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FORMULATION OF FUNCTIONAL FOODS USING CHIA SEEDS RICH IN
OMEGA-3 FATTY ACID AS A MAIN INGREDIENT

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ABSTRACT

Epidemiological studies strongly suggest that omega-3 fatty acids plays a significant role in the prevention of many chronic diseases, such as cardiovascular disease, inflammation, hyperlipidemia, and cancer. Omega-3 polyunsaturated fatty acids are considered essential because they cannot be synthesized in the human body and must be obtained through diet. However, although a ratio of omega-6/omega-3 fatty acids of about 4-1:1 is regarded as optimal for the prevention of different pathologies, Western diets often contain a ratio of 10-25:1, indicating a considerable omega-3 fatty acids deficiency. In light of this issue, in this PhD thesis it was considered the seeds of *Salvia hispanica* L. (common name chia), one of the best plant sources of omega-3 fatty acids, as raw material from which start to create a functional food. Indeed, chia seeds contain between 25% and 40% fats and up to 68% omega-3 alpha-linolenic acid (ALA). They also contain 20% of omega-6 linoleic acid, thus providing a good balance between the two essential fatty acids. ALA is able to improve the pathological condition in model animals like Spontaneously Hypertensive Rats (SHRs), by decreasing the systolic blood pressure and the Angiotensin Converting Enzyme (ACE) activity and expression, but also increasing the vasodilators level (Ogawa *et al.*, 2009; Sekine *et al.*, 2007). Chia seeds are considered a novel food ingredient in the European Union, and for this reason there is a restriction on the amount of use, initially of 5% (EFSA, 2009), now raised to 10% after the last 2013 revision (EU, 2013). This restriction is probably due to the lack of scientific analysis related to their *in vivo* effects in humans, considering that omega-3 fatty acids, for their chemical nature, such as the presence of unsaturations, are more prone to lipid peroxidation, especially in oxidized pathological subjects. According to the above considerations this work reports the main obtained results from the following activities and objectives. First step was the investigation of the *in vivo* effects following the long-term administration of chia seeds in oxidized pathological animal models. With the aim to formulate a functional food useful not only to maintain the health status of healthy people, but also to people with different pathologies, 5% of chia flour was administered for 5 weeks to SHRs, being the high blood pressure condition the major cause of worldwide death as reported from the latest report of the World Health Organization. At the end of the experimental period, parameters for antioxidant, inflammatory and hypertensive status were analysed. After that, the formulation and study of a functional food containing chia seeds and common/tartary buckwheat were performed. Considering that the *in vivo* study had shown the

necessity to increase the antioxidant capacity of chia seed-diet in order to avoid the *in vivo* oxidation increase after chia seed administration, for their high antioxidant activity common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.) were chosen as ingredients to combine with chia seeds in the functional food recipe. The results obtained in this study indicated that bread made with chia and tartary buckwheat flour was more acceptable in many nutritional aspects compared to the control (common wheat bread); it contained a higher amount of protein (20%), insoluble dietary fibres (74%), ash (51%), and alpha-linolenic acid (67.4%). Moreover, this bread possessed lower energy (14%) and carbohydrate contents (24%) compared to the control. Tartary buckwheat also improved the total antioxidant capacity of the bread (about 75%) and provided a considerable amount of flavonoids, which are healthy non-nutritional compounds. Overall, the combination of chia flour with tartary buckwheat flour allowed the creation of bread that might be useful for the following people categories: hypertensive, diabetics, patients with micro- and macro-inflammatory disease, but also to maintain the health status of healthy people by increasing the dietary omega-3 and antioxidant intake. In addition, both chia flour and tartary buckwheat flour contain no gluten and can be consumed by patients with celiac disease, thus making a first step in formulating foods suitable for celiacs and at the same time with high nutritional value to avoid cardiovascular disease risk associated with current gluten-free diet.

1. Introduction

1.1 Western dietary pattern and noncommunicable diseases

The food is constituted of a wide variety of chemicals, which may act singly on human metabolism, but more likely act in groups in a synergistic fashion. Although the human body is able to compensate the unbalance of these chemicals, if this becomes prolonged in time, can lead to the development of chronic diseases (Jacobs, 2012).

Diet is a complex aggregate of foods and behaviours which are generally typical of a certain geographical area and culture. Anyway, the study of nutrition and disease in aggregates of human beings - nutritional epidemiology - is hampered by the difficulty in accurately characterizing this complex aggregate, that is, in stating what people are eating. Most of this difficulty is inherent in the large day-to-day variability in what is eaten due to the integration of different cultures and globalization. The term “Coca-colonization,” a reference to the ubiquitous presence of Coca-Cola, Pepsi, and McDonald’s, describes a world that is moving toward a common diet, one accompanied by the more sedentary lifestyles (Zimmet, 2000). Indeed, several developing countries are changing their eating habits in relation to the consumerism spread, and conforming in this way to the habits of high-income countries. Whereby, many countries that historically sought and used national and international funds to combat micronutrient deficiencies and undernutrition are now facing the coexistence of under- and overweight people among the lower-income sectors of society.

For that reason, researchers talk about “dietary pattern” that literally is defined as ‘the quantities, proportions, variety, or combination of different foods, drinks, and nutrients in a diet, and a frequency with which they are habitually consumed’, but generally indicates a food habit cluster. For example are dietary pattern the Mediterranean diet and the Western diet. The first one indicates a typical dietary pattern originating in various countries surrounding the Mediterranean sea and characterized by a diet rich in plants foods like vegetables, fruits, nuts, and whole grain cereals, and with frequent use of fish, low-fat dairy products and vegetable oils, but low in refined cereal and sugar-rich products, and low in red and processed meats. Such Mediterranean dietary pattern also provides high amounts of micronutrients (essential minerals, vitamins, and fatty acids) and is rich in other bioactive components (e.g. antioxidants, phenolic compounds, phytoestrogens). The contrast to this plant food based diet is the Western dietary pattern, which is common in westernized

countries but spreading worldwide at a fast pace. It may vary slightly across populations, but the general characteristics are the dominance of red and processed meats, ‘junk foods’, refined cereals, confectionaries, sweets, sugar-containing beverages, and ultra-processed foods manufactured by the food industry. Biomarker studies have demonstrated that the Western dietary pattern is associated with low concentrations of micronutrients and likely provides less of other bioactive substances with health benefits found in plant foods. Furthermore, the Westernized dietary pattern includes many processed and ultra-processed foods that likely contribute components with potential adverse health effects; those added during the manufacturing process (like trans-fatty acids) or formed during prolonged heat treatment (like heterocyclic amines, acryl amide, or advanced glycation/lipid-oxidation end-products, Advanced Glycation End products – AGE, Advanced Lipid glycation End products - ALE) (Wirfält *et al.*, 2013).

Chronic diseases, or “noncommunicable diseases - NCD” as lately defined by the WHO (World Health Organization), kill 38 million of people (out of 56 million total) each year. In this definition are included four main types of diseases such as: cardiovascular diseases (like heart attacks and stroke), cancers, chronic respiratory diseases (such as chronic obstructed pulmonary disease and asthma) and diabetes; these 4 groups of diseases account for 82% of all NCD death. Cardiovascular diseases account for most of NCD deaths, or 17.5 million people annually, followed by cancers (8.2 million), respiratory diseases (4 million), and diabetes (1.5 million). All age groups and all regions are affected by NCDs. NCDs are often associated with older age groups, but evidence shows that 16 million of all deaths attributed to NCDs occur before the age of 70 (Fig. 1) (WHO, 2014 a). Of these “premature” deaths, 82% occurred in low- and middle-income countries.

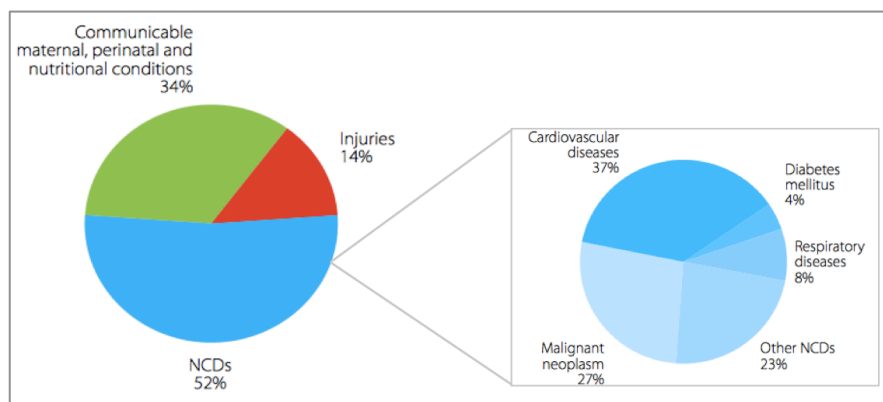


Figure 1. Proportions of global deaths under the age 70 years, by cause of death, comparable estimates, 2012, (WHO, 2014).

Children, adults and the elderly are vulnerable to the risk factors that contribute to NCDs, whether from unhealthy diets, physical inactivity, exposure to tobacco smoke or the effects of the harmful use of alcohol (WHO, 2015 a). WHO states that these diseases are driven by forces that include ageing, rapid unplanned urbanization, and the globalization of unhealthy lifestyles, like unhealthy diets that may show up in individuals as raised blood pressure, increased blood glucose, elevated blood lipids, and obesity. Moreover, WHO reports that NCDs, and in particular cardiovascular diseases (CVDs), are consistently associated with the Western dietary pattern, and CVD mortality rates are twice as high among segments of society that follow such a diet than among people who eat sensibly (O' Flaherty *et al.*, 2012).

In the recent years, the NCD death rates are strongly increased and this is probably also due to the fact that the world is shifting rapidly toward a diet linked with NCD, and mostly as regards changes in diet towards energy-dense ones high in fat, particularly saturated in fat, sugar and salt and low in unrefined carbohydrates. Moreover, this phenomenon involves those countries that historically possessed a healthy diet, such as the countries of the Mediterranean area. Indeed, Figure 2 shows that in Greece, Spain, and Italy the fat consumption increased, especially from animal products, and the cereal consumption decreased from 1964 to 2001 (Alexandratos, 2006).

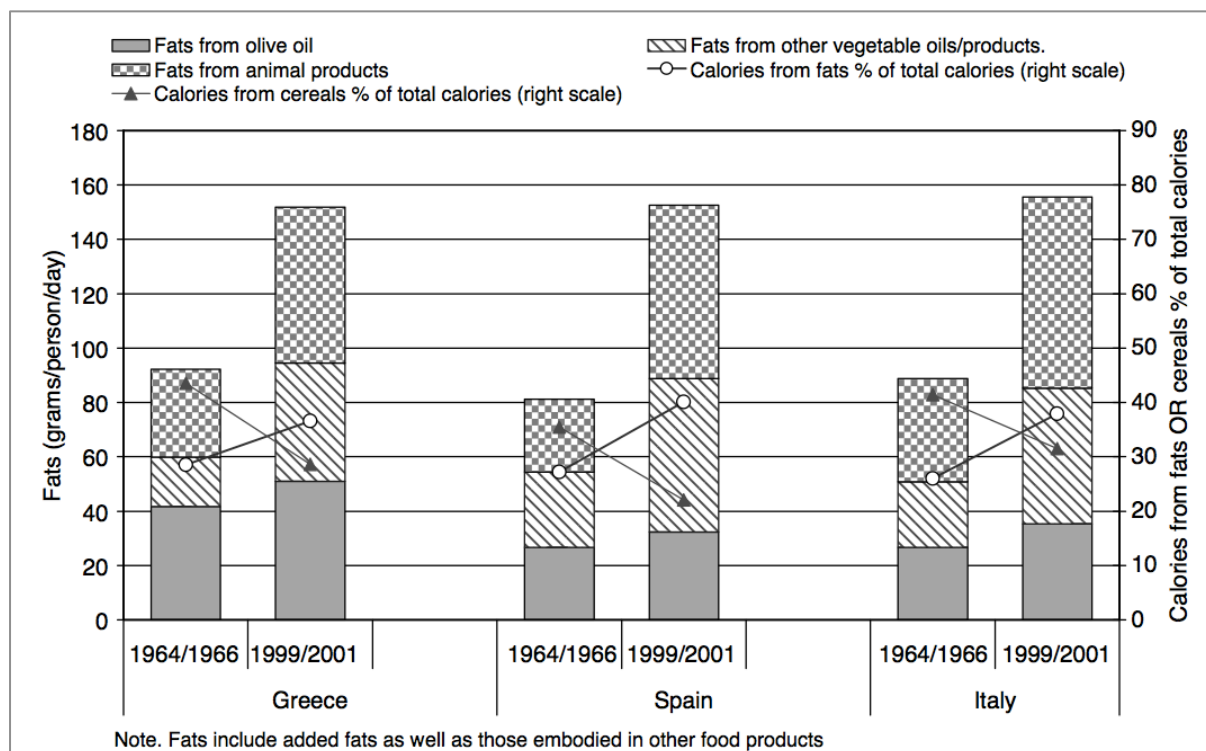


Figure 2. Greece, Spain, Italy: diet transition to increased fat consumption (Alexandratos, 2006).

In recent years, not surprisingly, the world obesity rate is dramatically increased, reaching the 13% of the world's adult population in 2014 (11% of men and 15% of women), and is more than doubled between 1980 and 2014 (Fig. 3). The risk for NCDs increases (such as, CVDs, diabetes, and some cancers) proportionally to the body mass index (BMI) increase (WHO, 2015 b). For that reason, obesity is probably the connection between Western dietary pattern and NCDs.

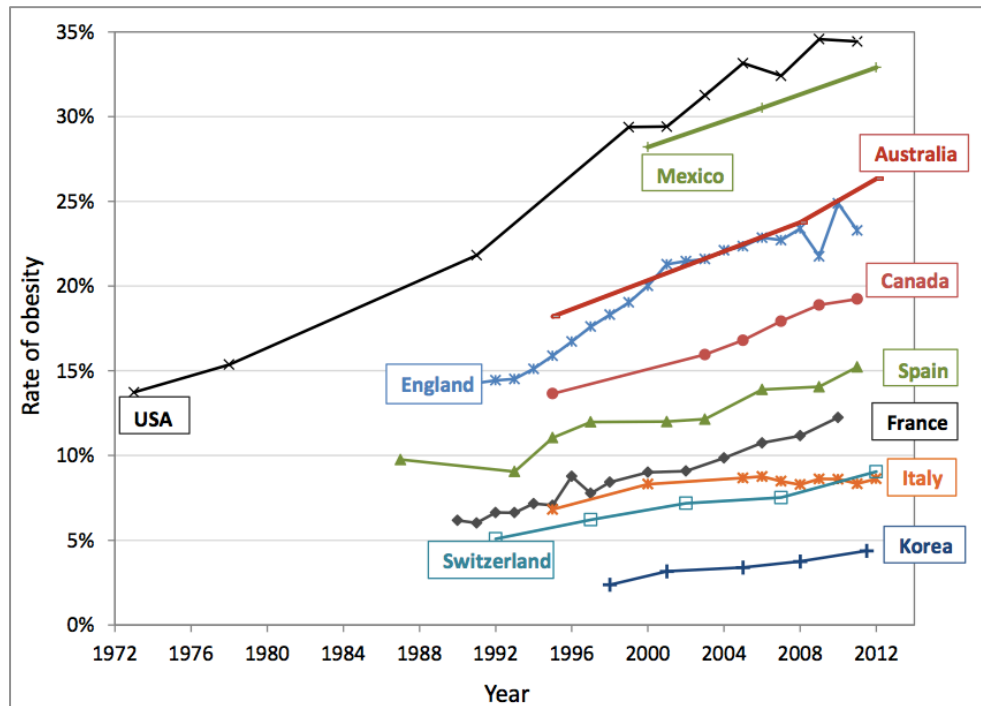


Figure 3. Obesity rates of some countries (OECD, 2014).

1.2 Hypertension and cardiovascular diseases

As seen in the previous chapter, unhealthy diet and physical inactivity are the most important and worldwide present behavioural risk factors. They may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, and overweight or obesity. Hypertension, also known as high or raised blood pressure, is a global public health issue. In 2009, WHO stated the hypertension as the first worldwide leading risk factor for mortality in 2004 (WHO, 2009) (Fig. 4).

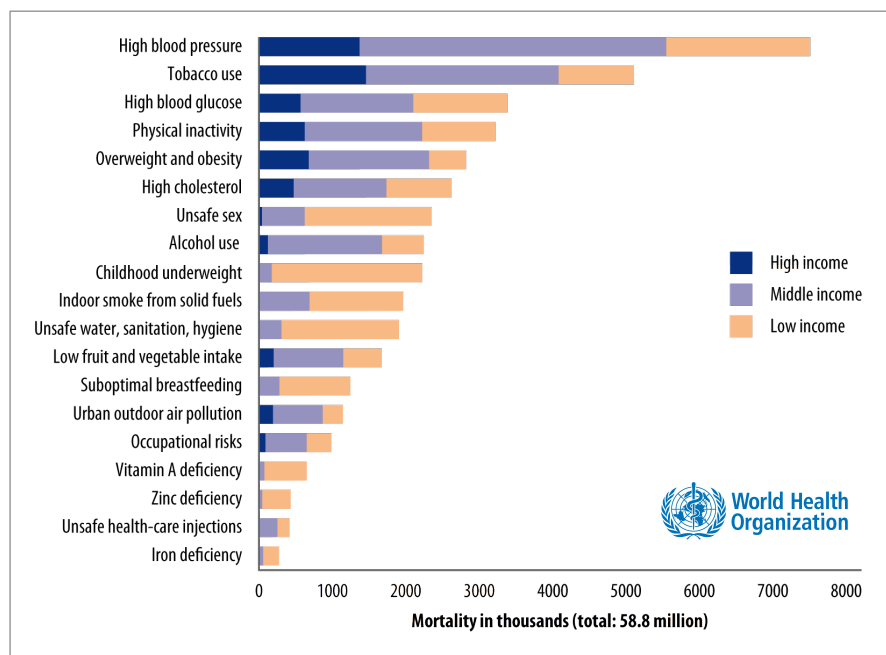


Figure 4. Deaths attributed to 19 leading risk factors, by country income level, 2004 (WHO, 2009).

Hypertension contributes to the burden of heart disease, stroke and kidney failure and premature mortality and disability. Indeed, the elevated blood pressure status can lead to cardiovascular diseases (CVDs), that are a group of disorders of the heart and blood vessels and generally they include: coronary heart disease (disease of the blood vessels supplying the heart muscle), cerebrovascular disease (disease of the blood vessels supplying the brain), peripheral arterial disease (disease of the blood vessels supplying the arms and legs), rheumatic heart disease (damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria), congenital heart disease (malformations of the heart structure existing at birth), deep vein thrombosis and pulmonary embolism (blood clots in the leg veins, which can dislodge and move to the heart and lungs). Cardiovascular diseases are the number one cause of death throughout the world: they killed 17.5 million people in 2012,

that is 3 in every 10 deaths. Hypertension was responsible for at least 45% of deaths due to heart disease (total ischemic heart disease mortality is shown in Fig. 5), and 51% of deaths due to stroke (total stroke mortality is shown in Fig. 5) (WHO, 2014 b).

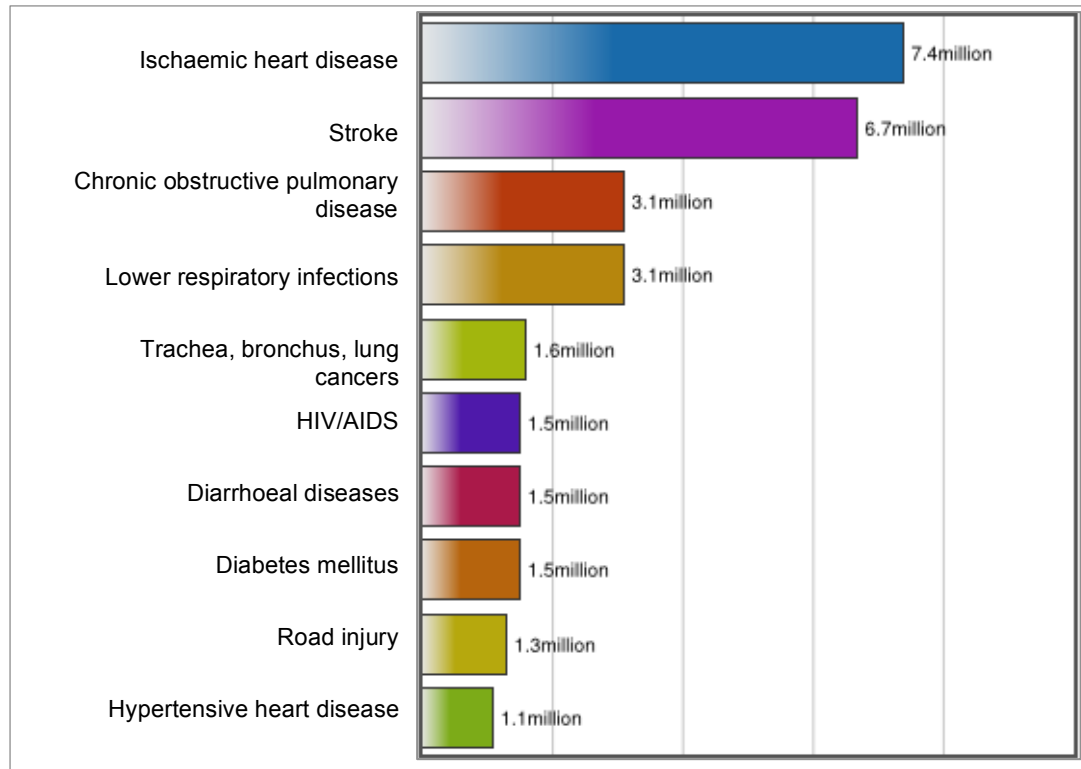


Figure 5. The 10 leading causes of death in the world, 2012 (WHO, 2014 b).

Blood pressure is measured in millimetres of mercury (mm Hg) and is recorded as two numbers usually written one above the other. The upper number is the systolic blood pressure - the highest pressure in blood vessels and happens when the heart contracts, or beats. The lower number is the diastolic blood pressure - the lowest pressure in blood vessels in between heartbeats when the heart muscle relaxes. Normal adult blood pressure is defined as a systolic blood pressure of 120 mm Hg and a diastolic blood pressure of 80 mm Hg. However, the cardiovascular benefits of normal blood pressure extend to lower systolic (105 mm Hg) and lower diastolic blood pressure levels (60 mm Hg). Hypertension is a condition in which the blood vessels have persistently raised pressure, and it is defined as a systolic blood pressure equal to or above 140 mm Hg and/or diastolic blood pressure equal to or above 90 mm Hg (Fig. 6). Normal levels of both systolic and diastolic blood pressure are particularly important for the efficient function of vital organs such as the heart, brain and kidneys and for overall health and wellbeing. Indeed, the higher the pressure in blood vessels the harder the heart has

to work in order to pump blood. If left uncontrolled, hypertension can lead to heart attack, an enlargement of the heart and eventually heart failure. Blood vessels may develop bulges (aneurysms) and weak spots due to high pressure, making them more likely to clog and burst. The pressure in the blood vessels can also cause blood to leak out into the brain: this cause a stroke. Hypertension can also lead to kidney failure, blindness, rupture of blood vessels and cognitive impairment (WHO, 2013).

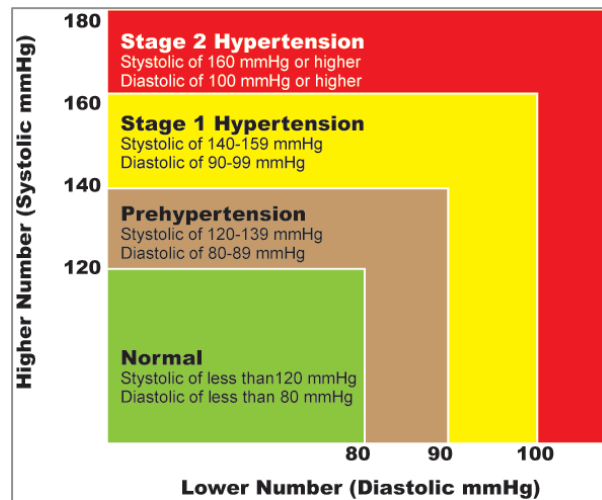


Figure 6. Stages of the raised blood pressure.

1.2.1 Systemic and cellular regulators of blood pressure: the renin-angiotensin-aldosterone system (RAAS), Endothelin-1 (ET-1), Bradykinin (BK) and Nitric Oxide (NO).

The best-known regulator of blood pressure and determinant of target-organ damage from hypertension is the renin-angiotensin-aldosterone system (RAAS). Overexpression of renin and its metabolic products predisposes individuals to developing hypertension and related target-organ damage. Renin is an aspartic protease that is synthesized in the juxtaglomerular cells of the kidney and is secreted into the circulation in response to various physiological stimuli. Renin cleaves angiotensinogen – a protein synthesized principally in the liver – to produce the decapeptide angiotensin I, which is biologically inactive. Angiotensin-converting enzyme – ACE – (also known as kininase II) is a non-selective dipeptidyl carboxypeptidase that accepts various substrates, including angiotensin I and bradykinin. ACE catalyses the hydrolysis of bradykinin to inactive products and converts the inactive decapeptide angiotensin I to the biologically active octapeptide angiotensin II (Fig. 7). Angiotensin II affects most of the biological functions of the RAAS by activating selective membrane-bound receptors. Indeed, angiotensin II activates the angiotensin II type 1 (AT₁) receptor: a classic seven transmembrane domain cell-surface receptor and is coupled to G protein. The AT₁ receptor facilitates the phosphorylation of various proteins, as well as the entry of calcium into the cell and the translocation of calcium within the cell. The downstream actions of the AT₁ receptor activation are vasoconstriction, aldosterone synthesis and release (which leads to sodium retention) and other functions that tend to elevate blood pressure and cause hypertrophy or hyperplasia of target cells (Zaman *et al.*, 2002).

Angiotensin II elevates blood pressure by various mechanisms, including direct vasoconstriction, potentiation of the activity of the sympathetic nervous system at both central and peripheral levels, stimulation of aldosterone synthesis and release with consequent sodium and fluid retention by the kidney, and stimulation of arginine vasopressin release. In addition to effects that obviously elevate blood pressure, angiotensin II has various actions that damage blood vessels; for example, stimulating NADH and NADPH activity, and thereby raising the oxidative potential of vascular tissue. Angiotensin II also has a role in the vascular-injury response, stimulating leukocytes adhesion to the site of injury and favouring superoxide and peroxynitrite formation, and proliferation and migration of various cell types towards the luminal site of injury. The cascade of events that follows causes

cellular components of the arterial wall to transform their phenotypes, resulting in atherosclerotic plaques or formation of fibrous neointima (Zaman *et al.*, 2002).

Because of these actions, and because angiotensin II and some of its constituent peptides stimulate the synthesis of the antithrombolytic agent PAI1, it is thought that activation of the RAAS predisposes to atherosclerosis and thromboembolytic events, including heart attack and stroke. Moreover, angiotensin II also causes hypertrophy of cardiac myocytes and both hypertrophy and hyperplasia of vascular-smooth-muscle cells by direct mechanisms, by elevating blood pressure and by stimulating the release of various cytokines and growth factors, usually in the setting of high blood pressure. Importantly, angiotensin II feeds back to renin through the AT₁ receptor, so that increased angiotensin II levels tend to inhibit renin release and normalize blood pressure after angiotensin II-stimulated increases in blood pressure. This feedback mechanism might function imperfectly in certain forms of hypertension and in cases in which the AT₁ receptor or ACE is blocked by pharmacological antagonists (Zaman *et al.*, 2002).

Aldosterone, the main circulating mineralcorticoid, is under the control of angiotensin II, but its secretion is also mediated by other factors, such as K⁺, adrenocorticotrophic hormone (ACTH), vasopressin, catecholamines, endothelin, serotonin and Mg²⁺. Aldosterone has a major role in blood-pressure and –volume regulation in normal subjects, and in the pathogenesis of hypertension and target-organ damage. Traditionally, it was thought that aldosterone is synthesized in the adrenal glomerulosa and acts principally on renal epithelial cells to promote Na⁺ and H₂O retention and K⁺ and Mg²⁺ loss. More recently, it has been shown that aldosterone is expressed in extra-adrenal tissues, including heart, brain and blood vessels, and has pathological effects on these non-epithelial tissues. These include cardiac and vascular fibrosis and remodelling, endothelial dysfunction and blood pressure-elevation, and are observed only in the presence of sodium overload (Fig. 7) (Zaman *et al.*, 2002).

