 2013; Salamone et al., 2012; Peng et al., 2011). In the present study no information is reported on the molecular mechanisms involved, however the reduction observed in the lipid accumulation following ACNs supplementation could be attributed to different factors. For example, ACNs could have regulated the expression of numerous genes including: fatty acid synthase, lipoprotein lipase. Moreover, ACNs may modulate the transcriptional activities of different nuclear receptors that control lipid homeostasis, including sterol regulatory elevated binding protein, and peroxisome proliferator-activated receptor (Guo et al., 2012; Liang et al., 2012; Wei et al., 2011).

Studies evaluating also the effect of the single ACNs have shown conflicting results, probably due to the specific role played by each single compound. In the present study the effect of the some ACNs (Mv, Dp, and Cy 3-glc) on lipid accumulation is reported in Figure 9.3.2 (A-C). These compounds were selected since they were the most representative compounds in the WB fraction. Moreover, as previously reported (see **study 3**), these compounds where the only ACNs absorbed in human plasma following blueberry intake.
In general, ANOVA revealed a significant effect of the treatment for Mv 3-glc and Dp 3-glc (p<0.0001). Concerning Mv 3-glc, the reduction in the lipid accumulation was shown at each

concentration tested with the exception of that at 1.25 µg mL^{-1}(Figure 9.3.2A). The reduction was high at the low doses (0.05-0.1 µg mL^{-1}, -63% and -53%, respectively) and significantly different compared to the control (FA). Dp 3-glc (Figure 9.3.2B) showed to reduce lipid accumulation at all the concentrations tested (from 0.05 till 10 µg mL^{-1}), but it was particularly highat the low doses (0.05-0.1 µg mL^{-1}, -59% and -52%, respectively; $p<0.0001$). On the contrary, no significant effect was observed for Cy 3-glc (Figure 9.3.2C) thus this compound may be not involved in the regulation of lipid accumulation.

This latter result seems in accordance with previous observation reported by Titta et al., (2010) in which Cy-3-glc was not able to counteract the lipid accumulation in rat adipocytes, but in contrast with other studies in which a role in the regulation of lipid metabolism was documented (Guo et al., 2012; Wei et al., 2011). Several studies have shown that cyanidin-rich diet decreased significantly cholesterol, triglyceride, ApoB plasma concentrations and aortic fatty streak area. Jia et al., (2013) documented that Cy-3-glc significantly reduced cellular lipid concentrations in lipid-loaded steatotic hepatocytes.

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Figure 9.3.2 *Effect of the single ACNs on lipid accumulation in THP-1 macrophages. A) malvidin, B) delphinidin and C) cyanidin 3-glucoside. 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.*
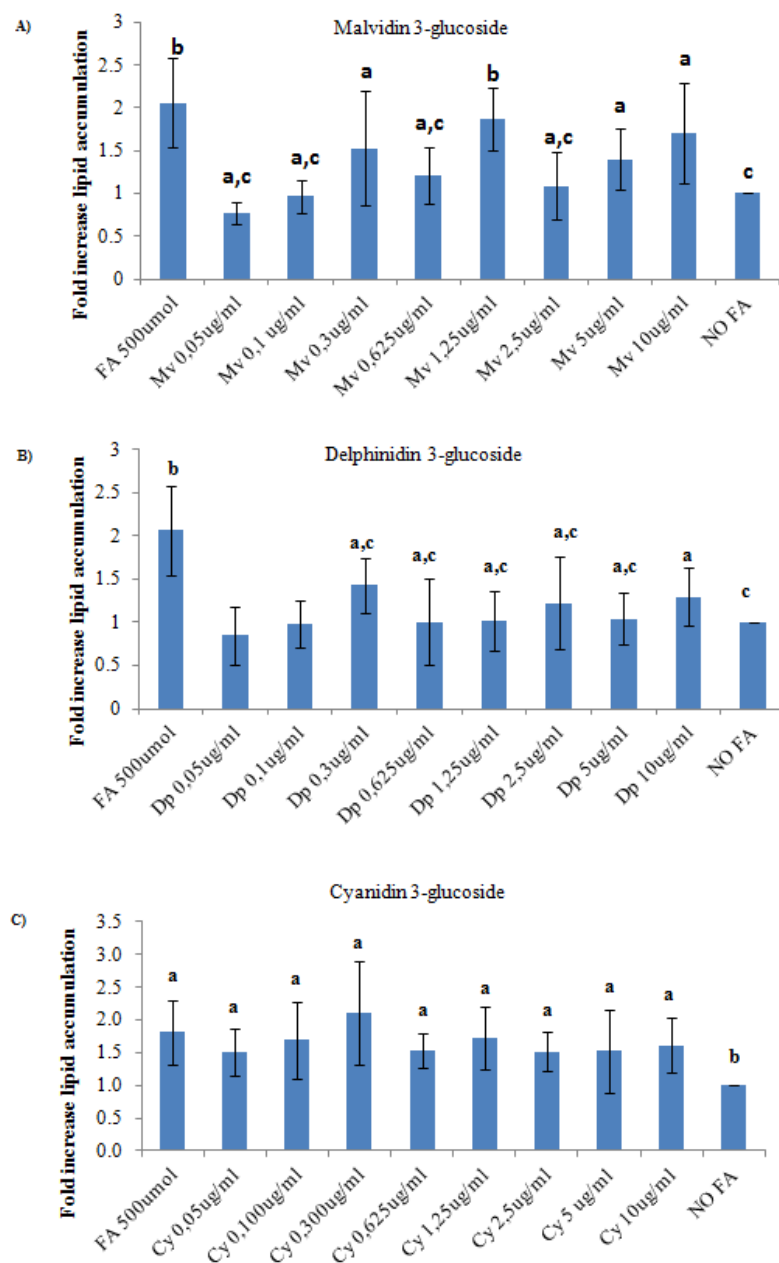
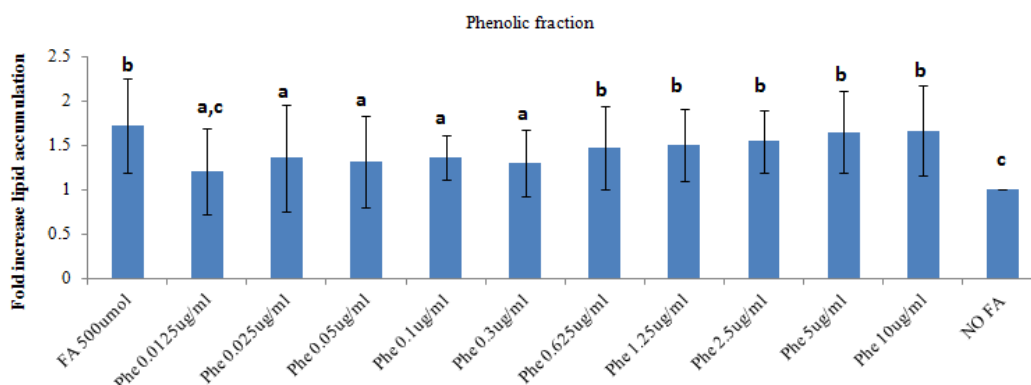*FA, fatty acids; Mv, malvidin; Dp, delphinidin; Cy, cyanidin FA, fatty acids; NO FA, fatty acids free*
[a,b,c] *Data with different letters are significantly different (P≤0.05)*

9.3.3 Effect of phenolic fraction and single compounds (anthocyanin metabolites) on lipid accumulation in THP-1 macrophages

Several studies documented the ability of phenolic compounds in the modulation of lipid accumulation. Cho et al., (2010) reported that a supplementation with caffeic acid or chlorogenic acid (0.02% wt/wt) was able to inhibit lipid metabolism and obesity-related hormones levels in high-fat fed mice.

The effect of Phe fraction on lipid accumulation in TPH-1 macrophages is reported in Figure 9.3.3. ANOVA revealed a significant effect of the treatment (p<0.0001). In particular, a significant reduction in the lipid accumulation was observed only at the low concentrations from 0.0125 µg mL^{-1} till 0.3 µg mL^{-1}, with respect to the control with FA, but not with respect to FA free control. No significant effect has been observed at higher concentrations (from 0.6 till 10 µg mL^{-1}).

Figure 9.3.3 *Effect of the phenolic fraction on lipid accumulation in THP-1 macrophages. 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.*

FA, fatty acids; Phe, phenolic fraction; NO FA, fatty acids free
[a,b,c] *Data with different letters are significantly different (P≤0.05)*

The effects at the low doses may be explained through the concept of hormesis. In fact, several plant antioxidants exhibit hormetic properties, by acting as 'low-dose stressors' that may prepare cells to resist more severe stress (Speciale et al., 2011; Rattan & Demirovic 2009). In fact, low doses of these phytochemicals activate signaling pathways that result in increased expression of genes encoding cytoprotective proteins, while high doses are instead cytotoxic. The most prominent example is the modulation of Nrf2/Keap1 pathway, the NF-κB pathway and the Sirtuin-FOXO pathway (Speciale et al., 2011).

The effects of the ACN metabolites on lipid accumulation in THP-1 macrophages are reported in Figure 9.3.4 (A-C). Syringic (Figure 9.3.4A) and gallic (Figure 9.3.4B) acids (the metabolic products of Mv and Dp 3-glc, respectively) showed to decrease lipid accumulation at all the concentrations. In particular, the higher effects were observed at the low concentrations as reported for Phe fraction. In particular, the maximum reduction in lipid accumulation was observed at 0.05 µg mL^{-1} for syringic acid (-64.8%, p<0.0001), and at 0.3 µg mL^{-1} for gallic acid (-59.3%, p<0.0001). On the contrary, protocatechuic acid (metabolic product of Cy 3-glc) showed (Figure 9.3.4C) to reduce the lipid accumulation only for some concentrations (0.05, 0.1 and 2.5µg mL^{-1}), by suggesting that this compound, like Cy 3-glc, is not particularly active in

the regulation of lipid metabolism but it cannot be exclude it is involved in other important pathways such as the inflammatory process as documented by Wei et al., (2012).

