PLANT FOODS AND CARDIOVASCULAR HEALTH:
LOOKING FOR MECHANISMS OF ACTION

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SUMMARY

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1.1 Plant foods and health

Plant foods are at the basis of dietary patterns that promote health and decrease risk of chronic diseases, as reported by important epidemiological evidences.

Plant foods, such as fruits, vegetables, legumes and whole grain cereals, tend to be a rich source of dietary fibre, vitamins, minerals, phytochemicals, antioxidants and other micronutrients, all factors able to contribute to the cardiovascular protective effects, although the physiological mechanism involved are far from being elucidated.

1.1.1 Whole-grain cereals

There is growing evidence that whole-grain cereal products protect against the development of chronic diseases such as obesity (Koh-Banerjee and Rimm 2003, Van de Vijver et al. 2009), metabolic syndrome (Sahyoun et al. 2006, Esmaillzadeh et al. 2005), type 2 diabetes (de Munter et al. 2007, Murtaugh et al. 2003), cardiovascular diseases (CVD) (Mellen et al. 2008) and cancers (Chan et al. 2007, Chatenoud et al. 1998, Larsson et al. 2005, Schatzkin et al. 2008).

A meta-analysis of seven prospective cohort studies found a consistent inverse association between whole, but not refined, cereal grains and the incidence of cardiovascular disease, including heart disease, stroke, fatal CVD (Mellen et al. 2008). Further, the results from the Nurses’ Health Study have demonstrated that increased intake of whole grains may protect against CHD (Liu et al. 1999) while other prospective data support the reduction of ischemic stroke risk (Liu et al. 2000 b).

A meta-analysis of 12 studies, evaluating the association between dietary fibre and CHD, shows that a regular intake of whole grain foods reduces the CHD risk by about 26%. A risk ratio of 0.74 (95% CI, 0.67 to 0.80) was found in individuals with the highest intake compared to individuals with the lowest intake of whole grains. It was observed that vegetable or fruit intake had a modest protective effect and, concluding, the authors suggest that three servings of whole grains per day may confer an important cardioprotective effect (Anderson et al. 2000). Sahyoun et al. (2006) also encourage elder and young adults to increase their daily intake to ≥ 3 servings/d, as the conclusion of their study on whole-grain intake and the metabolic syndrome and mortality from cardiovascular disease.
In the Iowa Women’s Health Study, that tested the relationship of whole grain fibre consumption with risk for all-causes mortality, the participants that consumed whole grain reported lower cancer, and CHD mortality, with a 17% lower mortality rate (RR of 0.83, 95% CI 0.73-0.94) for women who consumed an amount (fibre/2000 kcal) of 1.9 g from refined grain and 4.7 g from whole grain versus women who consumed predominantly refined grain fibre (4.5 g) and only 1.3 g whole grain fibre/2000 kcal (Jacobs et al. 2000). Other reviews, based on case-control and prospective studies (Jacobs et al. 1995, Jacobs et al. 1998 a) and follow-up analyses (Jacobs et al. 1999, Jacobs et al. 1998 b), reported findings on reduced cancer risk associated with higher whole grain intake.

In the Framingham Offspring Study cohort, a cross-sectional study of 2941 subjects, McKeown et al. (2002) examined the whole-grain and refined grain intake and metabolic risk factors for cardiovascular disease or type 2 diabetes. After adjustment for potential confounders, whole-grain intake resulted inversely associated with body mass index, waist-to-hip ratio, total cholesterol, LDL cholesterol, and fasting insulin concentration (McKeown et al. 2002).

1.1.2 Fruits and vegetables


The Nurses' Health Study and the Health Professionals' Follow-Up Study, both prospective cohort studies, included a total of 126399 healthy volunteers at recruitment, for a total of 84 251 women and 42 148 men for 14 and 8 years of follow up. The results, after adjustment for common cardiovascular risk factors, demonstrated that the subjects in the highest quintile of fruit and vegetable intake had a relative risk (RR) for coronary heart disease of 0.80 (95% confidence interval CI, 0.69 to 0.93) compared with those in the lowest quintile of intake. A dose-response analysis indicates that each increase of 1-serving/d in intake of fruits or vegetables lowered the risk for coronary heart disease of 4%. The most important contributor to the protective effect of total fruit and vegetable intake were green leafy vegetables, with a RR 0.77 (CI, 0.64 to 0.93), and vitamin C–rich fruits and vegetables, with a RR 0.94 (CI, 0.88 to 0.99) for each additional serving/d. An important finding consisted in the protective effect
on coronary heart disease demonstrated by green leafy vegetables and vitamin C–rich fruits and vegetables (Joshipura et al. 2001). Similar results were obtained by Liu et al. (2001) and Liu et al. (2000 a) while examining the relationship between fruit and vegetable intake and reduction of CVD risk in a large prospective cohort of women (Women’s Health Study) and in male (Physicians’ Health Study).

In the Framingham Study, a cohort study involving middle-aged men with more than 20 years of follow-up, fruits and vegetables were found marginally protective against the development of stroke. An increase of 3 servings/d of fruit and vegetables was associated with a RR of 0.75 (95% CI: 0.55, 1.03) for ischemic stroke (Gillman et al. 1995).

In the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study, the increased consumption of fruit and vegetables was inversely associated with stroke incidence, stroke mortality, ischemic heart disease mortality, and CVD mortality. The reduction (27%) of the incidence of stroke was achieved in the case of 3 servings/d compared with less that 1 time/d (RR 0.73; 95% CI, 0.57 to 0.95) (Bazzano et al. 2002).

Even if many comparable results concerning the protective effects of fruits and vegetables for risk of stroke have been reported (Bazzano et al. 2002, Gillman et al. 1995, Zhao and Chen 2001), some researchers did not find a protective effect on CHD with increased intake of fruit and vegetable (probably because of difficulties in collecting the data) (Rosengren et al. 1999, Sasazuki 2001).

The effects of higher intake of fruits and vegetables were also widely examined with respect to the reduction in risk of different forms of cancers. In the Nurses’ Health Study and the Health Professionals’ Study, the association between lung cancer risk and fruit and vegetable consumption was examined in 77283 women and 47778 men. For women the risk was lower for total fruit and vegetable consumption, with a RR for the highest versus lowest quintile of intake of 0.79 (95% CI, 0.59 to 1.06). For men results were not the same. However, for both sexes, after taking into account smoking status, the total fruit and vegetable consumption showed an inverse association with the risk of lung cancer, though not statistically significant (RR 0.63; 95% CI, 0.35 to 1.12 in the highest tertile of intake) (Feskanich et al. 2000).

The correlation between fruit and vegetable consumption and the incidence of colon and rectal cancers was also prospectively investigated in the Nurses’ Health Study (88764 women) and the Health Professionals’ Follow-up Study (47325 men) but resulted in no association. The authors noted that a significant reduction in colorectal cancer risk is more often found in case–control studies than in the more epidemiologically powerful prospective
cohort studies; nevertheless, they advocate a diet rich in fruit and vegetable because of protection against other diseases (Michels et al. 2000).

The relationship between breast cancer and fruit and vegetable consumption was examined by Gandini and colleagues (2000) in a meta-analysis of 26 published studies. The relative risk, based upon a random effects model, for ‘high consumption’ compared with ‘low consumption’ was 0.75 (95% CI, 0.66 to 0.85) in 17 studies on vegetable and 0.94 (95% CI, 0.79 to 1.11) in 12 studies on fruit consumption (Gandini et al. 2000). While these authors confirm the association between intake of vegetables and, to a lesser extent, fruits with breast cancer, Smith-Warner et al. (2001) found a non significant association in another meta-analytical study with data pooled from eight prospective studies.

In a multiethnic case-control study of African-American, white, Japanese, and Chinese men, Kolonel et al. (2000) examined the relationship between vegetables, fruits, and legumes and prostate cancer. After adjustment for common risk factors, they obtained an inverse association, mainly for advanced cases, with the intake of yellow-orange and cruciferous vegetables. The OR of the highest quintile for yellow-orange vegetables was of 0.67 (P for trend 0.01) and that of the highest quintile for cruciferous vegetables was of 0.61 (P for trend 0.006). An inverse correlation was also found with legume intake (OR of 0.62; P for trend 0.0002 for highest relative to lowest quintile of total legumes) while fruits were not related to cancer risk (Kolonel et al. 2000).

1.1.3 Legumes

Considering the data in literature, it can be noted that the correlation between legumes and CHD has been seldom assessed, if compared to cereals; moreover, the few existing studies are focused mainly on specific nutritional components of legumes and not on total dietary intake. The First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study (NHEFS), that involved 9632 participants, indicates a significant inverse relationship between total legume intake and the risk of CHD and CVD, with a reduction of 22% (RR 0.78; 95% CI, 0.68-0.90) and 11% (RR, 0.89; 95% CI, 0.80-0.98) respectively (Bazzano et al. 2001). Flight and Clifton (2006), in reviewing the literature, reported that - along with cereal grains - legumes are preventive against coronary heart disease and stroke.

Venn and Mann (2004) have examined the evidence for the role of whole grain foods and legumes in the management of diabetes, suggesting a strong evidence for a beneficial effect.
1.2 Mechanisms of action

Based on the existent epidemiological studies, there is strong evidence that plant foods play a role in CVD prevention, since they provide dietary fibre that helps lower blood cholesterol, antioxidants that help reduce lipoprotein oxidation, and methyl donors that help control plasma homocysteine, all strong risk factors for the development of CVD (Getz and Reardon 2007, Fardet 2010). However, the mechanisms of action are possibly broader than currently thought and need further in-depth examination, also considering that foods are biochemically complex as well as their ample content of nutrients, which may interact with one another.

In cereals it is agreed that the biological effect is result of the synergistic action of a large number of compounds, mainly contained in the bran and germ fractions (Jensen et al. 2006, Liu 2007). In wheat, functional compounds include fibre, polyphenols (especially phenolic acids such as ferulic acid and smaller amounts of flavonoids and lignans), n-3 fatty acids, sulphur amino acids, oligosaccharides (stachyose, raffinose and fructans), lignin, minerals, trace elements, vitamins B and E, carotenoids, alkylresorcinols, phytic acid, betaine, total choline-containing compounds, inositol, phytosterols, policosanol, melatonin. Each one of these compounds has physiological functions and, possibly, health benefits, among which reduction of oxidative stress, inflammation, hyperglycaemia, all leading to prevention of CVD (Fardet et al. 2008, Fardet 2010, Seal 2006).

In a recent publication, Fardet (2010) discusses new hypotheses for the health-protective mechanisms of whole-grain cereals. The author claims that antioxidant protection should not be reduced to free radical scavenging and antioxidant enzyme activation, but also the involvement of polyphenols in cell signalling and gene regulation, and of sulphur compounds, lignin and phytic acid in other different aspects of cell metabolism must be taken into account. Furthermore, whole-wheat is a rich source of methyl donors, such as betaine and choline, which may be involved in cardiovascular and/or hepatic protection, lipid metabolism and DNA methylation. Betaine, which is present up to 1% (w/w) in the wheat bran fraction (Zeisel et al. 2003) is probably primarily involved in lowering homocysteine; this might of interest considering that high consumption of whole grains, bran and germ, was associated with a significant decrease in plasma homocysteine in a cross-sectional study on healthy men and women (Jensen et al. 2006).

The beneficial effect of fruit and vegetable could be ascribed to several individual nutrients such as fibre, potassium, and folate, but especially it has been suggested that the reduced
CVD risk result from the high polyphenol content of these foods (Bazzano et al. 2002, Tribble 1999).

The oxidant stress, generated by excessive production of reactive oxygen species, is involved in the pathogenesis of many cardiovascular conditions, including hypercholesterolemia, atherosclerosis, hypertension and heart failure (Cai and Harrison 2000), and antioxidants such as dietary polyphenols might play an important role in the prevention of CVD (Tribble 1999, Chong et al. 2010). Polyphenols present in fruits and vegetables, chocolate, red wine, green tea and other plant foods have important free radical-scavenging properties in vitro. Although the protective effect of polyphenol-rich plant foods was initially ascribed to their antioxidant properties, involved in protection against lipid peroxidation, in the last years the mechanism of action of plant compounds has been discovered to be more complex than originally expected (Manach et al. 2005, Williams et al. 2004). For example, considering that polyphenols may exert modulatory action in cell metabolism, a reduction of oxidative stress could be related to a decreasing in the production of superoxide anion through the regulation of NADPH oxidase, the key enzyme of oxidative stress (Williams et al. 2004).

The protective action of legumes could be correlated, beside the high content in folates important for the metabolism of homocysteine, with their high content in vegetable protein and also in soluble fiber, which reduce total and low-density lipoprotein cholesterol levels as well as insulin resistance (Bazzano et al. 2001). By replacing protein from vegetable sources (soybean) for protein from animal sources the concentration of serum cholesterol can be reduced, as demonstrated in randomized clinical trials (Anderson et al. 1995). The low content in sodium and high content in minerals such as potassium, calcium, and magnesium, associated with a reduced risk of cardiovascular disease in epidemiologic studies (He et al. 1999, Sasaki et al. 1995, Ascherio et al. 1998) could be another explanation for such a beneficial effect (Bazzano et al. 2001).
1.3 Cardiovascular diseases

Atherosclerosis is a chronic inflammatory disease, associated with risk factors such as hyperlipidemia, diabetes, and hypertension. Endothelial dysfunction has been proposed to be the first step in atherosclerosis, with other possible causes including elevated and modified Low-density lipoprotein (LDL), free radicals, genetic alterations, elevated plasma homocysteine concentrations, and infectious microorganisms (Ross 1999).

As result of injury, the normal homeostatic properties of the endothelium are altered and some of the following modifications can occur: increased adhesiveness of leukocytes or platelets to the endothelium, increased permeability, increased pro-coagulant properties and formation of vasoactive molecules, cytokines, and growth factors. Subsequently, if this state persists, the inflammatory response stimulates migration and proliferation of smooth-muscle cells into the intima media, with resulting intermediate lesion later followed by thickening of the artery wall. The increased numbers of macrophages and lymphocytes resulting from continued inflammation, and their activation, entails the release of hydrolytic enzymes, cytokines, chemokines, and growth factors, which can induce further damage and eventually lead to focal necrosis (Ross 1999).

1.3.1 Inflammation, oxidative stress and CVD

Different data provide strong evidence that inflammation and oxidative stress are implicated in the pathogenesis of atherosclerosis (Libby 2007, Stocker and Keaney 2005).

C-reactive protein (CRP) is a marker of subclinical inflammation and represents a strong independent risk factor for cardiovascular disease (Ridker 2003). It is synthesized primarily by hepatocytes in response to different cytokines, such as interleukin (IL)-6, IL-1, tumour necrosis factor (TNF), and other (Fredrikson et al. 2004).

CRP induces oxidative stress by increasing production of superoxide from NAD(P)H oxidase via p38 kinase activation, for example in the endothelium (Qamirani et al. 2005). CRP can also induce the formation of ROS in vascular smooth muscle cells by affecting their pro-inflammatory activities (Ryu et al. 2007).

Hattori et al. (2003) have shown that the responses to CRP in vascular smooth muscle cells were much more complex. CRP induces parallel activation of the redox-responsive transcription factors NF-kappa B (NF-kB) and AP-1 and increases the activity of the MAP kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase
(JNK) and p38MAPK. Moreover, CRP increases the expression of monocyte chemoattractant peptide (MCP-1) and interleukin-6 (IL-6).

CRP is an active participant in atherosclerosis; it is present in atherosclerotic plaques, binds to low-density lipoprotein (LDL) promoting its uptake by macrophages, and also contributes to release the matrix metalloproteinase (implicated in plaque rupture) (Torzewski et al. 2000, Zwaka et al. 2001, Singh et al. 2008).

CRP levels are associated with diet, lifestyle and metabolic cardiovascular risk factors and many studies have found correlations with age, body mass index, smoking, insulin sensitivity, plasma triglycerides, fasting glucose, and low HDL cholesterol (Ridker et al. 2003, Freeman et al. 2002, Yudkin et al. 1999, Festa et al. 2000, Pradhan et al. 2001).