Endothelins are potent peptide-vasoconstrictors. Three isoforms of endothelins have been identified and designated as endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). ET-1 is mainly present in vascular endothelium whereas ET-2 and ET-3 are less widely distributed. ET-1 is responsible for most of the pathophysiological roles in the body. Endothelins are synthesized from a precursor molecule called pre-pro-endothelin. This pre-pro-endothelin is then cleaved by neutral endopeptidase to form ‘Big endothelin’ and finally cleaved to form endothelin by endothelin converting enzyme (ECE) found on endothelial cell membrane. Though endothelins have a short half-life of less than five minutes, they have a

much longer duration of action. The clearance of endothelins is mainly via lungs and kidneys. Five receptors for endothelins have been identified and cloned, of which endothelin A (ET_A) and endothelin B (ET_B) are found in mammals. Both are G protein coupled receptors. ET_A receptors are situated on smooth muscle cells and myocardium. ET_B is situated on endothelial cells, some smooth muscle cells and macrophages. ET_A receptor has affinity for both ET-1 and ET-2 whereas ET_B receptor has equal affinity for all the three endothelins. Endothelins act in a paracrine fashion on nearby cells and is not circulated in the body as a hormone. Endothelin being a potent vasoconstrictor is responsible for maintaining the tone of blood vessels and peripheral resistance. It has a role in the release of atrial natriuretic peptide (ANP), aldosterone, adrenaline and numerous pituitary and hypothalamic hormones. Endothelins cause natriuresis and diuresis by acting on ET_B receptors on tubular cells of kidney. Endothelins stimulate production of cytokines and growth factors like vascular endothelium derived growth factor, basic fibroblast growth factor, tumor growth factor- β and platelet derived growth factor. Since endothelins cause vasoconstriction and mitogenic effects, they have been implicated in myocardial fibrosis, atherosclerosis, congestive cardiac failure, hypertension, myocardial infarction, cerebral haemorrhage, stroke, pulmonary arterial hypertension, renal failure. In addition, hypercholesterolemia can induce endothelial dysfunction which can elevate endothelin levels. Endothelin in turn stimulate various growth factors and promotes neutrophil and platelet adhesion, thereby promoting growth and coronary thrombosis (Fig. 7) (Parida *et al.*, 2013).

Bradykinin (BK) is one of kinins, a pharmacologically active polypeptide, which is released in the tissues and body fluids as a result of enzymatic action of kallikreins on kininogens in blood (related to the blood clotting cascade) but can also be generated in other tissues and organs. Besides kallikrein, other proteases such as plasmin may also release bradykinin. Kinins are effectors of vasodilation, vascular permeability, NO release and arachidonic acid mobilization. They are important regulators of blood pressure, kidney function and heart function, and they are also involved in inflammation. The kinins include the BK (Arg-Pro-Pro-gly-Phe-Ser-Pro-Phe- Arg), kallidin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and methionyl-lysyl-BK (Met-Lys-Arg-Pro-Pro-Gly-Phe- Arg). Kallidin and methionyl-lysyl-BK are converted into BK by aminopeptidases present in plasma and urine. Plasma BK is rapidly (<15 s) inactivated by circulating kinases to a smaller stable peptide (BK1-5) form. Several peptidases can degrade kinins, including Angiotensin Converting Enzyme (ACE), a metalloproteinase which converts Angiotensin I to Angiotensin II and

destroys bradykinin. Once BK is released in the blood and body fluids, it may act on two BK receptors known as B₁ and B₂. Interaction between the kinins and their specific receptors can lead to the activation of several second messenger systems. The BK receptor stimulation of the intact cells or in tissues appears to initiate the second messenger pathways, such as the arachidonic acid products and the activation of calcium sensitive systems. The elevation of cellular inositol phosphates by BK involves G-protein coupled activation of phospholipase A₂ and C that are used in the synthesis of eicosanoids. The pharmacological actions of BK in regulation of systemic BP are vasodilation in most areas of circulation, reduction in total peripheral vascular resistance and regulation of sodium excretion from the kidney (Fig. 7) (Sharma, 2013).

Nitric oxide (NO) is a soluble gas with a half-life of ~6 - 30 s, continuously synthesized from the amino acid L-arginine in endothelial cells by the constitutive calcium-calmodulin-dependent enzyme nitric oxide synthase (NOS). There are two endothelial forms of NOS: constitutive NOS (cNOS; type III) and inducible NOS (iNOS, type II). Co-factors for NOS include oxygen, NADPH, tetrahydrobiopterin and flavin adenine nucleotides. Nitric oxide synthesized by constitutive nitric oxide synthase (iNOS), plays a primary role in the regulation of blood pressure. Another type of nitric oxide synthase (iNOS) is Ca²⁺-independent and inducible by immunological stimuli. Nitric oxide is thought to reduce blood pressure by evoking vasodilation either directly by causing relaxation of vascular smooth muscle or indirectly by acting in the rostral brainstem to reduce central sympathetic outflow, which decreases the release of norepinephrine from sympathetic nerve terminals. In patients with essential hypertension endothelium-dependent relaxation is decrease. Endocardial endothelial cells express the constitutive form of nitric oxide synthase and this enzyme is known to modulate myocardial contraction and coronary tone. A variety of agonists (e.g. acetylcholine, histamine, thrombin, serotonin, ADP, bradykinin, norepinephrine, substance P, and isoproterenol) can increase the synthesis and release of nitric oxide from the endothelium, although many of these same agonists (e.g. acetylcholine, serotonin, norepinephrine, and histamine) constrict vascular smooth muscle in the absence of endothelium. Vasoactive substances produced within the endothelium, such as bradykinin, may also stimulate nitric oxide release by autocrine and paracrine effects on endothelial B₂ kinin receptors. However, the principal physiologic stimulus for nitric oxide synthesis and release from the endothelium is likely the shear stress of blood flowing over the surface of the vessel by a non-receptor- dependent mechanism. Nitric oxide, released from the

endothelium as a gas or attached to other molecules, stimulates soluble guanylyl cyclase, producing increased concentrations of cyclic guanosine monophosphate (GMP). Cyclic GMP interacts with three types of intracellular receptor proteins: cGMP-dependent protein kinases (PKGs), cGMP-regulated ion channels, and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs). This means that cGMP can alter cell function through protein phosphorylation or through mechanisms not directly related to protein phosphorylation. Depending on the direction of nitric oxide release and the site of cyclic GMP activation, differing biological effects can be observed. NO and cGMP together comprise an especially wide-ranging signal transduction system when one considers the many roles of cGMP in physiological regulation, including smooth muscle relaxation, visual transduction, intestinal ion transport, and platelet function. For example, increased cyclic GMP in vascular smooth muscle cells underlying the endothelium activates GMP-dependent kinases that decrease intracellular calcium, producing relaxation, whereas increased cyclic GMP in platelets by action of nitric oxide released into the blood vessel lumen decreases platelet activation and adhesion to the surface of the endothelium. Nitric oxide also regulates the cellular environment within the vessel wall by inhibiting the activity of growth factors released from cells within the vessel wall and from platelets on the endothelial surface. More extensively, it is currently thought that at least one mechanism of cGMP-induced relaxation is the reduction of intracellular-free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$), since $[\text{Ca}^{2+}]_i$ is the signal for activation of myosin light-chain kinase (MLCK) and contraction in smooth muscle. This enzyme phosphorylates myosin regulatory light chains (MRLC), which then form cross bridges with actin thin filaments and so generate force. Cross bridges are dephosphorylated by myosin light-chain phosphatase (MLCP) resulting in relaxation. Calcium channel blockers predictably cause vasodilation because they reduce intracellular Ca^{2+} . Substances that increase cAMP like 2 agonist, may cause relaxation in smooth muscle by accelerating the inactivation of MLCK and facilitating the expulsion of calcium from the cell. Regulation of phosphodiesterase activity is an important component of control of cGMP concentration and hence activity of the NO–cGMP pathway. Nitric oxide also governs basal systemic, coronary, and pulmonary vascular tone by increased cyclic GMP in smooth muscle, by inhibition of a potent constrictor peptide, endothelin-1, and by inhibition of the release of norepinephrine from sympathetic nerve terminals (Fig. 7) (Tousoulis *et al.*, 2012).

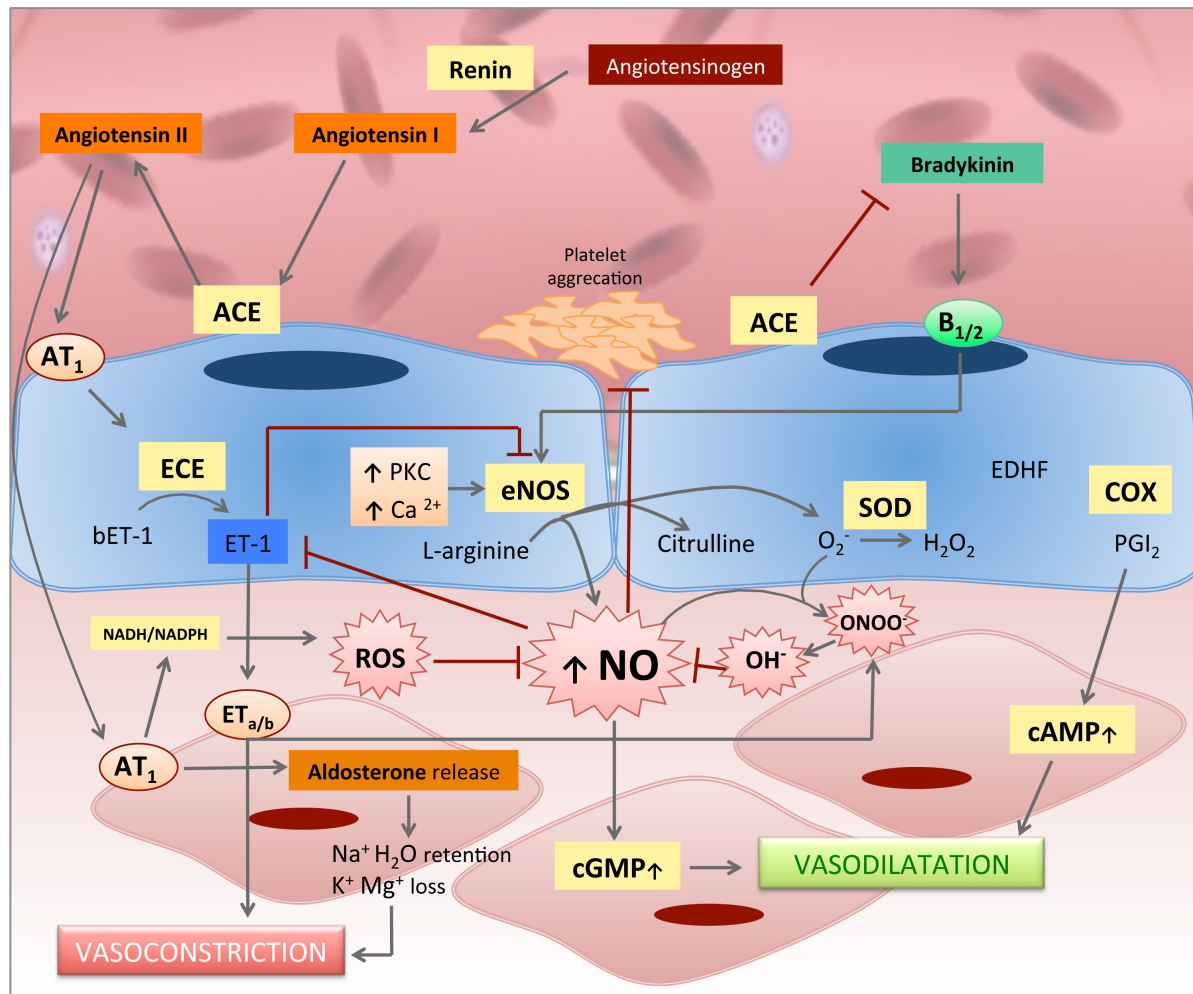


Figure 7. Some epithelial actions of RAAS, ET-1, BK and NO.

1.3 Correlation between gluten-free diet and cardiovascular risk

Celiac disease (CD), an autoimmune enteropathy characterized by a chronic inflammatory condition of the small intestine caused by permanent intolerance to gluten/gliadin (prolamin), is characterized by a complex interplay between genetic and environmental factors. The prolamin fractions in cereal grains (gliadin in wheat and similar alcohol-soluble proteins in other cereals, secalin in rye, hordein in barley) are the environmental stimuli responsible for the development of intestinal damage associated with CD. The prevalence of CD worldwide ranges between 0.5% and 3% of the general population (Norsa et al., 2013). The classical presentation of CD as a pediatric predominant disorder is characterized by small-intestinal villous atrophy and crypt hyperplasia (Fig. 8). However especially in adult-onset patients, a preserved mucosal architecture characterized by dense lymphocytic infiltration, with no villous atrophy or crypt hyperplasia can also be observed.

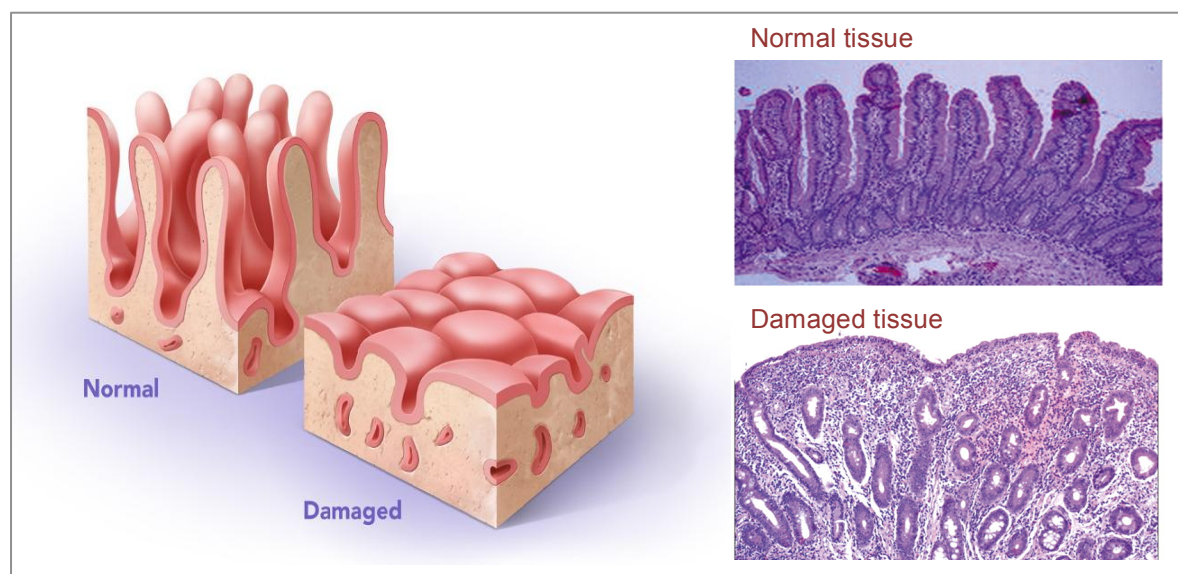


Figure 8. Celiac disease histologic pattern.

An interplay between innate and adaptive immune responses to ingested gluten is involved in CD. Developments in proteomics have provided an important contribution to the understanding of the biochemical and immunological aspects and the mechanisms involved in the toxicity of prolamins. It has been demonstrated that the gliadin sequence contains regions, which play a special role in CD pathogenesis: exert a cytotoxic activity or

immunomodulatory activity. Other regions trigger oxidative stress and induce the release of pro-inflammatory cytokines (Mamone et al., 2011).

The aetiology of CD is not fully understood. A previous CD genome-wide association study (GWAS) demonstrated risk variants in the human leukocytes antigen (HLA) region (Trynka et al., 2009). HLA-DQ2 and HLA-DQ8 risk alleles are necessary, but not sufficient, for celiac disease development. Anyway, other studies have shown that 6% of the European and USA population do not present these alleles. Therefore other no-HLA risk genes need to be investigated (Harmon et al., 2011).

Usually food proteins are degraded into small peptides and aminoacids by peptidases before they can be transported across the epithelium. The high proline (P) content in gliadin and similar proteins of wheat and related cereals, renders these proteins resistant to complete proteolytic digestion in the human intestine. Therefore toxic oligopeptides with high concentration of proline and glutamine (Q) are accumulated in the small intestine and can exert toxic effects in genetically susceptible subjects.

Different gluten peptides are involved in the CD process. Computer modelling studies have demonstrated that two groups of biologically active peptides derive from α -gliadin. The serine-containing group of peptides appears to be essentially cytotoxic, whilst the tyrosine-containing group has the capacity to trigger immunological reactions in CD patients (Cornell *et al.*, 2001). Both types of activity in celiac disease are possible if there is defective digestion of the active peptides. As far as concerns the serine-containing peptides (S), their activity is linked to the presence of PSQQ and QQQP motifs. The tyrosine-containing peptides (Y), sequences such as QQPY and/or QPYP are associated with immunological activity and hence toxicity. Some examples of peptides of α -gliadin that have been widely investigated are P31–43, P31–49, P44–55, P57–68, P57–89, and P63–76 (Fig. 9) (Ferretti *et al.*, 2012).

The “immunogenic” peptides such as P57–68, P57–89, and P63–76 cause an adaptive response that proceeds through their binding to HLA-DQ2 or -DQ8 of antigen presenting cells and the subsequent stimulation of T-cells (Fig. 9). The repetitive presence of these residues makes them a preferred substrate of the enzyme tissue transglutaminase (tTG), whose main function is to catalyse the covalent and irreversible cross-linking of a glutamine residue in glutamine-donor proteins with a lysine residue in glutamine-acceptor proteins, which results in the formation of DQ-“gluten” peptide complexes. However, apart from cross-linking its substrates, tTG can also hydrolyse peptide-bound glutamine to glutamic acid either at a lower pH or when no acceptor proteins are available, a process leading to an

enhanced immunogenicity of gluten peptides (Fig. 9). The DQ-“gluten” peptide complexes activate DQ2 or DQ8 restricted T-helper cells that proliferate and produce mainly Th1-type cytokines, particularly interferon-gamma (IFN- γ). The secretion of Th1 cytokines activates the release of enzymes such as matrix metalloproteinases that can damage the intestinal mucosa, with a loss of villous structure (Fig. 8). Th1 cytokines increase epithelial permeability which in turn will increase the passage of gluten peptides and peptide binding to DQ2 and DQ8 molecules on antigen-presenting cells, leading to a chronic feedback of the inflammatory process as long as gluten peptides are present in the intestinal lumen (Fig. 9). The interactions between toxic gluten peptides and specific cells in lamina propria, such as epithelial cells, macrophages and dendritic cells, induce an innate immune response by up-regulating the expression of different mediators such as interleukin-15 (IL-15) with subsequent massive increase of intraepithelial lymphocytes. These events contribute to the damage of the mucosal matrix. In particular IL-15, a major mediator of the innate immune response is involved in proliferation of crypt enterocytes, an early alteration of CD mucosa causing crypts hyperplasia (Fig. 9) (Ferretti *et al.*, 2012).

In addition to “immunogenic” effects, gliadin peptides may directly affect intestinal cell structure and functions as demonstrated *in vitro* on cultured cells and intestine biopsies. The effects exerted by “toxic peptides” P31–43, P31–49, P44–55 have been mainly investigated and different molecular mechanisms, strictly related, appear to be involved. Some α -gliadin peptides, in particular P31–43, possess the ability to penetrate cells. Thereby they are internalised by endocytic uptake. Peptide P31–43 accumulation in lysosomes leads to activation of some signal transduction pathways and to increased levels of free radicals (reactive oxygen species, ROS, and reactive nitrogen species, RNS) (Fig. 9). Thereby it has been assumed that oxidative stress is one of the mechanisms that can play a role in gliadin toxicity. Using different cell models, it has been reported that gliadin exposure reflects in an intracellular oxidative imbalance, characterized by an increase in the levels of lipid peroxidation products (4-hydroxy-2(E)-nonenal (4-HNE)), an increase in the oxidised (GSSG)/reduced (GSH) glutathione ratio and a decrease of protein-bound sulfhydryl groups (Luciani *et al.*, 2010). Significant structural perturbations of the cell plasma membrane were also detected. The increase in oxidative damage could induce alterations of cell morphology, cell proliferation, apoptosis and cell viability (Giovannini *et al.*, 2000).

The relationship between oxidative damage and celiac diseases is supported by several studies on intestinal cells and circulating cells. Increased levels of prostaglandin E2 (PGE₂)

were demonstrated in homogenized small bowel biopsy specimens from patients with active celiac disease (Giovannini *et al.*, 1994) while the levels of the antioxidant enzyme glutathione peroxidase and reductase were decreased in biopsies from celiac disease patients with consequent decreased levels of GSH (Stojiljković *et al.*, 2009). Several investigations reported that reactive nitrogen species such as nitric oxide (NO) also play an important role in the pathogenesis of celiac disease with enhanced NO. Inducible-nitric oxide synthase (iNOS) is constitutively expressed in human duodenal enterocytes; its activity is increased in patients with untreated celiac disease and is partially corrected when patients are treated with GFD. High levels of NO are present in serum and urine of children with celiac disease and correlate with an increased expression of iNOS in the small intestine (Högberg *et al.*, 2011; Ferretti *et al.*, 2012).

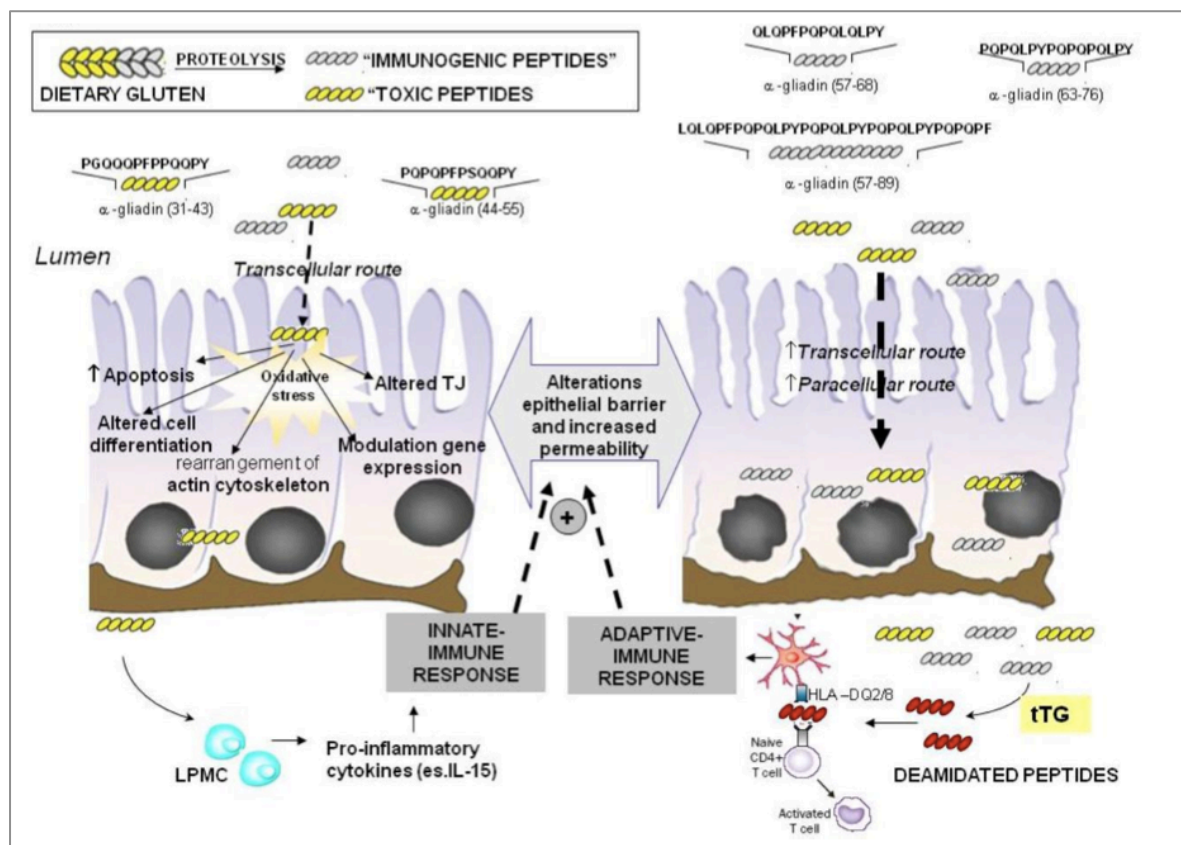


Figure 9. Intestinal epithelial damage in celiac disease: role of "toxic" and "immunogenic" peptides (Ferretti *et al.*, 2012).

An increase of markers of oxidative damage of lipids (thiobarbituric acid-reactive substances and lipid hydroperoxides), proteins (carbonyl groups) and DNA was demonstrated in intestinal cells and biological fluids of CD with respect to controls (Szaflarska-Poplawska

et al., 2010). Changes in vitamin E and antioxidant enzyme activities were observed also in circulating cells and plasma of patients. A decreased expression of the antioxidant and anti-inflammatory enzymes PON1 and PON3 mRNA was found in intestinal biopsy of celiac patients. All these decreased antioxidant defences may compromise the inflamed mucosa, rendering it more susceptible to oxidative tissue damage, hindering recovery of the mucosa and return of epithelial cell layer integrity (Ferretti *et al.*, 2012).