Figure 9.3.4 *Effect of the ACN metabolites on lipid accumulation in THP-1 macrophages. A) syringic acid, B) gallic acid and C) protocatechuic. 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.*
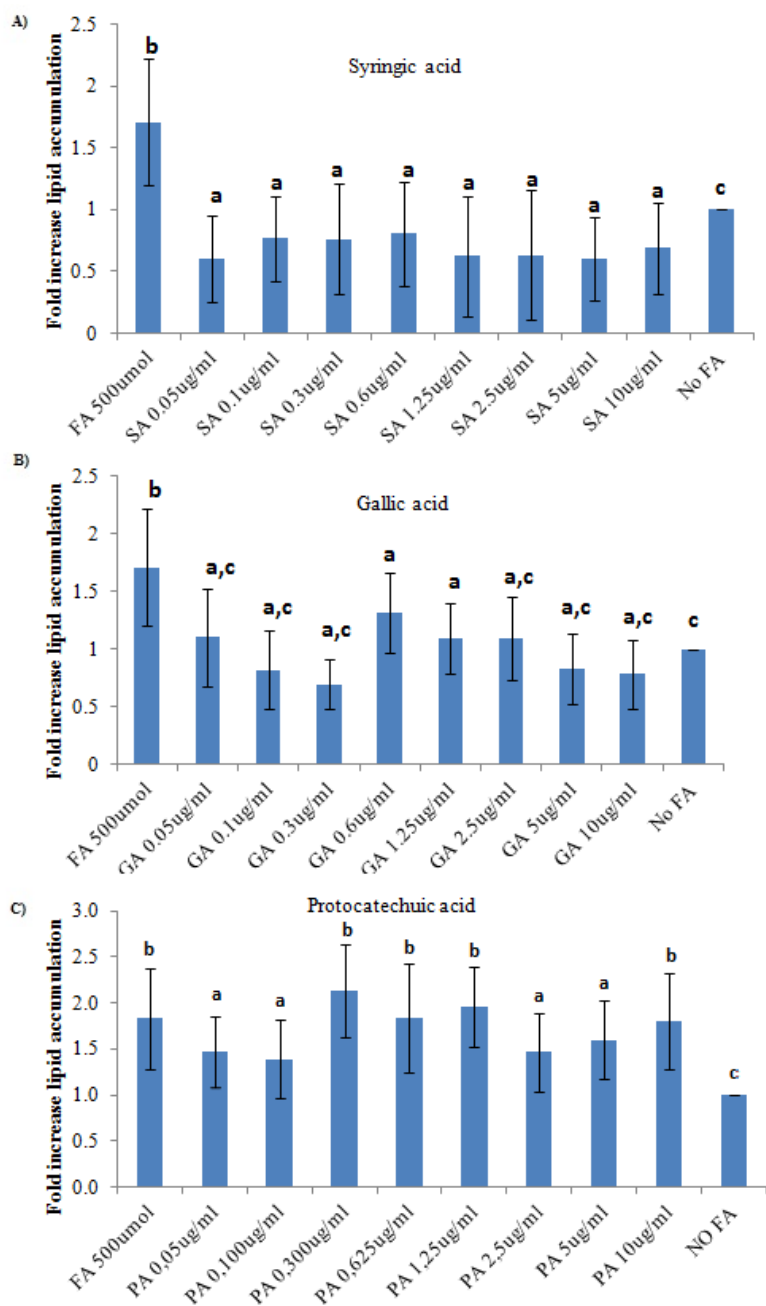
FA, fatty acids; SA, syringic acid; GA, gallic acid; PA, protocatchuic acid; NO FA, fatty acids free

[a,b,c] *Data with different letters are significantly different (P≤0.05)*

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9.3.4 Effect of wild bluebrry fractions in the modulation of the THP-1 attachment to HUVEC cells

In Figure 9.3.5 (A-B) are reported the results obtained by testing the capacity of WB fractions to counteract the development of atherosclerotic process. In particular, in this study it was investigated the ability of WB fractions to reduce THP-1 attachment to HUVEC following stimulation with pro-inflammatory cytokines as TNF-α.

Polyphenol compounds in general have shown to possess anti-inflammatory properties by acting on NF-kB and LPS response. These effects have been already demonstrated for different categories of polyphenols, and isolated form origins (Denis et al., 2013; Kostyuk et al., 2011; Liu et al., 2004; Youdin et al., 2002). For example, a grape-powder extract used in *in vitro* experiments on U937 macrophages reduced the LPS-induced production of inflammatory cytokines (Overman et al., 2010). A proanthocyanidin-rich cranberry fraction showed to decrease the LPS-induced cytotoxicity in macrophages and oral epithelial cells (La et al., 2009).

Wang et al., (2009) demonstrated that protocatechuic acid inhibited monocyte adhesion to TNF-α-activated mouse aortic endothelial cells, associated with the inhibition of VCAM-1 and ICAM-1 expression.

In a recent study performed in our laboratories, it has been documented that the same WB extract was able to reduce the inflammatory response induced by LPS on U937 by decreasing also the induction of TNF-α (unpublished data).

In the present study, we further demonstrated that ACN and Phe fractions reduced THP-1 attachment to HUVEC cells following stimulation with the pro-inflammatory cytokine. In particular, ACN fraction (Figure 9.3.5A) showed a positive and significant effect at the concentration of 10 µg mL^{-1} (-33%, p=0.04), while the Phe fraction (Figure 9.3.5B) was able to reduce significantly THP-1 attachment at the low doses (0.0125, 0.025, 0.05, 0.1 and 0.3 µg mL^{-1}, -52%, -49%, -45%, -49% and -51%, respectively). These results seem in accordance with the previous observations we obtained in the lipid accumulation study. Despite the lack of a mechanistic approach, the overall results suggest that WB fractions can exhibit an anti-inflammatory effect.

Figure 9.3.5 *Effect of the ACN and Phe fraction on THP-1 attachment to HUVEC cells following stimulation with TNF-α. A)ACN fraction, B)Phe fraction. Data are reported as fold increase in THP-1 attachment with respect to the control cells without TNF-α. Results are expressed as mean±standard error of the mean.*

Phe fraction

B)
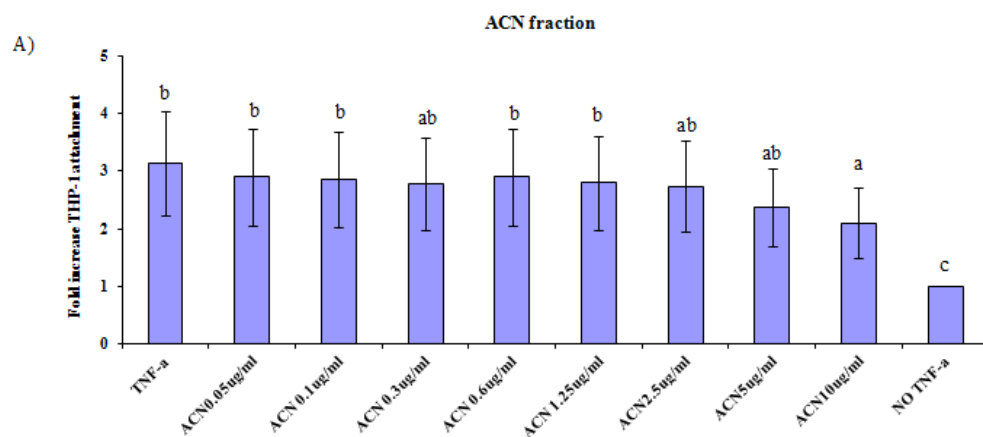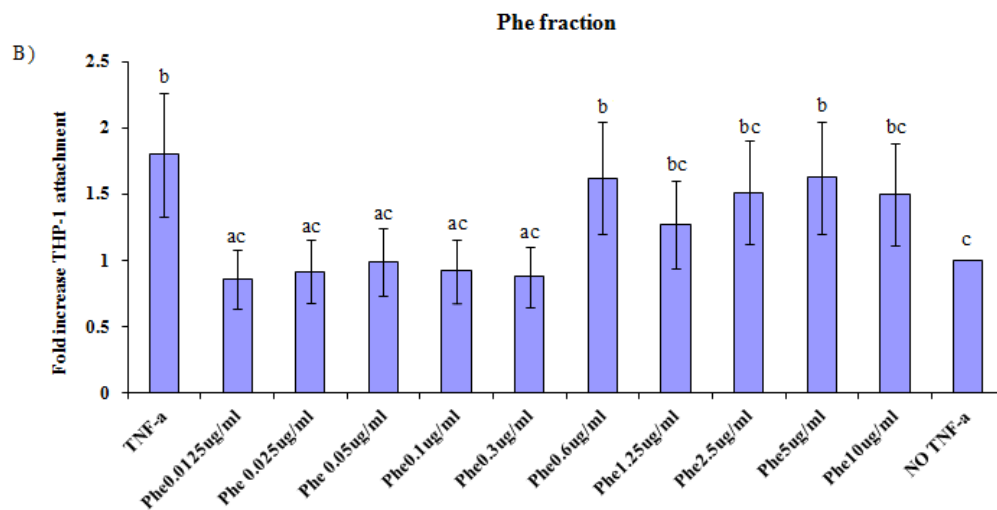
TNF-α, tumor necrosis factor alpha; ACN, anthocyanin; Phe, phenolic
[a,b,c] *Data with different letters are significantly different (P≤0.05)*

9.4 CONCLUSIONS

Several studies reported that ACNs display antiatherosclerosis effects; this may be attributed to their anti-inflammatory and/or antioxidant activity exerted through the regulation of the expression of genes involved in these responses.

In the present study it has been documented that ACN and Phe fractions, isolated from WB, could play an important role in the modulation of lipid accumulation and in the prevention of atherosclerosis. These effects were also observed at very low doses of ACNs and Phe compounds by suggesting that a protective effect could be reached also *in vivo* at physiological concentrations.

The results obtained supported the potential antiatherogenic and antiatherosclerotic effect of blueberry bioactives. Future *in vitro* studies will focus on the specific mechanisms involved in such modulation.