1.3.2 Nutrition and CVD
Among noncommunicable diseases, the major contributor to the global burden of disease is represented by CVD leading to 30% of global deaths as estimated by the World Health Organization (WHO 2007). Reporting the recommendations in preventing chronic diseases, WHO summarizes the evidences that affect the risk factors for CVD (WHO 2003).

In a recent review, Mente et al. (2009) have investigated dietary exposures in relation to CHD. The results from prospective cohort studies or randomized trials have shown that protective factors such as vegetables, nuts, and “Mediterranean” and high-quality dietary patterns were well associated with CHD risk reduction, as supported by strong evidence; conversely, intake of trans–fatty acids and foods with a high glycemic load were identified as harmful factors. Additionally, strong evidences were reported for monounsaturated fatty acids and “prudent” and “western” dietary patterns.

Intake of folate, whole grains, fish, marine ω-3 fatty acids, dietary vitamins E and C, beta carotene, alcohol, fruit, and fibre have moderate evidence of protective associations while the intake of supplementary vitamin E and ascorbic acid (vitamin C), saturated and polyunsaturated fatty acids, total fat, α-linolenic acid, meat, eggs and milk present insufficient evidence (Mente et al. 2009).

1.3.3 Antioxidants and CVD
According to the oxidative hypothesis of atherosclerosis (Berliner and Heinecke 1996), the oxidized low-density lipoproteins (LDL) play a central role in atherogenesis (Young and
The LDL undergo oxidative modification in the subendothelial space mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Patel et al. 2000). These modifications implicate many cell types, such as monocytes, macrophages, neutrophils, endothelial cells, smooth muscle cells and fibroblasts, including NADPH oxidase as one of the potential oxidative mechanisms (Young and McEneny 2001). NADPH oxidase is a membrane-associated enzyme able to catalyse the one-electron reduction of oxygen (Babior 1999).

The degenerative process includes different steps. First, when oxidation is minimal, the LDL are minimally oxidised and are characterized by intact ApoB-100, loss of polyunsaturated fatty acids and antioxidants with respect to native lipoprotein. The oxidation of phospholipids occurs on the surface of LDL particles (Parthasarathy et al. 1999). The minimally modified oxidised low-density lipoprotein (MM-LDL) can induce the expression of monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) by endothelial cells, leading to monocyte recruitment from the arterial wall and differentiation of monocytes into macrophages. Such forms of LDL are recognized by LDL receptors but not by scavenger receptors of macrophages. The advanced oxidised form of LDL are characterised by modified apoB and can activate endothelial cells with expression of adhesion molecules that, together with MCP-1, promote adhesion and entry of monocytes into arterial wall. Once differentiated into macrophages, monocytes internalize the oxidised LDL particles through scavenger receptors because of higher affinity, accumulating an important amount of intracellular lipids in the arterial wall. The result is the release of proinflammatory cytokines by macrophages, which further promote recruitment of monocytes and accumulation of foam cells, thus inducing a vicious circle (Lapointe et al. 2006).

The LDL oxidation process could be influenced by consumption of foods and nutrients with antioxidant properties (Lapointe et al. 2006). It is interesting to note that, in animal models, fruit and vegetable antioxidant extract has been shown to prevent both NAD(P)H oxidase expression and O2⁻- overproduction. It was also shown a decrease of cholesterol concentration, significant increase of plasma antioxidant capacity, and prevention of fatty streak formation in aortic arch in the heart from hypercholesterolemic hamster (Sutra et al. 2007). The oxidative stress and p22phox NADPH oxidase activation can be reduced by a moderate consumption of red wine, which also affect the progression of aortic lesions in an experimental model of diet-induced atherosclerosis (Qian et al. 2009).
Generally, the combination of antioxidant compounds that characterise the healthy food pattern of the Mediterranean diet appears effective to decrease the oxidation of LDL particle leading to cardiovascular protection (Lapointe et al.2006).

1.3.4 Betaine, choline and CVD

The relationship between betaine and choline and CVD could be explained through their effect on homocysteine, which can itself be an independent risk factor for cardiovascular disease (Craig 2004, Zeisel and Blusztajn 1994).

Betaine, a vital methylating agent (Craig 2004) and its precursor choline are important in maintaining the normal one-carbon metabolism and homocysteine homeostasis (James et al. 2002).

In humans with cardiovascular disease, there is a significant inverse relationship between plasma betaine and homocysteine concentrations in fasting (Schwahn et al. 2003 a) and postmethionine states (Holm et al. 2004). For example, the supplementation in healthy volunteers with 1.5–6 g/day betaine or 2.6 g/day choline (as phosphatidylcholine) can significantly lower fasting homocysteine (Olthof et al. 2003, Schwab et al. 2002, Steenge et al. 2003).

Chiuve et al. (2007) found an inverse association between total choline and betaine intake and total homocysteine in women, concluding that remethylation of total homocysteine may be more dependent on the betaine pathway when methyl sources are low as a result of either inadequate folate intake or heavier alcohol consumption.

In the ATTICA study, Detopoulou et al. (2008) found that, in free-eating and healthy adults, a major dietary intake of choline and betaine was independently associated with a reduction in inflammation indexes (C-reactive protein, interleukin-6, tumor necrosis factor-α, homocysteine) important players in cardiovascular disease progression.

Data suggest that non-alcoholic fatty liver disease (NAFLD), a common condition in people with type 2 diabetes, is associated with a high prevalence of cardiovascular disease risk (Targher et al. 2007, Targher et al. 2005). It has been shown that molecules that reduce the accumulation of fat in the liver, such as betaine and choline, are able to reduce the incidence of NAFLD (Patrick 2002, Angulo 2003). In an animal model betaine improved nonalcoholic fatty liver by reversing hepatic insulin resistance (Kathirvel et al. 2010).
1.4 Homocysteine

Homocysteine is an amino acid derived from methionine, an essential amino acid, that contain an readily oxidable thiol group (Jacobsen 2000). The concentrations in blood increase with age and sex and are affected by renal function (Gori et al. 2005, Powers et al. 2002, Selhub 1999, Mayer et al. 1996). In people with hyperhomocysteinemia, very high blood levels of homocysteine are independently associated with higher risk for atherosclerosis (Clarke et al. 1991).

At physiological pH in the presence of O2, the reactive sulfhydryl group (-SH) of homocysteine can undergo oxidation to different molecular disulfides (RSSR) (Jacobsen 2000). When methionine is in excess, the homocysteine can be irreversibly degraded to cysteine, via vitamin B6-dependent reactions (transulfuration pathway). This process generates cystathionine as intermediate product, catalyzed by cystathionine-βsynthase (CBS). Homocysteine is involved in two pathways to conserve methionine when the balance is negative (Figure 1). The remethylation into methionine requires vitamin B12 and, in most tissues, this process is catalyzed by the ubiquitous methionine synthase (MS) that uses methyltetrahydrofolate as the substrate. Methyltetrahydrofolate formation, in turn, is catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). In the liver betaine is a methyl donor for the enzyme betaine-homocysteine methyltransferase (BHMT) (Refsum and Ueland 1998).
1.4.1 Homocysteinemia

Elevated serum homocysteine concentration (hyperhomocyst(e)inemia or homocyst(e)inemia), as reported by epidemiologic studies, is associated with an increased risk of cardiovascular disease, stroke, Alzheimer disease, dementia, neural tube defects, and other metabolic disorders (Selhub 1999, Seshadri et al. 2002, Kittner et al. 1999, McCully 1969, Wilcken and Wilcken 1976).

Supplementation with folic acid lowers plasma homocysteine (van Oort et al. 2003) and betaine can improve the elevated fasting serum homocysteine and an elevated response of homocysteine after methionine loading (postmethionine), considered as two independent risk factors for cardiovascular disease (Steenge et al. 2003). However it must be noted that a recent meta-analysis of 8 intervention trials with homocysteine-lovering agents involving a total of 37485 individuals, found no effect on CVD events or cancer over a mean period of 5 years of follow-up (Clarke et al. 2010).

The normal mean homocysteine levels are 5 to 15 μM; when the homocysteine levels are higher they indicate mild, moderate, and severe hyperhomocysteinemia (16 to 30, 31 to 100, and higher than 100 μM) (Welch and Loscalzo 1998).
Homocysteinemia is the result of an imbalance in the methionine cycle because of genetic or nongenetic (nutritional) factors, for example deficiency of 5,10-methylenetetrahydrofolate reductase MTHFR or deficiency in B Vitamins or folates (Selhub 1999). Severe homocysteinemia (homocystinuria) can be caused by a deficiency of CBS (cystathionine β- synthase), MTHFR (methylenetetrahydrofolate reductase), or methylcobalamin synthesis (Craig 2004).

Chronic elevation of homocysteine results in parallel increases in intracellular SAH, and the ratio SAM(S-adenosylmethionine):SAH(S-adenosylhomocysteine) can influence the cellular methylation status important for pathogenesis of diseases related to homocysteinemia (James et al. 2002).

1.4.2 Hyperhomocysteinemia and oxidative stress

Homocysteine, as well as other thiols, has pro-oxidant activity and its oxidation to homocystine can generate a variety of reactive oxygen species, including superoxide anion radical and hydrogen peroxide. Such reaction is catalyzed by transition metals (Jacobsen 2000).

\[
\text{HOMOCYSTEINE (RSH)} + \text{O}_2 \rightarrow \text{HOMOCYSTINE (RSSR)} + [\text{O}_2^\cdot] \rightarrow \text{H}_2\text{O}_2
\]

There is also evidence that homocysteine affect nitric oxide (NO) production, which is lower when the exposure of endothelial cells to homocysteine increases (Stamler et al. 1993). In normal conditions, nitric oxide serves as an endothelium-dependent vasodilator and an antithrombotic agent in the vasculature (Upchurch et al. 1996). Homocysteine causes dysfunction of the vascular endothelium (Jacobsen 1998), reduces NO generation, impairs endothelium-dependent vasodilation (Tawakol et al. 1997) and also causes oxidation of low-density lipoprotein (LDL) (Heinecke et al. 1987). Also, the superoxide anion generated by oxidation of homocysteine is involved in the oxidative modifications of lipids (Olszewski and McCully 1993).

Further, homocysteine is able to attenuate GSHPx (glutathione peroxidase) (Lubos et al. 2007) and activate NADPH oxidase in human neutrophils and monocytes by phosphorylation and subsequently membrane translocation of p47phox and p67phox subunits, leading to

1.4.3 Hyperhomocysteinemia and CVD

Different studies had shown the link between hyperhomocysteinemia and vascular disease. In 1969, McCully made the first clinical observation about the implication of hyperhomocysteinemia in the pathogenesis of atherosclerosis (McCully 1969).

Mild homocysteinemia, a risk factor for premature arteriosclerotic disease, is characterized by mildly elevated fasting or postmethionine homocysteine concentrations, and occurs in 9–42% of subjects under 50 y of age who have peripheral or cerebral occlusive arterial disease, myocardial infarction, or thromboembolism (Franken et al. 1994). Mild homocysteinemia after a standard methionine load is present in young patients with coronary artery disease (21%), patients with cerebrovascular disease (24%), and patients with peripheral vascular disease (32%) (van den Berg and Boers 1996).

Different mechanisms for atherosclerosis induced by homocysteine have been suggested: endothelial dysfunction, activation of monocytes (resulting in secretion of cytokines), increased proliferation of smooth muscle cells, promotion of lipoprotein oxidation and platelet activation, and enhanced thrombus formation (Woo et al. 1997, Su et al. 2005, Mayer et al. 1996, Clarke et al. 1991).

1.5 Oxidative stress

The oxidative stress concerns the imbalance between production and elimination of reactive oxygen species (ROS) and represents an important factor for a wide variety of cardiovascular diseases, such as atherosclerosis and endothelial dysfunction (Cai and Harrison 2000).

Reactive oxygen species (ROS) are a large group of molecules, which include the molecular oxygen and its derivatives generated in all aerobic cells, and many of them are free radicals because of unpaired electrons (Cai and Harrison 2000). ROS produced naturally as consequence of aerobic metabolism are important for maintaining tissue oxygen homeostasis (Castro and Freeman 2001).

These molecules play a double role in disease promotion and prevention. At high concentrations ROS are detrimental to health because are implicated in the oxidation of
biological macromolecules, such as lipids, DNA, protein, carbohydrates. On the other end, at moderate concentrations, these signalling molecules have crucial roles in normal physiological processes such as in the response to growth factors, apoptotic elimination of damaged cells and also in the prevention of disease by helping the immune system (Castro and Freeman 2001, Seifried et al. 2007).

The regulation of ROS activity has been identified as an important factor for the development of some diseases, including cancer and cardiovascular disease (CVD) (Kunsch and Medford 1999, Seifried et al. 2007, Lum and Roebuck 2001). One of the mechanisms of defence is the enzymatic antioxidant system that includes catalase, glutathione peroxidase and superoxide dismutase. Beside these enzymes, the nonenzymatic activity of exogenous and endogenous antioxidants, including polyphenols, glutathione, thiols, some vitamins and metals, may represent an alternative for cell protection (Seifried et al. 2007).

![Cellular oxidative interactions](image)

**Figure 2.** Cellular oxidative interactions

1.5.1 ROS and signal transduction

The ubiquitous ROS are regulators of signalling cascades at different stages. Downstream cellular activities can be modulated by ROS directly or through activation of phospholipases
able to generate cofactors and cellular messengers that affect the activity of enzymes such as protein kinases and phosphatases (Lum and Roebuck 2001). They are capable of tyrosine and serine/threonine protein phosphatases inactivation, with a subsequent major activation of protein kinases (MAPK, PKC) (Whisler et al. 1995).

Being essential for the activity of signal transduction pathways, the controlled production of ROS can influence the function of the mitogen-activated protein kinases (MAPKs). The MAP kinase family, which plays a role in transferring signals from extracellular stimuli to the cell nucleus, consists of the extracellular signal–regulated kinases (ERK) subgroup that regulate cell proliferation, the c-Jun amino-terminal kinase (JNK), and the p38 MAP kinase, linked to stress responses (Seifried et al. 2007, Holbrook and Ikeyama 2002). JNK and p38MAPK, activated by environmental stresses and pro-inflammatory cytokines (tumour necrosis factor (TNF), interleukin-1 (IL-1), IL-2 and IL-17), can lead to apoptosis and promotion of inflammation (Chen et al. 2001, Chang and Karin 2001, Thomas and Huganir 2004).

As final targets of signal transduction pathways, PKC and MAP kinase pathways can activate two major redox-sensitive transcription factors AP-1, with important roles in normal development, and NF-kB, involved in the regulation of different physiological functions (Lum and Roebuck 2001).

1.5.2 NADPH oxidase

The superoxide anion (O2−), generated by neutrophils, eosinophils, and mononuclear phagocytes and B lymphocytes, is formed by the one-electron reduction of oxygen and implies the activity of NADPH as the electron donor.

$$2 \text{O}_2 + \text{NADPH} \rightarrow 2 \text{O}_2^- + \text{NADP}^+ + \text{H}^+$$

Subsequently, this anion triggers the generation of other different reactive oxidants, including oxidized halogens, free radicals, and singlet oxygen which have beneficial bactericidal implication but which need regulation to avoid the toxic effect (Chanock et al. 1994).