The only treatment for CD is lifelong adherence to a gluten-free diet (GFD), which is expected to result in reconstitution of the small intestinal villous structure, symptoms disappearance and improvement of nutritional status and general well being. Most of the emphasis on GFD focuses on strict avoidance of gluten, but it is well recognized that GF products are considered of lower quality and poorer nutritional value compared to the gluten-containing counterparts. Studies show that GF products often have a greater carbohydrate and lipid content than their gluten containing equivalents. Segura *et al.* analysed the nutritional composition of a range of GF breads and found that these are starchy-based foods with a high glycemic index (estimated between 83.3 and 96.1 vs. 71 for white wheat flour bread), with low protein and high fat content (Segura *et al.*, 2011). With regards to lipid content and composition, Caponio and co-workers showed that commercially available GF biscuits are richer in saturated fatty acids compared to the gluten containing equivalents (Caponio *et al.*, 2008). In a recent paper the nutritional compositions of 206 GF rendered products and 289 equivalent foods with gluten were compared (Miranda *et al.*, 2014). It was found a great nutritional content variability between GF rendered foods and their gluten-containing counterparts, mostly in the bread category. Indeed, GF breads had a great divergences in protein compositions, almost a third less protein than their equivalent with gluten, and in fat composition providing twice as much fat, which was mainly saturated fat (Fig. 10). The authors explained these findings because of the formulations of GF breads, whose ingredients provide less protein but more fat, and in the case of lipid fraction, this includes saturated fatty acids and cholesterol, which may indicate the frequent use of raw animal substances. The group of pasta had a similar nutrient profile than breads, presenting lower protein content and a greater amount of total and saturated fat than their equivalents with gluten; additionally, this group of GF products had more sodium and less fiber (Miranda *et al.*, 2014) (Fig. 10). As regards micronutrient and mineral contents, it has been shown that some commercially available GF products have a lower content of folates, iron and B vitamins or are not

Content	Foodstuff	Flour			Cereal bars			Pasta			Breads			Dough/Pastry/Pizza			Bakery		
		Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P
Energy (kJ)	With gluten	1,428	57.09	0.02	1,739	118	0.05	1,357	269	0.53	1,222	296	0.03	1,243	272	0.00	1,787	246	0.01
	Gluten-free	1,493	38.4		1,550	114		1,298	374		1,385	320		1,591	415		1,658	305	
Protein (g)	With gluten	9.77	1.69	<0.00	6.86	1.68	0.30	11.4	1.86	0.02	10.0	2.16	<0.00	2.00	1.71	<0.00	6.38	1.47	0.00
	Gluten-free	1.43	0.79		5.67	1.15		9.00	3.42		3.47	2.83		6.30	1.15		3.79	2.16	
Total carbohydrates (g)	With gluten	71.3	3.94	<0.00	64.0	8.79	0.14	64.9	11.90	0.951	55.8	12.3	0.08	59.5	18.7	0.31	53.0	8.72	0.04
	Gluten-free	82.7	4.27		55.3	2.31		65.1	18.56		61.2	14.8		54.1	18.1		49.3	9.95	
Simple carbohydrates (g)	With gluten	4.87	8.15	0.60	30.0	5.26	0.15	2.89	1.56	0.00	4.77	3.48	0.92	12.9	14.1	0.12	22.8	11.2	0.11
	Gluten-free	3.00	4.65		25.0	0.00		0.93	2.16		4.86	3.32		21.6	22.2		18.9	10.1	
Total lipids (g)	With gluten	1.61	0.65	0.66	13.1	5.40	0.39	2.16	0.69	0.03	3.86	2.15	0.00	4.83	5.08	<0.00	21.6	7.31	0.24
	Gluten-free	1.43	1.27		9.67	5.77		4.47	3.56		7.42	7.71		15.3	12.7		19.9	7.97	
Saturated lipids (g)	With gluten	0.08	0.28	0.33	5.43	2.57	0.72	0.35	0.49	0.02	0.85	0.66	<0.00	1.50	2.13	<0.00	8.67	5.61	0.05
	Gluten-free	0.28	0.49		6.00	.00		1.71	1.86		3.03	3.55		5.80	5.08		6.57	4.65	

Content	Foodstuff	Baby formulas			Flour			Cereal bars			Pasta			Cookies			Dough/Pastry/Pizza			Bakery		
		Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P	Mean	SD	P
Fiber (g)	With gluten	3.82	1.19	0.02	5.23	3.39	0.11	6.57	5.29	0.03	4.38	1.98	0.00	3.11	2.06	0.56	2.18	1.88	0.35	3.06	2.41	0.83
	Gluten-free	2.32	2.14		2.86	1.86		15.3	2.89		1.93	1.33		2.84	1.62		3.03	3.51		3.18	3.13	
Sodium (mg)	With gluten	17.5	15.6		0.00	0.00	0.04	0.00	0.00	0.03	56.4	103	0.01	327	168	0.00	342	307	0.01	248	112	0.01
	Gluten-free	-	-		525	530		177	63.5		283	298		186	157		145	229		414	362	
Salt (mg)	With gluten	43.9	38.8		0.00	0.00	0.04	0.50	0.71	0.00	141	257	0.01	817	421	0.00	856	767	0.01	608	292	0.00
	Gluten-free	-	-		1,312	1,324		350	0.00		707	745		466	392		362	573		1,035	904	
Cholesterol (mg)	With gluten	-	-		-	-		1.33	1.53		31.7	37.8	0.01	37.0	27.3	0.64	121	122	0.47	120	61.8	0.00
	Gluten-free	-	-		-	-		-	-		2.00	0.00		27.5	35.4		26.0	27.4		43.6	41.0	

Figure 10. Nutritional contents of GF products compared with the equivalent foods with gluten (Miranda *et al.*, 2014).

consistently enriched/fortified compared to their gluten containing counterparts. Thompson conducted two studies on US commercially available GF products. The author analysed the folate, iron and B vitamins (thiamine, riboflavin and niacin) composition of these products and compared them with the composition of their gluten containing counterparts, finding that GF products were significantly lower in folates, iron and B vitamins (Thompson *et al.*, 1999; Thompson *et al.*, 2000). Some studies have reported that GFD is associated with a lower intake of dietary fiber than gluten containing diet. A study conducted in the USA on adults showed that the diet of CD patients was low in fiber intake (Wild *et al.*, 2010). This phenomenon is likely to be related to the composition of many GF foods made with starches and/or refined flours with low content in fiber. Indeed, during the refining process, the outer layer of grain containing most of the fiber is removed, leaving the starchy inner layer. All these characteristics have a negative effect on health, and this should be seriously taken into account, because the limited choice of food products in the diet of children with CD induces a high consumption of packaged GF products, such as snacks and biscuits.

The higher fat intake of GF foods could be due to the ingredients used for an optimization of the texture and/or palatability of GF foods, but this condition can lead to the problems found in celiac patients. Indeed, already in 1996 it has been observed by Rea *et al.* that following a GFD resulted in an excess in energy, animal protein, and lipid intake, thus being responsible for the high percentage of overweight celiac children (Rea *et al.*, 1996). Subsequently, Hopman *et al.* studied the nutrient intake of 37 adolescents with CD (aged 13-16 years) following a strict GFD and compared the intakes with a control group in the same age category. In the CD group, the intake of saturated fat was significantly higher than recommended by the American RDAs (Hopman *et al.*, 2006). Furthermore, a study conducted by Valletta *et al.* showed that the frequency of being overweight in children with CD was nearly doubled after one year of GFD (Valletta *et al.*, 2010).

Recently, because of these data, it has been started to analyse if there is a correlation between GFD and the CVD risk factors. In a recent paper of Norsa and co-workers, three or more concomitant CVD risk factors (like body mass index, waist circumference, low density lipoprotein cholesterol, triglycerides, blood pressure and insulin resistance) were identified in 14% of children with CD on a GFD. Only 30.7% of the cohort did not have any risk factors. The most common CVD risk factors were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%). Also, in

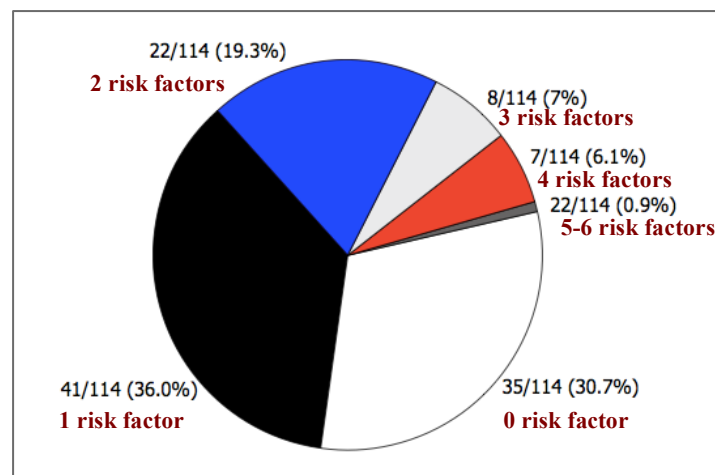


Figure 11. Risk factors for cardiovascular disease in pediatric patients with celiac disease in serological remission on gluten-free diets (Norsa *et al.*, 2013).

the present paper the authors demonstrated that GFD increases the prevalence of overweight and obesity in children with CD. Moreover, they showed that LDL cholesterol was the third most prevalent CVD risk factor in celiac children after at least one year of a GFD. They explain that this may be the result of a tendency in adult and adolescent patients to consume gluten-free products with high fat contents in order to compensate for the withdrawal of common gluten-containing carbohydrates from the diet. In addition, they found for the first time the presence of insulin resistance in CD children, although they specified that due to the lack of insulin levels before CD diagnosis, they were unable to assess whether such insulin resistance is directly related to the introduction of a GFD (Norsa *et al.*, 2013). However, previous publications have reported that available gluten-free products (e.g., gluten-free bread, pasta, pizza, etc.) have much higher glycemic indices than their gluten-containing equivalents, ingestion of which may lead to increased secretion of insulin (Berti *et al.*, 2004). In another recent paper, De Marchi and colleagues found a hyperhomocysteinemia in the majority of the studied CD patients, which persisted after gluten withdrawal (De Marchi *et al.*, 2013). Homocysteine is an amino acid in the blood. Too much of it is related to a higher risk of coronary heart disease, stroke and peripheral vascular disease (fatty deposits in peripheral arteries). Evidence suggests that homocysteine may promote atherosclerosis (fatty deposits in blood vessels) by damaging the inner lining of arteries and promoting blood clots. Folic acid and other B vitamins help break down homocysteine in the body. Homocysteine levels in the blood are strongly influenced by diet and genetic factors. Dietary folic acid and

vitamins B₆ and B₁₂ have the greatest effects. Several studies found that higher blood levels of B vitamins are related, at least in part, to lower concentrations of homocysteine. Other evidence shows that low blood levels of folic acid are linked with a higher risk of fatal coronary heart disease and stroke. So far, no controlled treatment study has shown that folic acid supplements reduce the risk of atherosclerosis or that taking these vitamins affects the development or recurrence of cardiovascular disease. As mentioned above, some GF products were significantly lower in folates and B vitamins (Thompson *et al.*, 1999; Thompson *et al.*, 2000). Then, GFD could be considered a CVD risk factor also because it may cause hyperhomocysteinemia.

In conclusion, there is a body of data published on cardiovascular complications in patients with CVD, however conclusions of some studies are at odds with each other. Anyway, it is important to emphasize that a correct GFD could suppress in part these risks, so why not do it?

1.4 Omega-3 fatty acids and cardiovascular diseases

Fatty acids (FAs) are carboxylic acids with a typical RCOOH structure, containing a methyl end, a hydrocarbon chain and a carboxylic terminus. The two main categories are saturated and unsaturated carboxylic acids with carbon chains containing between 2 and 36 carbon atoms. Saturated FAs (SFAs) do not contain any double bonds and are thus saturated with hydrogen atoms. They cover a part of the resting energy expenditure of the body, and are found to increase total and LDL-cholesterol production as well as to decrease insulin sensitivity. Moreover, they tend to be associated with metabolic diseases. Unsaturated FAs contain one or more double bonds between the carbon atoms. Monounsaturated FAs (MUFAs) can occur both in *cis* and in *trans* configuration, relating to the isomeric conformation of the hydrogen atoms. Limited epidemiologic and experimental evidence, although controversial, suggests that *cis*-MUFA diets favourably influence cardiovascular risk (Ros, 2003). *Trans*-MUFAs show physicochemical properties close to those of SFAs, due to their rather straight shape. They are even twice as active in raising LDL-cholesterol and decreasing HDL-cholesterol than SFA (Booker *et al.*, 2008). Polyunsaturated FAs (PUFAs) contain two or more double bonds in the molecule, and can be classified in two main classes: the methylene-interrupted polyenes or omega (n-9, n-6, n-3) FAs, and the conjugated FAs. The latter class consists of FAs with two or more conjugated double bonds (alternation of single and double bonds). In the omega FAs, the numbers 3, 6 or 9 correspond with the location of the first double bond, counted from the methyl end, which is also known as the omega (ω) or n-end. In addition, n-7 FAs have also been described comprising both a monounsaturated FA, Palmitoleic acid, as well as the conjugated linoleic acids Rumenic and Vaccenic acid. N-3 and n-6 PUFAs have received most attention in the last years. Linoleic acid (LA, n-6) and α -linolenic acid (ALA, n-3) are the respective basic n-6 and n-3 precursor FAs which can be converted into the longer-chain (LC) metabolites, such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They are termed essential FAs (EFAs), since mammals completely depend on their dietary intake due to a lack of $\Delta 12$ and $\Delta 15$ desaturases, enzymes only present in marine algae and plants, hindering *de novo* synthesis (Pottel *et al.*, 2014).

LCPUFA biosynthesis starts with linoleic acid (LA n-6, 18:2 $\Delta 9,12$) which can be either desaturated at the $\Delta 6$ position to continue along the n-6 pathway, or is desaturated at the $\Delta 15$ position, to produce α -linolenic acid (ALA n-3, 18:3 $\Delta 9,12,15$) which progresses down the n-

3 pathway. Most of dietary LA and ALA is β -oxidized for energy provisions, only a small portion is actually converted to other LCPUFAs. The synthesis of LCPUFAs starts with $\Delta 6$ desaturation, which introduces a double bond between carbons 6 and 7 of LA and ALA, respectively forming γ -linolenic acid (GLA n-6, 18:3 $\Delta 6,9,12$) and stearidonic acid (SDA n-3, 18:4 $\Delta 6,9,12,15$). Elongases alternate with different desaturases, to insert an ethyl group at each step. $\Delta 6$ elongase leads to respectively dihomo- γ -linolenic acid (DGLA n-6, 20:3 $\Delta 8,11,14$) and eicosatetraenoic acid (ETA n-3, 20:4 $\Delta 8,11,14,17$). The latter are then further desaturated by $\Delta 5$ desaturase to yield respectively arachidonic acid (AA n-6, 20:4 $\Delta 5,8,11,14$) and eicosapentaenoic acid (EPA n-3, 20:5 $\Delta 5,8,11,14,17$). Subsequent elongase, desaturase and β -oxidation activity of EPA finally lead to docosahexanoic acid (DHA n-3, 22:6 $\Delta 4,7,10,13,16,19$) production. In humans, the dietary EFAs have different biochemical roles and are not interconvertible, meaning that n-6 LA and its metabolites cannot be metabolized to n-3 FAs. In addition, the conversion of parental ALA to EPA (conversion of 6 %) and DHA (3.8 %) is restricted and varies within individuals (Fig.12).

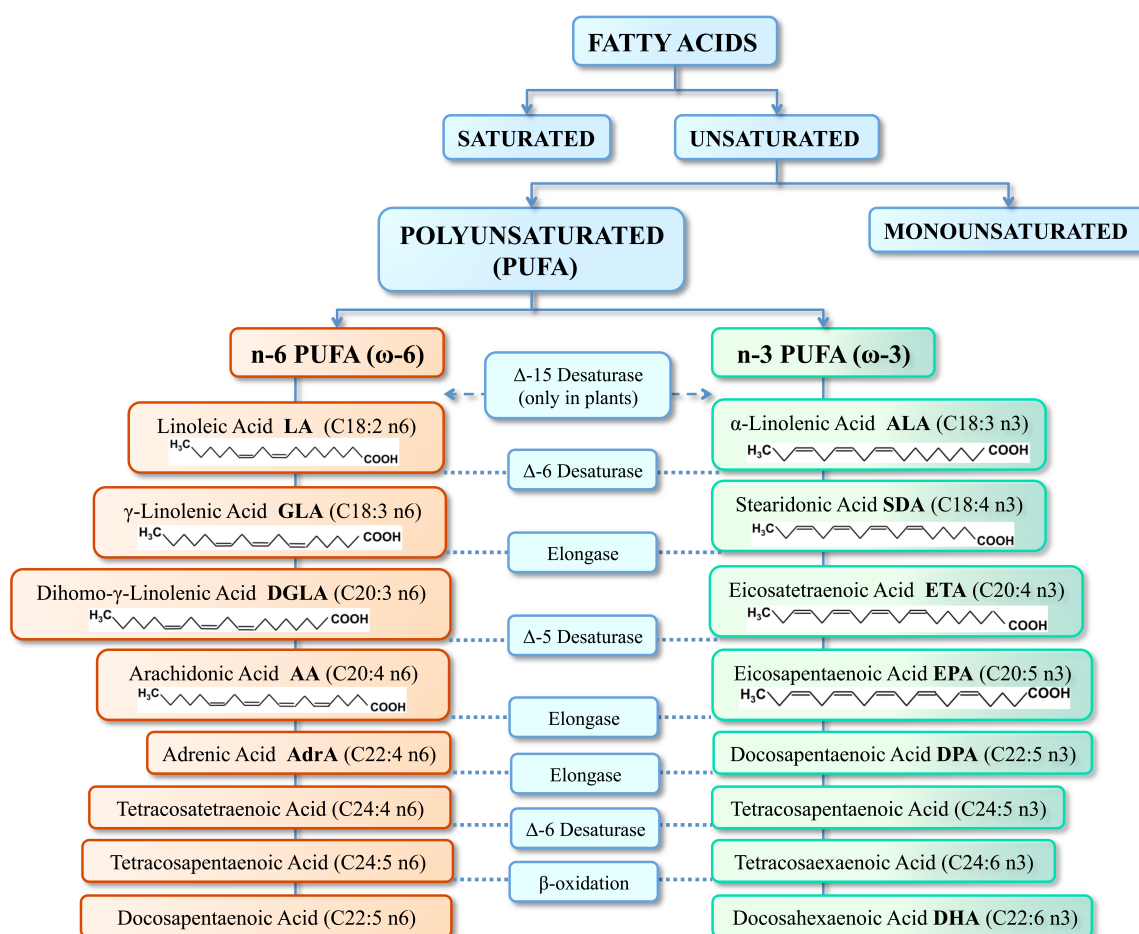


Figure 12. N-3 and n-6 PUFAs biosynthesis.

Sex hormones may account for the gender-specific differences in LCPUFA status, since estrogen and testosterone have been found to respectively stimulate and inhibit the conversion of fatty acids into their LC metabolites. Moreover, it has been reported a significantly greater precursor-product rate from plant-derived ALA to circulating n-3 LCPUFAs in non-fish-eaters compared to fish-eaters, suggesting an increased conversion. In addition, polyphenols, which are common constituents of plant-derived foods and major antioxidants in our diet, have been found to enhance LCPUFA metabolism (di Giuseppe *et al.*, 2009; Toufektsian *et al.*, 2011). Finally, a diet rich in n-6 FA can reduce the conversion rate as much as 50% due to the competition between n-6 and n-3 FAs for the same desaturation enzymes necessary for conversion (Pottel *et al.*, 2014).

In humans, FAs play a number of physiological roles. Most FAs act as energy substrates. About 25–35% of total energy intake in humans comes from dietary fat (Pottel *et al.*, 2014). Moreover, they play important and diverse roles in cellular and organelle membrane structure and function, tissue metabolism, and genetic regulation. With unique chemical structures and 3-dimensional configurations, n-3 PUFAs influence multiple relevant molecular pathways (Fig. 13), which individually or in sum might contribute to the observed effects on physiological risk factors and clinical events (Mozaffarian *et al.*, 2011).

Cellular and organelle functions are strongly influenced by membrane lipid environments. Lipid microdomains - for example, cholesterol and sphingolipid enriched “rafts” and caveolae in membranes - function as operational “platforms” to modulate numerous cellular functions, including signal transduction, protein and membrane trafficking, and ion channel kinetics. In cell culture and animal studies, the incorporation of n-3 PUFAs into membrane phospholipids alters the physicochemical properties of membrane rafts and caveolae, thereby influencing membrane-associated protein localization and function. Many such experimentally observed effects have been seen, including changes in caveolae-associated signaling protein H-Ras; suppression of protein kinase C- θ signaling and production of interleukin-2; and disruption of dimerization and recruitment of toll-like receptor-4 with subsequent inhibition of lipopolysaccharide-induced inflammation. Membrane-bound n-3 PUFAs might also enhance protein signaling efficiency as exemplified by the interaction between DHA and rhodopsin, a G-protein-coupled receptor critical in the visual system. Incorporation of n-3 PUFAs into cellular membranes with subsequent alteration of protein function and signaling might contribute to potential anti-inflammatory and anti-arrhythmic effects (Mozaffarian *et al.*, 2011).

In animal-experimental and *in vitro* studies, n-3 PUFAs directly affect myocyte electrophysiology (e.g., altering the function of membrane sodium channel, L-type calcium channel, and sodium–calcium exchanger). Such effects might contribute to reduced myocyte excitability and cytosolic calcium fluctuations, particularly in ischemic or damaged cells susceptible to partial depolarization and triggered arrhythmia. Accumulating evidence suggests that lipid microenvironments modulate ion channel function. Thus, as described previously, incorporation of n-3 PUFAs into and resultant changes in lipid membranes could contribute to effects on ion channels. Additionally, some evidence suggests that n-3 PUFAs might also directly interact with membrane channels and proteins. Whereas modulation of ion channels would be consistent with anti-arrhythmic effects seen in animal models and suggested by at least some human studies, the potential relevance of these experimentally observed influences on ion channels to health effects in humans is not established (Mozaffarian *et al.*, 2011).

Moreover, n-3 PUFAs are natural ligands of several nuclear receptors and transcription factors that regulate gene expression in multiple tissues. Non-esterified n-3 PUFAs or their acyl-CoA thioesters can bind and directly modulate activities of such receptors. Cytoplasmic lipid-binding proteins likely play important regulatory roles in this process by shuttling free fatty acids or fatty acyl-CoA into the nucleus to interact with the receptors. These receptors are central regulators of vital cellular functions, including lipid metabolism, glucose-insulin homeostasis, and inflammation. For example, effects of n-3 PUFAs on these pathways likely contribute to triglyceride-lowering and increased production of beneficial adipocytokines. N-3 PUFAs can also affect activation of transcription factors (Fig. 13). For example, by means of peroxisome proliferator-activated receptor- γ activation or reduced protein kinase-C translocation to the plasma membrane, n-3 PUFAs can reduce translocation of nuclear factor- κ B to the nucleus and inflammatory cytokine generation (Mozaffarian *et al.*, 2011).

Eicosanoids are bioactive lipid mediators derived from metabolism of PUFAs by cyclooxygenases, lipoxygenases, cytochrome P450s, and non-enzymatic pathways. Although the term “eicosanoids” has traditionally referred to the n-6 PUFA AA and its 20-carbon metabolites, it has also been applied to similar n-3 PUFAs-derived metabolites. N-3 PUFA consumption decreases production of AA-derived 2-series prostaglandins, thromboxanes, and 4-series leukotrienes in humans (Fig. 13). Because several AA-derived eicosanoids are considered to be pro-inflammatory or pre-thrombotic (e.g., leukotriene- B_4 , thromboxane- A_2), their lowering by n-3 PUFAs has been considered important for health benefits. Recently

identified classes of n-3 PUFA-derived eicosanoids (e.g., specialized pro-resolving mediators [SPMs] and CYP450-generated mono-epoxides from EPA and DHA [MEFAs]) possess unique bioactivities that might influence CVD (Fig. 13). Traditionally, it was thought that the

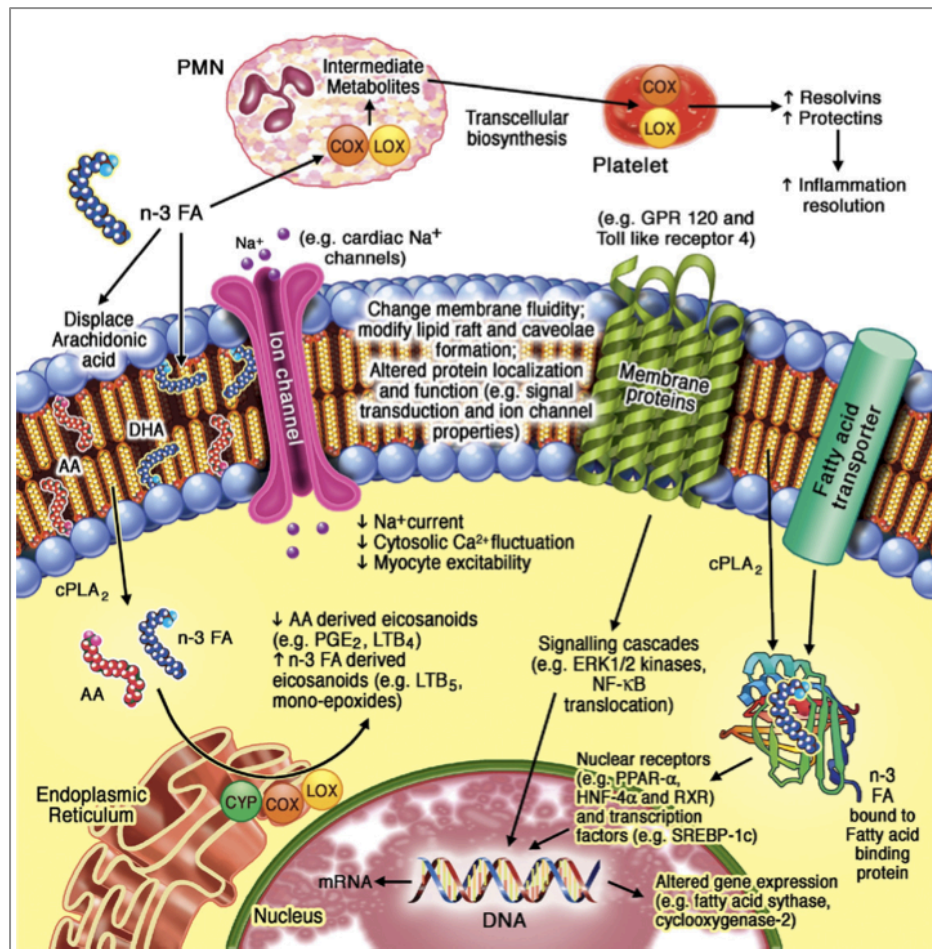


Figure 13. Molecular pathway affected by n-3 PUFAs (from Mozaffarian *et al.*, 2011).

breakdown of local pro-inflammatory mediators (e.g., prostaglandins, thromboxanes) was sufficient to end the inflammatory response. However, specific cellular “resolution programs” have recently been identified, the efficient functioning of which appears to be essential to ensure timely inflammation resolution and return to tissue homeostasis. Both n-3 PUFA-derived SPMs, such as resolvins, protectins, and maresins, and AA-derived lipoxins are key drivers of these resolution programs. SPMs and lipoxins reduce chronic inflammation in a range of animal models. MEFAs are potent vasodilators, modulate several ion channels, and reduce inflammation *in vitro*, with similar or stronger potency than analogous AA-derived eicosanoids. In recent experiments, n-3 PUFA-derived MEFAs possessed nearly 1,000-fold greater potency than their parents EPA or DHA in reducing effects of calcium

overload in rat ventricular myocytes; interestingly, AA-derived eicosanoids antagonized this effect. Short-term n-3 PUFA consumption (4 g/day for 4 weeks) increased EPA- and DHA-derived MEFA by $\cong 5$ - and 2-fold, respectively. Robust effects of SPMs and MEFA in multiple tissues and animal models suggest that they could play a key role in cardiovascular protection (Mozaffarian *et al.*, 2011).

Observational studies first reported in the 1970s showed that the Greenland Inuit population had a low incidence of coronary artery disease that was related to their distinctive dietary habits. The traditional Greenland Inuit diet derived its main energy source from cold-water marine fish and arctic mammals such as seals and whale. Thus in comparison with a typical Western diet, it contained much higher protein and fat (approx. 40% energy) and lower carbohydrates, but also substantially higher amounts of n-3 polyunsaturated fats, particularly EPA and DHA, rather than omega-6 polyunsaturated fats such as linolenic acid and arachidonic acid that are characteristic of Western diets. Indeed, although a ratio of omega-6/omega-3 fatty acids of about 4-1:1 is regarded as optimal for the prevention of different pathologies, Western diets often contain a ratio of 10-25:1, indicating a considerable omega-3 fatty acids deficiency (Simopoulos, 2011). The differences in dietary fats were consistent with the plasma fatty acids profile of the Inuit population. The metabolic consequences of the Inuit diet included a more favourable lipid and lipoprotein profile, typically lower levels of triglycerides, cholesterol, LDL-cholesterol and very low density lipoprotein cholesterol (VLDL-C), and higher concentrations of HDL-cholesterol; prolongation of bleeding times; reduced platelet aggregability and thrombosis; and altered eicosanoid profile due to the higher plasma EPA and reduced arachidonic acid levels (Mori, 2014).

From this observation many clinical and epidemiological studies provide support that the n-3 PUFAs EPA and DHA from fish and fish oils are cardioprotective, particularly in the setting of secondary prevention. Indeed, it has been demonstrated that EPA and DHA beneficially affect multiple cardiometabolic risk factors including blood pressure and cardiac function, arterial compliance, vascular reactivity and lipids, as well as having antiplatelet, anti-inflammatory, pro-resolving and anti-oxidative actions.

N-3 fatty acids reduce triglycerides in a dose-dependent manner across a wide range of n-3 fatty acid consumption. Mozaffarian *et al.* determined that plasma triglycerides are reduced by 0.33 mmol/L for a 1g per day increase in EPA plus DHA (Mozaffarian *et al.*,

2011). Reduced hepatic very low-density lipoprotein synthesis contributes to this effect, with implicated mechanisms including reduced fatty acid availability for triglyceride synthesis due to decreased *de novo* lipogenesis (DNL) (the process of converting carbohydrates into fat), increased fatty acid β -oxidation, and reduced delivery of non-esterified fatty acids to the liver; reduced hepatic enzyme activity for triglyceride synthesis; and increased hepatic synthesis of phospholipids rather than triglycerides. Triglyceride-lowering is linearly dose-dependent across a wide range of consumption but with variable individual responses, including greater absolute reductions among individuals with higher baseline levels (Fig. 14). At typical dietary doses, only modest triglyceride-lowering occurs and it is unlikely that this contributes appreciably to the reduced clinical risk seen with lower-dose fish oil supplements in randomized trials or habitual fish consumption in observational studies. Conversely, accrued modest benefits of reduced hepatic DNL, sustained over time from habitual n-3 PUFA consumption, could partly contribute to lower cardiovascular risk, for example mitigating development of hepatic steatosis and hepatic insulin resistance.