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APPENDIX 1. COPIES OF ABSTRACTS OF PAPERS

- **Del Bo' C**, Riso P, Brambilla A, Gardana C, Rizzolo A, Simonetti P, Bertolo G, Klimis-Zacas D, Porrini M. Blanching improves anthocyanin absorption from highbush blueberry (*Vaccinium corymbosum L.*) purée in healthy human volunteers: a pilot study. *Journal of Agricultural and Food Chemistry 2012; 60(36): 9298-304.*

- Riso P, Klimis-Zacas D, **Del Bo' C,** Martini D, Campolo J, Vendrame S, Møller P, Loft S, De Maria R, Porrini M. Effect of a wild blueberry (Vaccinium angustifolium) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors. *European Journal of Nutrition 2012;52(3):949-61.*

- **Del Bo' C**, Simonetti P, Gardana C, Riso P, Lucchini G, Ciappellano S. Horse meat consumption affects iron status, lipid profile and fatty acid composition of red blood cells in healthy volunteers. *International Journal of Food Science and Nutrition. 2013;64(2):147-54.*

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- Fracassetti D, **Del Bo' C**, Simonetti P, Gardana C, Klimis-Zacas D, Ciappellano S. Effect of time and storage temperature on anthocyanin decay and antioxidant activity in wild blueberry (*Vaccinium angustifolium*) powder. *Journal of Agricultural and Food Chemistry 2013;61(12):2999-3005*

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- Riso P, Vendrame S, **Del Bo' C,** Martini D, Martinetti A, Seregni E, Visioli F, Parolini M, Porrini M. Effect of 10-day broccoli consumption on inflammatory status of young *healthy smokers. International Journal of Food Science and Nutrition 2013, 65(1):106-11.*

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Blanching Improves Anthocyanin Absorption from Highbush Blueberry (*Vaccinium corymbosum* L.) Purée in Healthy Human Volunteers: A Pilot Study

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ABSTRACT: Blueberries (*Vaccinium corymbosum* L.) are rich sources of phenolics and anthocyanins (ACNs). We investigated the absorption of ACNs after consumption of one portion (300 g) of minimally processed blueberry purée (P) obtained from blanched (BL) or unblanched (NB) berries. A repeated-measures, crossover design study was conducted on healthy human volunteers. Blood was drawn between baseline and 24 h after BL-P or NB-P consumption, while urine were collected from the day before the experiment up to 48 h. Total plasma ACN content was not significantly different, while phenolics content was higher in BL-P with respect to NB-P. The maximum ACN absorption in plasma was observed after 1.5 h from the intake of the purées and was significantly higher ($p \leq 0.05$) after the intake of BL-P. Both products increased the excretion of hippuric acid in urine. In conclusion, blanching had no significant effect on total ACN content and enhanced their absorption from minimally processed purées.

KEYWORDS: *Blueberry, blanching, anthocyanins, absorption, human*

■ INTRODUCTION

Berry fruits (e.g., blueberries, blackberries, bilberries, blackcurrants, cranberries, and strawberries) are rich sources of polyphenol bioactive compounds, such as phenolic acids, flavanols, and flavonoids, including anthocyanins (ACNs). ACNs comprise the largest group of natural, water-soluble pigments that provide the bright colors to flowers and berry fruits. They are generally concentrated in the skins of fruits, and their content is usually proportional to color intensity. Blueberries contain different ACNs, such as cyanidin, peonidin, petunidin, malvidin, and delphinidin. These compounds possess significant antioxidant capacity and play a key role in the prevention of oxidative stress by scavenging reactive oxygen species and free radicals, as demonstrated in various *in vitro* and *in vivo* studies.[1−3] Moreover, ACNs play a potential beneficial role in human health by reducing risks of several diseases, such as cardiovascular diseases and cancer.[4−6] The health benefit is strictly dependent upon their absorption, metabolism, distribution, and excretion. Studies focusing on ACN absorption have shown that their bioavailability is lower than that of other flavonoids and less than 1% of consumed amounts of ACNs (180−215 mg/day) is generally absorbed.[7] Their absorption occurs from the stomach and small intestine by different mechanisms that may involve a specific enzyme, such as bilitraslocase.[8] ACNs enter in the circulatory system after passing through the liver, move in the blood, and are distributed to different tissues. They appear in plasma within 15−60 min following consumption, and their maximum concentration is in the order of nanomolar levels.[7,9] Mostly, ACNs reach the colon, where they are extensively metabolized by intestinal microbiota, which contribute to the bioavailability and bioefficacy of ACNs and phenolic compounds in the systemic circulation.[10] Moreover, we have recently documented that ACNs and phenolic compounds can positively modulate the composition of intestinal microbiota and promote the colonization of the gastrointestinal tract by beneficial bacteria (i.e., *Bifidobacterium* spp. and *Lactobacillus* spp.) at the expense of unhealthy bacteria.[10,11]

Particular attention must be paid to the chemical changes that ACNs undergo during processing, so that they may be able to exert and maintain their health benefits. Because the availability of blueberries is seasonal, processing for long-term storage is desirable by the food industry. The majority of berries, including blueberries, are consumed as processed foods: juices, purées, jams, syrups, jellies, and various ready-to-drink beverages. The influence of processing on the content of food polyphenols and ACNs has been previously studied.[12−20] Significant losses of phenolic compounds compared to fresh fruits have been observed in blueberry juices,[13−15] raspberry purée and jam,[16] strawberry jams,[17,18] canned fruit,[18] and nectar.[19] Hager et al. demonstrated that processing of berries canned in water or syrup reduced total ACNs by about 42 and 51%, respectively.[20] Patras et al. observed that ACNs from blackberry and strawberry purée were reduced by about 28% after a thermal treatment of 20 min.[12] ACN degradation in

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ORIGINAL CONTRIBUTION

Effect of a wild blueberry (*Vaccinium angustifolium*) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors

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Abstract

Purpose Wild blueberries (WB) (*Vaccinium angustifolium*) are rich sources of polyphenols, such as flavonols, phenolic acids and anthocyanins (ACNs), reported to decrease the risk of cardiovascular and degenerative diseases. This study investigated the effect of regular consumption of a WB or a placebo (PL) drink on markers of oxidative stress, inflammation and endothelial function in subjects with risk factors for cardiovascular disease.

Methods Eighteen male volunteers (ages 47.8 ± 9.7 years; body mass index 24.8 ± 2.6 kg/m^2) received according to a cross-over design, a WB (25 g freeze-dried powder, providing 375 mg of ACNs) or a PL drink for 6 weeks, spaced by a 6-week wash-out. Endogenous and oxidatively induced DNA damage in blood mononuclear cells, serum interleukin levels, reactive hyperemia index, nitric oxide, soluble vascular adhesion molecule concentration and other variables were analyzed.

Results Wild blueberry drink intake significantly reduced the levels of endogenously oxidized DNA bases (from 12.5 ± 5.6 % to 9.6 ± 3.5 %, $p \leq 0.01$) and the levels of H_2O_2-induced DNA damage (from 45.8 ± 7.9 % to 37.2 ± 9.1 %, $p \leq 0.01$), while no effect was found after the PL drink. No significant differences were detected for markers of endothelial function and the other variables under study.

Conclusions In conclusion, the consumption of the WB drink for 6 weeks significantly reduced the levels of oxidized DNA bases and increased the resistance to oxidatively induced DNA damage. Future studies should address in greater detail the role of WB in endothelial function. This study was registered at www.isrctn.org as ISRCTN47732406.

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Abbreviations

AACC	American Association for Clinical Chemistry
ACNs	Anthocyanins
AI	Augmentation index
AI@75	Augmentation index standardized for heart rate of 75 bpm
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease

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Horse meat consumption affects iron status, lipid profile and fatty acid composition of red blood cells in healthy volunteers

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Abstract

This study investigated the effect of moderate consumption of horse meat on iron status, lipid profile and fatty acid composition of red blood cells in healthy male volunteers. Fifty-two subjects were randomly assigned to two groups of 26 subjects each: a test group consuming two portions of 175 g/week of horse meat, and a control group that abstained from eating horse meat during the 90 days trial. Before and after 90 days, blood samples were collected for analysis. Horse meat consumption significantly ($p \le 0.05$) reduced serum levels of total and low-density lipoprotein cholesterol (-6.2% and -9.1%, respectively) and transferrin (-4.6%). Total $n-3$, long chain polyunsaturated fatty acids $n-3$ and docosahexeanoic acid content in erythrocytes increased ($p \le 0.05$) by about 7.8%, 8% and 11%, respectively. In conclusion, the regular consumption of horse meat may contribute to the dietary intake of $n-3$ polyunsaturated fatty acids and may improve lipid profile and iron status in healthy subjects.

Keywords: *horse meat, iron status, lipid profile, red blood cells, fatty acid composition, healthy subjects*

Abbreviations: *AOAC, Association of Official Analytical Chemists, BMI, body mass index, C, control group, CVD, cardiovascular disease, DHA, docosahexeanoic acid, EPA, eicosapentonoic acid, HDL, high-density lipoprotein, HM, test group, ICP-MS, inductively coupled plasma mass spectrometry, LC-PUFAs, long chain polyunsaturated fatty acids, LDL, low-density lipoprotein, MUFAs, monounsaturated fatty acids, PUFAs, polyunsaturated fatty acids, RBCs, red blood cells, SFAs, saturated fatty acids*

Introduction

Several studies have correlated the high consumption of red meat with an increased risk for cardiovascular disease (CVD) and colon cancer (Wei et al. 2004; Kelemen et al. 2005; Cross et al. 2007; Iqbal et al. 2008; Kontogianni et al. 2008; Zyriax et al. 2008). Nowadays, dietary recommendations provided by the Italian National Research Institute for Food and Nutrition, the American Heart Association and the United States Department of Agriculture Center for Nutrition Policy and Promotion and the Word Cancer Research Funds suggest limiting the intake of red meat to less than 500 g cooked weight per week and to encourage the intake of white meat (including chicken, game and turkey) for the low content in fat and cholesterol. This message is particularly important for Mediterranean population whose traditional diet does not include a high intake of red meat.