NADPH oxidases are situated in different cells of mesodermal origin. The most studied leukocyte NADPH oxidase represents a group of plasma membrane–associated enzymes which include: p40PHOX (PHOX for Phagocyte OXidase), p47PHOX, p67PHOX, cytosolic complex; p22PHOX, gp91PHOX and membrane bound components (which comprise cytochrome b558, a flavohemoprotein; Rap1A and Rac 2 low-molecular-weight guanine nucleotide-binding proteins that participate also in other processes).
In the resting cell, the NADPH oxidase is inactive and the subunits are distributed between the cytosol and the membrane. Once activated by appropriate stimuli, it occurs the phosphorylation of the subunit chiefly responsible for transporting $p47^{PHOX}$, and the entire cytosolic complex migrates to the membrane where it associates with cytochrome b558. This assemblage allows the transfer of electrons from the NADPH to FAD and then to oxygen, probably by involving the heme of cytochrome b558 as a second intermediate electron carrier. Activation requires also the involvement of two low-molecular-weight guanine nucleotide-binding proteins: Rac2 - located in the resting cell in the cytoplasm in a dimeric complex with Rho-GDI (Guanine nucleotide Dissociation Inhibitor) - and Rap1A - located in membranes. During oxidase activation, Rac2 binds guanosine triphosphate (GTP) and migrates to the membrane with the other cytosolic factors of the NADPH oxidase (Babior 1999).

**Figure 3. Activation of the leukocyte NADPH oxidase**

The phosphorylation, an essential step in the activation of the NADPH oxidase, first occurs in the cytosol, where the most of the phosphates are present, followed soon by the transfer of $p47^{PHOX}$ to the plasma membrane. Besides the phosphorylation of $p47^{PHOX}$ oxidase during activation, it has been shown that a phosphorylation of other two subunits $p67^{PHOX}$ (Heyworth et al. 1991) and $p40^{PHOX}$ (Chanok et al. 1994) also occurs.

In the phosphorylated subunit $p47^{PHOX}$, the phosphate groups were found linked to the 11 serines (El Benna et al. 1994); this induces a protein conformational change that renders the
SH3 domains accessible to the target $p_{22}^{PHOX}$ (Ago et al. 1999). The SH3-mediated interaction between $p_{47}^{PHOX}$ and $p_{22}^{PHOX}$ is strictly regulated and plays a key role in phagocyte oxidase activation.

The phosphorylation of $p_{47}^{PHOX}$ can be regulated by protein kinase. It was shown that PKC isoforms expressed in human neutrophils (Fontayne et al. 2002), as well as P21-activated kinase (Pak), Akt kinase, and mitogen-activated protein (MAP) kinases, are all implicated in the phosphorylation of $p_{47}^{PHOX}$ (Martyn et al. 2005).

1.5.2.1 The NADPH oxidase modulation

The production of superoxide anion ($O_2^-$) by NADPH oxidase can be modulated through transcriptional and post-transductional mechanisms.

The first regulation is either negative or positive when the expression of the core components of NADPH oxidase can be inhibited or not. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) can suppress the expression of NADPH oxidase subunits (Wassmann et al. 2002). Antioxidants supplied by fruits and vegetables can reduce the cardiac production of superoxide anion and also $p_{22}^{PHOX}$ subunit of NAD(P)H oxidase expression in an animal model (Sutra et al. 2007). Homocysteine are able to modulate positively NADPH (Ungvari et al. 2003), and also LPS (DeLeo et al. 1998), and CRP (Kobayashi et al. 2006).

The post-transductional regulation concerns the phosphorylation of the subunits of NADPH and the translocation of the cytosolic oxidase components. The phagocytic elements can modulate positively the NADPH oxidase activation (De Leo et al. 1998) with superoxide anion production.

For a negative modulation of oxidase, vitamin E was shown to be able to inhibit the phosphorylation of the subunit $p_{47}^{PHOX}$ in monocytes (Cachia et al. 1998) and subsequently decrease the superoxide anion production. Pignatelli et al. (2006) have demonstrated that the synergistic effect of quercetin and catechin can also lead to inhibition of PKC-dependent phosphorylation of the cytosolic sub–unit $p_{47}$ phox of NADPH oxidase.
1.6 Betaine
Betaine, also known as trimethylglycine, glycine betaine, lycine, and oxyneurine, is a zwitterionic quaternary ammonium compound (Figure 4). It is a methyl derivative of the amino acid glycine with a molecular weight of 117.2 and 3 chemically reactive methyl groups (Craig 2004).

![Figure 4. Structural formula of betaine](image)

First discovered in the juice of sugar beets (*Beta vulgaris*) in the 19th century, betaine can be found in microorganisms, plants, and animal tissues. Rich food sources are wheat, shellfish, spinach, and sugar beets (Koc et al. 2002, Zeisel et al. 2003).

Betaine plays important physiological roles; being an organic osmolyte it protects cells under stress and it acts, via transmethylation, as a source of methyl groups for use in many biochemical pathways (Figure 5) (Betaine Monograph).

Betaine is ingested in unknown amounts through intake of foods which contain the preformed molecule or its precursor choline (Zeisel et al. 2003). The choline introduced with the diet can have different fates: in part it can be irreversibly oxidized to betaine, whereas the remainder is transformed in acetylcholine and phospholipids (phosphatidylcholine) (Zeisel et al. 1991).
1.6.1 Betaine metabolism

Being an N-methylated amino acid, the cellular uptake and intracellular accumulation of betaine can be mediated by amino acid transport systems, in particular by betaine-gamma aminobutyric acid transporter and amino acid transport system A (Peters-Regehr et al. 1999, Wettstein et al. 1998, Weik et al. 1998). As shown in animal studies, dietary betaine has an increased absorption in the duodenum (Kettunen et al. 2001).

In human studies the absorption and distribution of betaine after intake is also rapid. Some data reveal a peak increase in serum betaine at 1–2 h (Schwahn et al. 2003b).
In normal human plasma concentrations of glycine betaine are usually between 20 and 70 µmol/l (higher in adult males than in females), and in renal disease and diabetes the concentrations are lowered and normal, respectively (Schwahn et al. 2003 b, Lever et al. 1994).

Betaine is mainly eliminated by metabolism and not excreted (Schwahn et al. 2003 b), except in subjects with renal disease or diabetes that present a high urinary excretion (Lever et al. 1994, Dellow et al. 2001, Dellow et al. 1999).

Regarding the sub-acute and sub-chronic effect of large doses of betaine in animals, the data show that trimethyl glycine is nontoxic at all doses studied and betaine is safe up to a daily intake of 9–15 g (average of 12 g)(Hayes et al. 2003).

Different enzyme reactions are involved in betaine catabolism, which occurs mainly in the mitochondria of liver and kidney cells. The majority of these reactions involve the methionine cycle (Figure 5) and the transfer of methyl groups. The transmethylation reactions, vital to biological processes, aim to conserve methionine, detoxify homocysteine, and produce S-adenosylmethionine (SAM), which plays an important role in maintaining the integrity of the liver (Barak et al. 1996). Generally, elevated total homocysteine and low SAM concentrations have been associated with chronic disease (Craig 2004). SAM is the methyl donor for 100 different transmethylation reactions; it is important also for DNA methylation and protection from CVD, considering that aberrant DNA methylation is associated with atherosclerosis (Dong et al. 2003).

Two pathways exist for the formation of methionine: via betaine or via 5-methyltetrahydrofolate (CH3-THF).

The first involves in liver and kidney the cytosolic enzyme betaine homocysteine methyltransferase (BHMT) that catalyses the methyl transfer yielding methionine and dimethylglycine (DMG), further metabolized to sarcosine and then to glycine.

The second involves the enzyme methionine synthetase (MS) that transfers a methyl group to the cofactor cobalamin (vitamin B-12) forming methylcobalamin, which in turn transfers, the methyl group to homocysteine to produce methionine. The enzyme methylenetetrahydrofolate reductase (MTHFR) regenerates the CH3-THF from methylene-THF (Barak et al. 1996).

BHMT, expressed in the kidney cortex and in hepatocytes in humans, pigs, and rats, is a zinc metalloenzyme with a distorted barrel shape and an optimum pH of 7.8 (Craig 2004), whose concentrations increases when diets are supplemented with betaine or choline, as shown in animal studies (Finkelstein et al. 1983).
Betaine is an important methylating agent in the liver. Even though another molecule - choline - is involved in the transmethylation process, its methyl groups cannot be used in transmethylation until they become "labile" through the oxidative conversion to betaine (Barak et al. 1996).

In healthy adults, betaine increases serum methionine, transmethylation rate, homocysteine remethylation, and methionine oxidation (Storch et al. 1991), and in animals it was demonstrated an enhanced production of SAM in red blood cell, a direct methyl donor in many important biological pathways such as synthesis of proteins, creatine, phospholipids, hormones, polyamines, carnitine, adrenaline, and DNA methylation (Craig 2004).

1.6.2 Physiological role of betaine

Betaine is involved in the protection of internal organs, and it has been established that the molecule improves vascular risk factors and enhance performance. As osmolyte, betaine protects cells, proteins, and enzymes from environmental stress; it is an organic osmolyte compatible with enzyme function. Different conditions of stress, such as high salinity or temperature and drought, induce betaine synthesis in mitochondria leading to its accumulation in the cells (Craig 2004).

Studying the effects of betaine on the structural dynamics of *Thermomyces (Humicola) lanuginosa* lipase, it was found that there is little or no binding of betaine to protein surfaces, allowing cells to control the surface tension of water without affecting the ionic strength of the environment (Soderlund et al. 2002).

It has been suggested an important role of osmolytes in modulating immune function in osmotically stressed liver macrophages (Kupffer cells); betaine has a regulatory role for TNF-α production by liver macrophages (Zhang et al. 1996 a), a modulation role of phagocytosis (Warskulat et al. 1996), and can reduces the induction of cyclooxygenase 2 and the increase of prostaglandin E2 formation (Zhang et al. 1996 b).

Betaine protects red blood cell (RBC) against hypoosmotic stress by regulating RBC membrane ATPases via conformational changes (Moeckel et al.2002). Functioning as an organic osmolyte, betaine protects early preimplantation mouse embryos against increased osmolarity in vitro (Hammer and Baltz 2002), modulates the response of porcine endothelial cells to hypertonicity and protects them from apoptosis (Alfieri et al. 2002).
In vitro, it was demonstrated that betaine affects the movement of water across the small intestinal epithelium; it also aids the osmoregulation of duodenal epithelium of broiler chicks (Kettunen et al. 2001).

As methyl donor, betaine is implicated in the methionine cycle (Figure 5) and it is the only molecule - besides methylfolate - that provides one-carbon units for homocysteine remethylation.

This second physiologic role is important to avoid the hypomethylation that can lead to disturbed hepatic protein (methionine) metabolism (in the case of elevated plasma homocysteine and decreased S-adenosylmethionine concentrations), and inadequate hepatic fat metabolism, causing plasma dyslipidemia (Craig 2004).

1.6.3 Betaine and health effects

1.6.3.1 Liver

Many diseases, including coronary, cerebral, hepatic, and vascular diseases, can be the result of an impaired hepatic transmethylation process (Craig 2004).

Nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver damages, ranging from simple steatosis (fat accumulation) to steatohepatitis (fatty inflammation), fibrosis (excessive fibrous tissue), and cirrhosis (serious liver damage). NAFLD has a high prevalence in the general population (10-39%), and can progress to cirrhosis and liver failure (Angulo 2002). NAFLD affects diabetics (50 percent), obese (57-74 percent), and morbidly obese individuals (90 percent).

Nonalcoholic steatohepatitis (NASH) is strongly associated with the major features of metabolic syndrome such as obesity, central fat accumulation, diabetes, dyslipidemia (depressed HDL levels, elevated triglycerides), hypertension, and cardiovascular disease (Patrick 2002).

It has been shown that betaine is able to mobilize hepatic cholesterol and phospholipids in rats, to treat hyperlipidemia, to synthesize carnitine, and to enhance the secretion of VLDL by methylation of phosphatidylethanolamine and generation of phosphatidylcholine (Craig 2004).

Abdelmalek et al. (2001) have shown that betaine is a safe and well tolerated molecule that leads to a significant biochemical and histological improvement (liver enzymes, steatosis,
necroinflammatory grade, and stage of fibrosis) in patients with NASH. Other researchers have concluded that betaine can be successfully employed to treat NAFLD and nonalcoholic steatohepatitis (NASH) (Patrick 2002, Neuschwander-Tetri 2001, Kathirvel et al. 2010, Angulo 2003).

1.6.3.2 Heart
Betaine is important for maintaining the heart health. Beside the improvement of non clinical and rather subjective parameters, such as improved sense of well-being, less fatigue, greater strength and endurance, and increased performance of physical and mental work, it has been shown improved cardiac function in subjects with cardiac deficit due to arteriosclerosis or rheumatic disease and congestive heart failure (Craig 2004).

Many years ago it was proposed to treat atherosclerosis with betaine to improve the ratio of serum phospholipids to serum cholesterol, the sense of well-being, exercise and activity, appetite, angina pain, dyspnoea (shortness of breath), and libido (Craig 2004). Furthermore, in human studies, a low-fat, low-cholesterol diet integrated with betaine, choline, liver extract, and vitamin B-12 resulted in a decrease in mortality of subjects with atherosclerosis (Craig 2004).

In animal studies, treatment of atherosclerosis with betaine showed a reduction in elevated total and ester-bound cholesterol, β-lipoproteins, total lipids in serum, and total cholesterol and triacylglycerols in the liver. Betaine was also shown to be implicated in the elimination of cholesterol through its transformation to biliary acids and intestinal excretion via the bile, and also to prevent the decrease of nicotinamide coenzymes and adenine nucleotides in the liver and myocardium (Craig 2004).

1.6.3.3 Homocysteinemia
As important methylating agent, trimethylglycine has been implicated in the treatment of, severe homocysteinemia and mild homocysteinemia.

In the case of severe homocysteinemia, when remethylation defects occurs, oral betaine supplementation (2–9 g per day) lowers homocysteine concentrations and improves some clinical conditions (Ogier de Baulny et al. 1998).

In the case of MTHFR deficiency (Holme et al. 1989, Al-Essa et al. 1999, Bonig et al. 2003), betaine has been shown to ameliorate homocysteine remethylation, and to normalize very low plasma methionine and elevate SAM.
Betaine, at a dose of 1.5–6 g/d (Schwab et al. 2002, Steenge et al. 2003, Olthof et al. 2003), can decrease mild homocysteinemia with a long term effect. The same effect was demonstrated for a mixture of vitamin B-6, folic acid, and betaine tested in patients with premature arteriosclerotic disease (Franken et al. 1994). Importantly, betaine appears to be highly effective in preventing the rise in plasma homocysteine concentration after methionine intake when compared to folic acid (Steenge et al. 2003, McGregor et al. 2002, Olthof et al. 2003).

In patients with renal disease, betaine can reduce postmethionine homocysteine concentrations but not fasting homocysteine (McGregor et al. 2002, van Guldener et al. 1999).

1.7 Choline

Choline is a quaternary amine (Figure 6) freely soluble in water and ethanol, insoluble in organic solvents. In alkaline solution it releases trimethylamine.

Choline is essential for normal cell function and it plays a critical role during fetal development. Choline and its metabolites are important dietary sources of methyl groups. Taking part in the process of homocysteine methylation, choline assures the structural integrity and lipid transport from liver as well as signalling functions of cell membranes, and cholinergic neurotransmission (Zeisel and Blusztajn 1994).
1.7.1 Choline metabolism

1.7.1.1 Absorption and transport

Choline is present in the cell membrane of all types of tissues, being an essential component of structural phospholipids. In the gut, part of the ingested free choline undergoes degradation mediated by gut bacteria, leading to formation of betaine and methylamines. The remaining portion is absorbed in the small intestine by a carrier-mediated process, which involves a saturable carrier in the brush border efficient at low luminal concentrations (Shils et al. 1999). When ingested as phosphatidylcholine, the choline is released by hydrolysis in the intestinal lumen through the action of phospholipases: phospholipases A2 (produced by pancreas) and phospholipases A1 and B (intestinal mucosa) and is then absorbed as lyssolecithin, which is reacylated in the enterocyte to yield phosphatidylcholine (Combs 2007).

Choline is transported to the tissues mainly as phospholipids associated with plasma lipoproteins (phosphatidylcholine is bound to chylomicra) and the uptake into the cells is realized through transport systems such as Na$^+$-dependent and Na$^+$-independent transporters, or by passive diffusion. The first system is characterised by a high-affinity while the second is characterised by a low-affinity.