N-3 PUFA consumption reduces resting heart rate (HR). Increased HR is a risk factor for cardiovascular death, particularly sudden death. Experimental studies suggest that HR lowering could result from direct effects on cardiac electrophysiological pathways, possibly mediated by effects on cardiac myocytes, autonomic nerve function or β -adrenoreceptor activity. N-3 PUFA might also lower HR by more indirect effects, such as by improving left ventricular diastolic filling or augmenting vagal tone. In short-term trials, n-3 PUFA consumption increases nitric oxide production, mitigates vasoconstrictive responses to norepinephrine and angiotensin II, enhances vasodilatory responses, and improves arterial compliance. Such effects could contribute to lowering of systemic vascular resistance and blood pressure (Mozaffarian *et al.*, 2011) (Fig. 14).

Randomised controlled trials in which participants have been provided fish meals or supplemented fish oils unequivocally demonstrate that n-3 fatty acids lower blood pressure. Three meta-analyses have investigated the effect of n-3 fatty acids on blood pressure. In an analysis of 31 placebo controlled trials involving 1536 subjects, Morris and co-workers showed a reduction in blood pressure of -3.0/-1.5 mmHg with an average dose of 4.8g per day n-3 fatty acids. The blood pressure-lowering effects were strongest in treated and untreated hypertensive individuals (a fall of -3.4/-2.0 mmHg) whereas there was no significant change in normotensive subjects. The authors predicted blood pressure was reduced -0.66/0.35 mmHg for each 1 g per day increase in n-3 fatty acids (Morris *et al.*,

1993). Appeal *et al.*, reported blood pressure fell -5.5/-3.5 mmHg in untreated hypertensive individuals and -1.0/-0.5 mmHg in normotensive with an average consumption of more than 3g per day of n-3 fatty acids (Appeal *et al.*, 1993). Finally, Geleijnse *et al.* examined 36 trials that included approximately 50% of participants with hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg), a mean trial duration of 11.7 years and a median dose of 3.7 g per day n-3 fatty acids. Overall, n-3 fatty acids reduced blood pressure by -2.1/-1.6 mmHg. The greatest effects on blood pressure were observed in individuals older than 45 years (a fall of -3.5/-2.4 mmHg) and those that were hypertensive ($\geq 140/90$ mmHg) (a fall of -4.0/-2.5 mmHg) (Geleijnse *et al.*, 2002).

The inhibitory effects of n-3 fatty acids on thrombosis are thought to account partially for the associated reduced risk for sudden cardiac death and reduced all-cause mortality. The antithrombotic and antiplatelet effects of n-3 fatty acids are due to modulation of vasodilator and vasoconstrictor prostanoids. Reduced platelet aggregation in vitro following n-3 fatty acids, particularly at large doses, is likely due to suppressed production of thromboxane A₂ (TXA₂) a vasoconstrictor and aggregator. Moreover, it has been suggested that an overall increase in total prostacyclin (PGI₂ and PGI₃) formation in conjunction with reduced total thromboxane (TXA₂ and TXA₃), could favourably alter thrombotic, endothelial and vascular responses following dietary n-3 fatty acids. In spite of the in vitro evidence that n-3 fatty acids have antithrombotic effects, it is currently thought that any benefits are more likely related to their antiarrhythmic rather than antithrombotic effects. This hypothesis is supported with the knowledge that cardiovascular clinical benefits of n-3 fatty acids have been noted with doses as low as 1g per day whilst changes in platelet aggregation have generally been observed (Mori, 2014).

Several trials have demonstrated improved flow-mediated arterial dilation, a measure of endothelial function and health, after n-3 PUFA supplementation. Because endothelial health is strongly linked to endothelial nitric oxide synthesis, experimental effects of n-3 PUFA on related biomarkers provide plausible biological mechanisms for such effects. Several although not all trials have also found that n-3 PUFA consumption lowers circulating markers of endothelial dysfunction, such as E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1. Thus, normalization of endothelial function could partly mediate protective effects of n-3 PUFA against CVD. Observational studies and small trials of n-3 PUFA and HR variability - a marker of autonomic function, circadian rhythms, and underlying cardiac health - have produced mixed findings, perhaps owing to variable

statistical power, n-3 PUFA dosing, durations of consumption, and methods for HR variability assessment. Overall, these studies suggest that n-3 PUFA might improve autonomic function, especially related to augmentation of vagal activity or tone (Mozaffarian *et al.*, 2011).

Among the most intriguing potential physiological effects of n-3 PUFA and also among the most challenging to document in humans is anti-arrhythmia. *In vitro* and animal experiments suggest that n-3 PUFA directly influence atrial and ventricular myocyte electrophysiology, potentially mediated by effects on membrane ion channels or cell–cell connexins. Confirmation of such effects in humans has been limited by absence of reliable physiological measures or biomarkers to quantify antiarrhythmic potential. In observational studies, n-3 PUFA consumption reduced risk of sudden cardiac death, suggesting that anti-arrhythmic effects seen in experimental studies could extend to humans. Overall, although evidence from *in vitro* studies, animal-experiments, and at least some human studies remains compelling, confirmation of clinically relevant anti-arrhythmic effects of n-3 PUFA has remained elusive (Mozaffarian *et al.*, 2011).

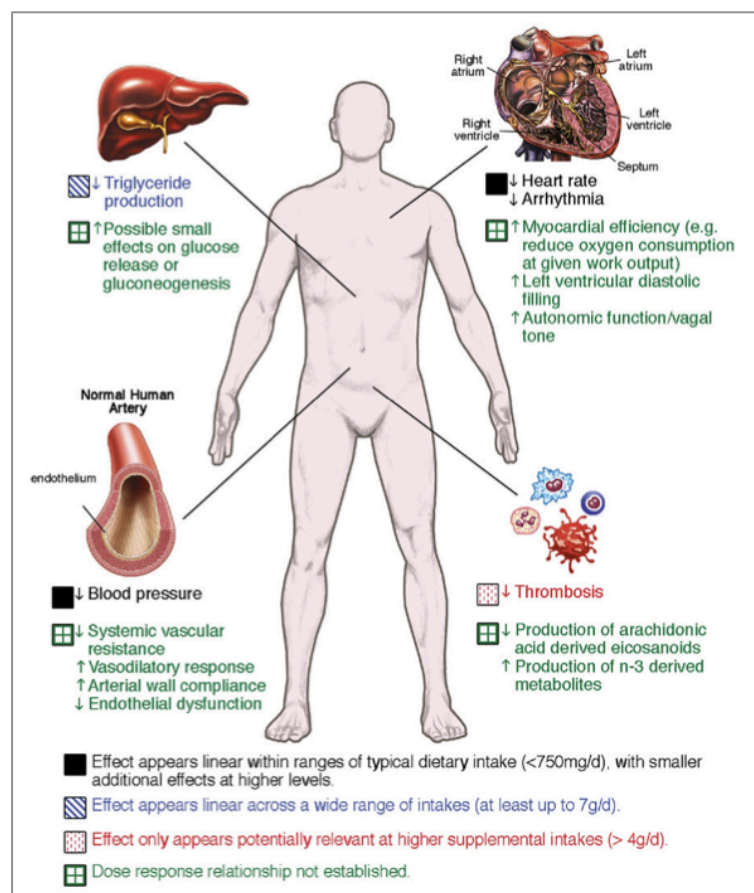


Figure 14. Physiological effects of n-3 PUFA that might influence CVD risk (from Mozaffarian *et al.*, 2011).

Alpha-linolenic acid (ALA) (18:3n-3) is the plant-derived n-3 fatty acid found in a relatively limited set of seeds, nuts, and their oils. ALA cannot be synthesized in humans and is an essential dietary fatty acid. Biochemical pathways exist to convert ALA to EPA and EPA to DHA, but such endogenous conversion is limited in humans: between 0.2% and 8% of ALA is converted to EPA (with conversion generally higher in women) and 0% to 4% of ALA to DHA (Burdge, 2004). Thus, tissue and circulating EPA and DHA levels are primarily determined by their direct dietary consumption.

There have been many epidemiological and clinical studies on the cardiovascular-protective effects of ALA. ALA intake can affect human coronary heart disease. Indeed, Djousse *et al.* (2005) showed that the intake of ALA reduced coronary artery calcification in their extensive multivariate model. The odds ratios for calcified plaque were reduced gradually by 65% from the lowest quintile of ALA intake to the highest (0.89 g/day), supporting a high correlation between the intake of ALA and anti-atherosclerotic effects. Higher intake of ALA may have reduced the risk of non-fatal acute myocardial infarction among 482 cases and it provides important protection against cardiovascular disease (Baylin *et al.*, 2003). Lowered plasma triacylglycerides were observed by dietary intake of ALA (0.81 and 0.69 g/day for the men and the women, as means) (Djousse *et al.*, 2003). Correlation between dietary intake of ALA and the risk of fatal coronary heart disease was studied to clarify the favourable effects of ALA. Prospective examination of the relationship between dietary ALA intake and the risk of sudden cardiac death, other coronary heart disease, and non-fatal myocardial infarction among 76,763 women showed an inverse association of the ALA intake with the risk of sudden cardiac death, but not with other coronary heart diseases, and non-fatal myocardial infarction during 18 years of a follow-up study. Five quintiles (0.37, 0.45, 0.52, 0.60, and 0.74 ALA% energy) of ALA intakes were designed. Women taking ALA in the second highest quintile showed a 38–40% reduced sudden cardiac death risk and the decreased risk appeared to be linear in nature. The authors concluded that their prospective data showed an inverse association between intake of ALA and sudden cardiac death risk among women (Albert *et al.*, 2005). Ascherio *et al.* (1996) reported that a 1% (% energy) increase in intake of ALA was associated with a 40% reduction in risk of non-fatal coronary artery disease in 43,757 subjects studied between 1986 and 1992. A meta-analysis of 27 studies, including a total of 251,049 subjects, concluded that a higher intake of ALA was related to a moderately lower risk of cardiovascular disease (Pan *et al.*, 2012). A case-control study for 10 years (1994–2004) concluded that the dietary intake

of ALA helped increase cardiovascular protection by lowering the risk of myocardial infarction in Costa Rica (Campos *et al.*, 2008). A cross-sectional study including 4584 subjects reported that there was an inverse relationship between the dietary intake of ALA and the risk of coronary artery disease (Djousse *et al.*, 2001). The dietary intake of ALA-enriched foods increased plasma ALA levels and induced the beneficial effects of lowering cholesterol or triglycerides (Bloedon *et al.*, 2008; Cunnane *et al.*, 1993; Dodin *et al.*, 2008; Egert *et al.*, 2009 a; Sioen *et al.*, 2009). Hypercholesterolemic adults taken 40g/day of ground flaxseed-containing baked products for 10 weeks showed a modest but short lived low density lipoprotein cholesterol lowering effect (Bloedon *et al.*, 2008). Fifty g/day of ground, raw flaxseed for 4 weeks lowered serum total cholesterol and low density lipoprotein cholesterol in healthy female volunteers (Cunnane *et al.*, 1993). Consumption of 40 g/day of flaxseed for 12 months increased ALA, EPA, and DHA in plasma in 199 healthy menopausal women (Dodin *et al.*, 2008). Intake of 4.4 g/ day of ALA for 6 weeks decreased fasting serum triacylglycerol concentration in 74 healthy volunteers (Egert *et al.*, 2009 a). ALA-enriched food supply (2.8–5 g/day of ALA) for 12 weeks decreased diastolic blood pressure in 59 healthy males (Sioen *et al.*, 2009). ALA is able to improve the pathological condition in model animals like spontaneously hypertensive rats (SHRs), by decreasing the systolic blood pressure and the Angiotensin Converting Enzyme (ACE) activity and expression, but also increasing the vasodilators level (Ogawa *et al.*, 2009; Sekine *et al.*, 2007). Moreover human studies have shown that ALA-enriched bread decrease systolic and diastolic blood pressure in mild hypertensive subjects, without changing the oxidative stress parameters, then without adverse effect (Takeuchi *et al.*, 2007). Although there is much evidence to associate the protective effect of ALA intake with cardiovascular diseases, other evidence shows no association of the ALA intake with the risk or risk reduction of cardiovascular diseases. Therefore, ALA might have benefits for cardiovascular health, but the overall evidence is still inconclusive. Thus, plant sources of n-3 fatty acids cannot currently be considered as a replacement for seafood-derived n-3 PUFA. Additional studies of ALA's effects are urgently needed, because of the lower cost and greater potential global supply of ALA as opposed to EPA+DHA.

In addition to potential cardiovascular benefits of fish consumption, concerns have been raised over potential harm from contaminants present in some fish species, such as methylmercury, dioxins, and polychlorinated biphenyls (PCBs). In most fish species, mercury levels are quite low; selected few species contain moderate levels (e.g., albacore

tuna, approximately 0.36 µg/g) or higher levels near the U.S. Food and Drug Administration action level of 1 µg/g (e.g., tilefish, swordfish, shark, Gulf of Mexico King mackerel). The environmental impact and long-term sustainability of aquaculture and commercial fishing are also relevant reason to search alternative sources of n-3 PUFAs (Mozaffarian *et al.*, 2011).

1.5 The lipid peroxidation problem

Free radicals were first described by Moses Gomberg more than a century ago. For a long time they were not considered to present in biological systems due to high reactivity and consequently short living time. More than 30 years later, Leonor Michaelis proposed that all oxidation reactions involving organic molecules would be mediated by free radicals (Lushchak, 2014). The term “free radicals” means any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Lobo *et al.*, 2010).

In living organisms under aerobic conditions more than 90% of oxygen consumed is reduced directly to water by cytochrome oxidase in electron-transport chain (ETC) via four-electron mechanisms without reactive species release. However, much less than 10% of oxygen consumed is reduced via one-electron successive pathways resulting in conversion of molecular oxygen to superoxide anion radical ($O_2^{\bullet -}$) followed by one-electron reduction with concomitant accepting of two protons to yield hydrogen peroxide (H_2O_2). Hydrogen peroxide molecule accepting one more electron is split up to hydroxyl radical ($HO^{\bullet}$) and hydroxyl anion (OH^-) (Fig.15). Finally, $HO^{\bullet}$ interacts with one more electron and proton resulting in formation of water molecule. In biological systems, this reaction is mainly realized through abstraction of hydrogen atom from different compounds such as proteins and lipids resulting frequently in initiation of chain processes. Summarizing, $O_2^{\bullet -}$, H_2O_2 , and $HO^{\bullet}$ collectively are called reactive oxygen species (ROS), but only $O_2^{\bullet -}$ and $HO^{\bullet}$ are free radicals, whereas H_2O_2 is not.

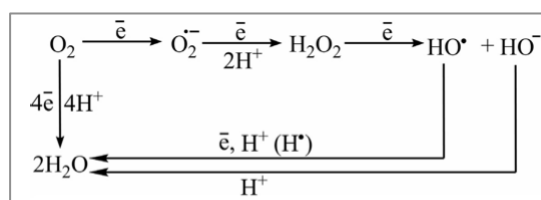


Figure 15. Reduction of molecular oxygen via four- and one-electron schemes (from Lushchak, 2014).

ROS include not only mentioned above $O_2^{\bullet -}$, H_2O_2 , and $HO^{\bullet}$, but also diverse peroxides, like lipid peroxides, and peroxides of proteins, and nucleic acids. Moreover, their homeostasis is

closely related to many other reactive species (RS), such as reactive carbonyl species (glyoxal, methylglyoxal). There are also many other RS of nitrogen (nitric oxide, peroxyxynitrite, and related compounds), carbon, sulfur, halogens, etc. (Lushchak, 2014).

As regards the ROS generation, it has been established that in eukaryotic cells over 90% of them are produced by ETC in mitochondria. Moreover, several oxidases are also rather powerful ROS producers. They oxidize carbohydrates, aldehydes, amino acids, heterocyclic compounds and others (e.g xanthine oxidase). There is also one more ROS source related to autoxidation of different small molecules of endo- and exogenous origin. Epinephrine (adrenalin) and norepinephrine provide a good example of endogenous molecules subjected to autoxidation coupled with ROS production, whereas many xenobiotics, especially different homo- and heterocyclic compounds, are clearly related to this sort of ROS generators (Lushchak, 2014).

The control of ROS steady-state level is provided not only via their production, but also via elimination. Living organisms possess multilevel and complicated antioxidant system operating either to eliminate ROS, or minimize their negative effects. There are several approaches to classify these systems and the mostly appreciated one is based on molecular masses. According to this system, antioxidants are placed in two groups: low molecular mass antioxidants (usually with molecular masses below one kilodalton) and high molecular mass antioxidants (with molecular mass higher than one or actually higher than ten kilodaltons). The group of low molecular mass antioxidants includes chemically different compounds such as vitamins C (ascorbic acid) and E (tocopherol), carotenoids, anthocyanins, polyphenols, and uric acid. Most of them are received by human organism as food or supplement components. However, one very important antioxidant glutathione (tripeptide c-glutamyl-cysteinyl-glycine, GSH) is synthesized by most living organisms and used to control ROS level either via direct interaction with them, or serving as a cofactor for ROS-detoxifying enzymes. Instead, the group of high molecular mass antioxidants includes enzymatic systems able to eliminate ROS, such as: superoxide dismutase (SOD), catalase (EC), glutathione-dependent peroxidases (GPx), and the enzymes of pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), which maintain the pool of NADPH. The described above high molecular mass system of ROS elimination operate with $O_2^{\cdot-}$ and H_2O_2 , but not with $HO^{\cdot}$. Actually, there is no known enzymatic system to eliminate $HO^{\cdot}$ formed, which is probably related with its high reactivity (Lushchak, 2014).

As described above, living organisms possess finely regulated systems to maintain very

low ROS levels, i.e. their production and elimination are well balanced resulting in certain steady-state ROS level. However, under certain circumstances this balance can be disturbed. There are several reasons for that: increased level of endogenous and exogenous compounds entering autoxidation coupled with ROS production; depletion of reserves of low molecular mass antioxidants; inactivation of antioxidant enzymes; decrease in production of antioxidant enzymes and low molecular mass antioxidants; and, finally, certain combinations of two or more of the listed above factors. So, under normal condition, ROS level fluctuates in certain range defined by concerted operation of system of their generation and elimination. Instead, when ROS level sharply increases and leaves the range of control conditions, it occurs a situation of “oxidative stress”. This term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defences is unfavourable. Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defences, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids (Lobo *et al.*, 2010).

Among these targets, the peroxidation of lipids is basically damaging because the formation of lipid peroxidation products leads to spread of free radical reactions. The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination. The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Several species can abstract the first hydrogen atom and include the radicals: hydroxyl ($\text{HO}^\bullet$), alkoxyl ($\text{RO}^\bullet$), peroxy ($\text{ROO}^\bullet$), and possibly $\text{HO}_2^\bullet$ but not H_2O_2 or $\text{O}_2^{\bullet-}$. The membrane lipids, mainly phospholipids, containing polyunsaturated fatty acids are predominantly susceptible to peroxidation because abstraction from a methylene ($-\text{CH}_2-$) group of a hydrogen atom, which contains only one electron, leaves at the back an unpaired electron on the carbon, $^\bullet\text{CH}-$. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom nearby to the double bond and thus facilitates $\text{H}^\bullet$ subtraction. The initial reaction of $^\bullet\text{OH}$ with polyunsaturated fatty acids produces a lipid radical ($\text{L}^\bullet$), which in turn reacts with molecular oxygen to form a lipid peroxy radical ($\text{LOO}^\bullet$). The $\text{LOO}^\bullet$ can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical. The LOOH formed can suffer reductive cleavage by reduced metals, such as Fe^{2+} , producing lipid alkoxyl radical ($\text{LO}^\bullet$). Both alkoxyl and peroxy radicals stimulate the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms. Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes. Injure to

mitochondria induced by lipid peroxidation can direct to further ROS generation (Catalá, 2009) (Fig.16).

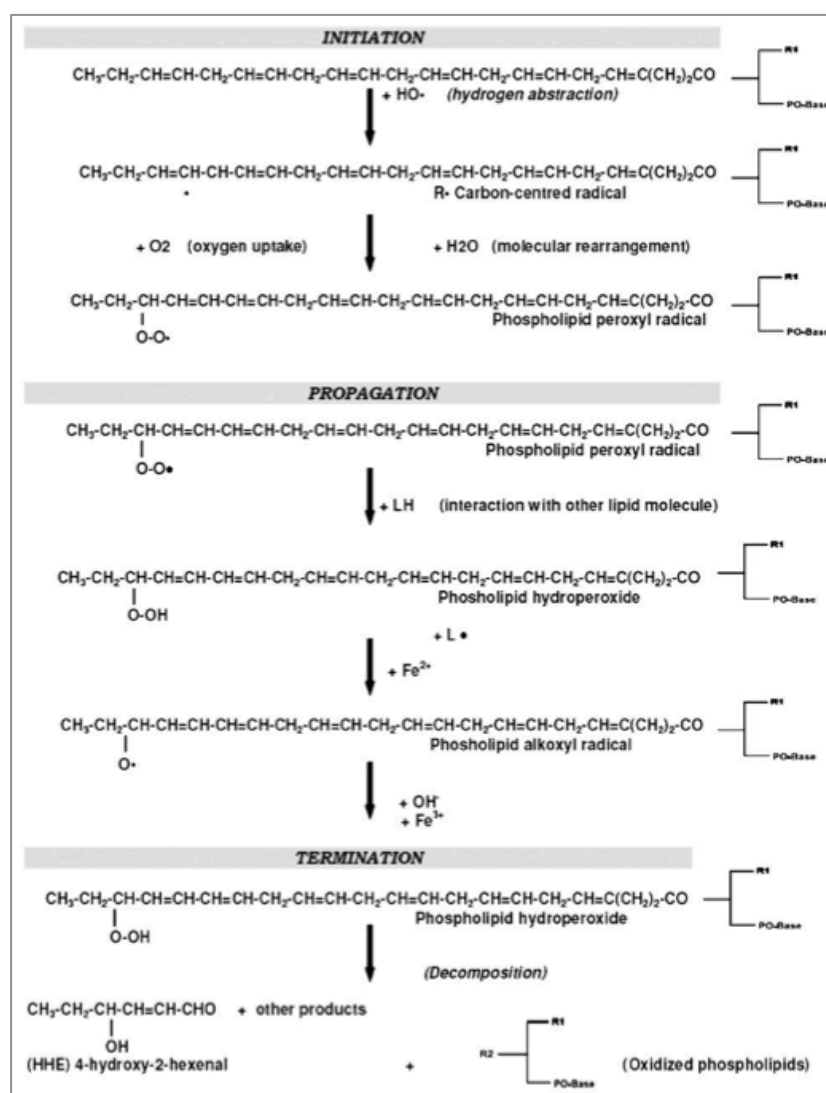


Figure 16. Chemical diagram of the steps in lipid peroxidation of phospholipids containing docosahexaenoic acid (22:6 n-3), R1= fatty acid, R2=fragmentation products of fatty acid oxidation (from Catalá, 2009).

Moreover, lipid peroxidation also propagates free radicals, which initially act locally with short half-lives. These local insults are followed by formation of more stable secondary breakdown products of lipid peroxides, such as alkanes, aldehydes, ketones, alcohols, and furans. Those products can then disseminate from their site of formation and diffuse across cellular membranes. Most secondary breakdown products of lipid peroxidation are reactive due to their electrophilic nature. They play powerful roles in cell signalling, mainly in cell

cycle regulation. The concentration of the secondary breakdown products can be quantified to assess the degree of *in vivo* lipid peroxidation. Some of those compounds include malondialdehyde (MDA), 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), F2-isoprostanes (F2-IsoPs)/8-isoprostaglandin F2 α , acrolein, hexenal, hydroxyoctadenoic acid (HODE), hydroxyeicosatetraenoic acid (HETE), 7 α -hydroxycholesterol, and 7 β -hydroxycholesterol. The *in vivo* concentrations of those specific lipid peroxidation biomarkers vary greatly as does the sensitivity of their assay methods. Although some lipid peroxidation biomarkers indicate the specific lipid substrate used to generate the measures breakdown product, others serve as an overall assessment of lipid peroxidation. For example, F2-IsoPs refer to a collection of compounds created by nonenzymatic, free radical attack of the arachidonic acid (AA) components in lipid membranes. Instead, the bulk of lipid peroxidation-derived MDA is produced by nonenzymatic, but also enzymatically, free radical peroxidation of PUFA containing at least three double bonds, such as AA or DHA, and serves as a biomarker for both n-3 and n-6 PUFA lipid peroxidation. In contrast to MDA which is correlated to both n-3 and n-6 PUFA, 4-hydroxy-2(E)-nonenal (HNE) is generated by autoxidation of n-6 PUFA and 4-hydroxy-2(E)-hexenal (HHE) is produced from autoxidation of n-3 PUFA. Alkenal species such as HHE and HNE are reactive substances and can be assayed by the unique adducts formed. For example, HNE readily reacts with cellular proteins forming adducts with cysteine, histidine, lysine, and serine (Kelley *et al.*, 2014).

Despite health benefits, there is concern that n-3 PUFA may increase oxidative stress due to their high degree of unsaturation. Indeed, because the most n-3 PUFA (ALA, EPA, DHA) have more double bonds than the most common n-6 PUFA (linoleic acid, LA, and arachidonic acid, AA), the n-3 PUFA would have a greater scavenging capacity than n-6 PUFA of the same chain length. Moreover, studies have shown that not only unsaturation but also the position of double bonds seems to be important in determining peroxidation of PUFA, and also for this issue, n-3 seems to be more effective than n-6 PUFA to act as scavenger molecules. There are many discrepancies among published results whether n-3 PUFA reduce or induce oxidative stress and lipid peroxidation. Indeed, from all the publications that have focused on the effects of n-3 PUFA on lipid peroxidation in humans, 27% reported an increase, 32% a decrease, and 41% no change in lipid peroxidation. In a recent review article, where the authors analysed results from 22 published human studies

regarding the effects of n-3 PUFA supplementation on markers of lipid peroxidation, 9 found no change, 8 a decrease, and 5 an increase in markers of lipid peroxidation (Kelley *et al.*, 2014). However, most of these analysed studies concern n-3 PUFA supplementation in association with antioxidant, mostly α -tocopherol used to prevent rancidity prior to ingestion. Therefore, probably the observed effects in these studies are distorted by the presence of the antioxidant. Moreover, this effect could be even greater in pathological subjects, when an already present oxidized status would be increased by the presence of n-3 PUFAs. Further studies are needed in this area considering that n-3 PUFA are being recommended as components of healthy diets.

1.6 Dietary antioxidant: the flavonoid quercetin

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. In terms of food, an antioxidant has been defined as any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate. The human diet contains an array of different compounds that possess antioxidant activities or have been suggested to scavenge ROS based on their structural properties. Antioxidants can be classified into two major groups, i.e., enzymatic and non-enzymatic antioxidants. Some of these antioxidants are endogenously produced which include enzymes, low molecular weight molecules and enzyme cofactors. Among non-enzymatic antioxidants, many are obtained from dietary sources. Dietary antioxidants can be classified into various classes, of which polyphenols is the largest class. Polyphenols consist of phenolic acids and flavonoids. The other classes of dietary antioxidants include vitamins, carotenoids, organosulfur compounds and minerals (Fig. 17) (Venkat Ratnam *et al.*, 2006).