Recent research has emphasized the nutritional value of horse meat. Compared to other meats, it is very low in fat and cholesterol (about 20% less), and it is a good source of polyunsaturated fatty acids (PUFAs) and is low in saturated fatty acids (SFAs),

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A single portion of blueberry (*Vaccinium corymbosum L*) improves protection against DNA damage but not vascular function in healthy male volunteers

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ARTICLE INFO

ABSTRACT

It has been suggested that anthocyanin-rich foods may exert antioxidant effects and improve vascular function as demonstrated mainly in vitro and in the animal model. Blueberries are rich sources of anthocyanins and we hypothesized that their intake could improve cell protection against oxidative stress and affect endothelial function in humans. The aim of the study was to investigate the effect of one portion (300 g) of blueberries on selected markers of oxidative stress and antioxidant protection (endogenous and oxidatively induced DNA damage) and of vascular function (changes in peripheral arterial tone and plasma nitric oxide levels) in male subjects. In a randomized cross-over design, separated by a wash out period ten young volunteers received one portion of blueberries ground by blender or one portion of a control jelly. Before and after consumption (at 1, 2, and 24 hours), blood samples were collected and used to evaluate anthocyanin absorption (through mass spectrometry), endogenous and H_2O_2-induced DNA damage in blood mononuclear cells (through the comet assay), and plasma nitric oxide concentrations (through a fluorometric assay). Peripheral arterial function was assessed by means of Endo-PAT 2000. Blueberries significantly reduced ($P < .01$) H_2O_2-induced DNA damage (−18%) 1 hour after blueberry consumption compared to control. No significant differences were observed for endogenous DNA damage, peripheral arterial function and nitric oxide levels after blueberry intake. In conclusion, one portion of blueberries seems sufficient to improve cell antioxidant defense against DNA damage, but further studies are necessary to understand their role on vascular function.

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Effect of Time and Storage Temperature on Anthocyanin Decay and Antioxidant Activity in Wild Blueberry (*Vaccinium angustifolium*) Powder

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ABSTRACT: This study evaluated the effects of storage on total and single anthocyanin (ACN) content, and total antioxidant activity (TAA) of freeze-dried wild blueberry (WB) powder maintained at 25, 42, 60, and 80 °C for 49 days. Storage reduced single and total ACN content at all of the temperatures; it was slower at 25 °C (−3% after 2 weeks), whereas it was faster at 60 °C (−60%) and at 80 °C (−85%) after 3 days. The values of half-life time ($t_{1/2}$) were found to be 139, 39, and 12 days at 25, 42, and 60 °C, respectively, utilizing the Arrhenius equation. No significant effects were detected on TAA by temperature increase. In conclusion, this study provides important information on the stability of WB powder at 25 °C; this is interesting scientific research for the food industry.

KEYWORDS: *wild blueberry powder, anthocyanins, total antioxidant activity, storage temperature*

■ INTRODUCTION

In recent years, several studies documented the beneficial effects of berries (i.e., cranberries, raspberries, and blueberries) on human health. Wild blueberries (*Vaccinium angustifolium*) have been reported to have a protective effect against chronic diseases, especially cardiovascular disease;[1−3] this has generally been attributed to their polyphenol content, anthocyanins (ACNs) in particular. These compounds are responsible for the blue and purple color of the berries and for these reasons are also used as natural food colorants in the food industry.[4] However, ACNs are labile in nature and susceptible to deterioration during processing and storage.[5] Blueberries are often quick-frozen at very low temperatures (−80 °C) for long-term preservation with minimal effects on quality.[6,7] The majority of berries, including blueberries, are consumed as processed foods, that is, juices, purees, jams, syrups, jellies, and various ready-to drink beverages to ensure extension of shelf life and consumption independent of the growing season.[8−11] Another common system to preserve blueberries is through the freeze-drying process, which has several advantages for the food industry such as reduction of storage space, size, and cost. Moreover, the freeze-drying process permits the standardization content of nutrient and phytochemicals useful for human health.

Several mechanisms of degradation during processing and storage have been documented. In freezing and cold storage, the retention of ACNs depends on the rate of freezing, temperature, the presence/absence of oxygen, and the food matrix.[5,12] Studies verified the stability or at least a slight increase in ACN content in berries/blueberries during cold storage[13,14] or storage in high-oxygen atmospheres.[15] On the contrary, a reduction was observed for extruded products such as cereal blueberry-rich products[16] and for thermally processed foods such as juices,[8,9,17] jams,[10,18] and purées.[11]

Anthocyanin degradation is high when these products are treated at higher temperature (up to 121 °C) and then refrigerated.[19,20] Concerning dry storage, the major parameters determining the stability of ACNs are water content, water activity (a_w), temperature, presence/absence of oxygen, light, and relative humidity.[11,19]

However, no data are available concerning the effect of storage on ACN content in freeze-dried wild blueberry powder. This is very important because the food industry uses the freeze-dried products as ingredients in many food formulations, such as jams, jellies, sauces, purées, toppings, syrups, juices, bakery, and dairy products. In addition, this information is extremely important for scientists that use freeze-dried products (frequently stored at room temperature for several months before using) as a feed ingredient in animal and human studies.

Moreover, in past studies, the ACN concentration was commonly quantified as total ACNs, and no information was reported on the fate of the single compounds contained in the blueberries.[8−11,17−20]

For these reasons, the objective of this study was to investigate, for the first time, the degradation kinetics of single ACNs contained in freeze-dried wild blueberry (WB) powder samples stored at different temperatures (25, 42, 60, and 80 °C) for 49 days. Total ACN content and total antioxidant activity (TAA) were investigated as well, under the above conditions.

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ARTICLE IN PRESS

Acute cigarette smoking impairs microvascular function in young moderate smokers: A potential model for studying vasoactive properties of food bioactives

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ARTICLE INFO

ABSTRACT

It has been suggested that several dietary compounds may improve endothelial dysfunction and oxidative stress induced by smoking. We investigated the effects of acute smoking on blood pressure, heart rate (HR), peripheral arterial function (reactive hyperemia index, RHI), and arterial stiffness in young smokers by Endo-PAT2000. Twenty subjects that smoked an average of 15 cigarettes per day participated in the study. Reactive Hyperemia Index, arterial stiffness, blood pressure and HR were assessed before and after smoking one cigarette. Acute smoking significantly reduced RHI by 28% while increased arterial systolic (+14%) and diastolic (+10%) blood pressure and HR (+13%) at 5 min, while no effect was observed after 30 min. Arterial stiffness was not significantly affected. A significant, positive correlation was found between total serum cholesterol concentration and post-smoking arterial stiffness values. This study demonstrates that acute cigarette smoking impairs RHI and vital signs in young moderate smokers as evaluated through a non-invasive technique. Additionally, the experimental model described, where a decrease of RHI is induced by cigarette smoking, may be useful to study the impact of dietary vasoactive compounds on endothelial function.

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1. Introduction

Recently, the study of the effect of diet in the modulation of oxidative stress-related processes in vivo is under investigation. Cigarette smoking is one condition that increases oxidative stress but also impairs endothelial function [1,2]. Endothelial dysfunction is described as the inability of the artery to sufficiently dilate in response to an appropriate stimulus and is considered an early phenomenon in atherogenesis [3]. This condition has been widely described in coronary and brachial arteries of healthy chronic moderate smokers and more often in passive smokers. The mechanism of endothelial dysfunction through smoking is poorly understood, but it seems to be attributed to the known/unknown substances (i.e. reactive oxygen and nitrogen species, nicotine, benzopyrene and acrolein) that constitute the particulate (tar) and gaseous phase of the cigarette [4]. These components may induce oxidative stress and inflammation with detrimental consequences on endothelial function. The increase in free radical species may reduce the production and bioavailability of nitric oxide (NO), the most important vasodilator produced by endothelial cells, with anti-atherosclerotic and anti-proliferative function [3]. Cigarette smoking may also participate in the uncoupling of endothelium-derived nitric oxide synthase, subsequently attenuating NO production and increasing superoxide generation [5].

The deleterious effects of cigarette smoking also involve an increase in arterial wall stiffness, one of the underlying pathophysiological mechanisms of the cardiovascular process [6]. Arterial stiffness has been related to the Framingham and other cardiovascular risk scores and it is considered a predictor of cardiovascular events in the general population, in patients with hypertension, end-stage renal disease, impaired glucose intolerance, and coronary artery disease [7]. Measurement of arterial stiffness provides information about the functional and structural vascular changes at the level of the aorta, muscular conduit arteries, peripheral branches, and microvascular components [7].

Differential Modulation of Human Intestinal *Bifidobacterium* Populations after Consumption of a Wild Blueberry (*Vaccinium angustifolium*) Drink

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ABSTRACT: Bifidobacteria are gaining increasing interest as health-promoting bacteria. Nonetheless, the genus comprises several species, which can exert different effects on human host. Previous studies showed that wild blueberry drink consumption could selectively increase intestinal bifidobacteria, suggesting an important role for the polyphenols and fiber present in wild blueberries. This study evaluated the modulation of the most common and abundant bifidobacterial taxonomic groups inhabiting the human gut in the same fecal samples. The analyses carried out showed that *B. adolescentis*, *B. breve*, *B. catenulatum/pseudocatelulatum*, and *B. longum* subsp. *longum* were always present in the group of subjects enrolled, whereas *B. bifidum* and *B. longum* subsp. *infantis* were not. Furthermore, it was found that the most predominant bifidobacterial species were *B. longum* subsp. *longum* and *B. adolescentis*. The results obtained revealed a high interindividual variability; however, a significant increase of *B. longum* subsp. *infantis* cell concentration was observed in the feces of volunteers after the wild blueberry drink treatment. This bifidobacterial group was shown to possess immunomodulatory abilities and to relieve symptoms and promote the regression of several gastrointestinal disorders. Thus, an increased cell concentration of *B. longum* subsp. *infantis* in the human gut could be considered of potential health benefit. In conclusion, wild blueberry consumption resulted in a specific bifidogenic effect that could positively affect certain populations of bifidobacteria with demonstrated health-promoting properties.