Choline is stored as phosphatidylcholine and sphingomyelins in essential organs such as brain, liver, and kidney. In placenta the concentration of acetylcholine is very high, probably to meet the requirement of the foetus (Combs 2007).

1.7.1.2 Metabolism

In tissues, phospholipase C can cleave the phosphatidylcholine and generate diglyceride and phosphorilcholine, which is subsequently converted to free choline by alkaline phosphatase. In peripheral tissues a second enzyme implicated in phosphatidylcholine metabolism is phospholipase B, which generates glycerclyphosphorylcholine that is further cleaved by glycerclyphosphorylcholine diesterase yielding free choline. In the brain it is active phospholipase D that release the free choline from phosphatidylcholine (Combs 2007).

Choline is irreversibly oxidized to betaine in the liver and kidney (Park and Garrow 1999). The enzyme choline dehydrogenase, found in the mitochondria, is involved in oxidation of choline to betaine aldehyde. This enzyme can also convert betaine aldehyde to betaine, though this reaction is mainly sustained by betaine aldehyde dehydrogenase, found in mitochondria
and in the cytosol. This enzyme system, namely choline oxidase, is present in some tissues, for example in liver and kidney, but absent from brain, muscle and blood (Combs 2007).

1.7.2 Synthesis of phosphatidylcholine
Choline is also utilized for the synthesis of phosphatidylcholine throug two different pathways. First, the cytosolic enzyme choline phosphotransferase (choline kinase) phosphorylates choline. In this reaction ATP serves as phosphate donor, and the resulting molecule, phosphorylcholine, can generate cytidine diphosphorylcholine (CDP-choline). Finally, after the combination of CDP-choline with diacylglycerol (mediated by phosphatidylcholine glyceride transferase), phosphatidylcholine is produced.
The second alternative pathway most active in liver and also in other tissues as brain and mammary gland involves the sequential methilation of phosphatidylethanolamine and represent the main way for de novo synthesis of choline phospholipids in adult mammals (Zeisel and Blusztajn 1994). This reaction is mediated by phosphatidylethanolamine N-methyltransferase and uses SAM as the methyl donor. The enzyme is membrane-bound and has at least two isoforms (Zeisel and Blusztajn 1994). The cell inner membrane enzyme is responsible for the first methyl group transfer, and the cell outer membrane enzyme is responsible for the second and third methyl group transfers. In the case of deficiencies of this methylating enzyme, the requirement for choline is higher (Combs 2007).
Choline, in small amount, can also be transformed in the neurotransmitter acetylcholine. The reaction is catalized by the enzyme choline acetyltransferase, found in nervous tissues and in placenta, and needs the presence of acetyl – CoA and choline (Combs 2007).

1.7.3 Choline, homocysteine, and folate
The metabolism of choline, methionine, and methyl-folate closely interact in methionine formation from homocysteine.
In one-carbon metabolism, choline can make available its methyl groups after oxidation to betaine. The methylation of homocysteine requires the enzyme betaine:homocysteine methyltransferase and betaine as methyl donor, while the alternative pathway requires 5-methylenetetrahydrofolate:homocysteine methyltransferase and uses a methyl group derived de novo from the 1-carbon pool (Finkelstein et al. 1983, Zeisel and Blusztajn 1994). The
resulting methionine is converted to S-adenosylmethionine (the active methylating agent) by methionine adenosyltransferase.

When metabolism of one of the methyl donor is perturbed, compensatory changes occurs in the other metabolic pattern. In animal studies, it was shown that a diet deficient in choline can lead to less SAM and total folate (Zeisel et al. 1989, Shils et al. 1999). In human studies, a low folate status can lead to neural tube defects in foetus (Rush 1994). It is also important the interrelationship between choline and homocysteine because plasma homocysteine concentration represents an independent risk factor for cardiovascular disease (Clarke et al. 1991).

1.7.4 Functions
Choline has an important role in the central nervous system, in the synthesis of membrane phosphatidylcholine and acetylcholine. Phosphatidylcholine is a structural compound of biological membranes, a precursor to ceramide and sphingolipids, important for transmembrane signalling, and a promoter of lipid transport playing and important role in lipid-cholesterol metabolism.

Moreover, acetylcholine is the main neurotransmitter in the parasympathetic nervous system (Combs 2007).

The platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is a choline-containing phospholipid with hormonal functions. The effect of PAF include activation of phosphoinositide–specific phospholipase C and release of fatty acids from phospholipids. This leads to subsequent generation of active metabolites, diacylglycerol and inositol-1,4,5-triphosphate, resulting in activation of PKC and arachidonic acid, involved in the formation of eicosanoids. As component of PAF, choline takes part in different biological functions such as activation of platelet aggregation, control of blood pressure, increase in vascular permeability; activation of monocytes, neutrophils and macrophages; stimulation of hepatic glycogenolysis. Moreover, it is also important in inflammation and allergy state and in childbirth induction (Zeisel and Blusztajn 1994, Combs 2007).

Finally, as choline plasmalogens, choline molecule is important in myocardial function.
1.7.5 Choline deficiencies

In humans and animal studies, a choline deficiency leads to liver dysfunction, with accumulation of triacylglycerol in hepatocytes (Zeisel et al. 1991, Bruni and Hegsted 1970, Blusztajn and Zeisel 1989, Yao and Vance 1988). Phosphatidylcholine is a component of very low-density lipoprotein (VLDL) (Yao and Vance 1988) and serve to deliver the triacylglycerols produced by the liver to tissues. When choline is insufficient, the synthesis of phosphatidylcholine is impaired and triglycerides accumulate. Methionine status can also influence the effect of choline deficiency (Combs 2007).

In animals, besides inducing liver dysfunction and fatty liver, choline deprivation impairs renal function by affecting free water reabsorption, sodium excretion, glomerular filtration rate, renal plasma flow, and increasing the risk of renal hemorrhage, but also infertility, depressed growth, hypertension, and decreased hematopoiesis (Zeisel and Blusztajn 1994).

It has been found that choline dietary deficiency causes the development of spontaneous hepatocarcinomas as well as carcinogen-induced hepatocarcinomas (Zeisel and Blusztajn 1994).

In healthy humans a 3-week choline-deficient diet resulted in decreased plasma choline and phosphatidylcholine concentrations, and also in decreased plasma and erythrocyte phosphatidylcholine. Choline stores in tissues were depleted with the development of signs of liver dysfunction, i.e. increased serum alanine transaminase (ALT) activity. The authors have concluded that choline is an essential nutrient for humans when excess methionine and folate are not available in the diet (Zeisel et al. 1991).

Different foods in human diet contain significant concentration of choline or choline esters, and a normal diet delivers sufficient amount. Generally, the demand for choline in normal adults is lower compared to the amount required in children for growth. Malnourished humans with poor stores of choline, methionine and folate, may also need more dietary choline (Zeisel and Blusztajn 1994).

1.7.6 Health effects

Choline can be beneficial in patients with diseases characterised by deficiencies of cholinergic neurotransmission. In animals, a choline supplemented diet leads to better cognitive
performance, increased electrophysiological responsiveness, and protection against neurotoxic agents and alcohol.

Supplementation of choline in humans has shown to improve the synthesis of acetylcholine, helping the treatment of tardive dyskinesia (inadequate neurotransmission at striatal cholinergic interneurons). Choline supplementation is also associated with better memory in subjects without dementia as well as lower memory losses in patients with Alzheimer's disease, where there is a deficiency of hippocampal cholinergic neurons (Combs 2007). In a large population-based study, choline intake was negatively associated with anxiety symptoms (Bjelland et al. 2009).

Some studies indicate that choline might be involved in the treatment of diseases such as hepatic steatosis or liver dysfunction (Combs 2007).

The toxicity of choline is very low, except for the salt choline chloride which may exert a deleterious effect through impaired utilization of vitamin B6 (Combs 2007).

1.8 Dietary sources of betaine and choline

In 1998, the Food and Nutrition Board of the Institute of Medicine had examined the choline requirements for the population and had established dietary recommendations for choline, estimating the intake of 550 mg / day for men and 425 mg / day for women as Adequate Intakes (AI) (Zeisel 2000). Generally, data on choline content in foods are sparse and therefore there are difficulties in estimating the levels of dietary intake. At present, for betaine there is no indication of Adequate Intake.

However, some databases on the content of betaine and choline in foods have been developed and may be useful in providing an estimate of intake from common foods.

Zeisel et al. (2003) have quantified both choline-containing compounds and betaine in 145 common foods by liquid chromatography and had provided choline data for the products analysed under the USDA National Food and Nutrition Analysis Program (NFNAP) and for establish the choline USDA database.

In their study, the authors investigated also different choline-containing compounds generally found in foods and tissues, such as choline, glycerophosphocholine, phosphocholine,
phosphatidylcholine and sphingomyelin, which can contribute to total choline intake. Foods such as beef and chicken liver (418 and 290 mg/100g, respectively), eggs (251 mg/100g), wheat germ (152 mg/100g), bacon (125 mg/100g) dried soybeans (116 mg/100g), and pork (103 mg/100g) were found to have the highest total choline content. Foods such as wheat bran (1339 mg/100g), wheat germ (1241 mg/100g), spinach (645 mg/100g), pretzels (237 mg/100g), shrimps (218 mg/100g), wheat bread (201 mg/100g) had the highest betaine content (Zeisel et al. 2003).

Later, the US Department of Agriculture, in collaboration with other institutions, created the USDA Database for the Choline Content of Common Foods (USDA 2008). The food sample units, more than 630 foods, were analysed for betaine and the choline-contributing compounds: free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin, using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS) (USDA 2008).

Slow et al. (2005) have examined the content of betaine and also its analogues as found in plants and other organisms. Beside the concentration of betaine, they measured proline-betaine, trigonelline, and dimethylsulphoniopropionate (DMSP) in 74 processed foods from New Zealand. Glycine betaine was found up to 150 µg/g in grain products such as pasta, bread, flour; fruits (oranges and orange juice) were rich in proline betaine, coffee in trigonelline whereas only a small number of foods contained DMSP, though in very small quantities (lower than 10 µg/g) (Slow et al. 2005).

De Zwart et al. (2003) surveyed the betaine content of a wide range of foods commonly found in the western diet. They also measured the content of betaine and its analogues proline betaine (stachydrine), trigonelline and dimethylsulfoniopropionate (DMSP). Shellfish, flour, and some vegetables, such as beetroot, spinach and silverbeet were a good source of glycine betaine. Citrus fruit, alfalfa sprouts were a source of proline betaine. Trigonelline was found in coffee, chickpeas, lentils and rolled oats, while DMSP was only found in some shellfish in important amount (De Zwart et al. 2003).

Authors sustain that the betaine content of the same food type from different sources can be highly variable, depending on the way in which food had been processed or cooked and the stress level of the organism grown during drought, or under salt stress (Slow et al. 2005, de Zwart et al. 2003).
1.9 Betaine and choline analysis

Different methods are available for quantification of betaine and choline and many of their analogues and multiple forms in foods and tissues. The demanding radioisotopic procedure has been proposed in choline and acetylcholine analysis, but later it was replaced by a procedure that uses high-pressure liquid chromatography (HPLC) with electrochemical detection (Ikarashi et al. 1985). Other accurate and inexpensive methods for the measurement of choline and acetylcholine include HPLC with fluorometric detection (Riccny et al. 1992) and HPLC with continuous-flow fast atom bombardment mass spectrometry (Ishimaru et al. 1993). To measure other choline compounds (choline, acetylcholine, phosphocholine, and glycerophosphocholine) a gas chromatography/ isotope dilution mass spectrometry (GC/IDMS) method was developed (Jenden 1973). That method, allowing the inclusion of an isotopically labelled internal standard for each metabolite, assures a good accuracy even when different matrixes are analysed but it cannot measure betaine. A high performance liquid chromatography (HPLC) method for the determination of betaine in tissues, which comprises derivatization and HPLC separation with UV quantification has been described in the literature (Mar et al. 1995), as well as a liquid chromatography/mass spectrometry (LC/MS) method for quantification of choline and some of its metabolites (Acevedo et al. 1996). For the simultaneous determination of betaine, choline, and dimethylglycine in plasma a liquid chromatography – tandem MS (LC-MS/MS) method is available (Holm et al. 2003). Further, a liquid chromatography/ electrospray ionization-isotope dilution mass spectrometry (LC/ESI-IDMS) method that can quantify choline, betaine, acetylcholine, glycerophosphocholine, cytidine diphosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin in various tissues and foods has been proposed by Koc et al. (2002). The LC/ESI-IDMS method is simpler and does not require isolation and derivatization compared to the GC/IDMS method (Koc et al. 2002). Recently, Graham et al. (2009) published a method for quantification of betaine and choline in the aleurone, bran, and flour fractions of wheat using NMR spectroscopy.
1.10 Polyphenols

1.10.1 Chemical structure and food sources
Polyphenols are secondary plant metabolites widely distributed in fruits, vegetables, and other plant products (Crozier et al. 2009). The skeleton of these molecules, characterised by aromatic rings and hydroxyl groups, are divided into several classes such as phenolic acids, stilbenes, flavonoids, lignans (Figure 7). A further distinction concerns the phenolic acids, which are classified in derivatives of benzoic acid and derivatives of cinnamic acid, and flavonoids that comprise flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (Figure 8) (Manach et al. 2004).

![Figure 7. Chemical structure of polyphenols](image)

\[
\text{Hydroxybenzoic acids} \quad \text{Hydroxycinnamic acids}
\]

\[
\begin{align*}
\text{Protocatechuic acid} & : R_1 = R_2 = \text{OH}, R_3 = \text{H} \\
\text{Gallic acid} & : R_1 = R_2 = R_3 = \text{OH}
\end{align*}
\]

\[
\text{Hydroxycinnamic acids}
\]

\[
\begin{align*}
\text{Cinnamic acid} & : R_1 = \text{OH}, R_2 = \text{H} \\
\text{Caffeic acid} & : R_1 = \text{H}, R_2 = \text{OH} \\
\text{Pelaric acid} & : R_1 = \text{OH}, R_2 = \text{OH}
\end{align*}
\]

\[
\text{Flavonoids}
\]

\[
\text{Colorogenic acid}
\]

\[
\text{Stilbenes}
\]

\[
\text{Resveratrol}
\]

\[
\text{Lignans}
\]

\[
\text{Sauceolamivoxil}
\]
Figure 8. Chemical structure of flavonoids

1.10.1.1 Flavonoids

The most ubiquitous class of flavonoids are flavonols, found in almost all plants, mainly as O-glycosides. The foods with the highest content are onions, curly kale, leeks, broccoli, blueberries, red wine and tea (Manach et al. 2004).
Much less distributed in foods are flavones, most significant sources of which are parsley and celery. These compounds are also present in glycosylated form (Crozier et al. 2009). The flavanones are found in tomatoes and aromatic plants, and predominantly in citrus fruits. These molecules are generally glycosilated by a disaccaride, for example hesperetin-7-O-rutinoside and narigenin-7-O-rutinoside commonly found in citrus peel (Manach et al. 2004). Isoflavones, presenting structural similarities to estrogens, are found almost exclusively in leguminose (particularly in soya). Flavanols range from the simple monomer (+)-catechin and its isomer (-)-epicatechin, to complex structures such as oligomeric and polymeric proanthocyanidins, known as condensed tannins. The monomers can be hydroxylated to form gallocatechins and also undergo esterification with gallic acid (Crozier et al. 2009). Flavanols in foods are generally not glycosylated. Catechins are found in fruits, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate in seeds of leguminose plants, in grapes, and especially in tea (Manach et al. 2004).

Anthocyanidins, widely distributed in plants, are responsible for red, blue, pink or purple colours and are present as sugar conjugates (anthocyanins). Rich sources of anthocyanins are fruits, especially the skin. Red wine, some varieties of cereals, and certain vegetables such as aubergines, cabbage, beans, onions and radishes are also a rich source of anthocyanins (Manach et al. 2004).