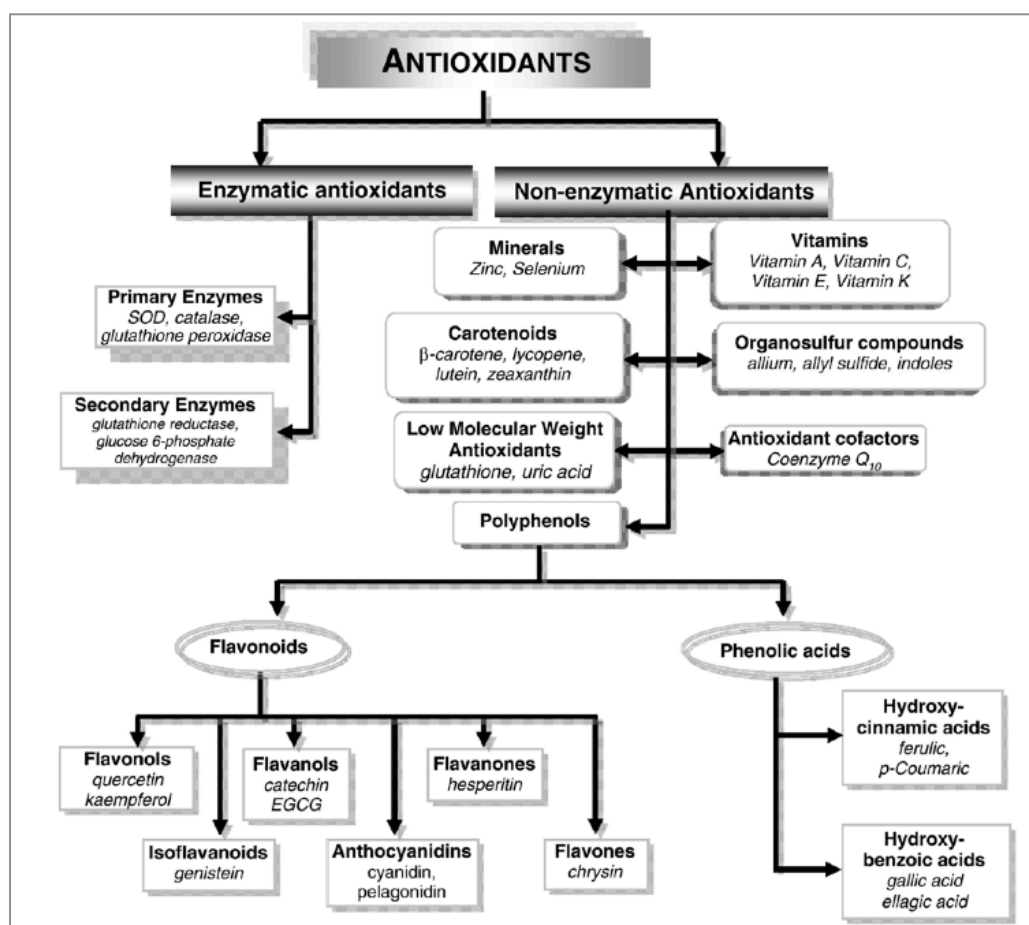


Figure 17. Classification of total antioxidants. Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ₁₀ are synthesized in the human body and they can also be derived from dietary sources (from Venkat Ratnam *et al.*, 2006).

Flavonoids are common components of plants. More than 8,000 polyphenolics, including over 4,000 flavonoids that have been identified in various plant species, have been isolated from almost all parts of the plant such as leaves, stems, roots, fruits or seeds. Flavonoids are formed in plants from the aromatic amino acids phenylalanine and tyrosine, and malonate. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings ($C_6-C_3-C_6$), which are labelled A, B and C (Fig.18). The presence of a double bond, a carbonyl and a hydroxyl group in the pyranyl ring C serves as a basis for their classification into several classes and subclasses. Substitution of A and B rings by hydroxyl groups distinguishes individual members of each class. The main subclasses of these $C_6-C_3-C_6$ compounds are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. Other flavonoid groups that are more minor dietary components are the chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones. The basic flavonoid skeleton can have numerous substituents. The majority of flavonoids occur naturally as glycosides rather than aglycones (Del Rio *et al.*, 2013).

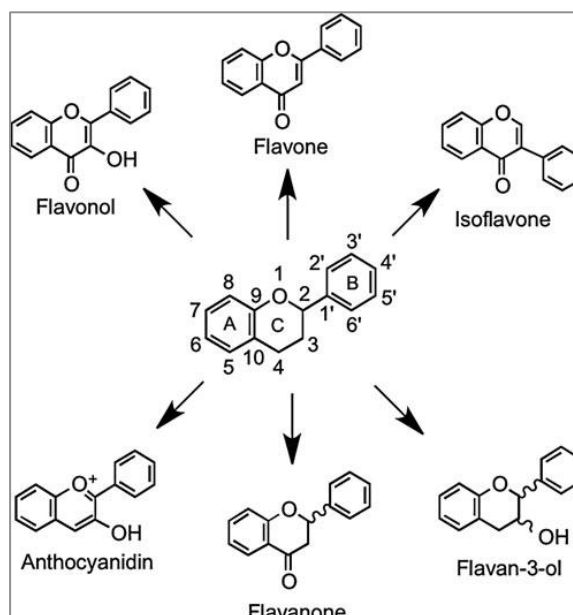


Figure 18. Structure of the flavonoid skeleton and the main subclasses (from Del Rio *et al.*, 2013).

Flavones, such as apigenin, luteolin, wogonin, and baicalein, are similar structurally to flavonols, except they lack oxygenation at C-3 (Fig. 18). A wide range of substitutions is possible with flavones, including hydroxylation, methylation, O- and C-glycosylation, and alkylation. In general, flavones are not distributed widely, although substantial amounts have been detected in celery (*Apium graveolens*), parsley (*Petroselinum hortense*), and some

herbs. Many flavones occur as 7-O-glycosides, although rooibos tea, a caffeine-free beverage prepared from leaves of the South African shrub *Aspalathus linearis*, contains small amounts of apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), luteolin-8-C-glucoside (orientin), and luteolin-6-C-glucoside (iso-orientin). Polymethoxylated flavones, such as nobiletin and tangeretin, occur in citrus species.

Isoflavones have the B-ring attached at C-3 rather than at the C-2 position (Fig. 18). They are found almost exclusively in leguminous plants with substantial quantities of daidzein and genistein occurring in soybean (*Glycine max*) principally as 7-O-(6"-O-malonyl)glucosides with lower amounts of the corresponding 7-O-(6"-O-acetyl)glucosides, 7-O-glucosides, and the aglycones. Fermented soy products can be rich in the aglycones as a result of hydrolysis of the glycosides, whereas products whose manufacture involves heating, such as soy milk and tofu, contain reduced quantities of isoflavones, mainly in the form of the daidzein and genistein glucosides, which form as a result of degradation of malonyl- and acetylglucosides. Because of their structural similarity to estrogen, isoflavones are classified as phytoestrogens, as are the non-flavonoid lignans, which are a diverse group of compounds that occur in high concentrations principally in cereal grains.

Flavanones such as naringenin and hesperetin are characterized by the absence of $\Delta^{2,3}$ double bond and the presence of a chiral center at C-2 (Fig. 18). In *planta* flavanones occur predominantly as the S- or (-)-enantiomer with the C-ring attached to the B-ring at C-2 in the α -configuration. Flavanones occur as hydroxyl, glycosylated, and O-methylated derivatives. They are present in especially high amounts in flavedo of citrus fruits. The most common flavanone glycoside is hesperetin-7-O-rutinoside (hesperidin). Flavanone rutinosides are tasteless, in contrast to flavanone neohesperidoside conjugates, such as hesperetin-7-O-neohesperidoside (neohesperidin) from bitter oranges (*Citrus aurantium*) and naringenin-7-O-neohesperidoside (naringin) from grapefruit (*Citrus paradise*), which have an intense bitter taste.

The most common anthocyanidin aglycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Fig. 18), which form conjugates with sugars and organic acids to generate a multitude of anthocyanins of differing colors, ranging from orange and red to blue and purple, and as a consequence, they are readily visible in fruits and flowers.

Flavan-3-ols, unusually, do not exist in *planta* predominantly as glycosides. They are the most complex subclass of flavonoids, ranging from the simple monomers to the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins.

The two chiral centers at C2 and C3 of the monomeric flavan-3-ol (Fig. 18) produce four isomers for each level of B-ring hydroxylation, two of which, (+)-catechin and (-)-epicatechin, are widespread in nature, while others such as (-)-epiafzelechin have a more limited distribution. Oligomeric and polymeric proanthocyanidins have an additional chiral center at C4 in the upper and lower units. Type B proanthocyanidins are formed from (+)-catechin and (-)-epicatechin by oxidative coupling between the C-4 of the upper monomer and the C-6 or C-8 of the adjacent lower or extension unit to create oligomers or polymers. Type A proanthocyanidins have an additional ether bond between C-2 in the B-ring of one monomer and C-7 in the A-ring of the other monomer. Proanthocyanidins can occur as polymers of up to 50 units. Proanthocyanidins that consist exclusively of (epi)catechin units are called procyanidins, and are the most abundant type of proanthocyanidins in plants.

Flavonols occur widely throughout the plant kingdom with the exception of fungi and algae. The most common flavonols, kaempferol, quercetin, isorhamnetin, and myricetin, are typically found as glycosides with conjugation occurring at the 5, 7, 3', 4', and 5' positions. Although the number of aglycones is limited, there are more than 200 sugar conjugates of kaempferol. There is information on the flavonol content of commonly consumed fruits, vegetables, and beverages with sizable differences in the amounts found in seemingly similar produce, possibly due to local growing conditions, seasonal changes, and varietal differences. Yellow and red onions (*Allium cepa*) are especially rich source of flavonols containing high concentrations of quercetin-4'-O-glucoside and quercetin-3,4'-O-diglucoside. The disaccharide quercetin-3-O-rutinoside is a common dietary component (Del Rio *et al.*, 2013).

Quercetin (3,3,4,5,7-pentahydroxyflavone) belongs to the flavonol subclass and is the most ubiquitous in the diet. Quercetin occurs abundantly in various plants, fruits, vegetables, teas and products made from these sources. It is estimated that 68-73% of flavonoids consumed in a typical Western diet is composed of quercetin (15-40 mg a day), primarily from apples and onions. Pure quercetin is an un-conjugated aglycone. However, in naturally occurring sources it primarily exists as quercetin glycosides. Quercetin forms the glycosides quercitrin and rutin by a 3-O glycosidic bond together with rhamnose and rutinose, respectively. Quercetin also forms the structural backbone of several other flavonoids, including hesperidin and naringenin. After *in vivo* administration, quercetin aglycone undergoes first-pass metabolism to quercetin-3-glucuronide, quercetin-3-sulfate, and isorhamnetin-3-glucuronide (3-O-methylated derivative), which are generally less active.

Tissues with β -glucuronidase activity convert quercetin conjugates to the aglycone, which is likely the only bioactive compound. In human plasma, quercetin is typically available in nanomolar concentration, but through supplementation can be increased to low micromolar concentration range (Islam *et al.*, 2014).

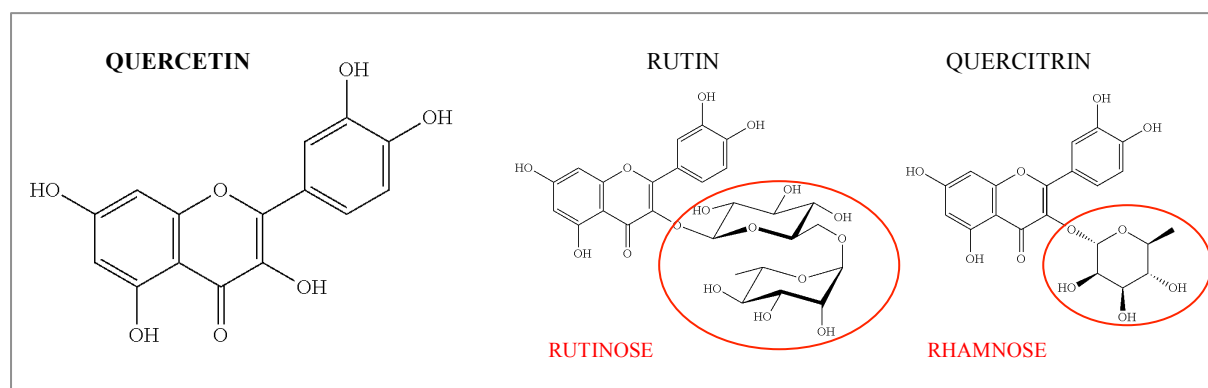


Figure 19. Structures of quercetin and quercetin glycosides, rutin and quercitrin.

There is a growing amount of evidence that quercetin exerts antioxidant activity through a number of mechanisms that include induction of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase and inhibition of prooxidant enzymes such NADPH oxidase. As an antioxidant, quercetin reduces NADPH oxidase-generated superoxide anion, as well as expression of the enzyme itself. Besides these, earlier studies have documented quercetin-induced scavenging of free radicals and/or chelating transition metal ions including superoxide anions, singlet oxygen, and lipid peroxy-radicals (Islam *et al.*, 2014).

Quercetin has been extensively studied for its potential cardiovascular and other health benefits in animal models. Although human clinical studies with pure isolated quercetin are limited, many cellular and animal studies show that quercetin possesses diverse cardiovascular effects such as anti-inflammatory, anti-oxidant, anti-atherogenic, anti-coagulative, and anti-hypertensive effects.

Human clinical trials on the effects of dietary quercetin on CVD risk factors and biomarkers have provided inconsistent results. In healthy human subjects, quercetin was found not to alter blood pressure. In a recent study in community-dwelling adults (n=1002, 60% women), Knab *et al.* (Knab *et al.*, 2011) showed that quercetin (500-1000 mg/day) supplementation for 12 weeks had negligible effects on blood pressure. However, in male smokers with high cardiovascular risk, quercetin (100 mg/day $\times$ 10 weeks) significantly

decreased systolic as well as diastolic blood pressure (Lee *et al.*, 2011). Similarly, a randomized, placebo-controlled study by Egert *et al.* (Egert *et al.*, 2009 b) showed that quercetin (150 mg/day $\times$ 6 weeks) reduced blood pressure in obese pre-hypertensive subjects with Apo E3 phenotype. In another randomized, placebo-controlled study in stage 1 hypertensive men and women (n=22), quercetin (730 mg/day $\times$ 4 weeks) vs. placebo treatment significantly reduced ($p<0.01$) blood pressure (Edwards *et al.*, 2007).

There have been extensive *in vitro* animal studies to test the effects of quercetin and its metabolites in isolated blood vessel preparations. Acute treatment of isolated rat aorta with quercetin produced a vasodilator effect in healthy rat aortic rings (Duarte *et al.*, 1993; Khoo *et al.*, 2010; Li *et al.*, 2012), coronary arteries (Ibarra *et al.*, 2002), resistance vessels (Perez-Vizcaino *et al.*, 2002), mesenteric and pulmonary arteries (Menendez *et al.*, 2011), and portal veins (Chiwororo *et al.*, 2010). Similar vasodilatory effects have also been reported in isolated blood vessels from spontaneously hypertensive rats (Machha *et al.*, 2005).

Endothelium-dependent vasodilation involves increased eNOS activity, NO production, and cyclic 3,5- monophosphate (cGMP) production]. In turn cGMP is metabolized by cyclic nucleotide phosphodiesterase (PDEs) and thus NO activity and endothelium-dependent vasodilation are strongly dependent on PDE activity. The effects of quercetin on NO metabolism depend on the status of oxidative stress and published results are conflicting. Administration of high dose of quercetin with diet to healthy rats increased NOS activity with concomitant endothelium-dependent relaxation (Benito *et al.*, 2002). Recently, Khoo *et al.* (Khoo *et al.*, 2010) showed that quercetin caused vasorelaxation in rat aortic ring segments and increased cGMP levels. Moreover, in the same study, *in vitro* experiments in bovine aortic endothelial cells revealed that quercetin treatment rapidly increased Ca^{2+} , stimulated eNOS phosphorylation at Ser1179, and increased production of NO. Similarly, Li *et al.* (Li *et al.*, 2012) found that quercetin exerted vasodilatory effect in rat aortic rings in an endothelium-dependent manner. Moreover, quercetin induced dose-dependent phosphorylation of eNOS at Ser 1179 and increased NO production in endothelial cells by an AKt-independent and cAMP/PKA-dependent mechanism. The contributions of cAMP/PKA-dependent mechanisms in quercetin-induced vasodilation have also been reported in rat-isolated portal vein *via* smooth muscles (Chiwororo *et al.*, 2010). In a number of hypertensive rat models, quercetin significantly reduced endothelium-dependent vasodilatory capacity with accompanying increased NO metabolites. In a recent study with porcine isolated coronary artery, quercetin and its metabolites, quercetin-3-sulfate and quercetin 3-glucuronide

prevented lipopolysaccharide (LPS)-induced contractile dysfunction with 3- to 10-fold increase in nitrite production and expression of inducible NO synthase (iNOS) (Al-Shalmani *et al.*, 2011). NO and ET-1 interplay as natural counterparts in maintaining the vascular tone. Imbalance between these two mediators contributes to endothelial dysfunction leading to progression of CVDs. Endothelial dysfunction is associated with elevated levels of ET-1. Quercetin reduced plasma ET-1 in ApoE knockout mice (Loke *et al.* 2010). In human umbilical vein endothelial cells, quercetin (0.1-1 μ M) decreased oxidative stress-induced ET-1 mRNA expression and ET-1 secretion (Nicholson *et al.* 2008; Nicholson *et al.* 2010). Quercetin has also been reported to inhibit vascular endothelial dysfunction induced by ET-1. The protective effect of quercetin against ET-1-induced endothelial dysfunction is thought to be mediated by down-regulation of p47phox through PKC inhibition (Romero *et al.* 2009).

Dyslipidemia, primarily characterized by increased plasma levels of low-density lipoprotein (LDL), total cholesterol, triglycerides, phospholipids, and reduced level of plasma high-density lipoprotein (HDL), is a significant risk factor for CVD. Published data of human interventional studies investigating the effects of quercetin on lipoprotein metabolism are conflicting and inconclusive. Quercetin (150 mg/day $\times$ 6 weeks) treatment decreased plasma HDL cholesterol ($p < 0.01$) and apoA1 ($p < 0.01$) and increased the LDL:HDL ratio ($p < 0.05$) in overweight and obese volunteers with metabolic syndrome traits related to apolipoprotein (apo) E4 genotype (Egert *et al.*, 2010). In another double-blind, cross-over study in humans with APOE genotype 3/3, 3/4, and 4/4, quercetin (150 mg/day $\times$ 8 weeks) increased HDL levels and decreased postprandial triacylglycerol plasma levels (Pfeuffer *et al.*, 2011). In a randomized, double blinded, placebo-controlled parallel design study, Lee *et al.* (Lee *et al.*, 2011) have documented that intake of 100 mg quercetin capsules daily for 10 weeks significantly reduced serum total cholesterol ($p < 0.05$) and LDL-cholesterol ($p < 0.01$) in healthy male smokers ($n=49$, placebo $n=43$). Furthermore, greater increases in HDL-cholesterol were observed in subjects receiving quercetin-rich supplementation than the placebo. In contrast, several other randomized, placebo-controlled trials involving healthy men or women reported no effects on lipid parameters.

There are a limited number of human interventional studies that addressed the effects of quercetin on inflammatory biomarkers. Lee *et al.* (Lee *et al.*, 2011) recently studied the effects of quercetin-rich supplementation derived from onion peel extract on cardiometabolic risk factors including inflammatory status in male smokers who confer higher CVD risks. Daily supplementation of 400 mg quercetin along with 512 mg of other mixed flavonoids of

unknown composition for 10 weeks did not affect the plasma inflammatory markers IL-6 or VCAM-1. The authors explained that lack of effects of quercetin might be related with insufficient doses and/or duration of treatment. Similarly, in a randomized placebo-controlled trials involving a large group of community adults (n=1002), quercetin supplements (500 mg and 1000 mg/day for 12 weeks) did not alter risk factors including inflammatory markers such as C-reactive protein (CRP) and IL-6. However, another recent study by Boots *et al.* (Boots *et al.*, 2011) reported that quercetin reduced inflammatory biomarkers (plasma ratios of TNF-/IL-10 and IL-8/IL-10) when given at a dose of 2000 mg orally within 24 hours to untreated sarcoidosis patients (n=12, placebo, n=6).

In a double-blind study by Conquer *et al.* (Conquer *et al.*, 1998), intake of a quercetin supplement (quercetin 1.0 g/day for 28 days) that contained a mixture of bioflavonoids increased plasma quercetin concentration (~23-fold vs. control), but failed to alter any CVD or thrombogenic risk factors in healthy subjects. Similarly, oral intake of 220 g onion/day (114 mg of quercetin/day for 2 weeks resulting mean plasma concentration of quercetin 1.5 $\mu\text{M/L}$) by healthy volunteers did not affect platelet aggregation, thromboxane B₂ production, factor VII, and other hemostatic variables (Janssen *et al.*, 1998). The inconsistent observations in human studies on the effects of quercetin on CVD biomarkers have been due to a number of confounding factors that include study design, type of compound (pure or food source), dosage, and duration of study, individual life-style and dietary variations.

The bioavailability of quercetin in the normal diet is hard to determine since many food items contain quercetin of varying amounts depending on factors such as the geographical region harvested, size of portion, and other unknown physiological factors. Several studies have demonstrated that the achievable plasma levels of quercetin range from 0.01 $\mu\text{mole/L}$ to 1.5 $\mu\text{mole/L}$ (Alwaili *et al.*, 2010; Vanhoutte *et al.*, 2009; Moon *et al.*, 2000). A number of *in vitro* studies have suggested that the effective levels of quercetin are between 1 $\mu\text{mole/L}$ and 10 $\mu\text{mole/L}$ (Kobuchi *et al.*, 1999; Alcocer *et al.*, 2002; Tribolo *et al.*, 2008). This might explain the failure of several clinical trials to observe effects of quercetin on CVD risk factors. The bioavailability and bioefficacy of quercetin is generally low due to its very poor aqueous solubility and scant stability in biological fluids. Several research efforts have been recently devoted to overcome these limitations by designing and testing appropriate modified forms and formulations able to improve the quercetin bioavailability. The most common supplement form of quercetin is an aglycone that has significantly lower bioavailability and is

not found extensively in nature. The orally administered quercetin aglycone is poorly absorbed, and the bioavailability of quercetin administered in capsule form to humans was reported to be less than 1%. Quercetin is generally found in plants as glycosides (glucosides and rutinosides), and these glycosides have better solubility in water than quercetin aglycone due to the more hydrophilic nature of their sugar moieties (Hollman *et al.*, 1995; Hollman *et al.*, 1997).

1.7 Functional raw ingredients: chia seeds and buckwheat

1.7.1 Chia seeds (*Salvia hispanica* L.)

Salvia hispanica L., whose common name is chia, that is biannually cultivated plant that blooms during the summer months, is categorized under the mint family (*Labiatae*), superdivision of *Spermatophyta*, and kingdom of *Plantae*. It is native from southern Mexico and northern Guatemala. It has been cultivated from tropical to subtropical regions. Chia seeds have long been used by the Aztec tribes and they are important not only as food, but also for medicines. The chia was rediscovered in the last decade of the XX century as best plant source of omega-3, more than source of fibers, proteins and antioxidants. Chia can grow up to 1m tall and has opposite arranged leaves. Chia flowers are small flower (3-4mm) with small corollas and fused flower parts that contribute to a high self-pollination rate. The seed are small (1.87 ± 0.1 mm length, 1.21 ± 0.08 mm width, and 0.88 ± 0.04 mm thickness), with an oval, flattened shape and ranged in color from dark coffee to beige with small darker spots (Fig. 20); the white seeds are greater in weight, width, and thickness than the darker ones. Clean and dry seeds can be kept for years, as they contain antioxidants that prevent the deterioration of the essential oils held within. Wild and domesticated chia differs little. Currently, only *Salvia hispanica* but not other species of the genus *Salvia* can be grown domestically. To prevent the misidentification of *Salvia hispanica* and other species of *Salvia*, clear understanding of the morphological and genotypical differences among them had been proposed as solutions (Mohd Ali *et al.*, 2012). It grown in light to medium, clay and sandy soils, and even in arid soils that have good drainage but are not too wet. The plant is semitolerant to acid soils and drought. Chia grown mainly in mountainous areas and has little tolerance to abiotic phenomena, such as freezing and sunless locations. The leaves contain essential oils that act as insect repellents, thus the plant can be grown without pesticides or other chemical compounds. In Mexico, it grows easily and mainly in juniper, oak, pine, and pine-oak forests, spreading by seed dispersal, with the wild type an average height of 1.9 m. In the state of Jalisco, Mexico, chia is grown on farmland from late spring to early summer. In European countries, it is grown in greenhouses during March and April, where germination lasts for approximately 2 weeks, and is then transferred to pots when the plants are high enough. As the plant is resistant to very dry areas, this crop is very attractive for developing countries such as Bolivia, Colombia, and Argentina, where it is grown in provinces including Salta, Jujuy, Tucumán, and Catamarca. The harvested seed continues to

be much more profitable than the cultivation of beans. The commercial yield per hectare is normally 500-600 kg of seeds; however, experimental plots located in Salta, Argentina, provide a yield of around 2500 kg per hectare, with the assistance of irrigation and nitrogen fertilization. This type of crop is a substitute for tobacco crops in northwestern Argentina and southern Bolivia, where they can be rotated in the fields so that soil nutrients are not depleted (Muñoz *et al.*, 2013).

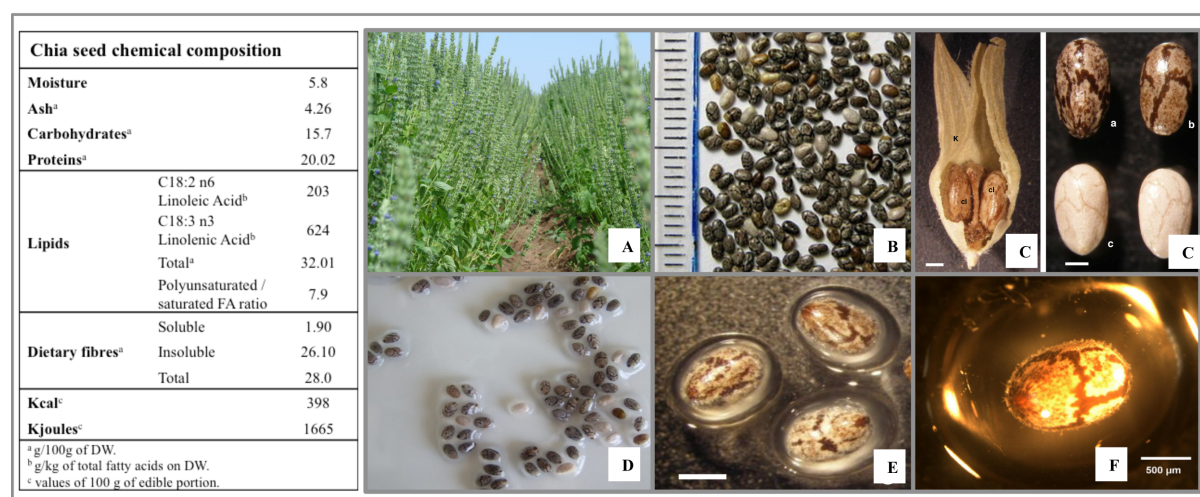


Figure 20. Chia seed chemical composition, from Costantini *et al.*, 2014. *Salvia hispanica* L.: A, plants; B, seed measure; C, seed conformation; D, E and F, seed mucilage.