KEYWORDS: bifidobacteria, *Bifidobacterium longum* subsp. *infantis*, wild blueberry, human study, microbiota, diet, prebiotic

■ INTRODUCTION

The human intestinal microbiota is strongly emerging as a main protagonist in the maintenance of host homeostasis.[1] Particularly, specific bacterial populations in the human gut, such as bifidobacteria and lactobacilli, are classically recognized as beneficial for human health, and their reduction has been associated with several definite host dysfunctions.[2]

Intestinal microbiota markedly depends on diet,[3] and numerous nutritional interventions have been shown to selectively modify specific bacterial groups, including bifidobacteria. For instance, certain dietary compounds, named prebiotics, have been reported to selectively increase the beneficial components of microbiota, such as bifidobacteria.[4] According to the Food and Agriculture Organization of the United Nations (FAO), a prebiotic is *"a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota"*.[5]

Although most studies have been conducted on the effect of isolated prebiotic molecules or prebiotic-fortified food products, the ability to stimulate the growth of bifidobacteria in the human gut (conventionally referred as bifidogenic effect) has been also recognized for conventional foods that are naturally rich in prebiotics, such as banana,[6] apple,[7] whole grain breakfast cereals,[8] red wine,[9] and coffee.[10] Interestingly, polyphenolic food components including anthocyanins (ACNs) have been documented to have possible effects on intestinal micro-

organisms,[1,9] suggesting that also ACNs could potentially be prebiotic molecules.

Berries are rich sources of ACNs, which together with flavonoids, phenolic acids, folate, minerals, and fiber are important contributors to the in vivo biological activities of these fruits.[12] It is well-known that absorption of ACNs is quite limited, so that most of the compounds reach the colon, where they are widely metabolized by the intestinal microbiota.[13] Thus, the regular intake of ACN-rich products, such as berries, may affect the different bacterial groups with potential, yet undetermined, benefits.[11] In this context, we recently reported that intestinal bifidobacteria significantly increased in healthy subjects following a 6 week consumption of a wild blueberry (*Vaccinium angustifolium*) drink.[14]

Bifidobacteria are among the very first colonizers of the human intestine immediately after birth, soon becoming the predominant component of the microbiota in breast-fed infant intestine, where they have been purported to play a key role in the maturation of the host's immune system.[15] Bifidobacteria are also abundant in the adult human population, comprising up to 15% of the normal intestinal microbiota.[16] Additionally, the reduction of intestinal bifidobacteria that occurs in the

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Research Article

Modulation of plasma antioxidant levels, glutathione *S*-transferase activity and DNA damage in smokers following a single portion of broccoli: a pilot study

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Abstract

BACKGROUND: Broccoli is a rich source of bioactive compounds (i.e. glucosinolates, carotenoids, vitamin C and folate) that may exert an antioxidant effect and reduce oxidative damage. The objective of this pilot study was to investigate the effect of broccoli consumption on carotenoids, vitamin C and folate absorption, glutathione *S*-transferase (GST) activity, and oxidatively induced DNA damage in male smokers.

METHODS: Ten healthy subjects consumed a single portion of steamed broccoli (250 g) with cooked pasta. Blood was drawn at baseline and at 3, 6 and 24 h from consumption.

RESULTS: Broccoli significantly ($P \leq 0.01$) increased plasma level of vitamin C and folate (+35% and 70%, respectively) at 3 h, and β-carotene (+8%) at 6 h. A modulation of GST activity occurred in plasma 6 h after broccoli consumption. A significant ($P \leq 0.01$) reduction of the levels of H_2O_2-induced DNA damage (−18%) was observed in blood mononuclear cells 24 h after broccoli intake in GSTM1 positive, but not in GSTM1 null subjects.

CONCLUSION: One portion of broccoli increased plasma antioxidant levels, modulated plasma GST activity and improved cell resistance against H_2O_2-induced DNA damage in healthy smokers. These results support the importance of consuming fruit and vegetable regularly.
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Keywords: broccoli; antioxidants; glutathione *S*-transferase, DNA damage; smokers

INTRODUCTION

Brassica vegetables include red and white cabbage, cauliflower, Savoy cabbage, Brussels sprouts, watercress and broccoli. Several epidemiological studies reported a positive association between high intake of Brassica vegetables and reduction of cancer risk for kidney, prostate, bladder, colon, rectum and lung.[1–6] The protective mechanisms of action of compounds present in these vegetables can depend on their ability to reduce the levels of free radicals and/or improve the endogenous antioxidant defence system, which could be particularly important in subjects with high exposure to oxidative stress, such as smokers. Cigarette smoke contains a large amount of reactive oxygen species (ROS) and other substances that may cause oxidative damage to DNA, such as oxidised bases and strand breaks.[7] These DNA lesions, including the promutagenic 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) could be implicated in early steps of cancer development.[8] We recently documented that the intake of broccoli increased resistance to *ex vivo* H_2O_2-induced DNA strand breaks in blood mononuclear cells (BMCs) of smokers and non-smokers.[9] Our results indicated that broccoli bioactives may either act as free radical scavengers or increase the endogenous

defence against oxidative stress, in smoker subjects.[10] The distinctive protective effects of broccoli are likely attributable to glucosinolates (GLs), a wide class of compounds whose major metabolic breakdown products are isothiocyanates (ITCs). These substances are involved in the induction and modulation of phase II detoxifying enzymes such as glutathione *S*-transferases (GSTs; EC 2.5.1.18), which detoxify polycyclic aromatic hydrocarbons from smoke and, in turn, catabolise ITCs, preparing them for excretion.[11,12]

A polymorphism in the *GSTM1* gene results in lack of GSTM1-1 protein. Pharmacokinetic studies suggest that lack of GSTM1 enzyme is associated with more rapid excretion of the ITC sulforaphane; therefore, individuals who have this

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RESEARCH ARTICLE

Effect of 10-day broccoli consumption on inflammatory status of young healthy smokers

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Abstract

This study evaluated the effects of 10-day broccoli (250 g/day) intake on dietary markers and markers of inflammations in young male smokers. A dietary intervention study with a repeated measures crossover design was conducted. Circulating levels of carotenoids, folate, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 6 receptor (IL-6sR) and adiponectin were measured. Broccoli intake significantly increased plasma levels of folate (+17%) and lutein (+39%), while no significant effect was observed for TNF-α, IL-6, IL-6sR or adiponectin. Plasma CRP decreased by 48% (post-hoc analysis, $p < 0.05$) following broccoli diet; this resulted to be independent from the plasma variations in lutein and folate. An inverse correlation between lycopene, TNF-α and IL-6sR was observed at baseline. In conclusion, broccoli consumption may reduce CRP levels in smokers, consistent with epidemiologic observations that fruit and vegetable intake is associated with lower circulating CRP concentrations.

Introduction

Cruciferous vegetables are rich sources of glucosinolates (precursors of isothiocyanates, ITCs), flavonoids (e.g. kaempferol, quercetin, isorhamnetin), carotenoids, vitamins (e.g. vitamin C, K1, folate), minerals (e.g. selenium, calcium, magnesium), lipoic acid, protease inhibitors and fiber (Moreno et al., 2006). These compounds may be largely responsible for the cardioprotective and chemopreventive activity of cruciferous vegetables, as reported by numerous epidemiologic studies (Van Poppel et al., 1999; Verhoeven et al., 1996). The protective actions of cruciferous components are mediated by different mechanisms, such as modulation of detoxifying enzymes and cell signalling (Conaway et al., 2002; Zhang, 2004); control of cell cycle and induction of apoptosis (Conaway et al., 2002); protection against oxidative damage (Verhagen et al., 1997); and anti-inflammatory activities (Heiss et al., 2001; Zhang, 2004). The latter is likely of utmost importance, because chronic inflammation correlates with endothelial dysfunction, insulin resistance and oxidative stress, therefore increasing the risk of cancer, diabetes, cardiovascular disease and other pathologies such as arthritis, pulmonary diseases, neurological diseases (such as Alzheimer's disease) and autoimmune diseases (Aggarwal et al., 2006; Danesh et al., 2000; Giugliano et al., 2006; Pepys & Hirschfield, 2003).

Many environmental factors, e.g. viruses, bacteria, pollutants, cigarette smoke and other stressors contribute to increase inflammation in the body through the action of both reactive oxygen species and specific molecules such as interleukins and chemokines (Aggarwal et al., 2006).

Diet can modulate inflammation chiefly by limiting the production of pro-inflammatory eicosanoids and by restoring the proper balance of pro- and anti-inflammatory cytokines (Giugliano et al., 2006). Indeed, adequate intakes of fruits, vegetables, nuts, whole grains and long-chain omega-3 fatty acids are associated with lower levels of inflammation, while dietary patterns high in refined starches, sugars, saturated and trans-fatty acids and poor in antioxidants, fiber, and omega-3 fatty acids lead to the opposite situation (Ajani et al., 2004; Clifton, 2003; Gao et al., 2004; Mozaffarian et al., 2004; Pischon et al., 2003; Van Herpen-Broekmans et al., 2004).

While several epidemiologic and some intervention studies in humans have reported positive effects of fruit and vegetable consumption on the inflammatory status, no specific information is available on the association between Brassicaceae intake and inflammation in humans (Ajani et al., 2004; Esmaillzadeh et al., 2006; Gao et al., 2004; Jacob et al., 2008; Sanchez-Moreno et al., 2003; Van Herpen-Broekmans et al., 2004; Watzl et al., 2005). We have previously demonstrated that the intake of broccoli (250 g/d for 10 days) is able to affect markers of oxidative stress (Riso et al., 2010). In the present paper, we report on five validated biomarkers of inflammation, i.e. two visceral proteins [C-reactive protein (CRP) and adiponectin], two major cytokines [tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)], and one cytokine receptor (IL-6sR) that we analyzed in samples from the same intervention study.