1.10.1.2 Phenolic acids
Hydroxycinnamic acids, the first class of phenolic acids, are the most common in foods. Important acids are p-coumaric, caffeic, ferulic, and sinapic, generally present in bound forms. Caffeic acid is widely distributed in all parts of fruits and ferulic acid in cereal grains. Chlorogenic acid, deriving from the combination of caffeic and quinic acids, are found mainly in coffee and also in different fruits (Manach et al. 2004).

The second group of phenolic acids are hydroxybenzoic acids, but the concentration of these acids in foods are low and only some fruits, tea and onions have a significant content.

1.10.2 Bioavailability of polyphenols
The absorption into the circulatory system of flavonoids ingested with foods occurs in the small intestine, but only for a few components (Crozier et al. 2009). Prior to passage into the bloodstream as aglycons, the glycoside conjugates of flavonoids require the hydrolysis by
lactase phloridizin hydrolase (LPH). LPH is situated on the brush-border of the mammalian small intestinal epithelial cells and possesses a specific action on flavonoid-\(O-\beta-D\)-glucosides. The released aglycone is able to enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane (Day et al. 2000). An alternative route for hydrolysis appears to be mediated by a cytosolic \(\beta\)-glucosidase (CBG), which implies the transport of the polar glucosides into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter SGLT1 (Gee et al. 2000). However, despite some evidence on the existence of two defined way, recent research indicates that SLGT1 does not transport flavonoids and that glycosylated flavonoids, and some aglycones, have the capability to inhibit the glucose transporter (Kottra and Daniel 2007).

Before absorption into the circulatory system, intestinal enzymes conjugate the aglycones. Such metabolism includes methylation, sulfation, and glucuronidation through the respective action of catechol-\(O\)-methyltransferases (COMT), sulfotransferases (SULT), and uridine-5\'-diphosphate glucuronosyltransferases (UGTs). However, some of the metabolites are transported back into the lumen of the small intestine through the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters, and multidrug resistance protein (MRP) and P-glycoprotein (P-gp). Later, during the transport from the portal to systemic circulation, the metabolites are subject to phase II metabolism in the liver. Finally, the resulting metabolites are excreted back into the small intestine through the enterohepatic cycle (Crozier et al. 2009).

If not absorbed in the small intestine, the dietary polyphenols and their metabolites that reach the colon are extensively metabolized by the local microbial ecosystem. The polyphenol metabolism consists in deglycosylation, ring fission and production of different aromatic acids (Van Duynhoven et al. 2010) such as hydroxyphenylacetic acids - deriving mainly from flavonols - hydroxyphenylpropionic acids - deriving mainly from flavanones and flavones - and phenylvalerolactones and hydroxyphenylpropionic acids, deriving from flavanols (Manach et al. 2004). These compounds can be absorbed into the colon and can be eliminated in the urine.

The result of the structure and nature of the secondary metabolites compared with the native dietary form, and also the low concentrations that actually reach different cells, could determine the overall bioactivity (Crozier et al. 2009) besides the fact that the metabolites and
polyphenols can bind to proteins (Manach et al. 2004). In the last years many investigations focused on phenolic metabolites in order to gain insight on the physiological role and significance by taking into account the above considerations.

It has been reported in vitro that protocatechuic acid, the main metabolite formed from cianidin-3-glucoside (Vitaglione et al. 2007), is a potent anticancer agent in human breast, lung, liver, cervix, and prostate cancer cells at concentrations lower than 10 µmol/L (Yin et al. 2009).

Plasma enterolactone, which derives from the colonic metabolite of lignan, has been shown to reduce the risk of breast (Adlercreutz 2002, Hulten et al. 2002) and prostate cancer (Stattin et al. 2002, Chen et al. 2007), and the risk of coronary events (Vanharanta et al. 1999). Interesting findings have also been found in protection of cardiovascular health by improvement of platelet function through the anti-thrombotic effect of some anthocyanins and their in vivo metabolites of colonic origin. At physiological concentrations, dihydroferulic acid, 3-(3-hydroxyphenyl) propionic acid, delphinidin-3-rutinoside and the mixture of all the compounds showed activity indicating also synergistic effect of different phenolics (Rechner and Kroner 2005).

1.10.3 Polyphenols and health effects

1.10.3.1 Polyphenols and CVD

A large number of epidemiological studies demonstrate that diets rich in fruits and vegetables protect against the incidence of cardiovascular disease (Liu et al. 2000 a, Joshipura et al. 2001, Lichtenstein et al. 2006, Mursu et al. 2008, Holt et al. 2009). The health effects could be attributed to polyphenols present in fruits, vegetables and plant products, considering the results of different humans and animals studies showing that the intake of these molecules reduces endothelial dysfunction and hypertension, dyslipidemia, atherosclerosis, inflammatory state, platelet activation and thrombosis (Galleano et al. 2009, Bertelli and Das 2009, Dohadwala and Vita 2009, Corti et al. 2009, Desch et al. 2010).

1.10.3.2 Mechanisms of action

Food polyphenols represent a class of reducing agents, and a well-known mechanism involved in the health benefits of these molecules is the free radical-scavenging property. The
scavenging of free radicals consists in the reaction of one-electron donation involving the phenolic OH groups that reduce the radicals. In biological tissues, the main role of polyphenols is to protect against lipid peroxidation, which consist in the inhibition of the free radical chain reaction. This is considered as one of the most important antioxidant property of this class of molecules.

Another action of polyphenols is the metal chelation. The capacity to sequester iron and copper, both involved in free radical-producing reactions, depends on the “iron chelation sites” that is specific for different polyphenols (Fraga et al. 2010).

However the biological action of these molecules attributed to their antioxidant activity is not the only mechanism for body defence. Flavonoids, and also their metabolites, are able to exert modulatory effects in cells by interacting with intracellular signalling cascades. The polyphenols, selectively affect the state of different components, for example that of the protein kinase signalling cascades (phosphoinositide 3-kinase (PI 3-kinase), Akt/PKB, tyrosine kinases, protein kinase C (PKC), and MAP kinases), and are able to change the cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression (Maggi-Capeyron et al. 2001, Schroeter et al. 2005, Kong et al. 2000, Gamet-Payrastre et al. 1999, Spencer et al. 2003, Agullo et al. 1997). These effects are very important in prevention against some chronic states such as cancer, inflammation, and neurodegeneration.

Maggi-Capeyron et al. (2001) have shown that wine phenolics (gallic acid, caffeic, protocatechuic, paracoumaric, sinapic acids, ferulic acid) can inhibit the transcriptional activity of AP-1 (activator protein-1) implicated in the expression of a wide variety of genes involved in the regulation of proliferation and apoptosis and in cellular response to stress or in inflammation processes (Maggi-Capeyron et al. 2001). Green tea flavanols, trans-resveratrol and quercetin are also able to inhibit the activity of AP-1 (Aggarwal and Shishodia 2006).


Pasten et al. (2007) have reported that the polyphenols catechin and quercetin are cardiovascular protectors through activation of the MAPK signalling pathways and
consequent suppression of PAI-1 expression in human coronary artery endothelial cells \textit{in vitro}.

Feng et al. (2005) reported in an \textit{in vitro} study molecular evidence for anticarcinogenic potential of chlorogenic acid through regulation of ROS-mediated NF-kB, AP-1, and MAPK signaling pathways. That phenolic compounds also increased the activities of detoxifying enzymes GST (glutathione \textit{S}-transferases) and NAD(P)H quinone oxidoreductase. Gallic acid, \textit{p}-coumaric acid and ferulic acid can regulate the gene expression of cardiac antioxidant enzymes in rats through increase in the levels of nuclear transcription factor \textit{erythroid 2p45 (NF-E2)-related factor 2 (Nrf2)}, an important transcription factor that promotes the encoding for antioxidant enzymes (Yeh et al. 2009). Curcumin and trans-resveratrol can also activate this target for chemoprevention Nrf2 and protect the cells against carcinogenesis (Lee and Surh 2005). Anthocyanins, in particular malvidin, were shown to induce the programmed cell death in human gastric adenocarcinoma AGS cells through the MAPK pathway (Shih et al. 2005).

1.11 Aim of the study

Given the importance of vegetable foods in the prevention of CVD and in the maintenance of vascular health, this PhD researcher project was aimed to investigate the role of specific vegetable components, such as betaine, choline and polyphenol metabolites on different mechanisms putatively related to cardiovascular health. Betaine and choline are two methyl donors that convert homocysteine to methionine while polyphenols diminish the oxidative stress. Specifically, the work has been focused on 1) the evaluation of the role of betaine and choline on inflammatory markers and 2) on the role of phenolic metabolites on the modulation of NADPH oxidase activity, the key enzyme of oxidative stress through its role in reactive oxygen species production.

In particular, the research work addressed two questions: 1) whether betaine and choline intake from the diet are related to inflammation, and 2) what is the effect of metabolites derived from berries and coffee polyphenols on the reduction of superoxide anion production in a cell line suitable as an \textit{in vitro} model for atheroma. The two research lines hereby described resulted into two research papers, resumed below.
**Paper I**

*Survey on betaine and choline content in food products consumed in Italy, dietary intake and relation with traditional and emerging CVD risk factors.*

The aim of the work was to determine the content of betaine and free choline in products commonly consumed in Italy in order to estimate the daily intake in the Italian population and to assess the association with markers of inflammation and components of the metabolic syndrome in a cross-sectional study of a healthy population living in Northern Italy.

**Paper II**

*Effect of polyphenol metabolites on superoxide anion production in cultured human promonocyte cells THP-1*

The aim of this *in vitro* study was to investigate the effect of different colonic metabolites derived from berries and coffee polyphenols, tested at the low concentrations actually found in the bloodstream after absorption, on the modulation of NADPH oxidase-dependent superoxide anion production in monocytes THP-1.
CHAPTER 2:

Survey on betaine and choline content in food products consumed in Italy, dietary intake and relation with traditional and emerging CVD risk factors

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2.1 Introduction

Cardiovascular diseases are intimately linked to inflammatory status. Different inflammatory biomarkers, such as C-reactive protein, interleukin-6, and tumor necrosis factor–α, have been related to cardiovascular risk (Cesari et al. 2003, Ridker et al. 1998) and atherosclerosis, a leading cause of vascular complications, is itself characterised by an increased inflammatory state (Ross 1999). It has been shown that homocysteine, independently associated to CVD, is also a factor related to subclinical inflammation (Su et al. 2005, Shai et al. 2004); the same is true for the metabolic syndrome that represent an important risk for CVD and, besides dislipidemia, insulin resistance and hypertension, is accompanied by subclinical inflammation (Feldeisen and Tucker 2007).

Betaine and choline are two quaternary amines widely distributed in foods. Betaine is an osmolyte and a methyl donor with an important role in the protection of liver, kidney and heart health (Craig 2004). Choline has several biological functions, being involved in maintaining the integrity of cell membrane, cholinergic neurotransmission, transmembrane signalling and lipid metabolism (Zeisel and Blusztajn 1994). Moreover, as a precursor of betaine, choline represents a source of methyl groups.

Both molecules are introduced through the diet (Slow et al. 2005, Zeisel et al. 2003) or may be synthesised endogenously. Choline derives from de novo synthesis (Zeisel et al. 1991) and can be oxidized via a two steps process to generate betaine, which can further donate its methyl group to homocysteine. This leads to the conversion of homocysteine into methionine and the subsequent generation of the methyl donor S-Adenosyl-Methionine (SAM) (Craig 2004).

There are few epidemiological studies considering both betaine and choline intake in relation to chronic diseases, mainly due to lack of food composition data (Slow et al. 2005, USDA
Detopoulou et al. (2008) observed that a diet rich in betaine and choline was associated with a reduction of inflammatory markers in healthy adults. Betaine and choline intakes were inversely associated with plasma total homocysteine concentration in the Framingham Offspring Study (Cho et al. 2006).

High levels of betaine have been reported in spinach, beet, bread, pasta, flour, wheat bran and germ. High levels of total choline have been reported in beef and chicken liver, eggs, wheat germ, bacon, soybean (Slow et al. 2005, Zeisel et al. 2003). Recently Graham et al. (2009) determined betaine and choline contents in different wheat fractions and confirmed that whole grain wheat contains high concentration of both molecules.

The aim of this work was to determine the content of betaine and free choline in products commonly consumed in Italy in order to estimate the daily intake of these putatively beneficial dietary components in the Italian population and to assess the association with markers of inflammation and components of the metabolic syndrome.

2.2 Materials and methods

2.2.1 Italian database generation

In order to decide the foods to be included in the composition database, as a first estimation of betaine and choline intake we used the IEO Italian database of food composition (Salvini et al. 1998) to which values on the content on betaine, free choline and total choline extrapolated from an USDA database (USDA 2008) were added. To this purpose, generic food items of the IEO database that were also present in the USDA database were assigned the USDA betaine and choline content. For foods for which values were expressed on moist form “as eaten” in the IEO database we calculated the corresponding values by applying the proportion between fresh and dry weight. To vegetables present in the IEO database whose values were missing in the USDA list, the average value of vegetables from the same family was assigned. Cooked foods were assigned values equivalent to their fresh counterpart. Fish products were classified on the fat content basis: to lean fish (fat content < 3%) was associated the value of tilapia, whereas to fat fish (fat content > 3%) the value of salmon. Similarly, the value reported for shrimps was applied to all crustaceous and shellfish categories. For meat products the values of beef and chicken were used, whereas for game meats such as duck, pheasant, pigeon and others no values were assigned. For different categories of oils, except olive oil, the value for
corn oil was assigned. In some cases of missing data in the USDA database, the New Zealand database of Slow et al. (2005) was applied. The resulting database included the content of betaine, free and total choline expressed as mg/100g of more than 700 foods and was then used to obtain intake estimates based on the food consumption data from 1240 24h recalls registered in northern Italy used for the validation study of the northern Italian EPIC cohort (Pala et al. 2003). In addition to total intake, we used the resulting data to tentatively identify the theoretical main food contributors for the Italian diet.

The foods identified as potential contributors that did not have a corresponding value in the USDA database were then directly analysed to quantify their betaine and free choline content and used to integrate the missing values for the IEO database. Betaine and free choline were quantified in a total of 28 cereal-based products and 23 vegetables.

Finally, the resulting complete database was applied to estimate betaine and choline intake in a set of food records registered in an Italian population to identify the main contributors to intake of betaine, free and total choline.

2.2.2 Betaine and choline quantification

Reagents and standards

Anhydrous betaine was obtained from Fluka Biochemika (Steinheim, Germany) and choline base solution 50% (w/w) was obtained from Sigma (St. Louis, USA). Hexane, acetonitrile, formic acid, was purchased from Carlo Erba (Milan, Italy).

Samples and sample preparation

Cereal products, such as pasta, bread, flour, biscuits, cereals, breadsticks, toasted bread, crackers, focaccia, pizza, salty snacks, cakes and croissants, were analysed in a total of 28 items. Vegetables such as spinach, beet and others were analysed in a total of 23 items. All products were purchased locally in supermarkets according to a sampling plan that considered leading brand and items for each food category.

Before extraction, a preliminary treatment to ameliorate the homogenization and extraction process was performed according to the food type. Samples with non-homogeneous structure and high moisture (breads, sliced bread loaves, focaccia, snack, croissant, tart, Madeira cakes and pizza) were desiccated overnight at ambient temperature before being milled in a food processor. Foods with a consistent texture (cornflakes, biscuits, crackers, breadsticks, toasted
bread, sponge cake) were directly milled to obtain a fine granulometry. Other foods, such as flour, breadcrumbs and pasta were extracted without any preliminary treatment. For vegetables, all the products were chopped in a food processor. All samples were then analyzed for residual moisture content, according to AOAC standard methods.