Chia seeds are considered a pseudocereal and oilseed due to their high oil content. Chia seeds, which contain between 25% and 40% fats and up to 68% omega-3 α -linolenic acid, are among the plant sources with the highest contents of α -linolenic acid, compared to camelina (36%), perilla (53%) and flax (57%) (Ayerza *et al.*, 2011). They also contain 20% of omega-6 linoleic acid, thus providing a good balance between the two essential fatty acids. Furthermore, their protein content is higher (19–23%) than most of the traditionally utilised grains, including wheat (14%), corn (14%), rice (8.5%), oats (15.3%) and barley (9.2%). Chia seeds contain all of the essential amino acids, in particular leucine, lysine, valine, and isoleucine (4.15, 2.99, 2.85, and 2.42 g/100 g proteins, respectively) (Sandoval-Oliveros *et al.*, 2012). Despite this high protein content, there is no evidence of any adverse effects or allergenicity caused by whole or ground chia seeds (EFSA, 2009). Moreover, chia seeds are rich in dietary fibre (up to 30% of the total weight) and expel a natural exudate in aqueous solution that surrounds the seed, which is a branched polysaccharide composed of xylose, glucose and glucuronic acid and has a high molecular weight ($0.8\text{--}2 \times 10^6$ Da). This exudate can absorb up to 10 times its weight in water, allowing for a slower absorption of sugar into

the body (Lin *et al.*, 1994; Muñoz *et al.*, 2012 a; Muñoz *et al.*, 2012 b). Another key feature of chia seed is that it does not contain gluten (Bueno *et al.*, 2010). Finally, it is an important source of vitamins and minerals. Indeed, chia is a good source of B vitamins. Compared with other cereals, the seed has higher niacin content than corn, soybeans, and rice. As for its thiamine and riboflavin contents, they are similar to those found in rice and corn. Moreover, chia is an excellent source of minerals, and contains 6 times more calcium, 11 times more phosphorus, and 4 times more potassium than 100 g of milk, besides possessing magnesium, iron, zinc, and copper. Chia contains 13-354 times more calcium, 2-12 times more phosphorus, and 1.6-9 times more potassium than 100 g of wheat, rice, oats, and corn. The iron content of chia is also quite high compared with most other seeds: it has 6 times more iron than spinach, 1.8 times more than lentils, and 2.4 times more than liver (Muñoz *et al.*, 2013) (Fig. 21). Chia seed has a number of compounds that can act as antioxidants, an asset that makes the seed even more attractive. Among the most important are the phenolic compounds and the tocopherols. These compounds are primary and synergic antioxidants and make a proportionally greater contribution to the antioxidant activity of chia. Total amount of tocopherols in chia seed (238-427 mg/kg) is similar to that in peanut oil (398.6 mg/kg) but lower than in flaxseed (588.5 mg/kg), sunflower (634.4 mg/kg), and soybean (1797.6 mg/kg). the major phenolic compounds found in chia seeds are chlorogenic and caffeic acid, followed by myricetin, quercetin, and kaempferol. Caffeic and chlorogenic acid protect against free radicals and inhibit the peroxidation of fats. These compounds present in chia seed have much stronger antioxidant properties than those of ferulic acid, vitamin C (ascorbic acid), and vitamin E (α -tocopherol) (Muñoz *et al.*, 2013).

Vitamins	Units	Quantity
Vitamin C	mg	1.60
Thiamine	mg	0.62
Riboflavin	mg	0.17
Niacin	mg	8.83
Folate	μ g	49.00
Vitamin A	IU	54.00
Vitamin E (α -tocopherol)	mg	0.50

Macroelements	
Calcium	631 mg
Potassium	407 mg
Magnesium	335 mg
Phosphorus	860 mg
Microelements	
Selenium	55.2 μ g
Copper	0.924 mg
Iron	7.72 mg
Manganese	2.723 mg
Molybdenum	0.2 mg
Sodium	16 mg
Zinc	4.58 mg

Figure 21. Vitamins and mineral contents (per 100 g). From Muñoz *et al.*, 2013.

Today, the chia seed is used for different purposes in different countries, such as Mexico, Argentina, Chile, New Zealand, Japan, the United States, Canada, and Australia. In 2000, the US Dietary Guidelines recommend that chia seed can be used as a primary food not exceeding 48 g/day. In 2009, it was approved as a Novel Food by the European Parliament and Council of Europe. According to scientific opinion, chia does not cause any adverse allergenic, antinutritional, or toxic effects (EFSA, 2009). In 2013, the European Parliament authorized an extension of use of chia seeds, from 5% (authorized in the Council Directive of 2009) to 10% in foodstuffs, and a daily intake not more than 15 g (Fig. 22) (EU, 2013). Some of most important applications of the seeds include their use as a nutritional supplement and as an ingredient in cereal bars, biscuits, pasta, bread, snacks, and yogurt, among others, that include their use even in cake formulations. Another important characteristic of this seed is its use in the production of oil. It is light in color and has the advantage of containing a large quantity of essential oils. The main application of this product is in the production of capsules that provide a nutritional supplement of omega-3. Oil can also be extracted from chia leaves, and can be consumed as a condiment or used as a fragrance. Another application is using the mucilage of chia in food industry. This component is composed mainly of polysaccharides in form of soluble fiber (Muñoz *et al.*, 2013). In a recent study, it was demonstrated that the mucilage could be a new source of polysaccharides with the potentials of generating different polymer blends to produce films and coating with improved properties (Muñoz *et al.*, 2012a).

L 21/34	EN	Official Journal of the European Union	24.1.2013
COMMISSION IMPLEMENTING DECISION of 22 January 2013 authorising an extension of use of Chia (<i>Salvia hispanica</i>) seed as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council <i>(notified under document C(2013) 123)</i> <i>(Only the English text is authentic)</i> <i>(2013/50/EU)</i>			
USES OF CHIA (<i>SALVIA HISPANICA</i>) SEED			
Baked products	not more than 10 %		
Breakfast cereals	not more than 10 %		
Fruit, nut and seed mixes	not more than 10 %		
Pre-packaged Chia seed as such	not more than 15 g per day		

Figure 22. Commission implementing decision of 2013 about the extension of the use of chia seed (EU, 2013).

Chia seed production is a major contributor to the Argentine economy being responsible for 24% of its agricultural industry. In 2008, Argentina contributed approximately 4% of the world grain production. Although chia seed has been commercialized for a long time in Argentina, however, due to the comparatively small-scale production there, problems in its availability and sustainability as an edible oil source in the global market exist. The current planting and production of chia seed oil are yet to fully meet the world market demand (Mohd Ali *et al.*, 2011).

Some chia seed-animal studies were performed to evaluate the related health benefits. A comparative study using flaxseed, rapeseed, and chia seed as chicken feed had been conducted. Eggs from hens fed with chia had the highest ALA content as compared to hens fed with flaxseed or rapeseed (Antruejo *et al.*, 2011). Due to the easier availability and lower price of flaxseed over chia, an attempt to replace chia with flaxseed in laying hen's feed was carried out. The incorporation of flaxseed in the diet resulted in a slight decrease of the n-3 ALA content of egg yolk (Ayerza *et al.*, 2000). However, the high antinutritional content of flaxseed affected the poultry meat quality. Besides the utilization of chia in poultry animal targeted for human consumption, it had also been used for animal nutrition by itself. Other than chia seed oil, studies had been done using other grain oil such as flaxseed in broiler feed which also resulted in an increase of fatty acid quality level in broiler's meat (Betti *et al.*, 2009). Ayerza and Coates (Ayerza *et al.*, 2007) and Fernandez and colleagues (Fernandez *et al.*, 2008) conducted studies concerning the effects of chia seed feeding on rat plasma. Their findings indicated that serum triglycerides (TG) and low-density lipoprotein (LDL) were significantly decreased whereas high-density lipoprotein (HDL) and n-3 PUFA levels were increased. They also noted that no adverse effects were observed on the rat's thymus and IgE serum level. Furthermore, chia seed feeding was tested in pigs and rabbits, which resulted in an increase of PUFA in meat fats as well as aroma and flavour (Coates *et al.*, 2009; Masoero *et al.*, 2008; Dalle Zotte *et al.*, 2011). These are desirable characteristics of human food. In summary, the incorporation of chia seed into animal feed results in an increase of ALA and a decrease of cholesterol levels in meat and eggs. Hence, it is a good substitute source of PUFA to fish and other seed oils. Moreover, atypical organoleptic characteristics such as flavor and smell from marine sources were not found in chia (Ayerza, 2002). This showed the superiority of chia seed against other nutritional sources.

In addition, chia seed-human clinical trials were performed to evaluate the real health

benefits. To date, only four clinical trials have been carried out. Among these trials, only that of Nieman *et al.* (Nieman *et al.*, 2009) showed no health benefits from chia seed. This difference could be due to the treatment durations employed and also the actual biochemical components of the dietary chia seed used in the various studies. Nevertheless, later studies (Jin *et al.*, 2010; Martha *et al.*, 2012; Vuksan *et al.*, 2010) demonstrated well the benefits of chia on human health. The study of Vuksan and co-workers was conducted on 20 healthy diabetic patients who were given bread made with chia flour and additional whole seeds to be sprinkled on foods at home. The total consumption of chia seeds was 37 g per day. The study subjects had their blood pressure measured in order to observe any changes. Results showed a small decrease in blood glucose, although the most important findings were as follows: blood became less prone to coagulation, which is a risk factor that may lead to heart attack and strokes; decreased levels of internal inflammation as measured by C-reactive protein (CRP); and chia had an effect on blood pressure, decreasing systolic blood pressure by an average of 6 mmHg (Vuksan *et al.*, 2010). Finally, more research conducted to evaluate the physiological effects and medical benefits of chia seed and its components on human beings need to be done to demonstrate the immense potential of this small seed.

1.7.2 Common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.)

Buckwheat (*Fagopyrum esculentum* Moench) is derived from the Anglo-Saxon boc (beech) and whoet (wheat) because it resembles the beech nut. It is classified as a pseudocereal because of the similarity to conventional cereals in its use and chemical composition. Historically, it was a very popular food during the 17th-19th centuries, although it was later neglected during the 20th century in Western countries because of competition from wheat. Yet it is well recognized as a potential functional food source in some countries, such as China, Japan and Taiwan. Buckwheat has a powerful ecological adaptability that allows the plant to grow in almost all kinds of extreme environments. Major cultivation areas are located in Asia and in particular in Southeast Asia, where crops are grown on marginal and fairly unproductive land. Tartary and common buckwheat exhibit different growth behaviours: tartary buckwheat is a frost-tolerant crop and is generally grown at higher altitudes, whereas common buckwheat is grown at lower altitudes (Ahmed *et al.*, 2014).

Buckwheat is a dicotyledon and belongs to the family *Polygonaceae*. Its seeds are brown in colour, irregularly shaped and have four triangular surfaces. Plants usually are at

least 1m tall, and may take 11-12 weeks to mature. The cultivation of buckwheat has numerous advantages, such as being easy to cultivate, having a short growing period (70-90 days) and a longer storage time without alteration due to its phenolic and antioxidant properties. Many species of buckwheat are grown around the world; however, only nine have agricultural and nutritional value. Out of these, only two species are used as food around the world: common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.) (Fig. 23). China, the Russian Federation, Ukraine and Kazakhstan are the leading producers of common buckwheat; with production also in Slovenia, Poland, Hungary and Brazil (Ahmed *et al.*, 2014).

Buckwheat contains a variety of nutrients in its grains. The main compounds are proteins, rutin, polysaccharides, dietary fibre, lipids, polyphenols and micronutrients. The total content of these compounds depends on different factors, such as the species and the environment. Common buckwheat (*Fagopyrum esculentum* Moench), and especially tartary buckwheat (*Fagopyrum tataricum* Gaertn.), contain more rutin (a quercetin-3-rutinoside; 10 and 40 mg/g, respectively) than most fruits, vegetable and grain crops (Li *et al.*, 2001). Rutin is metabolised *in vivo* into quercetin, which has a strong antioxidant activity capable of scavenging free radicals and chelate metals, in turn inhibiting lipid peroxidation (Boots *et al.*, 2011). It has been proven that, in bread made with tartary buckwheat flour, the rutin concentration decreased, whereas the quercetin concentration increased and remained stable during processing (Vogrinčič *et al.*, 2010). Buckwheat bran and hulls have 2-7 times higher antioxidant activity than barley, triticale and oats. In addition, 32 free and 24 bound phenolic compounds were found in buckwheat flour. Among these six flavonoids are the most present, especially in hulls: rutin, quercetin, orientin, vitexin, isovitexin and isoorientin. The general composition of tartary buckwheat in terms of crude protein, fibre, fat, and ash is essentially the same as common buckwheat, but it is rarely consumed because of its bitter taste, which is caused by the enzymatic degradation of the high rutin content (Bonafaccia *et al.*, 2003).

Buckwheat is considered a pseudocereal with high nutritional value due to its protein content. Indeed, although it has lower protein content than chia seeds (10.6 and 10.3 g/100 g of DW in common and tartary buckwheat, respectively), it has a balanced amino acid composition, with high levels of essential amino acids, such as leucine and lysine (6.92, 5.84, and 7.11, 6.18 g/100g proteins in common and tartary buckwheat, respectively) (Bonafaccia *et al.*, 2003). However, the protein content in buckwheat is significantly higher than in rice, wheat sorghum, millet and maize. Similarly, its protein content is the second highest after oat

flour. The main disadvantage of buckwheat is its low protein digestibility (79.9%) because of anti-nutritional factors present in common buckwheat, including protease inhibitors (such as trypsin inhibitors) and tannins. Trypsin inhibitors in buckwheat seeds are resistant to thermal processing, especially at elevated temperatures and due to acidic conditions. Germination of buckwheat seeds considerably reduces the activity of protease inhibitors; therefore seedlings and buckwheat plants are a source of food with improved utilization of proteins. Evidence in the literature has shown that buckwheat resistant proteins can reduce the concentration of cholesterol in the serum by increasing the faecal excretion of steroids, which is induced by the binding of steroids to undigested proteins (Takahama *et al.*, 2011). Buckwheat protein isolates can also be used as a functional food ingredient to treat hypertension, obesity and constipation. These proteins lower the activity of angiotensin converting enzyme (ACE) and directly control hypertension. No or low gluten types have been identified in buckwheat, thus contributing as an ingredient in the gluten-free diet people suffering from celiac disease (Ahmed *et al.*, 2014) (Fig. 23).

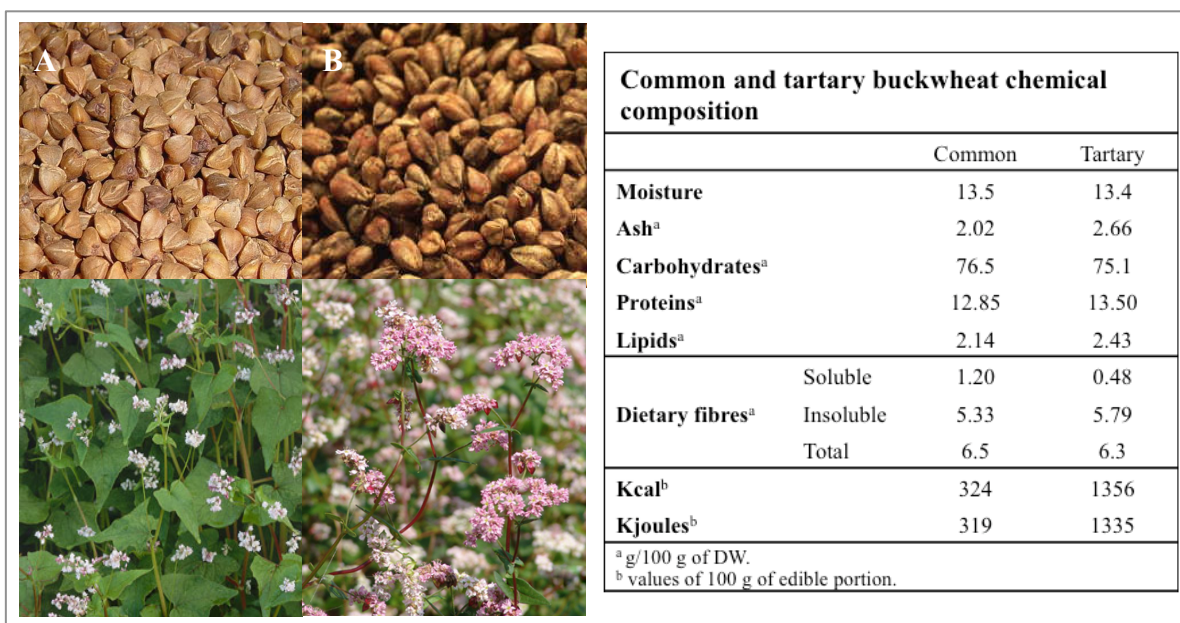


Figure 23. Common and tartary buckwheat appearance and chemical composition A: Common buckwheat, seeds on the top, flowered plant on the bottom. B: tartary buckwheat, seeds on the top, flowered plant on the bottom.

Seeds of common buckwheat contain 15-37 mg/g total lipids. The major fatty acids of common buckwheat are palmitic, oleic, linoleic, stearic, linolenic, arachidic, behenic, and

lignoceric. Buckwheat contains about the same amount of total lipids as wheat rye, but it is nutritionally superior in fatty acids composition to cereal grains with 800 mg/g unsaturated fatty acids, out of which 40 mg/g of fatty acids are polyunsaturated. In comparison with other cereals such as rice, wheat flour or maize, buckwheat contains higher levels of zinc, copper and manganese. The bioavailability of zinc and copper from buckwheat is especially high. A major quantity of these minerals exists in bran portions, followed by endosperm. Moreover, buckwheat grains contain higher levels of vitamin B₁ (thiamine), B₂ (riboflavin), E (tocopherol), and B₃ (niacin and niacinamide) compared with most cereals. Generally, tartary buckwheat has more vitamin B₁, B₂, and B₃, but less vitamin E than common buckwheat (Ahmed *et al.*, 2014).

Buckwheat is also nutritionally significant for its dietary fibre and resistant starch contents, which have beneficial effects via decreasing the glycemic and insulin indexes (Skrabanja *et al.*, 2001). A considerable portion of buckwheat dietary fibre is soluble. Soluble non-starch polysaccharides of buckwheat contain xylose, mannose, galactose and glucuronic acid. Bran fractions obtained by the milling of buckwheat are especially rich in dietary fibre (130-160 mg/g), but buckwheat flours contain considerably lower amounts of fibre (17-85 mg/g). Starch is the major component of buckwheat. Indeed, buckwheat flour contains 700-910 mg/g of starch depending on the flour types, and starch consists of 250 mg/g amylose and 750 mg/g amylopectin. The inclusion of 30 g buckwheat in the daily diet has been sufficient to produce clinically relevant reductions in serum total and LDL-cholesterol, triglycerides and increases in HDL-cholesterol, thus reducing the risk of cardiovascular diseases (He *et al.*, 1995).

Fagopyrin is a photo-sensitive substance found in buckwheat plants, belonging to the naphthodianthrone and structurally related to hypericin. The fagopyrin found in buckwheat grains are unique, but the concentration is very low and isolation is difficult. It can be utilized in the treatment of type II diabetes. Six fagopyritols, representing two distinct series differing in bonding positions, have been found in buckwheat seeds. Fagopyritols are carbohydrates compounds which were first identified in buckwheat and are ex-galactosyl derivatives of D-chiro-inositol. These are fagopyritol A1, fagopyritol A2, fagopyritol A3, fagopyritol B1, fagopyritol B2, and fagopyritol B3. Fagopyritol B1 and A1 are the major fagopyritols accumulated in buckwheat seeds (Fig. 24). Buckwheat is the richest source of these carbohydrates. The bran milling fractions may contain 26 mg of fagopyritols per g DW, whereas dark and light buckwheat flours contain 7 and 3 mg/g DW respectively. These

compounds have important healthy actions because they are isomers of D-chiro-inositol, which has a positive effect on blood glucose levels and insulin activity (Ahmed *et al.*, 2014).

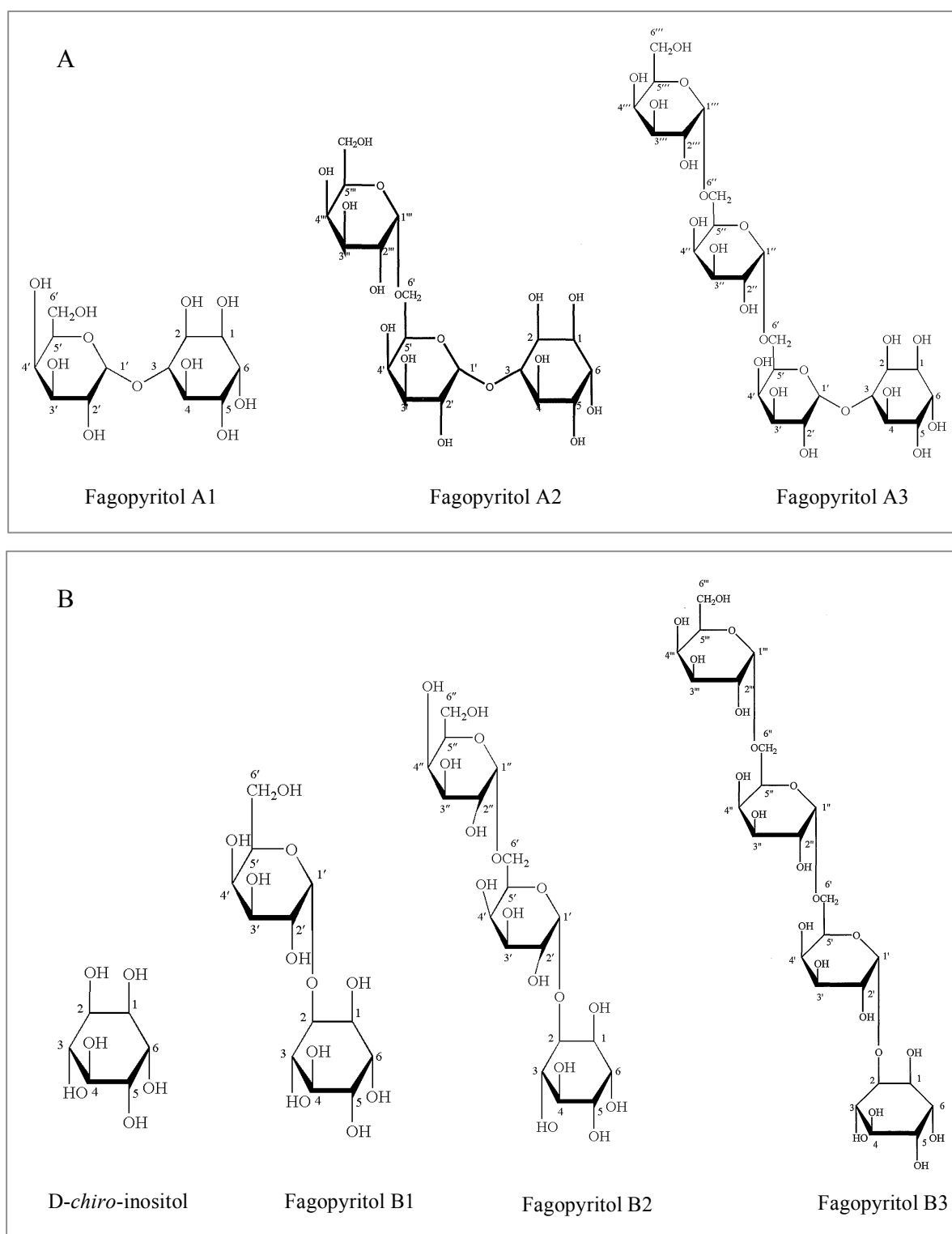


Figure 24. Fagopyritols found in buckwheat: A, A series; B, D-*chiro*-inositol and B series.

1.8 How ‘functional foods’ and ‘nutraceuticals’ can act against cardiovascular diseases?

The notion that foods not only provide basic nutrition, but can also prevent diseases and ensure good health and longevity is now attained greater prominence. Nutrient-gene interactions are a predominant factor in promoting beneficial or detrimental effects in cardiovascular health and hypertension. Various terms have been used interchangeably to designate foods for disease prevention and health promotion. The term ‘nutraceuticals’, the hybrid of ‘nutrition’ and ‘pharmaceutical’, was introduced in 1989 by the US Foundation for Innovation in Medicine and referred to “any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease”. The interest in nutraceuticals for cardiovascular prevention was particularly stimulated after the observations of a close association between the consumption of particular dietary factors, as indicated by higher plasma levels, and a reduced cardiovascular event rate. In 1994, the US Institute of Medicine’s Food and Nutrition Board defined ‘functional foods’ as “any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains”. In the case of functional foods for CVD, they contain physiologically active components either from plant or animal sources, marketed with the claim of their ability to reduce heart disease risk focusing primarily on established risk factors, that is, blood cholesterol, diabetes, and hypertension. Functional foods are suspected to exert their cardioprotective effects mainly through lipid lowering effects, antioxidant actions, and/or decreased homocysteine levels. Vegetable and fruit fibers (with pectin), garlic and oily seeds (walnut, almonds, etc.), and fish oils have lipid-lowering effects in humans, through both inhibition of fat absorption and suppression of hepatic cholesterol synthesis. Homocysteine increases the risk of both cardiovascular and cerebrovascular disorders by enhancing arteriolar constriction and decreasing endothelial vasodilation. A higher intake of folate, antioxidant vitamins, whole grains, and phytochemicals has been reported to abrogate the deleterious vascular effects of homocysteine in the heart. A significant cardiovascular benefit of phytochemicals (polyphenols in wine, grapes, and teas), vitamins (ascorbate, tocopherol), and minerals (selenium, magnesium) in foods is thought to be the capability of scavenging free radicals produced during atherogenesis (Fig. 25) (Alissa *et al.*, 2012).

Despite the high levels of interest in the diet and health relationship, the traditional approach in nutritional epidemiology has mainly focused on the effects of individual nutrients or foods. However, individuals do not consume nutrients in isolation but, rather,

Functional foods	Bioactive compounds	Potential mechanism
- Nuts	- Tocopherols, omega-3 fatty acids	Lowering blood cholesterol
- Legumes	- Fiber and polyphenols	
- Fruits and vegetables	- Fiber (pectin)	
- Margarine	- Phytosterols	
- Fish oil	- Omega-3 fatty acids	
- Whole grains	- Fiber and phytochemicals	
- Soy proteins	- Genistein and daidzein	
- Dark chocolate	- Flavonoid	
- Fish	- Omega-3 fatty acids	Inhibition of LDL-C oxidation
- Green leafy vegetables, fruits	- Carotenoids	
- Citrus fruits and vegetables	- Vitamin C	
- Tomato	- Lycopene	
- Extravirgin olive oil	- Polyphenolics and oleic acid	
- Green tea	- Tea polyphenolics	
- Soy proteins	- Genistein, daidzein, and glycitein	
- Dark chocolate	- Flavonoid	
- Pomegranate	- Polyphenols	
- Fish	- Omega-3 fatty acids	Lowering blood triglycerides
- Fish	- Omega-3 fatty acids	Decreasing blood pressure
- Legumes	- Fiber	
- Whole grains	- Fiber and phytochemicals	
- Citrus fruits	- Ascorbic acid	
- Ginseng	- Ginsenosides	
- Onion and garlic	- Quercetin	
- Green and black teas	- Tea polyphenols	
- Grapes and red wines	- Grape polyphenols	Lowering blood homocysteine
- Dark chocolate	- Flavonoid	
- Fruits and vegetables	- Folate	
- Whole grains	- Phytochemicals	
- Citrus fruits and vegetables	- Fiber and phytochemicals	
- Nuts, seeds, and oils	- Vitamin C	
- Tomatoes	- Vitamin E	Antioxidant action
- Green leafy vegetables, fruits	- Lycopene	
- Vegetable oils	- Carotenoids	
- Citrus fruits and vegetables	- Tocopherol, tocotrienols	
- Soy proteins	- Vitamin C	
- Green and black teas	- Genistein and daidzein	
- Grapes and red wines	- Tea polyphenols	Anti-inflammatory action
- Nuts, seeds, and oils	- Anthocyanins, catechins, cyanidins, and flavonols, myricetin and quercetin	
- Fish	- Vitamin E	
- Legumes	- Omega-3 fatty acids	
- Grapes and red wines	- Polyphenols	
- Fish	- Anthocyanins, catechins, cyanidins, and flavonols, myricetin, and quercetin	
- Nuts	- Vitamin C	Endothelial function
- Citrus fruits and vegetables	- Omega-3 fatty acids	
- Grapes and red wines	- Polyphenols	
- Dark chocolate	- Vitamin C	
- Grapes and red wines	- Anthocyanins, catechins, cyanidins, and flavonols, myricetin, and quercetin	Platelets aggregation
- Dark chocolate	- Flavonoid	
- Grapes and red wines	- Anthocyanins, catechins, cyanidins, and flavonols, myricetin and quercetin	

Figure 25. Potential cardiovascular protective effects of functional foods (from: Alissa *et al.*, 2012).

meals consisting of a variety of foods in combinations of nutrients that are likely to be interactive or synergistic. Indeed, much less concern has been focused on dietary patterns because of their complex nature. Anyway, whole food and phytonutrient concentrates of fruits, vegetables and fiber with natural combinations of balanced phytochemicals, nutrients, antioxidants, vitamins, minerals, and appropriate macronutrients and micronutrients are generally superior to single-component or isolated artificial or natural substances for the prevention and treatment of hypertension and cardiovascular disease. So, although many of these functional foods have been found to have high therapeutic potential, future studies should include well-designed clinical trials assessing different combinations of these nutrients to realize possible additive and/or synergistic effects on health outcomes. Many functional foods have antioxidant and anti-inflammatory activities, by mechanisms that may require further investigation. Therefore, these functional foods should be incorporated into a healthy diet to provide cardiovascular benefits and hence lower cardiovascular risk (Alissa *et al.*, 2012).