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CHAPTER

11

Preventive Effects of Broccoli Bioactives: Role on Oxidative Stress and Cancer Risk

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List of Abbreviations

p0010 **8-oxodG** 8-oxo-7,8-dihydro-2'-deoxyguanosine
p0015 **AhR** aryl hydrocarbon receptor
p0020 **ARE** antioxidant response element
p0025 **Bcl-2** B-cell lymphoma 2
p0030 **CYP** cytochrome
p0035 **ER** estrogen receptors
p0040 **FPG** formamidopyrimidine DNA glycosylase
p0045 **GLs** glucosinolates
p0050 **GSH** glutathione
p0055 **GST** glutathione-S-transferase
p0060 **HDAC** histone deacetylases
p0065 **HO-1** heme oxygenase (decycling) 1
p0070 **I3C** indole-3-carbinol
p0075 **IkB** inhibitor kappa B
p0080 **ITCs** isothiocyanates
p0085 **KEAP1** Kelch-like ECH-associated protein 1
p0090 **MAPK** mitogen-activated protein kinase
p0095 **NF-kB** Nuclear Factor kappa B
p0100 **NQO** NADPH quinone reductase
p0105 **Nrf2** nuclear factor-erythroid-2-related factor 2
p0110 **NUDT1** nucleoside diphosphate linked moiety X-type motif 1
p0115 **OGG1** 8-oxoguanine DNA glycosylase
p0120 **PBMCs** peripheral blood mononuclear cells
p0125 **ROS** reactive oxygen species
p0130 **SERMs** selective estrogen receptor modulators
p0135 **SFN** sulforaphane
p0140 **UGTs** UDP-glucuronosyl transferases
p0145 **XRE** xenobiotic response element

s0005
INTRODUCTION

p0150 Cruciferous vegetables belong to the botanical family known as Brassicaceae, a family of plants including the Brassica genus with a large number of accessions. They are characterized by a peculiar phytochemical composition; the most commonly consumed products within this class are broccoli, cauliflower, cabbage, and Brussels sprouts. Other brassica vegetables such as kale, watercress, turnip, mustard, and radish are consumed primarily in specific regions.[1]

p0155 A large number of studies, including cell culture, animal models, and epidemiological studies, have documented the importance of this class of vegetables for cancer prevention.[1] However, data from human intervention studies are relatively limited. The present chapter will discuss the available evidence on the role of cruciferous vegetables, with focus on broccoli, modulation of oxidative stress, and markers of cancer risk.[1]

s0010
CRUCIFEROUS VEGETABLES INTAKE AND CANCER RISK

p0160 Epidemiological evidence has linked brassica vegetable consumption to a reduced risk for a variety of cancers.[1-3] Most of the evidence comes from case-control studies, and appears to be most conclusive for an inverse association with lung and gastric cancers.[2]

p0165 Although it is extremely difficult to discriminate the contribution of a single food without incurring methodological biases and confounding factors when obtaining and evaluating epidemiological data, a few studies have attempted to focus specifically on the association between broccoli consumption and cancer risk.

p0170 Inverse associations between cruciferous vegetable intake and lung cancer have been repeatedly reported in case-control and cohort studies,[1] as recently reviewed

1

APPENDIX 2. TITLES OF THE PAPERS SUBMITTED

- Taverniti V, Fracassetti D, **Del Bo' C**, Lanti C, Minuzzo M, Klimis-Zacas D, Riso P. Guglielmetti S. Wild Blueberry anthocyanins exert immunomodulatory effects on human Caco-2 intestinal cells and U937 macrophages. *Journal of Agriculture and Food Chemistry.*

- **Del Bo' C,** Porrini M, Fracassetti D, Campolo J, Klimis-Zacas D, Riso P. A single blueberry (*Vaccinium corymbosum*) portion prevents peripheral arterial dysfunction induced by acute cigarette smoke in young healthy volunteers. *European Journal of Nutrition.*

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APPENDIX 3. COPIES OF ABSTRACTS OF ORAL COMMUNICATIONS AND POSTERS

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Regulation of adipose tissue gene expression *in vitro* and *in vivo* studies: review on the role of anthocyanins.

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Anthocyanins (ACNs) are a group of natural pigments found in red-blue fruits and vegetables with a wide range of biological activities including: antioxidant, anti-inflammatory and anticarcinogenic activities. *In vitro* and *in vivo* experiments suggest that ACNs can inhibit adipogenic development and fat accumulation[1-2]. Lipogenesis consists of fatty acids and triglycerides synthesis and occurs both in liver and fat tissue. The molecular pathways involved in the adipocyte differentiation it is not well known; however the adipocyte differentiation transcription factor PPARγ could be involved in the regulation of lipogenesis[1]. To our knowledge, only few reports exist suggesting that food factors directly modulate the adipocyte function including adipocytokine secretion or adipocyte specific gene expression. We present a review on the role of ACNs in the regulation of adipose tissue, reporting also data we obtained through a high ACNs Moro juice supplementation in a mice model[3]. Further studies are required to clarify the effect of dietary ACNs on adipose tissue gene expression during differentiation (from preadipocyte into mature adipocyte) and to confirm results obtained in the animal model through human studies.

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"Nutrizione e Rischio Cardiovascolare" Annual Meeting Società Italiana di Nutrizione Umana, Napoli, 12th-13th October, 2011.

Effect of wild blueberries bioactive compounds on immunomodulation: preliminary *in vitro* study

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Wild blueberries (WB) are one of the fruits having the highest antioxidant potential due to their polyphenol content, particularly anthocyanins. These compounds may be beneficial for human health because of their antioxidant, anti-inflammatory and immunomodulatory potential.

This study aims to understand the effect of WB (*Vaccinium angustifolium)* components, such as anthocyanins and phenols on immunomodulation, in an inflammatory state model through a human intestinal Caco-2 cell line.

A reporter gene system was prepared by transfecting Caco-2 cells with a vector where the reporter gene luciferase is under the control of an ELAM gene promoter and five NF-κB repeated transcription factor binding sites. Caco-2 transfected cells were stimulated with interleukin (IL)-1β and supplemented with different concentrations of WB extracts. The immunomodulatory activity of the samples under study was monitored in real time by quantification of bioluminescence with a luminometer. The WB fractions were separated by a polymeric SPE cartridge. Three different fractions were obtained: soluble fraction (eluted with 0.1% HCl), phenolic fraction (eluted with 100% ethyl acetate) and anthocyanin fraction (eluted with 0.1% acidified methanol), which were further characterized.

Results suggest that anthocyanins can attenuate Caco-2 cell inflammatory state. In fact, lower bioluminescence emission (i.e. inhibition of NF-κB activation) was observed as a consequence of anthocyanins addition to the system (5 mg/L, 25 mg/L and 50 mg/L), with a 50.2 % reduction at the highest concentration tested as compared to the control. On the contrary, the phenolic fraction did not show a dose-response effect (2.5 mg/L, 12 mg/L and 24 mg/L) and a trend towards increased inflammation was observed following the addition to Caco-2 cells of increasing amounts of the soluble fraction (organic acids: 1.1 mg/L and 2.2 mg/L; sugars: 69 mg/L and 138 mg/L). On the whole, these data suggest a positive role of anthocyanins from WB on immunomodulation.

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"Nutrizione e Rischio Cardiovascolare" Annual Meeting Società Italiana di Nutrizione Umana, Napoli, 12th-13th October, 2011.

Effect of wild blueberry *(Vaccinium angustifolium)* consumption on markers of oxidative stress and endothelial function in subjects with risk factors for cardiovascular disease

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Background: Wild blueberries (WB) (*Vaccinium angustifolium*) are rich sources of polyphenols such as anthocyanins (ACNs) capable of counteracting oxidative stress, influencing vasomotor tone and modulating gene expression associated with disease processes such as cardiovascular disease (CVD).

Objective: The objective of the study was to investigate the effect of consumption of a WB product on lipid profiles, markers of oxidative stress (endogenous and oxidatively-induced DNA damage in mononuclear cells), soluble vascular adhesion molecule 1 (s-VCAM-1) and nitric oxide concentration and endothelium mediated changes in peripheral arterial tone in subjects with at least one risk factor for CVD.

Methods: Twenty male volunteers were recruited and randomized in a cross-over design. Subjects received a WB drink (25 g of lyophilised WB powder corresponding to 148 g of raw fruits) providing 375 mg of ACNs or a placebo drink (without ACNs) for 6 weeks each. A six week wash-out period was scheduled. At the beginning and at the end of each treatment, blood samples were collected. Serum lipid and cholesterol profiles were measured by validated laboratory methods. The resistance to H_2O_2-induced DNA damage and endogenously oxidized DNA bases (formamidopyrimidine DNA glycosilase (FPG) sensitive sites) were evaluated in blood mononuclear cells by the comet assay. Peripheral arterial function was assessed by using finger plethysmography (Endo-PAT2000) while plasma nitric oxide and s-VCAM-1 analysis was performed by commercial kits. All variables were examined by a two way ANOVA for repeated measures.

Results and Conclusion: Six weeks of WB drink significantly reduced the levels of H_2O_2-induced DNA damage (from $45.8 \pm 7.9\%$ to $37.2 \pm 9.1\%$, $p \leq 0.01$) and the levels of endogenously oxidized DNA bases (from $12.5 \pm 5.6\%$ vs $9.6 \pm 3.5\%$, $p \leq 0.01$), while no effect was found after PL drink. No significant difference was observed in the total group for the peripheral arterial tone even though more than half of the subjects had an improvement, following the intervention with the WB drink. No statistically significant difference was also observed for lipid profile, nitric oxide and s-VCAM-1 concentrations. Considering the high inter-individual variation observed in endothelial function response, further studies may be necessary to demonstrate an effect.

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11th European Nutrition Conference, Madrid, 26th- 29th October, 2011.