*Betaine and choline extraction*

For the extraction, a previously published method (De Zwart et al. 2003) was adopted. After sample preparation, 25 g of food were weighed in triplicate and mixed with 500 ml of bidistilled water in a blender (Braun, Czech Republic) for 2 - 5 min. After homogenization, 5 ml of sample were centrifuged for 10 min at 15000 x g. One ml of the supernatant was added with an equal volume of hexane to eliminate hydrophobic compounds and then centrifuged again under the same conditions. Finally, the aqueous phase was filtered through nylon filters (0.45 µm) before HPLC MS/MS analysis. When non directly injected, samples were stored at -80° C until analysis. To check for efficiency of extraction, three subsequent extractions on the residual pellets were performed and the supernatants analysed as described below.

*HPLC MS/MS analysis*

An HPLC Waters 2695(Milford, USA) equipped with a Micromass Quattromicro API triple-quadrupole tandem mass spectrometer was used for molecular identification and quantification. Separation was carried out with a Waters Spherisorb CN 5 µm (2.1 x 250 mm) column. Betaine and choline were eluted with isocratic water/formic acid 1% at a flow rate of 0.25 ml/min. Identification and quantification was performed in positive-ion mode. The source temperature was 120 °C, the collision gas was argon and collision energy was 14 eV. The capillary voltage was 3 kV and the cone voltage 30 V. The desolvation temperature was 350 °C.

The two molecules were analysed by Multiple Reaction Monitoring mode, with the specific transitions (parent mass-daughter mass m/z) of 118 - 58 for betaine and 104 - 60 for choline. Each sample was injected in triplicate and the results averaged.

2.2.3 Subjects

The population in this analysis included 469 healthy subjects living in the Parma area for which data on clinical biochemistry and functional anthropometry were gathered. Information
on diet was collected via 3-day dietary questionnaires. From the total 469 participants 177 were excluded in the final analysis because of missing data and/or implausibly low food intake, according to the Goldberg Cutoff (Goldberg et al. 1991). The final number of participants with a complete dataset was 265. All volunteers gave their informed consent to the study, which was cleared by the local ethical committee.

**Statistical analysis**

To characterize clinical and dietary data we used descriptive statistics. We calculated the mean intake and the main food contributors for betaine and choline by linear regression analysis, then we assessed the association between betaine, free choline and total choline and intermediate CV risk markers by GLM analysis, using as predictor variables the quartiles of intake of betaine, free and total choline after adjustment for energy intake with the residual method (Willet and Stampfer 1986).


All statistical analyses were performed using the statistical package, SPSS 17.0.

2.3 Results

2.3.1 Preliminary study: betaine and choline intake in the EPIC cohort

The Italian database contains 789 foods of which only 76 did not receive an associated value from literature. The application of this preliminary database to the 1240 24h food records from the EPIC validation study allowed a first rough estimate of the theoretical mean content of the 3 molecules in different food groups. Betaine was found at high levels in cereal products (52.69 mg/100g) and vegetables (29.60 mg/100g); free choline was found at high levels in offal (48.54 mg/100g) and legumes (40.07mg/100g), while total choline in eggs
(462.43 mg/100 g) and offal (274.59 mg/100 g). The main contributors to betaine and choline intake in this free-living population were cereal products, fresh fruits, canned foods, juices and vegetables. The mean betaine intake was (mg/day) 196±94; the mean free choline intake was 73±23, and the mean total choline intake was 266±83. The women had lower intake than men but this difference disappeared after adjustment for energy intake. Examining the food contributors for betaine and choline, cereal products (semolina pasta and bread) and vegetables (spinach and beetroot) resulted the main contributors to betaine intake. The main contributors to free choline intake resulted bread, pasta and red wine, while the main contributors to total choline resulted eggs, bread and meat.

2.3.2 The betaine and choline content in foods. Database integration

We included in our database the betaine and free choline content of foods identified as major contributors and some variety of vegetables mostly consumed in Italy: 28 cereal (Table 1) and 23 vegetable products (Table 2).

*Extraction of betaine and choline from foods and HPLC-MS/MS analysis*

The applied extraction volume and the removal of lipophilic compounds ensured an exhaustive extraction of analytes (98.2±0.14% for betaine and 96.7±0.38% for choline, n=3). A one step procedure allowed a better accuracy and precision.

Figure 1 shows the chromatograms of standards. An example chromatogram of betaine and choline in a bread extract is reported in Figure 2.
Figure 1. Typical (MRM) chromatograms of betaine (m/z 117.9) and choline (m/z 103.88) reference standards.

Figure 2. Typical chromatogram of betaine (m/z117.9) and choline (m/z 103.88) in a wheat bread “type 0” sample.
Betaine and free choline content of foods

The betaine and free choline content of the foods analysed are shown in Table 1 and Table 2. All data are reported in mg/100g of edible portion (Fresh Weight).

Table 1. Content of betaine and free choline in cereal food products

<table>
<thead>
<tr>
<th>Food</th>
<th>Betaine (mg/100g FW)</th>
<th>Choline (mg/100g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pasta</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta (penne), cooked</td>
<td>77.5 ± 1.6</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>Pasta (fusilli), cooked</td>
<td>74.7 ± 1.3</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td>Fresh egg pasta, cooked</td>
<td>49.7 ± 0.4</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>Pasta, whole-wheat, cooked</td>
<td>91.0 ± 0.5</td>
<td>13.2 ± 0.3</td>
</tr>
<tr>
<td>Dry egg pasta, cooked</td>
<td>41.1 ± 1.1</td>
<td>32.0 ± 2.2</td>
</tr>
<tr>
<td><strong>Bread</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bread, white</td>
<td>51.1 ± 0.5</td>
<td>14.4 ± 0.1</td>
</tr>
<tr>
<td>Milk bread</td>
<td>53.2 ± 0.5</td>
<td>14.6 ± 0.3</td>
</tr>
<tr>
<td>Bread, common</td>
<td>62.7 ± 1.0</td>
<td>14.2 ± 0.4</td>
</tr>
<tr>
<td>Bread, whole-wheat</td>
<td>103.9 ± 2.1</td>
<td>18.2 ± 0.6</td>
</tr>
<tr>
<td>Sliced loaves</td>
<td>51.8 ± 0.9</td>
<td>11.3 ± 0.4</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>81.6 ± 1.9</td>
<td>19.6 ± 0.1</td>
</tr>
<tr>
<td><strong>Baked products and flour</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melba toast</td>
<td>79.3 ± 2.1</td>
<td>18.2 ± 0.7</td>
</tr>
<tr>
<td>Melba toast, whole-wheat</td>
<td>135.5 ± 6.8</td>
<td>21.5 ± 0.5</td>
</tr>
<tr>
<td>Cracker, salted</td>
<td>42.3 ± 1.6</td>
<td>16.0 ± 0.1</td>
</tr>
<tr>
<td>Pizza (plain baked dough, with tomato sauce)</td>
<td>36.3 ± 0.8</td>
<td>14.5 ± 0.0</td>
</tr>
<tr>
<td>Pizza (thin with cheese and tomato sauce)</td>
<td>29.5 ± 0.4</td>
<td>16.4 ± 0.2</td>
</tr>
<tr>
<td>Focaccia</td>
<td>35.0 ± 0.6</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td>Breadstick</td>
<td>46.2 ± 1.8</td>
<td>11.2 ± 0.3</td>
</tr>
<tr>
<td>Flour, wheat, “type 00”</td>
<td>93.3 ± 4.1</td>
<td>10.7 ± 0.2</td>
</tr>
<tr>
<td>Breakfast cereals and cakes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornflakes (maize, brown rice, whole-wheat)</td>
<td>50.3 ± 3.9</td>
<td>12.9 ± 0.4</td>
</tr>
<tr>
<td>Frollino cookie, whole-wheat</td>
<td>66.9 ± 5.7</td>
<td>11.8 ± 0.2</td>
</tr>
<tr>
<td>Frollino egg cookie</td>
<td>39.9 ± 1.5</td>
<td>9.4 ± 0.2</td>
</tr>
<tr>
<td>Cookies (low fat)</td>
<td>34.5 ± 0.9</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Sponge cake</td>
<td>25.0 ± 1.0</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>Tart (no jam)</td>
<td>25.4 ± 0.8</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>Cake Margherita</td>
<td>10.6 ± 0.2</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Sponge cake (wheat, oat, barley), cherry jam</td>
<td>34.8 ± 1.3</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>Croissant</td>
<td>29.4 ± 1.6</td>
<td>10.8 ± 0.2</td>
</tr>
</tbody>
</table>

¹Values are reported as mean ±SD, n=3.
The betaine and choline content of cereal samples ranged from 10.6 mg to 135 mg and from 4 mg to 32 mg per 100g of food (FW), respectively. Wholemeal foods contained higher levels of betaine and choline with respect to refined foods, in agreement with the findings of Zeisel et al. (2003) and Graham et al. (2009).

### Table 2. Content of betaine and free choline in vegetable products

<table>
<thead>
<tr>
<th>Food</th>
<th>Betaine (mg/100g FW)</th>
<th>Choline (mg/100g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field mushroom Agaricus campestris</td>
<td>29.3±1.38</td>
<td>7.3±0.10</td>
</tr>
<tr>
<td>Fennel Phoeniculum vulgare</td>
<td>0.0</td>
<td>11.1±0.07</td>
</tr>
<tr>
<td>Beetroot Beta vulgaris L. var. cruenta L. Salisb</td>
<td>667.2±36.62</td>
<td>4.8±0.35</td>
</tr>
<tr>
<td>Spinach Spinacia oleracea L.</td>
<td>195.4±6.94</td>
<td>9.2±0.2</td>
</tr>
<tr>
<td>Beta vulgaris L. var. cycla (L.) Ulrich</td>
<td>182.6±8.93</td>
<td>9.5±0.36</td>
</tr>
<tr>
<td>Beet Beta vulgaris L. var. cycla (L.) Ulrich</td>
<td>233.5±17.72</td>
<td>12.9±1.14</td>
</tr>
<tr>
<td>Tomato Pachino</td>
<td>0.0</td>
<td>4.5±0.07</td>
</tr>
<tr>
<td>Tomato Ramato</td>
<td>0.0</td>
<td>2.4±0.14</td>
</tr>
<tr>
<td>Tomato ripe</td>
<td>0.0</td>
<td>3.0±0.07</td>
</tr>
<tr>
<td>Tomato S. Marzano</td>
<td>0.0</td>
<td>3.4±0.18</td>
</tr>
<tr>
<td>Tomato, without peel, canned</td>
<td>0.0</td>
<td>5.7±0.9</td>
</tr>
<tr>
<td>Chicory Witloof Cichorium intybus, cv witloof</td>
<td>0.0</td>
<td>1.9±0.01</td>
</tr>
<tr>
<td>Chicory Catalogna Cichorium intybus, cv catalogna</td>
<td>tr²</td>
<td>12.1±0.19</td>
</tr>
<tr>
<td>Chichorium intybus Chioggia</td>
<td>0.0</td>
<td>10.2±0.32</td>
</tr>
<tr>
<td>Chichorium intybus Radicchio</td>
<td>tr²</td>
<td>13.7±0.28</td>
</tr>
<tr>
<td>Carrot Daucus carota</td>
<td>0.0</td>
<td>2.0±0.16</td>
</tr>
<tr>
<td>Cabbage Brassica oleracea cv capitata</td>
<td>0.0</td>
<td>6.0±0.09</td>
</tr>
<tr>
<td>Onion Allium cepa</td>
<td>0.0</td>
<td>3.4±0.24</td>
</tr>
<tr>
<td>Lettuce Lactuca sativa</td>
<td>0.0</td>
<td>4.2±0.21</td>
</tr>
<tr>
<td>Courgette Cucurbita pepo</td>
<td>tr²</td>
<td>12.5±0.17</td>
</tr>
<tr>
<td>Pepper Capsicum annuum</td>
<td>tr²</td>
<td>8.4±0.29</td>
</tr>
<tr>
<td>Aubergine Solanum melongea</td>
<td>0.0</td>
<td>2.0±0.12</td>
</tr>
<tr>
<td>Potatoes</td>
<td>tr²</td>
<td>28.2±1.68</td>
</tr>
</tbody>
</table>

¹ Values are reported as mean ±SD, n=3.  
² Traces, value <1.

Concerning the betaine and choline content of vegetables, there is a uniform distribution of choline, while betaine is present either in trace or in huge amounts in different vegetables. Further, as previously noticed, the same family of vegetables contains approximately the same betaine amount. For example Chenopodiaceae are rich in betaine.
2.3.3 Association of betaine and choline intake with intermediate risk factors for CVD

Population characteristics and estimated intake in the Italian diet.

Table 3 shows the clinical characteristics of the study participants. The subject’s sample of 125 female and 140 male was aged 51±11 and 56±13 y respectively and had a BMI (kg/m²) of 25.52±3.75 with an SBP and DBP of 131±21 and 83±13 (mean ± SD) (Table 3). The descriptive statistic of energy and nutrient intakes and food sources are reported in Table 4 and Table 5.

Table 3. Characteristics of the population

<table>
<thead>
<tr>
<th>Gender</th>
<th>Female (125)</th>
<th>Male (140)</th>
<th>Total (265)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Age</td>
<td>51</td>
<td>11</td>
<td>56</td>
</tr>
<tr>
<td>Weight</td>
<td>64,1</td>
<td>10,6</td>
<td>78,1</td>
</tr>
<tr>
<td>BMI</td>
<td>24,87</td>
<td>4,10</td>
<td>26,11</td>
</tr>
<tr>
<td>Waist</td>
<td>88</td>
<td>10</td>
<td>94</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>123</td>
<td>18</td>
<td>138</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>78</td>
<td>13</td>
<td>88</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>88</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>Serum insulin (mU/L)</td>
<td>7,4</td>
<td>3,9</td>
<td>8,6</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>69</td>
<td>15</td>
<td>57</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>74</td>
<td>39</td>
<td>93</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>2,3</td>
<td>3,2</td>
<td>3,2</td>
</tr>
<tr>
<td>Physical activity level PAL</td>
<td>1,72</td>
<td>0,28</td>
<td>1,67</td>
</tr>
</tbody>
</table>
Table 4. Descriptive of nutrient intake