In conclusion, functional foods and nutraceuticals may allow an optimal nutritional dietary intake of healthy compounds that can be useful in the prevention and treatment of CVD and their risk factors, such as hypertension. However, so far few studies have been done in relation to the combination of these healthy compounds in a diet or in a functional food to understand possible additive and/or synergistic effects of these against CVD. Therefore, future research should focus on this issue, even if it is a very complex research topic.

2. Objectives of the present work

Epidemiological studies strongly suggest that omega-3 fatty acids plays a significant role in the prevention of many chronic diseases, such as cardiovascular disease, inflammation, hyperlipidemia, and cancer. Omega-3 polyunsaturated fatty acids are considered essential because they cannot be synthesized in the human body and must be obtained through diet. However, although a ratio of omega-6/omega-3 fatty acids of about 4-1:1 is regarded as optimal for the prevention of different pathologies, Western diets often contain a ratio of 10-25:1, indicating a considerable omega-3 fatty acids deficiency. In light of this issue, in this PhD project it was considered the seeds of *Salvia hispanica* L., one of the best plant sources of omega-3 fatty acids, as raw material from which start to create a functional food. Indeed, chia seeds contain between 25% and 40% fats and up to 68% omega-3 alpha-linolenic acid (ALA). They also contain 20% of omega-6 linoleic acid, thus providing a good balance between the two essential fatty acids. ALA is able to improve the pathological condition in model animals like Spontaneously Hypertensive Rats (SHRs), by decreasing the systolic blood pressure and the Angiotensin Converting Enzyme (ACE) activity and expression, but also increasing the vasodilators level (Ogawa *et al.*, 2009; Sekine *et al.*, 2007). Chia seeds are considered a novel food ingredient in the European Union, and for this reason there is a restriction on the amount of use, initially of 5% (EFSA, 2009), now raised to 10% after the last 2013 revision (EU, 2013). This restriction is probably due to the lack of scientific analysis related to their *in vivo* effects in humans, considering that omega-3 fatty acids, for their chemical nature, such as the presence of unsaturations, are more prone to lipid peroxidation, especially in oxidized pathological subjects.

According to the above considerations this work reports the main obtained results from the following activities and objectives:

A) Investigation of the *in vivo* effects following the long-term administration of chia seeds in oxidized pathological animal models. With the aim to formulate a functional food useful not only to maintain the health status of healthy people, but also to hypertensive people with different pathologies, 5% of chia flour was administered for long-term (5 weeks) to SHRs, being the high blood pressure condition the major cause of worldwide death as reported from the latest report of the World Health Organization. As controls wheat and corn flour were used, the first one because have an insignificant content of fatty acids, instead the second one because is rich in omega-6 fatty acids. Weight was recorded weekly, instead at

the end of the experimental period, parameters for antioxidant, inflammatory and hypertensive status were analysed (Fig. 26).

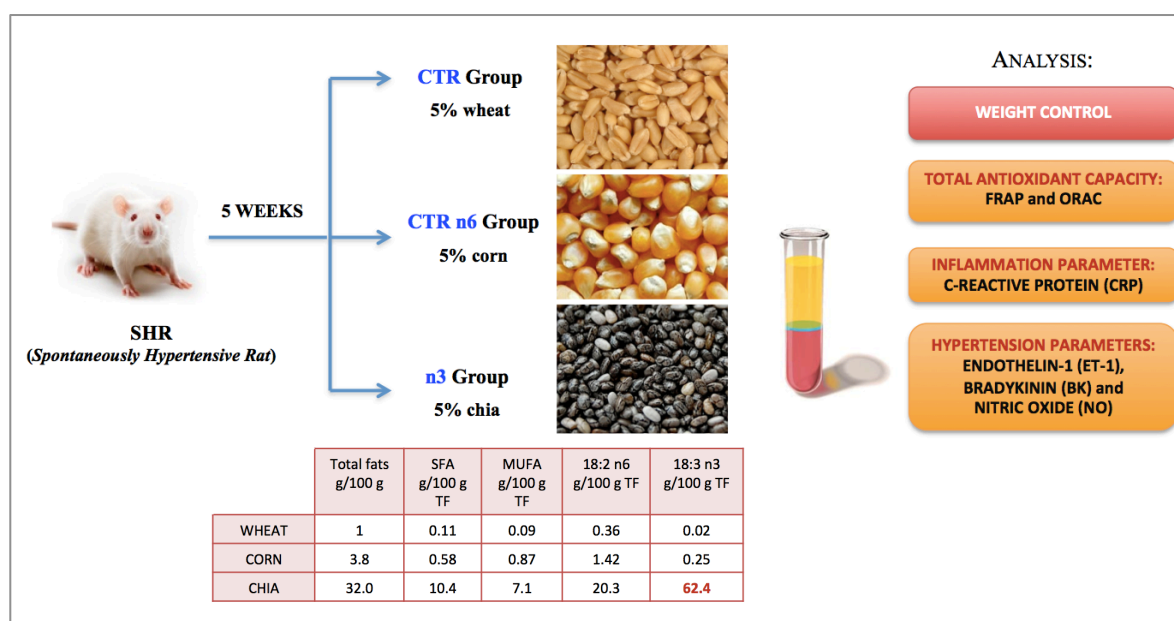


Figure 26. Experimental design of *in vivo* study.

B) Formulation and study of a functional food containing chia seeds and common/tartary buckwheat. Considering that the *in vivo* study had shown the necessity to increase the antioxidant capacity of chia seed-diet in order to avoid the *in vivo* oxidation increase after chia seed administration, for their high antioxidant activity common and tartary buckwheat were chosen as ingredients to combine with chia seeds in the functional food recipe. Taking into account that wheat bread represents a staple food for the majority of the world population and contributes substantially to the intakes of certain nutrients, in the present work different types of experimental loaves were formulated by replacing wheat flour with whole chia seed flour and whole buckwheat flours. Despite the fact that, in two previous papers, these raw materials have been used individually for bread formulation (Iglesias-Puig *et al.*, 2013; Vogrinčič *et al.*, 2010), in the present study, we combined 10% of the chia seed flour with buckwheat flours for the first time to obtain gluten-free breads. Both omega-3 fatty acids and flavonoids have been demonstrated to have many beneficial actions against several diseases, so the purpose of the present work was to obtain a bread with improved nutritional value and healthy features that can be useful not only to celiac patients, but also to maintain the health status of people with different pathologies, such as hypertension and CVD, pathologies also related to celiac disease (see 1.3 section).

3. Materials and methods

3.1. Materials

Spontaneously hypertensive rats (SHRs) were purchased from Charles River Laboratory, Lecco, Italy. Casein, salt mixture, vitamin mixture, coline and methionine were purchased from Laboratories Dottori Piccioni, Milano. Tubes containing ethylene-diamine-tetra-acetic acid (EDTA), sodium citrate or heparin were purchased from Venosafe Terumo, Leuven Belgium. The endothelin-1 (ET-1) and bradykinin (BK) levels were analyzed by immunoassay kits (ENZO life Sciences). Corning Spin-X concentrators were purchased from, Corning Incorporated, MA, USA. Plasma nitric oxide (NO) metabolite (NO₂ and NO₃) levels were analysed by colorimetric assay kit (ENZO life Sciences). Hydroxyl radical antioxidant capacity (HORAC) assays were manufactured by Oxford Biomedical Research (Oxford, MI, USA). The C-reactive protein (CRP) levels were analyzed by immunoassay kits (Alpha diagnostic international). Commercial Italian wheat flour type “0”, as legally defined in the Italian Government Official Bulletin (2001), yeast and salt were purchased from a local market, and whole common buckwheat (*F. esculentum* Moench) and whole tartary buckwheat (*F. tataricum* Gaertn.) were bought from Rangus mill (Vrhopolje pri Šentjerneju, Slovenia). Chia seeds (*S. hispanica* L.) were obtained from Alesco S.r.l. (Pisa, Italy). All of the chemical reagents (methanol, ethanol, chloridric acid, Folin Ciocalteau reagent, sodium carbonate, gallic acid, iron (III) chloride hexahydrate, 2,4,6-(2-tripyrityl)-s-triazine, acetic acid, sodium acetate trihydrate, ferrous sulphate heptahydrate, rutin hydrate, aluminium chloride hexahydrate, potassium acetate, chloroform, BF₃–methanol, and *n*-hexane) were purchased from Sigma–Aldrich (St. Louis, USA).

3.2. Experimental animal and diets

For the *in vivo* study fifteen male SHRs, twenty-five week-old were used. In the experimental period, the rats were maintained in individual cages (1 rat/cage) in a temperature-controlled room (23±2°C) with a 12-h light/dark cycle with free access to food and tap water. After an acclimatization cycle of 7 days, an experimental period of five weeks was started, and SHRs were randomly divided into three groups (n=5), namely “CRT” rats, “CRT n6” rats and “n3” rats, and every day, they were fed with a diet formulated in accordance with AIN-93 (48% wheat flour, 17% sucrose, 14% casein, 10% corn oil, 4% salt mixture, 1% vitamin mixture, 0.6% coline, and 0.4% methionine) and integrated with the 5% of grounded daily wheat flour, corn flour, and chia flour respectively (this last one in

agreement with the restriction of the European food safety authority of that time; EFSA, 2009). Pellet intakes were recorded daily, while body weights were recorded weekly over the five-week experimental period. The treatment was stopped 1 day before the end of the experiment to study the long-term effects of the diet without acute administration effects. At the end of the experimental period, animals were fasted overnight, anesthetized with isoflurane and sacrificed. Blood was drawn from the heart in tubes containing ethylenediamine-tetra-acetic acid (EDTA), sodium citrate or heparin. Plasma was separated by centrifugation at $3000 \times g$ for 20 min at 4°C , and the supernatant was utilized for analysis. All experiments and surgical procedures were performed in accordance with the Italian Laws (D.L.vo 116/92 and following additions, which enforce EU86/609 directives: Council Directive 86/609/EEC of 24 November 1986 on the approximation of law, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes) and with the approval of the Health Ministry (authorization number DGSAF 0009805-A-23/05/2012) and the animal care committee of Tuscia University.

3.3. Plasma and serum biochemical analysis

The antioxidant capacity was analyzed using the ORAC and FRAP methods on plasma. The oxygen radical absorbance capacity (ORAC) was determined using the hydroxyl radical antioxidant capacity (HORAC) assay kit according to the manufacturer's instructions. The HORAC assay is based on the oxidation of fluorescein by hydroxyl radicals *via* a classic hydrogen atom transfer (HAT) mechanism. Free radicals are generated by hydrogen peroxide (H_2O_2). The hydroxyl radicals thus generated quench the fluorescence of fluorescein over time. The antioxidants block the hydroxyl radical mediated oxidation of fluorescein until all of the antioxidant activity in the sample is exhausted, after which the H_2O_2 radicals react with and quench the fluorescence of fluorescein. The area under the fluorescence decay curve (AUC) is used to quantify the total hydroxyl radical antioxidant activity in a sample and is compared to a standard curve obtained using various concentrations of gallic acid. The final results are expressed as mmol gallic acid equivalents (GAE) per L of plasma. Unlike other antioxidant activity assays, the fluorescent HORAC assay provides a direct measurement of antioxidant capacity against hydrophilic chain-breaking hydroxyl radicals.

A ferric reducing antioxidant power (FRAP) assay was performed according to the method described by Benzie and Strain (1999), which was adapted for 96-well plates and an

automatic reader (Infinite 2000, Tecan, Salzburg, Austria). The method is based on the reduction of the Fe^{3+} -2,4,6-tripyridyl-s-triazine (TPTZ) complex to its ferrous form at a low pH. Briefly, 160 μL of FRAP assay solution (consisting of 20 mM ferric chloride solution, 10 mM TPTZ solution, and 0.3 M acetate buffer at pH 3.6) was prepared daily, mixed with 10 μL of the sample, standard, or blank, and dispensed into each well of a 96-well plate. The absorbance was measured at 595 nm at 37 °C after 30 min of incubation. All of the analyses were conducted in triplicate. The final results are expressed as $\mu\text{mol Fe}^{2+}$ equivalents per L of plasma, and the results were obtained using a standard curve with different concentrations of ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$).

The ET-1 Enzyme-linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of ET-1 in plasma. Briefly, the procedure involves the following steps: samples and standards are added to wells coated with a monoclonal antibody specific for ET-1. The plate is then incubated. After that, the plate is washed, leaving only bound ET-1 on the plate. A solution of horseradish peroxidase (HRP) labeled monoclonal antibody to ET-1 is then added. This binds the ET-1 captured on the plate. The plate is then incubated. The plate is washed to remove excess HRP labeled antibody. 3,3',5,5' tetramethylbenzidine (TMB) substrate solution is added. The substrate generates a blue color when catalysed by the HRP. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signals is directly proportional to the level of ET-1 in the sample (Fig. 27). Results are expressed as pg/mL plasma.

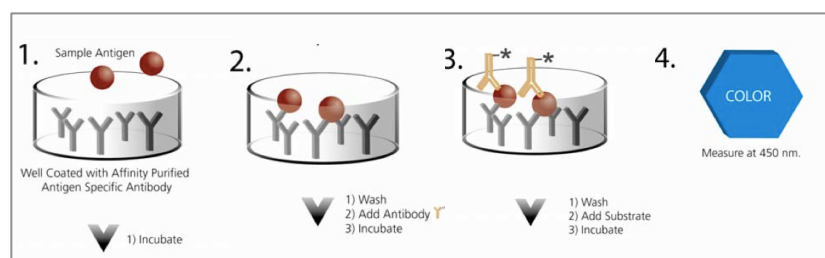
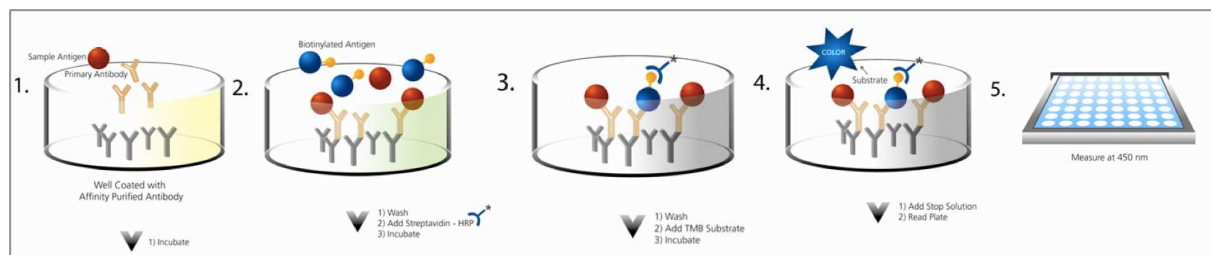


Figure 27. ET-1 Enzyme-linked Immunosorbent Assay (ELISA) kit procedure.

The BK Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of BK in plasma. Briefly, the procedure involves the following steps: standards and samples are added to wells coated with a goat anti-rabbit IgG antibody. A blue solution of BK conjugated to biotin is then added to the wells. A yellow solution of rabbit polyclonal antibody to BK is then added, and the plate is incubated at room temperature. During this incubation the antibody binds, in a competitive manner, the BK in

the sample or conjugate. The plate is then washed, leaving only bound BK. A solution of streptavidin conjugated to HRP is added to each well, to bind the biotinylated BK. The plate is again incubated. The plate is washed to remove excess HRP conjugate. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signals is inversely proportional to the level of BK in the sample (Fig. 28).



Results are expressed as ng/mL plasma.

Figure 28. BK Enzyme-linked Immunosorbent Assay (ELISA) kit procedure.

The plasma NO metabolite (NO_2 and NO_3) levels were analyzed through a colorimetric assay kit (nitric oxide detection kit). NO_2 and NO_3 are stable metabolites of NO after deproteinization of the plasma through concentrator-based filtration. The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme Nitrate Reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540nm. The conversion of NO into nitrate and nitrite by the reactions outlined below (Fig. 29), varies in each system. The interaction of NO in a system is measured by the determination of the total nitrate and nitrite concentrations in the sample. This kit allows for the total determination of both NO products in the sample by conversion of all the sample nitrate into nitrite, followed by the determination of the total concentration of nitrite in the sample. Results are expressed as $\mu\text{mol/L}$ plasma.

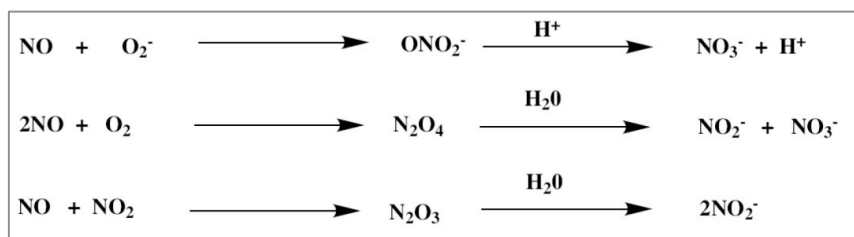


Figure 29. Conversion of NO into nitrate and nitrite.

The C-reactive protein (CRP) levels were analyzed in the serum by immunoassay kits. The Rat CRP ELISA kit is based on the binding of Rat CRP in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to HRP enzyme. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of CRP present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of CRP in samples and control is calculated from a curve of standards containing known concentrations of CRP. Results are expressed as mmol/L plasma.

3.4. Bread-making procedure

Six types of bread samples were prepared in this study based on different wheat-buckwheat/chia flour ratios: wheat flour bread (100:0), wheat flour and chia bread (90:10), common buckwheat bread (100:0), common buckwheat and chia bread (90:10), tartary buckwheat bread (100:0), and tartary buckwheat and chia bread (90:10). Whole chia flour at a rate of 10% was integrated into the experimental bread, in accordance with the maximum threshold approved by the EU in the recent revision of the European Commission (EU, 2013). Chia seeds were ground daily to avoid the oxidation of the resulting whole chia flour. A basic bread formula, based on flour weight, was used, as follows: 1000 g of flour mixture, water at 30 °C up to a consistency of 500 Brabender units (BU), 20 g yeast, 20 g sourdough starter (obtained from a mixture of 50 g yeast, 90 g flour of reference, and 150g water and left to ferment for 10h at 5°C) and 15g salt. Water absorption (percentage of water required to yield a dough consistency of 500 BU) was determined in a Brabender farinograph-resistograph (Duisburg, Germany), according to the AACC method number 54-21.02 (AACC International, 2008). The ingredients were kneaded for 5 min and rested for 30 min (first rising at 30 °C, 85% relative air humidity). After that, the doughs were divided into 350 g per piece (three loaves were produced for each bread-type), manually sheeted and rolled, and then put into steel pans for 60 min (second rising at 30 °C, 85% relative air humidity). The breads were baked in an electric oven for 30 min at 220 °C. The bread quality attributes were evaluated after cooling for 2 h at room temperature.

3.5. Bread physical characteristics

Loaf volume was determined by the rapeseed displacement method, according to

AACC method number 10-05.01 (AACC, 2008), and specific volumes were obtained by dividing the volume by the loaf weight (expressed as mL/g). Colour was determined by the crust and crumb using a Minolta Colorimeter (Chroma Meter CR400, Konica Minolta Sensing, Osaka, Japan). The established parameters were a hue angle of 10° and illuminant D65. The instrument was calibrated before each analysis with white and black standard tiles. The CIELab colour system was used to determine the following parameters: L^* (lightness, black = 0, white = 100), a^* (- green, + red), and b^* (- blue, + yellow) (Fig. 30). Crust colour was determined on eight pre-selected locations on the crust of each loaf, whereas crumb colour was determined on four points on the two central slices of each bread type sample.

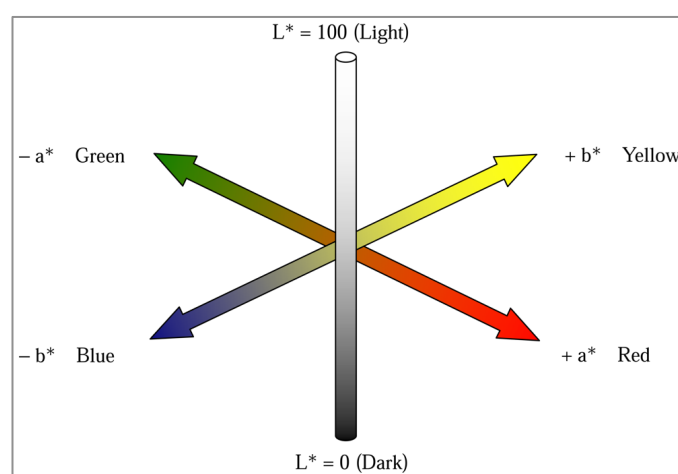


Figure 30. CIELab colour system.

3.6. Chemical analysis of the flour and bread

3.6.1. Proximate composition

The proximate compositions of flour and breads for moisture, ash, soluble and insoluble fibres were respectively determined on dry matter using the methods of the ICC number: 110/1, 104/1, and 156 (ICC, 2010).

The ICC 110/1 standard method is for the determination of the moisture content in cereals and cereal product. It provides the determination of the weight loss suffered by the sample when dried at a temperature of 130 to 133°C under precisely fixed conditions.

The ICC 104/1 standard method is for the determination of ash in cereal and cereal products. The method involves weighing the sample, which may have to be preground, into crucibles, and place them into a muffle furnace. The ashing is carried out at 900 °C, and is completed when the cool residue is white or nearly white. As the ash quantity has to be related to dry matter, the moisture content of the test substance has to be determined

separately.

The ICC 156 standard method is for the determination of total dietary fibre in cereal foodstuffs and food in general. The method involves that samples (duplicates at least, but preferably two duplicates), defatted if necessary, are gelatinized in the presence of heat stable alpha amylase, and then enzymatically digested with protease and amyloglucosidase to remove digestible protein and starch. Four volumes of ethanol are added to precipitate soluble dietary fibre. Total residue is filtered off and washed with ethanol and acetone. The residue is weighed after drying. The remaining material is analysed for protein and ash content, respectively. Subtracting the amounts measured for protein, ash and a blank control from the dry weight of the filtered residue yields a value for total dietary fibre content.

Protein content was determined both in flour and bread samples by the Kjeldhal method, and the 5.70 and 6.25 conversion factors were used for wheat and buckwheat/chia, respectively (AACC, 2008). When determining protein according to the Kjeldahl method, the sample is first treated (digested) with concentrated H_2SO_4 , which leads to the formation of ammonium sulphate. Through alkalisation with NaOH, the ammonia is displaced from the ammonium sulphate and over-distilled into a boric acid receiver via steam distillation. This solution is then titrated with HCl or H_2SO_4 to determine the nitrogen content (and hence protein) in the sample.

The fat contents of the flour and bread samples were measured utilising a Soxhlet extractor (Velp Scientifica, Monza-Brianza, Italy) according to AOAC official methods 920.39 (AOAC, 1997). According to this procedure, cereals are extracted by repeated washing (percolation) with an organic solvent, diethyl ether, under reflux in a special glassware, to obtain the total fats. In this method the sample is dried, ground into small particles and placed in a porous cellulose thimble. The thimble is placed in an extraction chamber, which is suspended above a flask containing the solvent and below a condenser. The flask is heated and the solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. At the end of the extraction process, which lasts a few hours, the flask containing the solvent and lipid is removed. In some device a funnel allows to recover the solvent at the end of the extraction after closing a stopcock between the funnel and the extraction chamber. The solvent in the flask is then evaporated and the mass of the remaining lipid is measured. The percentage of

lipid in the initial sample can then be calculated.

The carbohydrate content (%) was calculated by subtracting the contents of ash, fat, fibre and protein from 100% dry matter.

Calorie contents were calculated using the following specific energy factors: 9 for fats, 4 for carbohydrates and 4 for proteins, calculated based on 100 g of the edible portion (carbohydrate, fat, and protein content in the edible portion: data not shown). Kjoule contents were calculated using the 4.184 factor on the calorie obtained.

3.6.2. Fatty acid profile

The flour and bread samples (dry matter and edible portions) were dissolved with 10 mL chloroform/methanol (2:1 v/v). Then, 500 μ L of each extract was dried by nitrogen and transmethylated by BF_3 (Supelco, Palo Alto, CA, USA), before being heated under reflux with methanol at 72°C for 30min. The obtained fatty acid methyl esters (FAMEs) were extracted with n-hexane and evaporated by nitrogen to dryness. Transmethylated samples were dissolved in n-hexane, and their compositions were determined by capillary gas chromatography. A capillary column SPTM-2330 (Supelco), 30 m $\times$ 0.32 mm $\times$ 0.2 μ m, was fitted to an Agilent 6890 Series GC System with helium carrier gas flow-rate of 20 cm/s and used for analysis. The conditions were as follows: column temperature, hold 0.5 min at 50 °C, then 160 °C at 10 °C/min; hold 0.5 min at 160 °C, then to 250 °C at 2 °C/min and hold; injection temperature 240 °C, flame ionization detector (FID) 250 °C, carrier gas high-purity helium, and injection 2 μ L (split-splitless mode).

3.6.3. Extract preparations for phenol and antioxidant activity determination

After cooling, the breads were sliced (slices about 1.5 cm thick) and kept frozen (-80 °C) until analysis. The slices were freeze-dried and then manually crumbed with a traditional stone mortar to obtain bread powder. Flour and powdered bread samples were extracted with a solvent consisting of methanol:water (80:20, v/v) in a ratio 1:25 (w/v) and 1:9 (w/v). The 1:25 and 1:9 mixtures were shaken at room temperature for 8 h and 2 h, respectively, and then centrifuged at 1000g for 10 min. After centrifugation, the 1:25 extracts were used for the determination of total phenols and flavonoids, and the 1:9 extracts, for the determination of total antioxidant capacity.

3.6.4. Determination of total phenols

Total phenols (TP) were determined using a modification of the Folin–Ciocalteu standard method (Singleton et al., 1965). Briefly, the assay was conducted by mixing 4 mL of deionised water, 0.25 mL of extracts, 0.25 mL of Folin–Ciocalteu reagent, and 0.5 mL of Na_2CO_3 . After 30 min at room temperature, the absorbance of the mixture was measured at 725nm on Uvikon spectrophotometer (942, Kontron Instruments, Zurich, Switzerland). The final total phenols were expressed as gallic acid equivalents (GAE)/g of dry weight (DW).

3.6.5. Determination of total flavonoids

The total flavonoid contents in flour and bread samples were determined using the aluminium chloride colorimetric method described by Qin, et al. (2010), based on the method of Woisky et al. (1998). Briefly, the appropriate dilutions of extractions (0.5 mL) were mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride hexahydrate (AlCl_3), 0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of deionised water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm on Uvikon spectrophotometer (942, Kontron Instruments, Zurich, Switzerland) against a deionised water blank. The total flavonoid contents were expressed as mg rutin equivalents (RE)/g of DW.

3.6.6. Total antioxidant capacity determination

FRAP and ORAC assay was performed as done in plasma samples and described in the 3.3 section.

3.7. Statistical analysis

The mean and standard deviation (SD) of the three replicates were calculated for all the analysed data. Statistical analysis was performed with the XLSTAT 2013 4.03 (Addinsoft SARL, New York, USA) software using one-way ANOVA. Fisher's least significant differences test was used to describe statistical differences between means at the $p < 0.05$ significance level.

4. Results and Discussion

4.1 Investigation of the *in vivo* effects following the long-term administration of chia seeds in SHR.

The first aim of the present project has been to study the hypotensive effect in hypertensive animal model, SHR, after a long-term chia seed-feeding. The evaluation of the hypotensive power was performed considering some plasma biochemical parameters as the endogenous vasoconstrictor endothelin-1 (ET-1) and some endogenous vasodilators like nitric oxide (NO) and bradykinin (BK). Furthermore, it was analyzed the plasma antioxidant capacity, by ORAC and FRAP methods, and the inflammatory condition analyzing the C-reactive protein (CRP) in the serum.