Effect of a single blueberry portion on oxidative oxidative stress and endothelial function: a pilot study

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INTRODUCTION: Blueberries *(Vaccinium corymbosum)* are important sources of dietary bioactive compounds such as anthocyanins (ACNs) that could protect the integrity of DNA from reactive species, reduce the inflammatory state and improve endothelial function.
OBJECTIVE: The objective of the study was to investigate the effect of one portion (300g) of blueberry (providing about 300mg ACNs) on markers of oxidative stress (endogenous and oxidatively induced lymphocyte DNA damage) and endothelial function/action (nitric oxide and endothelium-mediated changes in peripheral arterial tone).
METHOD/DESIGN: Ten healthy male subjects were randomized in a cross-over design and received one portion of fresh blueberries ground by blender or placebo (purple jelly). The products were consumed early in the morning and blood was collected before consumption and at different times (1h, 2h and 24h). The resistance to H_2O_2-induced oxidative damage and endogenous DNA damage (formamidopyrimidine DNA glycosilase (FPG) sensitive sites) were evaluated in peripheral blood mononuclear cells by means of the comet assay. Peripheral arterial function was assessed using a new non-invasive plethysmographic method (Endo-PAT 2000), while plasma nitric oxide analysis was performed by commercial kits. All variables were examined by a two-way ANOVA for repeated measures design.
RESULTS: The consumption of one portion of blueberry significantly reduced ($p < 0.01$) the levels of H_2O_2-induced DNA damage (-18%) after 1h of intake with respect to that obtained following the placebo. No significant differences were observed for endogenous DNA damage, nitric oxide and peripheral arterial function following blueberry intake.
CONCLUSIONS: This pilot study showed that one portion of blueberry may improve the resistance against H_2O_2-induced DNA damage. Further analysis, on a larger group of subjects, could be useful to clarify the effect of blueberry consumption on the modulation of endothelial function/action.

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The 8OTH European Atherosclerosis Society Congress, Milano, 25th-28th May, 2012.

Effect of acute cigarette smoking on blood pressure and peripheral endothelial function in young healthy male smokers: preliminary data.

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Aim: Smoking is one of the major risk factors for atherosclerosis associated with premature coronary and artery diseases. Endothelial dysfunction is an early event in atherosclerosis, and seems mainly related to the decreased production or availability of nitric oxide in smokers. The objective of the study is to investigate the effect of a single cigarette on blood pressure and peripheral arterial function in young moderate smokers (approximately 15 cigarettes/day).
Methods: The study includes young, male, healthy volunteers recruited from the student population of the University of Milan. Sixty subjects were screened to check their eligibility taking into account smoking, physical activity, alcohol consumption, dietary habits, and endothelial function (PAT score $\geq$ 1.67). Blood pressure, heart rate and peripheral arterial function (determined through finger plethysmography; Endo-PAT2000), were assessed before and after smoking or non-smoking a single cigarette.
Results: Preliminary results on 12 subjects showed that smoking increased systolic (from 111.3±7.20 mmHg to 128.6±6.53 mmHg, $p<0.001$) and diastolic pressure (from 71.4±7.9 mmHg to 82.0±6.5 mmHg $p<0.001$), heart rate (from 56.9±10.0 beats/min to 71.8±16.5 beats/min; $p=0.001$) and reduced peripheral arterial function (from 2.16±0.30 PAT score to 1.87±0.30 PAT score; $p=0.039$). No significant difference was observed without smoking.
Conclusions: From these preliminary data, smoking a single cigarette seems to increase blood pressure and heart rate, and reduce peripheral arterial function in young healthy male smokers. Since anthocyanin-rich foods have been suggested to modulate endothelial function, their role in counteracting smoke related effects is under evaluation.

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Minimally-processed frozen purées from Vaccinium corymbosum L.: Influence of berry blanching on the phenolic profile

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Pigmented fruit, especially blueberries, are a natural source of phenolic phytochemicals which could be exploited through proper technologies, limiting by -product loss and detrimental impact of processing on fruit bioactive compounds. Frozen blueberry purée could be a versatile product, processed without pomace loss and potentially rich in all the bioactive compounds which are characteristic of the whole berry. The aim of this research was to study the influence of the mild thermal pretreatment of steam blanching on the phenolic profile of blueberry fruits and frozen purée after thawing (24 h at 4°C). Purées were prepared by processing IQF blueberries cv Brigitta either after thawing at 20°C for 3h (NB), or after steam-blanching for 3min and tap water -cooling in a pilot steam blanching tunnel (BL). Then 300g aliquots of purées were packed in 400mL plastic vessels, sealed under partial vacuum using a 25 μm thick polypropylene film and frozen and stored at −20°C. Chlorogenic acid and anthocyanins were quantified in berries and purées samples after extraction in formic acid-water (5:95 v/v) and in formic acid -water-acetone (5:35:60 v/v/v) and gradient -RP-HPLC and diode array detection. Fourteen anthocyanin compounds were identified; in NB berries delphinidin and malvidin glycosides made up more than 70% of total anthocyanins. Steam blanching did not affect chlorogenic acid berry concentration, as well as delphinidin-3-galactoside and delphinidin-3-arabinoside contents, whereas did it decrease the berry contents of malvidin-3-galactoside, malvidin-3-glucoside and malvidin-3-arabinoside. The NB purées were characterized by lower amounts of chlorogenic acid and delphinidin-3-galactoside and higher amounts of malvidin glycosides, compared to BL ones. However, in BL berries there were no quantitative changes for chlorogenic acid and all the fourteen anthocyanin compounds at the end of processing (puréeing, freezing and thawing). In contrast, processing NB berries resulted in lower amounts of chlorogenic acid and anthocyanin compounds, except for malvidin-3-galactoside and malvidin-3-arabinoside, which did not change. Our results indicated that berry blanching acts on anthocyanins pattern in two ways: firstly by glucosides and malvidine glycosides thermal degradation in berries and, secondly, protecting delphinidin and petunidin from oxidative degradation during processing.

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Oxidants and Antioxidants in Biology. Cell Signaling and Nutrient – Gene Interactions – Oxygen Club of California, Alba, 20th-23th June, 2012.

Immunomodulatory potential of wild blueberries *(Vaccinium angustifolium)* in human intestinal epithelial Caco-2 cells: preliminary data

Daniela Fracassetti[1], **Cristian Del Bo'[1]**, Simone Guglielmetti[1], Patrizia Riso[1], Valentina Taverniti[1], Dorothy Klimis-Zacas[2] and Marisa Porrini[1]

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Wild blueberries (WB) are a rich source of polyphenols, particularly anthocyanins, which may be beneficial for human health because of their antioxidant, antiinflammatory and immunomodulatory potential. This study aims to investigate the immunomodulatory potential of three WB fractions (anthocyanin, phenolic and soluble fraction) in an inflammatory state model, based on human intestinal Caco-2 cells. A reporter gene system was prepared by transfecting Caco-2 cells with the pNiFty-SEAP reporter- construct containing an engineered ELAM promoter with 5 NF-kB binding sites and an insect luciferase reporter gene. The transfected Caco-2 cells were stimulated with interleukin (IL)-1β to mimic an inflammatory state and then supplemented with 5 mg/L, 25 mg/L, 50 mg/L and 100 mg/L of the anthocyanin, phenolic and soluble fractions. Immunomodulatory activity for each fraction was monitored in real time by quantification of bioluminescence with a luminometer. The addition of anthocyanins at the concentration of 50 and 100 mg/L reduced the bioluminescence, and therefore NF-κB activation, up to 70% and 85% ($p \leq 0.05$), respectively. No dose-response was observed after supplementing Caco-2 cells with both phenolic and soluble fractions. These data suggest that only the anthocyanin fraction can modulate Caco-2 cells inflammatory state in the experimental conditions adopted. Future experiments will address supplementation of Caco-2 cells with anthocyanin standards to elucidate the compound(s) responsible for the observed immunomodulatory effect.

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Oxidants and Antioxidants in Biology. Cell Signaling and Nutrient – Gene Interactions – Oxygen Club of California, Alba, 20th-23th June, 2012.

The intake of a single blueberry (*Vaccinium corymbosum L.*) portion affects blood pressure and peripheral arterial function in smokers: preliminary data.

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Cigarette smoking is one of the primary risk factors for the development of endothelial dysfunction, an early event in atherosclerosis, due to an increase of oxidative stress and the impairment of nitric oxide production. Anthocyanin-rich foods, have been suggested to offer protection against oxidative stress and modulate endothelial function. This study aims to investigate whether a single portion of blueberry improves endothelial dysfunction induced by acute cigarette smoking. Fifteen healthy smokers were recruited for a crossover study. Three types of conditions were assessed: 1-blueberry treatment (300g of blueberry) + smoking (1 cigarette); 2-control treatment (300mL of water with 27g of sugars; the same amount present in blueberry) + smoking (1 cigarette); 3- smoking only (1 cigarette). Peripheral arterial function (determined through Endo-PAT2000) and blood pressure were evaluated. Preliminary results on 8 subjects showed that acute smoking caused a reduction in peripheral arterial function (from 2.22 ± 0.3 to 1.62 ± 0.4 RHI, $p<0.01$) and an increase in systolic blood pressure (from 112.3 ± 7.9 to 129.7 ± 6.7 mmHg, $p<0.001$) and diastolic blood pressure (from 71.2 ± 7.8 mmHg to 81.3 ± 6.0 mmHg, $p\leq0.01$) with respect to baseline. The consumption of blueberry, but not of the control drink, seems to counteract the observed effects of smoking by reducing the impairment of peripheral arterial function ($-4.0\pm10.3\%$ vs $-26.2\pm17.1\%$ RHI, $p<0.01$) and the increase of systolic blood pressure ($+9.1\pm7.1\%$ vs $+16.6\pm4.9\%$ mmHg, $p<0.05$).

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5th International Symposium on Nutrition, Oxygen Biology and Medicine, Paris, 5th-7th June, 2013.

A single blueberry (*Vaccinium corymbosum L.*) portion counteracts peripheral arterial dysfunction, induced by acute cigarette smoking, in healthy volunteers

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Cigarette smoking is one of the primary risk factors for the development of endothelial dysfunction, an early event in atherosclerosis, due to an increase of oxidative stress and the impairment of nitric oxide production. Anthocyanin-rich foods, have been suggested to offer protection against oxidative stress and modulate endothelial function. This study aims to investigate whether a single portion of blueberries counteracts endothelial dysfunction induced by acute cigarette smoking. Sixteen healthy smokers were recruited for a crossover study. Three types of conditions were assessed: 1- S: smoking only (1 cigarette); 2- BS: blueberry treatment (300g of whole blueberries) + smoking (1 cigarette); 3- CS: control treatment (300mL of a sugar drink; the same amount present in blueberry) + smoking (1 cigarette). Each condition was separated by one week of wash-out period. Blood pressure, heart rate and peripheral arterial function (reactive hyperemia index, RHI) was measured 20 min after smoking by a finger plethysmography named Endo-PAT2000.