<table>
<thead>
<tr>
<th>Gender</th>
<th>Female</th>
<th>Mean: SD</th>
<th>Male</th>
<th>Mean: SD</th>
<th>Total</th>
<th>Mean: SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Energy (kcal)</td>
<td>2151.37: 350.76</td>
<td>2656.94: 409.34</td>
<td>2418.46: 458.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbohydrates (% of energy)</td>
<td>49.43: 6.42</td>
<td>48.75: 6.40</td>
<td>49.07: 6.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fat (% of energy)</td>
<td>34.87: 5.01</td>
<td>31.84: 4.83</td>
<td>33.27: 5.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sugars (g)</td>
<td>96.33: 28.91</td>
<td>99.81: 32.68</td>
<td>98.17: 30.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total fibre (g)</td>
<td>19.4: 7.17</td>
<td>22.32: 6.59</td>
<td>20.94: 7.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total fats (g)</td>
<td>83.87: 19.08</td>
<td>94.05: 19.46</td>
<td>89.25: 19.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saturated fat (% on total)</td>
<td>35.80: 5.03</td>
<td>35.00: 5.14</td>
<td>35.38: 5.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monounsaturated fat (% on total)</td>
<td>50.91: 4.72</td>
<td>51.39: 4.76</td>
<td>51.16: 4.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyunsaturated fat (% on total)</td>
<td>13.29: 2.73</td>
<td>13.61: 3.29</td>
<td>13.46: 3.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cholesterol (mg)</td>
<td>299.95: 96.98</td>
<td>336.18: 128.54</td>
<td>319.09: 115.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcohol (g)</td>
<td>12.23: 11.36</td>
<td>30.51: 20.31</td>
<td>21.88: 19.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin C (mg)</td>
<td>119.78: 62.75</td>
<td>133.73: 72.48</td>
<td>127.15: 68.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beta-carotene (µg)</td>
<td>2949.49: 1529.61</td>
<td>3249.89: 1990.59</td>
<td>3108.19: 1791.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin E (mg)</td>
<td>11.01: 3.31</td>
<td>11.83: 3.55</td>
<td>11.44: 3.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folate (µg)</td>
<td>277.94: 86.16</td>
<td>314.97: 92.97</td>
<td>297.50: 91.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Betaine (mg/day)</td>
<td>193.61: 66.77</td>
<td>262.70: 83.76</td>
<td>230.11: 83.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free choline (mg/day)</td>
<td>83.20: 22.09</td>
<td>107.18: 24.84</td>
<td>95.87: 26.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total choline (mg/day)</td>
<td>255.18: 69.31</td>
<td>313.39: 85.90</td>
<td>285.93: 83.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Descriptive of food sources (grams/ day)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Female</th>
<th>Mean: SD</th>
<th>Male</th>
<th>Mean: SD</th>
<th>Total</th>
<th>Mean: SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sweets, cakes, biscuits</td>
<td>108.02: 65.55</td>
<td>97.85: 59.32</td>
<td>102.65: 62.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bread, pizza</td>
<td>140.46: 60.50</td>
<td>181.86: 81.29</td>
<td>162.33: 75.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cereals, flours</td>
<td>69.90: 37.32</td>
<td>105.6: 51.17</td>
<td>88.76: 48.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Legumes, potatoes</td>
<td>47.46: 48.40</td>
<td>59.75: 68.75</td>
<td>60.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sweets, cakes</td>
<td>88.45: 64.01</td>
<td>75.98: 53.64</td>
<td>81.86: 58.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcoholic beverages</td>
<td>135.6: 119.6</td>
<td>320.2: 211.5</td>
<td>232.8: 196.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chocolate</td>
<td>3.6: 4.9</td>
<td>3.1: 8.7</td>
<td>3.3: 7.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese</td>
<td>45.6: 27.2</td>
<td>45.1: 25.5</td>
<td>45.4: 26.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fruits</td>
<td>248.1: 138.0</td>
<td>260.7: 155.8</td>
<td>254.8: 147.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry fruits</td>
<td>2.5: 4.9</td>
<td>2.5: 6.6</td>
<td>2.5: 5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk</td>
<td>137.9: 104.2</td>
<td>135.9: 116.0</td>
<td>136.9: 110.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fish</td>
<td>15.1: 26.1</td>
<td>13.2: 33.3</td>
<td>14.0: 30.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soft-drink</td>
<td>25.1: 59.9</td>
<td>25.9: 61.8</td>
<td>25.5: 60.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fruit juices</td>
<td>37.1: 68.7</td>
<td>44.7: 91.1</td>
<td>41.1: 81.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spices</td>
<td>1.3: 1.9</td>
<td>1.4: 2.8</td>
<td>1.4: 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eggs</td>
<td>12.8: 14.6</td>
<td>13.7: 16.5</td>
<td>13.3: 15.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vegetables</td>
<td>191.7: 98.5</td>
<td>215.4: 98.8</td>
<td>204.2: 99.2</td>
<td></td>
</tr>
</tbody>
</table>
There were significant differences in the consumption of some food items in males and females. The highest consumption of bread and pizza, cereals and flours, and alcoholic beverages was observed in men (Table 5). Also, significant differences between males and females were noticed in the case of energy, energy from fat, total fibre, total fats, cholesterol, and alcohol (Table 4). The mean daily intake (mg) for betaine, free choline and total choline, were respectively 230.11±83.56, 95.87±26.42, 285.93±83.60. The mean quartile intakes varied from 149.81 to 320.71 mg/d for betaine, from 74.94 to 121.80 mg/d for free choline, and from 206.77 to 376.46 mg/d for total choline.

Dietary intake of betaine was predicted by the consumption of cereal products and flours, bread and pizza, fresh and cured meat, vegetables and milk (40% of total intake). Free choline intake was predicted by a wider range of foods, such alcoholic beverages, legumes and potatoes, bread and pizza, vegetables, fruits, milk, dry fruits and cereal- flours with a total contribution of 63%. For total choline, the highest predictors were eggs and egg products, followed by fresh and cured meats, alcoholic beverages, legumes and potatoes, milk, fish, fruits and other foods, for a total contribution of 56% (Table 6). Given that foods with the highest betaine content are wheat bran and germ, spinach, beets, and wheat bread, while foods with the highest total choline content are beef and chicken liver, eggs, wheat germ, these relations seem reasonable.

<table>
<thead>
<tr>
<th>Betaine</th>
<th>Free Choline</th>
<th>Total Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food</td>
<td>Cumulative percentage</td>
<td>Food</td>
</tr>
<tr>
<td>Cereal and flours</td>
<td>18.3</td>
<td>Alcoholic beverages</td>
</tr>
<tr>
<td>Bread and pizza</td>
<td>35.7</td>
<td>Legumes and potatoes</td>
</tr>
<tr>
<td>Fresh and cured meat</td>
<td>37.5</td>
<td>Bread and pizza</td>
</tr>
<tr>
<td>Vegetables</td>
<td>38.9</td>
<td>Vegetables</td>
</tr>
<tr>
<td>Milk</td>
<td>39.6</td>
<td>Fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry fruits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cereals and flours</td>
</tr>
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<td></td>
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</tbody>
</table>
Association of betaine and choline intake with components of the metabolic syndrome

In this study, betaine intake showed a positive and significant association with waist girth, DBP, glycaemia at 60 min after glucose challenge, plasminogen activator inhibitor-1 (PAI-1), and C-reactive protein (CRP). Free and total choline intake did not show associations. Choline and betaine were not associated with any of the following variables: fasting glycaemia and insulinaemia, HDL, Triglycerides, AST and ALT, fibrinogen, and TNF-α. All these associations were not changed after stratification for sex, whereas we found a positive association after introducing the new variables energy from carbohydrates, folates, glycaemic load, and a negative association with energy from fats, vitamins C and E (Table 7).

Further, after adjustment for covariates, betaine intake was positively associated with CRP (Table 8), the following covariates resulting significant (p <0.05): age, glucose 120, TEAC, AST, ALT, polyunsaturated fatty acids and leucocytes, with a total contribution of (R²)=26.5%.
**Table 7.** Characteristics of the population by percentile group of betaine

<table>
<thead>
<tr>
<th></th>
<th>1st quartile</th>
<th>2nd quartile</th>
<th>3rd quartile</th>
<th>4th quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean §</td>
<td>SE</td>
<td>Mean &amp;</td>
<td>SE</td>
</tr>
<tr>
<td>Age</td>
<td>54</td>
<td>1</td>
<td>53</td>
<td>2</td>
</tr>
<tr>
<td>BMI</td>
<td>25.61</td>
<td>.46</td>
<td>24.71</td>
<td>.43</td>
</tr>
<tr>
<td>Waist</td>
<td>91</td>
<td>1</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>132</td>
<td>2</td>
<td>127</td>
<td>2</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>85</td>
<td>1</td>
<td>79</td>
<td>2</td>
</tr>
<tr>
<td>Glycaemia (mg/dL)</td>
<td>91</td>
<td>1</td>
<td>91</td>
<td>1</td>
</tr>
<tr>
<td>Post-load plasma glucose (60 min) (mg/dL)</td>
<td>122</td>
<td>5</td>
<td>136</td>
<td>5</td>
</tr>
<tr>
<td>Post-load plasma glucose (120 min) (mg/dL)</td>
<td>92</td>
<td>3</td>
<td>102</td>
<td>4</td>
</tr>
<tr>
<td>Insulinaemia (mU/L)</td>
<td>8.4</td>
<td>.6</td>
<td>6.9</td>
<td>.4</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>220</td>
<td>5</td>
<td>211</td>
<td>5</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>66</td>
<td>2</td>
<td>62</td>
<td>2</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>82</td>
<td>6</td>
<td>76</td>
<td>5</td>
</tr>
<tr>
<td>AST transaminase (U/L)</td>
<td>24</td>
<td>1</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>ALT transaminase (U/L)</td>
<td>23</td>
<td>1</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor - 1 (ng/mL)</td>
<td>13.71</td>
<td>1.21</td>
<td>10.32</td>
<td>.83</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>2.2</td>
<td>.3</td>
<td>2.3</td>
<td>.5</td>
</tr>
<tr>
<td>Betaine (mg/d)</td>
<td>149.81</td>
<td>3.99</td>
<td>204.06 §</td>
<td>1.23</td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
<td>46.73</td>
<td>.83</td>
<td>48.26</td>
<td>.70</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>13.96</td>
<td>.27</td>
<td>14.48</td>
<td>.30</td>
</tr>
<tr>
<td>Fats (% of energy)</td>
<td>35.80 £ $</td>
<td>.60</td>
<td>34.60 £ $</td>
<td>.62</td>
</tr>
<tr>
<td>Total fibre (g)</td>
<td>20.25</td>
<td>.91</td>
<td>19.71</td>
<td>.81</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>22.43</td>
<td>1.99</td>
<td>19.87</td>
<td>2.19</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>146.67 £</td>
<td>10.73</td>
<td>122.81</td>
<td>7.75</td>
</tr>
<tr>
<td>Beta-carotene (µg)</td>
<td>3239.79</td>
<td>240.61</td>
<td>3111.97</td>
<td>237.90</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>12.53 £  $</td>
<td>.52</td>
<td>11.42</td>
<td>.38</td>
</tr>
<tr>
<td>Folates (µg)</td>
<td>307.86</td>
<td>13.55</td>
<td>277.48</td>
<td>9.84</td>
</tr>
<tr>
<td>Glycemic load (g eq)</td>
<td>164.15</td>
<td>5.12</td>
<td>158.82</td>
<td>4.73</td>
</tr>
<tr>
<td>Glycemic Index</td>
<td>57.81</td>
<td>.43</td>
<td>56.57</td>
<td>.39</td>
</tr>
<tr>
<td>Total antioxidant capacity (mmol)</td>
<td>6.54</td>
<td>.40</td>
<td>5.68</td>
<td>.27</td>
</tr>
</tbody>
</table>

Significantly different <0.05 from; §: first quartile; &: second quartile; £: third quartile; $: fourth quartile; SE: Standard error.
### Table 8. Betaine association with CRP after adjustment for covariates

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of the square</th>
<th>df</th>
<th>Mean of the square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted model</td>
<td>1277.42</td>
<td>13</td>
<td>98.263</td>
<td>7.870</td>
<td>.000</td>
</tr>
<tr>
<td>Intercept</td>
<td>162.852</td>
<td>1</td>
<td>162.852</td>
<td>13.042</td>
<td>.000</td>
</tr>
<tr>
<td>Quartile of Betaine</td>
<td>104,657</td>
<td>3</td>
<td>34,886</td>
<td>2.794</td>
<td>.041</td>
</tr>
<tr>
<td>Covariates:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>287,043</td>
<td>1</td>
<td>287,043</td>
<td>22.989</td>
<td>.000</td>
</tr>
<tr>
<td>Post-load plasma glucose (120 min)(mg/dL)</td>
<td>88,474</td>
<td>1</td>
<td>88,474</td>
<td>7.086</td>
<td>.008</td>
</tr>
<tr>
<td>Total antioxidant capacity (mmol vitE/L)</td>
<td>99,610</td>
<td>1</td>
<td>99,610</td>
<td>7.978</td>
<td>.005</td>
</tr>
<tr>
<td>Glycemic Index (%)</td>
<td>35,397</td>
<td>1</td>
<td>35,397</td>
<td>2.835</td>
<td>.094</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>86,223</td>
<td>1</td>
<td>86,223</td>
<td>6.905</td>
<td>.009</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>70,699</td>
<td>1</td>
<td>70,699</td>
<td>5.662</td>
<td>.018</td>
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<tr>
<td>Polyunsaturated fatty acids (% of energy)</td>
<td>108,015</td>
<td>1</td>
<td>108,015</td>
<td>8.651</td>
<td>.004</td>
</tr>
<tr>
<td>WBC</td>
<td>347,082</td>
<td>1</td>
<td>347,082</td>
<td>27.797</td>
<td>.000</td>
</tr>
<tr>
<td>Error</td>
<td>2934.267</td>
<td>235</td>
<td>12,486</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6070.017</td>
<td>249</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted total</td>
<td>4211,689</td>
<td>248</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.4 Discussion

In this study we developed an Italian database on the betaine and choline content of common foods. This included molecular quantification for more than 40 food items, which allowed us to better characterise the Italian population and to study the correlation between betaine and choline intake and CVD risk factors. Cereal products are a good source of betaine and free choline, ranging from 10.6 mg to 135 mg and from 4 mg to 32 mg per 100g of food (FW), respectively. In particular wholemeal foods contain higher levels of betaine and choline with respect to refined foods. In vegetables, choline is widely distributed while betaine was found at high levels in vegetables of Chenopodiaceae family as beetroot (667.2 mg/100g) and spinach (195.4 mg/100g). Comparing betaine content with data reported in other databases, some difference can be noted. For example, the betaine content (mg/100g FW) of white and whole-wheat bread in the USDA database is 31 and 38, while the content for refined and whole-wheat bread in this study is 62.7 and 103.9, respectively. De Zwart et al. (2003) found
that pasta has an average betaine content of 35.2 mg/100g, whereas betaine content in pasta samples considered in this study is about 2 folds higher. The content of beetroot are higher in our study relative to USDA data (130 mg/100g). Generally it must be considered that betaine content can vary in foods and some factors able to influence the concentration include growing and osmotic stress conditions of crops (Craig 2004), food processing (De Zwart et al. 2003) as well as the variety of grain and the milling systems (Likes et al. 2007). This underlines the importance of national databases with the molecular content of local foods.

Regarding intake data, the strength of this work is the use of a database specifically designed to include molecular content of the national foods, and the use of diet records, which give a more accurate estimate of food consumption compared to food frequency questionnaires.

In the present survey on 265 persons we found that the mean daily intakes were of 230.11±83.56, 95.87±26.42, and 285.93±83.60 for betaine, free choline and total choline, respectively. Beside the mean intake of betaine and free choline, we assessed the amount of total choline considering that the molecule in foods can be present bound as esters in phosphocholine, glycerophosphocholine, sphingomyelin, and phosphatidylcholine. Comparing the dietary intake with some data from literature we found a lower intake with respect to a Greek population for betaine and total choline (Detopoulou et al. 2008). In a sample of New Zealanders, betaine intake has been reported higher than in our Italian sample (Slow et al. 2005). Finally, it can be noticed that choline intake in our participants is lower than the Adequate Intake, given that the level for choline (Zeisel et al. 1991) is 550 mg/d for men and 425 mg/d for women. Choline is widely present in foods and we found that choline intake derives from a great number of food items, whereas for betaine the main food sources in the Italian diet are almost limited to cereal products. In a New Zealand population the intake of betaine is also attributed to grain-based products (Slow et al. 2005), whereas in a Greek population consuming a Mediterranean diet, it was attributed to vegetables (particularly spinach) and legumes (Detopoulou et al. 2008).

Relevant findings from our study are that betaine intake was positively associated with component of the metabolic syndrome (Eckel et al. 2005). These factors are waist girth, DBP, glycaemia at 60 min after glucose challenge, but also PAI-1 and CRP that, while not part of the metabolic syndrome definition, are normally associated with insulin resistance. Further, the highest quartile of betaine intake had higher energy from carbohydrates, folates and glycaemic load, and less energy from fats, vitamin C and E. After adjustment for covariates,
betaine intakes were positively associated with the inflammatory biomarker C-reactive protein, an independent risk factor of cardiovascular events (Li and Fang 2004).

Choline and its metabolite betaine are two important methyl-donors involved in the transformation of homocysteine to methionine (Craig 2004, Zeisel and Blusztajn 1994). Supplementations with high doses of betaine (1.5-6 g/day) or choline (2.6g/day) decreased plasma homocysteine, as shown in intervention studies in healthy population (Steenge et al. 2003, Olthof et al. 2003, Olthof et al. 2005). Cho et al. (2006) assessed the relation between dietary choline and betaine and plasma total homocysteine in 1960 subjects, finding that higher dietary intakes were related to lower homocysteine. The effects of dietary choline and betaine were studied on different inflammatory biomarkers. Detopoulou et al. (2008) reported that higher dietary intakes of choline and betaine in a Greek population reduce several biomarkers of inflammation. In the cross-sectional ATTICA study, with more than 3000 persons, the authors found that plasma C-reactive protein, interleukin-6, and tumor necrosis factor-α concentrations in the highest tertile were significantly lower that in the lowest tertile of betaine and choline intake. For betaine it was also found that participants with the highest consumption had lower concentration of homocysteine (Detopoulou et al. 2008).

Despite many evidences in cardiovascular protection, some other studies with different outcomes must be considered. The EPIC cohort demonstrated no relation of dietary intake of choline or betaine with cardiovascular disease risk (Dalmeijer et al. 2008). The ARIC study showed no relation between dietary choline or choline plus betaine and incident coronary heart disease (Bidulescu et al. 2007). The results of these two large-scale studies are not far from our study, considering that we also found no association for choline. The low dietary range of the molecular intake, as suggested by Dalmeijer et al.(2008), can represent a possible hypothesis of these association. In our population the intake is also low and similar to the intake revealed in the participants from EPIC cohort (Dalmeijer et al. 2008).

There are many potential mechanisms that concern the consumption of diets lower in choline and betaine and increase in biomarkers of inflammation and cardiovascular risk in healthy humans. Beside the methylation and the removal of homocysteine, it can be included the pathway of under-methylation of DNA, when the low dietary intakes of choline and betaine alter epigenetic regulation of genes that accelerate the atherogenic process (Dong et al. 2002, Zaina et al. 2005). It has also been reported that choline deficiency is associated with leaky mitochondria, major leakage of reactive oxygen species and DNA damage (Vrablic et al. 2001, Da Costa et al. 2006), leading to increased state of inflammation. Detopoulou et al.
(2008) hypothesise that inflammation can be reduced by increasing S-adenosylmethionine (SAM) and decreasing S-adenosylhomocysteine (SAH).

Metabolic syndrome is linked to dietary intake and recognises protective the 'healthy' pattern (Feldeisen and Tucker 2007, Esmailzadeh et al. 2007). In this study the major betaine contributors were cereal products such wheat pasta, white bread, but not whole foods. Furthermore, compared with the lowest quartile of betaine intake, participants who consumed >320 mg/100g in the highest quartile had higher intake of energy from carbohydrates, folates, glycaemic load and the lowest intake of energy from fats, vitamins C and E. Given that the major food contributors to betaine intake are cereal products, it can be argued that the inverse association between betaine intake and component of metabolic syndrome might at least partially depend on diet and might be attributable to consumption of refined wheat, known be associated with the development of metabolic syndrome (Feldeisen and Tucker 2007).
CHAPTER 3:
Effect of polyphenol metabolites on superoxide anion production in cultured human promonocyte cells THP-1

3.1 Introduction

Atherosclerosis is a multifactorial chronic disease accompanied by chronic low-grade inflammation (Ross 1999). A characteristic of this inflammatory state consists in the damage of the vascular endothelial and smooth muscle cells, sustained by reactive oxygen species (ROS). As a consequence, anticoagulant, anti-inflammatory and vasorelaxation properties of the endothelium are impaired whereas dysfunction arises from the recruitment of monocytes, macrophages, growth factors, with generation of atherosclerotic plaques (Seifried et al. 2007).

It has been shown that the superoxid anion O$_2^-$ and its metabolites are involved in smooth muscle cell proliferation (Hidaka et al. 1992), endothelial dysfunction such as increased vascular permeability, and leukocyte adhesion (Lum and Roebuck 2001) increasing CVD risk. The leading system responsible for O$_2^-$ production is NADPH oxidase, present in leukocytes and vascular cells (Chanock et al. 1994).

Antioxidants have been shown to protect cells against superoxide anion production through at least two distinct processes: direct free radical scavenging or modulation of cell free radical generation through regulation of NADPH activity in monocytes (Cachia et al. 1998). For example, polyphenol mixtures from olive oil wastewaters decreased superoxide anion production by scavenging and depressing O$_2^-$ in a model of human macrophages implicated in atheroma (Leger et al. 2000). Shafiee et al. (2003) found that polyphenols from grape seed rich in procyanidins lowered superoxide anion production in THP1 cells.

The inhibition of the release of reactive oxygen species was also demonstrated in other cell models, for example in human neutrophils (Limasset et al. 1993).

Therefore, endothelial NADPH oxidase could represent a candidate target of dietary flavonoids, and particularly of their metabolites. Steffen et al. (2008) have reported that in human endothelial cells only the O-methylated metabolite of (-) epicatechin results in a decrease in superoxide anion production, while its native parent polyphenol proved to be an O$_2^-$-scavenger that did not inhibit NADPH oxidase activity.
This is one of the few works that investigates the effects of polyphenol metabolites on $O_2^-$ inhibition and in particular on NADPH oxidase activity. In previous works on monocytic cell no research has taken into consideration the effect of polyphenol metabolites but only that of the parent compounds as present in food sources.

Moreover, polyphenols are normally studied as aglycones and sugar conjugates presented to cells at non-physiological concentrations, rather than metabolites at low concentration levels, as they actually occur in the circulatory system. Probably, to identify the true bioactivity effects of polyphenols, we should research the effect of phenolic metabolites formed in the small intestine and hepatic cells, and the catabolic products of the colonic microflora (Crozier et al. 2009).

Accordingly, the aim of our study was to investigate the effect of some colonic metabolites on the modulation of NADPH oxidase-dependent superoxide anion production in monocytes THP-1, a cell line implicated in atheroma. We focused our research on the catabolites deriving from the metabolism of coffee and berry, two food sources known to have a high content of antioxidants (Gomez-Ruiz et al. 2007, Halvorsen et al. 2006), working at physiological concentrations. We also investigated the synergistic effect of the molecules deriving from the same parent compound by testing their mixtures.

3.2. Materials and methods

We tested on the THP-1 cell line the molecules Dihydrocaffeic acid (DHC), Dihydroferulic acid (DHF), Feruloylglycine (FG) individually at 3µM and as a mixture at 9µM; Protocatechuic acid (PA), 3-(3-hydroxyphenyl) propionic acid (33HPP), Homovanillic acid (HVA), 3-hydroxyphenylacetic acid (3HPA) at 5 µM and 20 µM as mixture formulation.

3.2.1 Chemicals and reagents

All culture reagents were purchased from Gibco (Paisley, UK). Lucigenin, phorbol 12-myristate 13-acetate (PMA), and differentiation agents were obtained from Sigma (St. Louis, MO, USA). DHF, DHC, PA, HVA, 3HPA, were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). FG were obtained from TAKAO, 33HPP were supplied by FLUOROCHEM (Derbyshire, UK). The ethanol utilized for dissolution of the molecules was obtained from Carlo Erba, Milan.
3.2.2 THP-1 cell culture and in vitro test with polyphenol catabolites

Catabolites were tested together, according to the class of native molecules, on a line of human THP-1 monocytes obtained from a human diffuse hystocytic lymphoma.

DHC, DHF, FG, derived from coffee phenolic catabolism, were prepared as a solution to obtain a final concentration of 3µM for each metabolite. When tested all together, the total concentration in the plate was 9µM. The second group of molecules, PA, 33HPP, HVA, 3HPA were prepared to reach a final concentration in the medium of 5µM and 20µM respectively for single metabolite and mix formulations.

The cell cultures were grown at a concentration of 2.0 – 2.5 x 10^5 cells/ml in RPMI 1640 GLUTAMAX red phenol supplemented with SVF (10%), Fungizone (0.05%), Penicill in/Streptomycin (1%) in an humidified incubator (5% CO2) at 37°C. The medium was changed every 2nd and 3rd day. The differentiation of promonocytes to monocytes was induced with retinoic acid (1mM), 1,25 dihydroxycholecalciferol (0,1mM), and interferon-γ (100UI/µl) at a concentration of 3.0 x 10^5 cells/ml. After 72h, the differentiated cells, adherent and unable to proliferate, were changed of medium and added in a Petri plate with different test catabolites alone or in mix and incubated for 3 and 24h. For each food, catabolites were tested under the same conditions.

To establish the optimal time of incubation, in a previous experiment test molecules (DHC3µM, DHF 3µM, DHC3µM and DHF3µM ) at physiological concentrations were incubated for 1, 3 and 24 h in duplicate.

3.2.3 Determination of superoxide anion production

The cultures treated as described above were recovered by scraping the cell layer into a tube containing 5 ml of RPMI 1640 and centrifuged for 10 min at 1500rpm. The cellular precipitate was resuspended and homogenized in RPMI 1640 without red phenol (1x10^6 cells/ml). The samples were incubated with lucigenin 10^-4 M for 30 min at 37°C and subsequently stimulated with PMA (final concentration of 10^-7 M) for chemiluminescence assay. Such stimulation of cells causes a gradual increase in O2^- production as shown in Figure 1.
Figure 1. Typical course of O$_2^-$ production in PMA and non PMA stimulated cells.

The resulting O$_2^-$-dependent luminescence was immediately recorded for 1h at 37°C using a Wallac Luminometer (Wallac Co., Turku, Finland) (Vacher et al. 1994, Delbosc et al. 2002). At the end of the experiment, the cellular medium was pooled for protein determination (Lowry et al. 1951).

3.2.4 Expression of results

The results of bioluminescence was expressed as luminescence unit/protein (LU/µg protein) in THP-1 cells. The 100% effect corresponded to the response of cells activated with PMA and incubated with only the solvent vehicle of polyphenol solution. The volume was standardized considering the conditions in which the solvent was employed at a maximum concentration for preparation and incubation of catabolites. Experiments were performed in triplicate comparing the percentage of O$_2^-$ production to control (100%).

All data were expressed as means ±SD for three experiments.
3.3 Results

3.3.1 Time course of inhibition

As a first step the single molecules DHC, DHF, and their mixture were tested respectively at a concentration of 3µM and 6µM. The effect on O2⁻ inhibitation in THP-1 cells was assessed after 1, 3 and 24h of incubation. A small inhibitory effect was observed at 3h for all the samples. At 24h, DHC and the mixture showed a minimum inhibition. After 1h of incubation inhibition was present only when the molecules were tested together. Results are reported in Figure 2.

Figure 2. Time course of inhibition

3.3.2 Superoxide anion production in THP-1 cells

Considering the results of the inhibition kinetic, all the catabolites were incubated at 3 h and 24h. The results (Mean±SD) are reported in Table 1.
Table 1. % O2<sup>−</sup> production in THP1- cells<sup>1</sup>

<table>
<thead>
<tr>
<th></th>
<th>24 H INCUBATION</th>
<th></th>
<th>3H INCUBATION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>DHC 3µM</td>
<td>98,7</td>
<td>11,1</td>
<td>103,7</td>
<td>19,3</td>
</tr>
<tr>
<td>DHF 3µM</td>
<td>101,9</td>
<td>4,8</td>
<td>96,31</td>
<td>3,8</td>
</tr>
<tr>
<td>FG 3µM</td>
<td>104,1</td>
<td>16</td>
<td>94,6</td>
<td>4,1</td>
</tr>
<tr>
<td>MIX 9µM</td>
<td>115,9</td>
<td>16,9</td>
<td>109,5</td>
<td>4</td>
</tr>
<tr>
<td>PA 5µM</td>
<td>107,2</td>
<td>11,6</td>
<td>107,6</td>
<td>9</td>
</tr>
<tr>
<td>33HPP 5µM</td>
<td>116,3</td>
<td>30,7</td>
<td>110,8</td>
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</tr>
<tr>
<td>HVA 5µM</td>
<td>94,3</td>
<td>10,6</td>
<td>102,7</td>
<td>9,6</td>
</tr>
<tr>
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<td>103</td>
<td>10,6</td>
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<tr>
<td>MIX 20µM</td>
<td>104,4</td>
<td>21,1</td>
<td>106,2</td>
<td>18,3</td>
</tr>
</tbody>
</table>

<sup>1</sup>values are reported as mean±SD, n=3.

The O2<sup>−</sup> production in monocytes after 3 h of incubation (Figure 3) with coffee and berry catabolites was above the control sample (100%) and a negligible inhibition was found for both DHF and FG.

At 24 h of incubation (Figure 4) the inhibition percent was also above 100% (indicating activation), and only DHC, HVA showed an inhibitory effect, although too low to be considered of biological relevance.

At physiological concentrations, the catabolites did not present inhibitory effect also when tested as a mixture.

![Coffee Catabolites 3H](image)

![Berry Catabolites 3H](image)

**Figure 3.** Coffee and berry catabolites at 3 h of incubation.
3.4 Discussion

In this study we found that the colonic catabolites of coffee and berries are not capable to protect the monocyte cells THP-1 against PMA-stimulated production of O2^- through modulation of NADPH system. The bioluminescence assay of catabolites tested at physiological concentration (3 and 5 µM) did not show a significant anion superoxide inhibition. Similar negative results were obtained at higher concentrations (9 and 20 µM) and when catabolites were tested as a mixture to evaluate the synergistic effect.

Protection of monocytes against superoxide anion production was achieved in different studies when the molecules tested were native polyphenols from foods. In our study we demonstrate that the polyphenol catabolites, i.e. the molecules which are likely those that may reach the cell in the internal body compartments, are not able to exert a similar effect on NADPH oxidase modulation hopeful target for atherosclerosis prevention.

NADPH oxidase is a complex system whose core is composed of five components and whose activation needs the phosphorylation of some components and the transfer of all the components to the plasma membrane. In the resting cell, the proteins p47phox, p67phox, p40phox, Rac 2 are present in the cytosol, while cytochrome b558 (the complex p22phox, gp91phox), and Rap1A are present on the membrane (Babior 1999). PKC is a protein able to
phosphorylate the p47 subunit (Chanock et al. 1994), and its inhibition leads to oxidase inactivation and $\mathrm{O}_2^-$ decreased production. Pignatelli et al. (2006) found a synergistic effect of quercetin and catechin to inhibit PKC-dependent phosphorylation of the cytosolic subunit p47 phox of NADPH.

Some of the mechanisms suggested by previous research for cellular modulation and $\mathrm{O}_2^-$ production are the expression and phosphorylation of proteins for assembling the NADPH system and activation of protein kinase C (Leger et al. 2000, Cachia et al. 1998).

Our study is the first to look at the effect of colonic catabolites at physiological concentrations on NADPH oxidase modulation in THP-1 cell line, assessed both individually and in mixture to investigate any possible synergistic effect. Our data suggest that a reduction of oxidative stress through inhibition of $\mathrm{O}_2^-$ production was not achieved with molecules derived from berries and coffee when the experimental design takes into account the metabolic fate of dietary polyphenols. These results strongly suggest that the protective effect of polyphenols in vascular dysfunctions such as atherosclerosis, whose characteristic is an inflammatory state and excessive ROS production, is likely to be due to other mechanism of action which should be further investigated in order to highlight the potential role of antioxidant-rich foods and beverages to the risk of cardiovascular disease.
CHAPTER 4: OVERALL CONCLUSIONS

Contrary to the hypothesis, the outcomes of the survey on dietary intake of betaine showed a positive correlation with some components of the metabolic syndrome and with the inflammatory biomarker C-reactive protein, an independent risk factor of cardiovascular events, whereas dietary choline showed no association.

Moreover, the results obtained in our *in vitro* study demonstrated that polyphenol catabolites of colonic origin are not capable to reduce the oxidative stress through modulation of NADPH system. These data seem to indicate that the protection exerted by dietary sources of these classes of molecules, such as whole grain cereals, fruits and vegetables, is not directly related to the compounds assessed in this study, or at least, is not specifically related to the mechanisms investigated. Further studies are warranted in order to elucidate the role of betaine and choline, as well as the role of phenolics, in the beneficial effects of whole grain cereals, fruits and vegetables on risk of cardiovascular disease and atherosclerosis.
CHAPTER 5: REFERENCES


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methionine levels in chronic haemodialysis patients. Journal of Internal Medicine, 245, 175–183.