Data were analyzed by one way ANOVA with time (before and after smoking) or treatment (S vs BS vs CS) as dependent factors. Post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test. A p-value $\leq$ 0.05 was considered significant. Data are expressed as mean values and standard deviation (SD) or standard error of the mean (SEM).

Acute smoking impaired peripheral arterial function (from 2.23 ± 0.28 to 1.59 ± 0.27 RHI, p=0.0001) and, increased systolic blood pressure (from 116.0 ± 6.9 to 131.7 ± 6.2 mmHg, p=0.0001), diastolic blood pressure (from 76.1 ± 8.2 to 83.5 ± 7.7 mmHg, p=0.005) and heart rate (from 63.3 ± 11.4 to 70.7 ± 11.8 beat/min, p=0.047) at 5 min from smoking. This effect was transitional and no effect was observed after 30 min.

The consumption of blueberry, but not of the sugar drink, reduced the impairment of RHI (-4.4 ± 0.8% BS vs -22.0 ± 1.1% S, p<0.01) and the increase of systolic blood pressure (+8.4 ± 0.02% BS vs +13.1 ± 0.02% S mmHg, p<0.05). The effect on RHI and systolic blood pressure was significantly different with respect to CS treatment (BS vs CS, p= 0.01), while no effect was observed between S vs CS treatment for both the variables. Diastolic blood pressure and heart rate were unaffected following BS and CS treatments.

In conclusion, the consumption of a portion of whole blueberries counteracted peripheral arterial dysfunction induced by acute cigarette smoking. These results support the role of ACN-rich food in the modulation of endothelial function, suggesting the importance to consume fruit regularly.

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10th International Comet Assay Workshop, Porto, Portugal, 18th – 20th September, 2013.

DNA damage evaluated through comet assay in fresh versus cryopreserved peripheral blood mononuclear cell samples from a dietary intervention study.

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Endogenous and oxidatively induced DNA damage evaluated through the comet assay in peripheral blood mononuclear cells (PBMCs) is widely utilized as biomarker of oxidative stress in numerous dietary intervention studies.[1-2] The analysis can be performed on fresh PBMCs or for logistic reasons on cells adequately cryopreserved until analysis. In this regard, information on how cryopreservation can affect results on DNA damage is often not considered. This is particularly important in studies where samples are analyzed before and after different treatments as in dietary intervention studies with repeated measures design.

To this aim we planned to compare results obtained on formamidopyrimidine DNA glycosylase (FPG)-sensitive sites and hydrogen peroxide-induced DNA strand breaks in cryopreserved human PBMCs with respect to results obtained in fresh samples. For the comparison, we used the samples obtained from a dietary intervention study in which 20 male volunteers received, for a period of 6 weeks, a wild blueberry or a placebo drink.[3] In particular fresh PBMCs were used to analyze FPG-sensitive sites and H_2O_2-induced strand breaks; contemporary aliquots of cells were stored for 12 months at -80°C in an appropriate medium. Results presented are relative to a subgroup of 9 subjects. The intake of the wild blueberry drink, but not placebo drink, significantly reduced FPG-sensitive sites (from $16.4 \pm 5.3\%$ to $11.0 \pm 2.8\%$, p=0.02) and H_2O_2-induced strand breaks (from $48.8 \pm 11.8\%$ to $40.0 \pm 10.1\%$, p=0.03) as evaluated in fresh PBMCs. Cryopreservation maintained the trend in the reduction of FPG-sensitive site levels following wild blueberry treatment (from $18.8 \pm 2.9\%$ to $15.9 \pm 4.0\%$, p=0.04). On the contrary, a greater resistance against H_2O_2-induced DNA damage have been observed in cryopreserved samples, thus the protective effect of wild blueberry treatment demonstrated in the fresh samples was not evidenced in stored samples (from $27.1 \pm 3.1\%$ to $26.1 \pm 2.5\%$, p=0.48).

In conclusion, the levels of oxidized bases, measured as FPG-sensitive sites, seem comparable in fresh and frozen PBMCs even after 1 year storage. On the contrary, cell protection against oxidative insult seems consistently different between fresh and frozen cells. The analysis of samples from the whole group of subjects will enable to demonstrate the effect of storage on both endpoints of DNA damage.

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Anthocyanins from Wild Blueberry (*Vaccinium aungustifolium*) 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-κB-binding sites followed by the firefly luciferase reporter gene *luc*. The effect of WB fractions was evaluated in the presence of IL-1β, a pro-inflammatory cytokine. Caco-2 cells were supplemented with 25, 50 and 100μg/mL of the water soluble fraction and 5, 25, 50 and 100μg/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 μg/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-κB induced by IL-1β in Caco-2 cells in a dose-dependent manner. Specifically, the concentration of 50 and 100μg/mL decreased the bioluminescence by 68.9% and 85.2% respectively (p≤0.05). Neither the phenolic nor the water soluble fractions showed a modulation in the activation of NF-κB. 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-κB in presence of IL-1β 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.

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"Comprendere e Applicare i LARN" Annual Meeting Società Italiana di Nutrizione Umana, Firenze, 21th-22th October, 2013.

The acute effect of cigarette smoking on inflammatory markers in young moderate smokers: a potential model for studying the protective properties of food bioactives.

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Background: We recently documented that acute cigarette smoking is able to impair peripheral arterial function (reactive hyperemia index, RHI) in young healthy smokers. The intake of a single portion of blueberry counteracts this deleterious effect. The causes of this transient endothelial dysfunction may be related to an increase of oxidative stress and inflammation.

Aim: This study aims to investigate whether the temporary impairment of vascular function evaluated through the acute experimental design may be correlated with the modulation of some markers of endothelial function and inflammation such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8).

Methods: Sixteen young healthy volunteers participated in a crossover study. Three types of treatments were studied; 1) smoking (1 cigarette); 2) blueberry treatment (300 g of whole blueberries + smoking; 3) control treatment (300 mL of water and 27g of sugars; the same amount present in blueberry + smoking). Each condition was separated by a one week of wash-out period. Cigarette was smoked 1 h 45 min after blueberry or control drink consumption. RHI was measured by Endo-PAT2000 at the baseline and 20 min after. Blood samples were collected at baseline and after 20 min, 1 h, 1.30 h, 2 h and 24 h from smoking. Markers of inflammation and endothelial function were measured in serum by Bio-plex Pro TM Array.

Results: Blueberry consumption, but not the control drink, counteracted the impairment of RHI ($-4.4\pm0.8\%$ blueberry treatment *vs* $-22.0\pm1.1\%$ smoking treatment, $p<0.01$). Preliminary results on a subgroup of 6 subjects showed an apparent increase in serum circulating levels of VEGF, ICAM-1, VCAM-1, 20 min from cigarette smoking, while a decrease occurred for IL-8 levels.

Conclusions: In conclusion, blueberry consumption reverses the peripheral arterial dysfunction following acute cigarette smoking. The selected biomarkers modulated after smoking suggest their direct or indirect involvement in the transient process of endothelial dysfunction. The feasibility of the repeated measurements of such markers in the short term and the role of blueberry in their modulation will be discussed.

APPENDIX 4. AWARDS

Riunione Nazionale

Comprendere e applicare i LARN
21-22 ottobre 2013
Palazzo degli Affari
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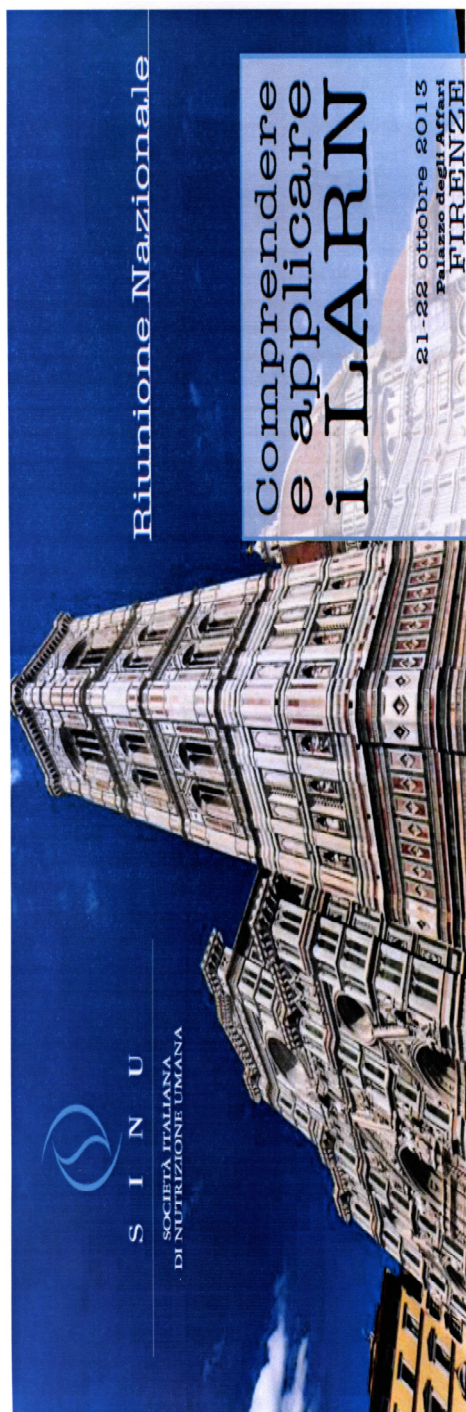
In occasione della Riunione Nazionale SINU "*Comprendere e applicare i LARN*" - Firenze, 21-22 Ottobre 2013

si ringrazia il Dott.

CRISTIAN DEL BO

vincitore del premio Giovani Ricercatori SINU per il contributo scientifico selezionato, dal titolo:

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