Pellet intakes were recorded daily, and no differences between groups and between individual rats were found (data not shown). The results shown in Figure 31 indicate that there were no weight differences between groups (panels A and B), even if the chia seed diet was higher in fats (Fig. 26). Even better, there was the following decrease trend between groups: PUFA n3 < CTR PUFA n6 < CTR; even if it was not statically significant. This result is consistent with the results found by Chicco and co-workers, which demonstrated that Wistar rats fed a ‘sucrose rich diet’ (SRD) supplemented with chia seeds, showed a weight gain slightly lower than in the group of rats fed the SRD alone, even though a similar energy intake was recorded in both the SRD and SRD + chia seed groups. So, probably higher amount of chia seeds can lead to a greater weight loss (Chicco *et al.*, 2009).

Both the analysis for the total antioxidant capacity (FRAP panel C, and ORAC panel D) shown that the experimental group was more oxidized than the control CTR group. However, there was no difference between PUFA n3 group and CTR PUFA n6 group for both the total antioxidant capacity analysis. This is probably due the higher intake of omega-3 and omega-6 fatty acids that determined the spread of radical species already present in these pathological subjects. However, even if the experimental group was more oxidized than control group, this condition does not activate an inflammatory response, indeed there was no difference between groups for C-reactive protein (panel E). Similarly, Poudyal *et al.* demonstrated that Wistar rats fed high carbohydrate and high fat diet possess a CRP amount almost doubled compared to the rats that have followed the same diet, but supplemented with 5% of chia seeds. Further studies are needed to understand the real action of chia seeds, but these preliminary data may reflect an anti-inflammatory action related to the great ALA amount found in chia seeds (Poudyal *et al.*, 2012).

In the present study for the first time was tested the functional activity of chia seeds against hypertension in pathological animal models, SHR. The found results shown that among biochemical hypertension parameters, only the endogenous vasoconstrictor ET-1 was significantly decreased (panel F), while for the vasodilators, NO and BK (panels G and H), no significant differences were observed, even if there was a trend of increase for NO, but it was not statically significant. Previously, Poudyal and colleagues have tested the hypotensive activity of chia seeds, but they used Wistar rats fed high carbohydrate and high fat diet, and they found no differences in the systolic blood pressure after 5% chia seed supplement. In the present work an animal model more pertinent was used, and the positive result for the endogenous vasoconstrictor ET-1 suggests that chia seeds may have a hypotensive role at higher concentrations.

The *in vivo* evaluation of chia flour hypotensive role has allowed to obtain the following directions: first and foremost we found that in oxidised pathological animals, probably, the high intake of omega-3 fatty acids determined the spread of radical species and an increased oxidative stress. For this reason, chia seeds need to be associated with a high antioxidant activity ingredient in order to avoid the omega-3 oxidation. Furthermore, this association would allow to increase the chia seed amount from 5% to 10% in line with the recent European commission revision (EU, 2013), and solving in this way the second problem found in the *in vivo* analysis that is the insufficient amount of ALA to obtain the hypotensive effect found in literature (0.27 g/day of this trial against 0.45 g/day of that found in literature; Ogawa *et al.*, 2009 and Sekine *et al.*, 2007).

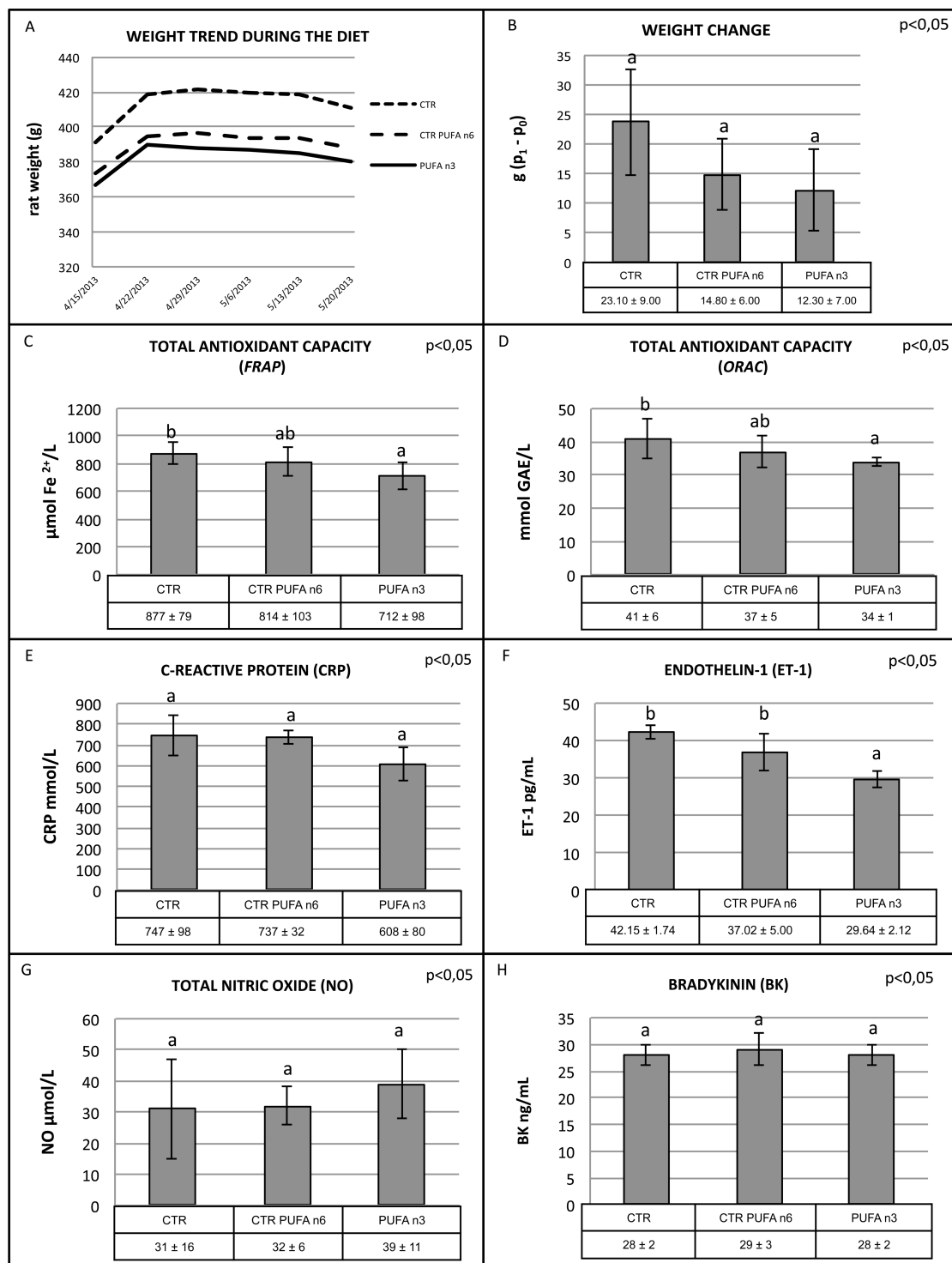


Figure 31. *In vivo* effects of long-term administration of chia seeds in SHR. Weight trend during the diet (panel A), and weight changes at the end of the experimental period (panel B). The plasma total antioxidant capacity determined by FRAP (panel C) and ORAC (panel D), plasma inflammation parameter C-reactive protein (panel E), and plasma blood pressure parameters (ET-1, panel F; NO, panel G; BK, panel H). Each value is expressed as the mean ± S.D. (n=5). Different letters (a and b) denote significant differences ($p < 0.05$) within each panel.

4.2. Functional food formulation: gluten-free bread with chia and buckwheat

From the directions of the *in vivo* study, buckwheat was chosen as high antioxidant activity ingredient, because it has a high content of the flavonoid rutin which amounts to 10 mg/g in common buckwheat and 40 mg/g in tartary buckwheat, a Slovenian landrace. Rutin is metabolized *in vivo* into quercetin, which has a strong antioxidant activity capable of scavenging free radicals and chelate metals, in turn inhibiting lipid peroxidation. Moreover, in a previous study we demonstrated that pasta containing tartary buckwheat improved the oxidative status and normalized some blood pressure parameters in SHR_s (Merendino *et al.*, 2014). In light of these considerations and taking into account that wheat bread represents a staple food for the majority of the world population, three experimental breads were made in which 10% of chia flour was integrated in three different bread recipe based on wheat flour, common buckwheat flour and tartary buckwheat flour (three experimental bread 90:10 and three controls 100:0, Figure 32).



Figure 32. Experimental bread loaves: wheat bread, A; wheat bread and chia, B; common buckwheat bread, C; common buckwheat bread and chia, D; tartary buckwheat bread, E; tartary buckwheat bread and chia, F.

4.2.1. Physical characteristics

The values of loaf volume and of specific volume for the different experimental bread types are shown in Table 1, and the major volume changes are displayed in Fig. 32. The specific volume, which is the ratio between the volume and weight, has been adopted in the literature as the most reliable measure for bread. Higher weights and volumes exert positive economic effects for the production of breads because consumers are often attracted by bread loaves with higher volume. This means that any reduction of loaf size during the baking process is undesirable. It is also known that the loaf volume of buckwheat/wheat mixed bread is decreased in correlation with the higher percentage of buckwheat flour in the flour mixture (Vogrinčič *et al.*, 2010). The results reported in Table 1 show that the specific volume of 100:0 wheat bread was higher than 100:0 common buckwheat bread and 100:0 tartary buckwheat bread. The 10% chia flour addition allowed for a slight increase in the specific volumes of all three bread formulations; however, these increases were not statistically significant. The absence of gluten in the flours had a great influence on the bread's rheological properties. For example, because of their low carbon dioxide binding activity during rising, the volumes of gluten-free breads are mostly lower (Houben *et al.*, 2012). One of the procedures by which the improvement of the specific volume of gluten-free bread can be effected is the inclusion of gums, pectin, carboxymethylcellulose, agarose, xanthan, β -glucan or insoluble fibres into the dough (Kang *et al.*, 1997). In the present study, it is assumed that the presence of the chia seed mucilaginous matrix, which is composed of xylose, glucose and glucuronic acid (see Section 1.7.1), allowed a slight increase in the specific volumes of all the three bread types containing chia (Capitani *et al.*, 2013; Lin *et al.*, 1994; Muñoz *et al.*, 2012 a). Most likely, a higher rate of chia flour would create a significant increase in the specific volume of buckwheat bread. Moreover, we observed that the chia flour's inclusion in all three bread types enabled a substantial decrease in water absorption (Table 1) and that effect could be attributed to the natural mucilage expelled, allowing the achievement of a 500 BU dough consistency with a smaller amount of water. These results did not coincide with those reported by Iglesias-Puig *et al.* (2013), which failed to detect significant differences in the water absorption after the inclusion of 5% chia whole flour into wheat bread. This difference could be due to the lower percentage of chia flour included into the bread and the insufficient mucilage amount to reach the 500 BU dough consistency with a smaller amount of water. In any case, further studies are needed to confirm this hypothesis. The values relating to the colour analysis of the crumbs and the crusts of the six experimental

bread samples are listed in Table 1, and the colour changes are shown in Fig. 32. Such analyses have shown considerable differences regarding the lightness (L^*) between wheat bread samples and buckwheat bread samples, both in the crumb and crust. Indeed, these values were significantly lower compared to the wheat bread L^* values, whereas there were no significant differences in the buckwheat bread samples 100:0 and 90:10. However, even in the wheat bread samples, the 10% chia flour inclusion conferred a significant change in the crumb lightness. This change is clearly visible in Fig. 32. For the redness parametre (a^*), there were no significant differences in the crusts of the six experimental bread samples, whereas some changes were detected in the crumb. The addition of chia flour to wheat bread led to a significantly high increase of a^* parametre, involving a chromaticity increase in the presence of chia pigments, as can be seen in Fig. 32. The visual perception of common buckwheat bread samples appeared brownish. Indeed, the a^* value was considerably higher than that of the standard white bread, and the chia seed addition did not change this parametre. On the other hand, the tartary buckwheat bread appeared greenish to visual perception; the values found for the redness parametre were low and tended towards 1, whereas these samples showed the highest values for the yellowness parametre b^* . The chia seed's inclusion in tartary buckwheat bread effected a slight increase in the a^* parametre, but this difference was not statistically significant.

Table 1. Physical characteristics of experimental bread samples.

	Wheat		Wheat and chia		Common buckwheat		Common buckwheat and chia		Tartary buckwheat		Tartary buckwheat and chia	
Water absorption (%) (at 500 BU)	52.5	48.0	75.0	75.0	68.3	68.3	75.0	75.0	68.3	68.3	68.3	68.3
Loaf volume (mL)	784 ± 52a	820 ± 38a	417 ± 19b	417 ± 19b	428 ± 24b	428 ± 24b	343 ± 35c	343 ± 35c	397 ± 5bc	397 ± 5bc	397 ± 5bc	397 ± 5bc
Specific volume (mL/g)	2.7 ± 0.2a	2.8 ± 0.1a	1.40 ± 0.05b	1.40 ± 0.05b	1.49 ± 0.08b	1.49 ± 0.08b	1.2 ± 0.1b	1.2 ± 0.1b	1.34 ± 0.01b	1.34 ± 0.01b	1.34 ± 0.01b	1.34 ± 0.01b
Colour parameters												
Crumb												
<i>L*</i>	71.8 ± 6.6a	63.4 ± 5.2b	48.01 ± 0.01c	48.01 ± 0.01c	45.9 ± 0.9c	45.9 ± 0.9c	46.5 ± 0.4c	46.5 ± 0.4c	46.0 ± 0.6c	46.0 ± 0.6c	46.0 ± 0.6c	46.0 ± 0.6c
<i>a*</i>	0.1 ± 0.7c	1.8 ± 0.3b	4.12 ± 0.02a	4.12 ± 0.02a	4.19 ± 0.07a	4.19 ± 0.07a	1.09 ± 0.07b	1.09 ± 0.07b	1.58 ± 0.03b	1.58 ± 0.03b	1.58 ± 0.03b	1.58 ± 0.03b
<i>b*</i>	18.2 ± 2.1a	14.4 ± 1.1b	9.39 ± 0.06c	9.39 ± 0.06c	10.2 ± 0.2c	10.2 ± 0.2c	20.1 ± 0.4a	20.1 ± 0.4a	19.8 ± 0.1a	19.8 ± 0.1a	19.8 ± 0.1a	19.8 ± 0.1a
Crust												
<i>L*</i>	62.6 ± 8.0a	60.2 ± 8.9a	43.6 ± 4.3b	43.6 ± 4.3b	44.7 ± 5.7b	44.7 ± 5.7b	41.1 ± 3.1b	41.1 ± 3.1b	41.7 ± 3.4b	41.7 ± 3.4b	41.7 ± 3.4b	41.7 ± 3.4b
<i>a*</i>	11.2 ± 3.3a	8.8 ± 5.0a	12.0 ± 1.9a	12.0 ± 1.9a	10.7 ± 2.6a	10.7 ± 2.6a	9.5 ± 2.2a	9.5 ± 2.2a	10.3 ± 1.4a	10.3 ± 1.4a	10.3 ± 1.4a	10.3 ± 1.4a
<i>b*</i>	31.1 ± 2.1a	26.8 ± 4.4b	22.0 ± 0.2c	22.0 ± 0.2c	20.7 ± 1.0c	20.7 ± 1.0c	21.4 ± 2.2c	21.4 ± 2.2c	23.0 ± 1.8c	23.0 ± 1.8c	23.0 ± 1.8c	23.0 ± 1.8c

Volume and colour data are means ± SD (*n* = 3). Means with different letters within a row are significantly different (*p* < 0.05).

4.2.2. Nutritional evaluation

Table 2 shows the macronutrient content, expressed as g/100 g, and the energetic value of the four flour kinds and the six experimental breads. Although the chia flour had the lowest moisture content, the inclusion of 10% chia flour in the bread formulations resulted in a notable moisture increase of about 6%, 3% and 5% in wheat, common and tartary buckwheat breads, respectively. Similarly, Inglett and co-workers found increased water holding capacities in oat products dry-blended with increasing amounts of ground chia seeds (Inglett *et al.*, 2014). Such increases are probably due to the ability of the mucilage and fibre to absorb and retain the moisture (Muñoz *et al.*, 2012 b; Vázquez-Ovando *et al.*, 2009). The carbohydrate content of chia flour was determined to be about 80% lower than that of wheat and buckwheat flour. For this reason, the carbohydrate contents of the 90:10 bread formulations were significantly lower, by about 8–12%, compared to 100:0 bread formulations. On the other hand, the protein contents of chia flour turned out to be over 30% higher than the other flour types. This increased protein content was also found in the 90:10 bread samples, as each of them was significantly higher than the equivalent 100:0 bread sample. Furthermore, the fat contents were considerably higher in the chia flour, compared to the other raw materials, since the chia seed is also an oilseed. Consequently, the fat content was 1.5–2 times higher in the 90:10 bread samples than the 100:0 bread samples. Concerning the fibre content, the chia flour had the highest concentration; the total fibres were 4–8 times higher compared to the other analysed flours, mostly due to an increased amount of insoluble fibres. Such a fibre concentration in the chia flour allowed for a substantial increase in the total, insoluble and soluble fibres in 90:10 bread formulations. Finally, the ash content of chia flour turned out to be greater than that of other flours; its inclusion in 90:10 bread formulations led to a significant increases in their ash contents.

In the present paper, the found values for the contents of carbohydrates, proteins, lipids, total dietary fibres and ash in the chia and buckwheat flour are consistent with those in the literature (Bonafaccia *et al.*, 2003; Porras-Loaiza *et al.*, 2013; Qin *et al.*, 2010). It should be emphasised that, although the amount of chia flour integrated in these formulations was only 10%, the chia flour was consistently able to improve the nutritional composition of the experimental bread. Indeed, in a previous paper, Pizarro and co-authors showed that including chia flour at 15% of the total flour in a pound cake generated a significant increase in the protein, lipid and ash contents, compared to control cake without whole chia flour (Pizarro *et al.*, 2013). In addition, concerning the total dietary fibres, Rendón-Villalobo *et al.*,

(2012) showed a 30% increase in corn tortillas supplemented with 10% chia seed powder.

Despite the higher fat content in chia flour, the energy value of 90:10 bread did not differ substantially from that of 100:0 bread. On the contrary, it was consistently lower (255 kcal/100 g) than that of 100:0 wheat bread (270 kcal/100 g). This condition may be due to the higher fibre and protein contents of 90:10 experimental breads. Based on the nutritional assessment, the best between the three raw materials fortified with chia flour can be classified into the following order: tartary buckwheat flour > common buckwheat flour > wheat flour. Indeed, the experimental bread made with tartary buckwheat flour and chia (90:10) showed a lower carbohydrate content and an increased protein, lipid, total dietary fibre and ash contents, compared with the other experimental breads and with wheat bread.

Therefore, this nutritional evaluation has shown that bakery products containing chia and buckwheat flour may be useful for diabetics and the obese because they have the lowest carbohydrate content, and the highest protein and insoluble fibre contents. Moreover, according to the literature, the use of buckwheat flour in bread may contribute to the lowering of the glycemic and insulin indexes, primarily in three different ways: by the formation of indigestible starch after the heating of buckwheat flour (Skrabanja *et al.*, 2001), by the inhibition of digestive enzymes, such as α -amylase, due to the presence of proanthocyanidins in buckwheat flour, and by the amelioration of insulin resistance through D-chiro-inositol (Takahama *et al.*, 2011). The high dietary fibre content in bread fortified with chia seeds may have different healthy functions, including the following: increasing post-meal satiety, decreasing subsequent hunger, contributing to recovery from constipation, and finally functioning as prebiotics. In addition, both chia flour and buckwheat flour contain no gluten. Hence, chia/ buckwheat bread can also be ingested by celiac disease patients.

Table 2. Chemical composition (g/100g) and energetic value of raw material and experimental bread samples.

	Moisture	Carbohydrates ^a	Proteins ^a	Lipids ^a	Dietary fibre ^a		Ash ^a	kcal ^b	Kjoules ^b
					Soluble	Insoluble			
Raw material samples									
Wheat flour	14.2 ± 0.5a	81.3	13.40 ± 0.04b	1.20 ± 0.02d	1.29 ± 0.08b	2.21 ± 0.17c	3.5	0.60 ± 0.09d	331
Common buckwheat flour	13.5 ± 0.1b	76.5	12.85 ± 0.06c	2.14 ± 0.01c	1.20 ± 0.09b	5.33 ± 0.43b	6.5	2.02 ± 0.06c	324
Tartary buckwheat flour	13.4 ± 0.2b	75.1	13.50 ± 0.06b	2.43 ± 0.06b	0.48 ± 0.04c	5.79 ± 0.45b	6.3	2.66 ± 0.05b	319
Chia flour	5.8 ± 0.5c	15.7	20.02 ± 0.01a	32.01 ± 0.42a	1.90 ± 0.12a	26.10 ± 2.09a	28.0	4.26 ± 0.07a	398
Experimental bread samples									
Wheat bread	30.1 ± 0.4e	81.1	11.10 ± 0.10e	1.59 ± 0.06f	1.30 ± 0.08bc	2.91 ± 0.23c	4.2	2.01 ± 0.04f	270
Wheat and chia bread	32.0 ± 0.6d	74.2	12.58 ± 0.06d	2.43 ± 0.01e	1.39 ± 0.12ab	7.02 ± 0.55b	8.4	2.43 ± 0.07e	255
Common buckwheat bread	40.2 ± 0.2b	73.2	12.90 ± 0.20c	3.20 ± 0.01d	1.40 ± 0.10ab	6.00 ± 0.48b	7.4	3.30 ± 0.03d	229
Common buckwheat and chia bread	41.5 ± 0.3a	64.7	13.80 ± 0.20ab	6.00 ± 0.02b	1.52 ± 0.13a	10.48 ± 0.83a	12.0	3.54 ± 0.09c	230
Tartary buckwheat bread	38.6 ± 0.1c	71.5	13.76 ± 0.14b	3.42 ± 0.01c	1.00 ± 0.08d	6.40 ± 0.51b	7.4	3.89 ± 0.04b	236
Tartary buckwheat and chia bread	40.5 ± 0.2b	62.9	14.04 ± 0.01a	6.60 ± 0.02a	1.20 ± 0.09c	11.21 ± 0.90a	12.4	4.09 ± 0.05a	233
									975

Data are means ± SD ($n = 3$). Means with different letters within a column of the same group (raw material samples and experimental bread samples) are significantly different ($p < 0.05$).

^a Of DW.

^b Values of 100 g of edible portion.

4.2.3. Fatty acid content

Table 3 shows the fatty acid content of the raw material and of the six experimental bread samples. As already pointed out in the nutritional evaluation, the chia flour had the highest lipid content and consequently the fatty acid amount. In particular, its polyunsaturated/saturated ratio was approximately 8/1; this high ratio is particularly due to the alpha-linolenic acid (C18:3 n3) content, the quantity of which exceeded 60% of the total fatty acids (624 g/kg). The fatty acid amount found in common and tartary buckwheat flour and chia flour corresponded with those reported in the literature (Bonafaccia *et al.*, 2003; Porras-Loaiza *et al.*, 2013). As expected, the 10% addition of chia flour into the experimental bread led to a considerable increase of alpha-linolenic acid, which turned out to be found in 12–13 times the amount in 90:10 buckwheat bread than in their 100:0 counterparts. This increase was even greater in 90:10 wheat bread, since wheat flour also has an insignificant amount of alpha-linolenic acid. It should be noted that common and tartary buckwheat flour both contained a substantial amount of linoleic acid (C18:3 n6), and although these values were not comparable to those in chia flour, they did lead to a greater amount of linoleic acid in the 90:10 buckwheat bread samples. For that reason, the omega-6/omega-3 ratio of 90:10 buckwheat bread samples was approximately halved (1:1.4) compared to that in the 90:10 wheat bread sample (1:3). However, the omega-3 amount was always higher (67.5 g/kg and 67.4 g/kg in 90:10 common buckwheat and 90:10 tartary buckwheat) compared to controls (5.3 g/kg and 5.6 g/kg in 100:0 common buckwheat and 100:0 tartary buckwheat). Moreover, the polyunsaturated/saturated ratio was significantly greater in all 90:10 bread samples; at its peak in the 90:10 buckwheat bread samples, this ratio was about 5 times greater than in the 100:0 counterparts. Table 3 shows the fatty acid evaluation in the edible portion of total fatty acids and the total omega-6 and omega-3 fatty acids. This analysis showed a significant increase in the amount of total fatty acids, especially of omega-3 fatty acids, in the 90:10 bread samples. The found results showed, for the first time, that experimental wheat bread fortified with 10% chia seed flour had a total lipid content of 2.4% on DW and an alpha-linolenic acid content of 62.5% of total fatty acids. Chia seeds are one of the best sources for alpha-linolenic acid supplementation in the diet, considering that they possess a higher content of this essential omega-3 fatty acid than the other two major vegetal sources, flax and perilla seeds (Sargi *et al.*, 2013). Indeed, wheat bread containing 10% flaxseed flour had a total lipid content of 1.48% on DW and an alpha-linolenic acid content of 50.25% of total fatty acids (Menteş *et al.*, 2008). The data obtained in this study show that the 1:3 omega-

6/omega-3 ratio in chia seeds was maintained in 90:10 wheat bread. Moreover, in agreement with previous published results (Bonafaccia *et al.*, 2003), a ratio of 1:1.4 was found in the buckwheat experimental breads for the high omega-6 content in buckwheat flours. This relationship between the unsaturated fatty acids is of great importance in terms of diet and the balance of fats in the body. Therefore, consuming the experimental bread would help to increase the omega-3 intake in the dietary habits of Western populations, which are characterised by a high intake of omega-6 and a lower intake of omega-3 fatty acids.

Table 3. Fatty acids contents of raw material and experimental bread samples.

	C16:0 ^a palmitic acid	C18:0 ^a stearic acid	C18:1 ^a oleic acid	C18:2n6 ^a linoleic acid	C18:3n3 ^a linolenic acid	P/S ^b	Total FA ^c	Total n6 FA ^d	Total n3 FA ^d
<i>Raw material samples</i>									
Wheat flour	0.12 ± 0.01d	n.d. ^e	0.08 ± 0.01c	0.36 ± 0.03c	0.02 ± 0.01 b	3.2	1.00 ± 0.01b	n.d. ^e	n.d. ^e
Common buckwheat flour	16.0 ± 0.9b	2.0 ± 0.1b	32.7 ± 2.5b	31.4 ± 2.9b	5.3 ± 0.6 b	2.0	1.8 ± 0.2b	56.5 ± 2.8b	9.5 ± 3.1b
Tartary buckwheat flour	14.0 ± 0.1c	2.2 ± 0.1b	31.8 ± 1.4b	30.7 ± 2.0 b	5.9 ± 0.5 b	2.6	1.7 ± 0.2b	52.0 ± 2.1b	10.0 ± 0.4b
Chia flour	69.0 ± 1.9a	35.3 ± 0.5a	71.4 ± 3.3a	203 ± 10 a	624 ± 23 a	7.9	30.1 ± 1.6a	6110 ± 304a	18782 ± 836a
<i>Experimental bread samples</i>									
Wheat bread	0.11 ± 0.02e	n.d. ^e	0.09 ± 0.01d	0.40 ± 0.02d	0.03 ± 0.01d	3.9	1.5 ± 0.1c	n.d. ^e	n.d. ^e
Wheat and chia bread	7.0 ± 0.4 d	3.6 ± 0.3a	7.15 ± 0.45c	19.3 ± 1.2c	61.8 ± 2.4b	7.7	2.2 ± 0.6c	42.4 ± 2.0c	136.0 ± 5.4b
Common buckwheat bread	16.2 ± 0.7a	2.0 ± 0.1b	31.4 ± 1.9b	31.6 ± 1.8b	5.3 ± 0.7c	2.0	2.8 ± 0.7bc	88.0 ± 3.3b	14.8 ± 0.6c
Common buckwheat and chia bread	8.3 ± 0.3c	3.7 ± 0.1a	35.4 ± 2.2a	48.6 ± 2.6a	67.5 ± 2.9a	9.7	5.4 ± 2.1ab	262.4 ± 9.6a	364.5 ± 18.6a
Tartary buckwheat bread	14.6 ± 0.8b	2.1 ± 0.1b	31.7 ± 3.0b	30.4 ± 1.9b	5.6 ± 0.8c	2.2	3.1 ± 1.6abc	94.0 ± 4.7b	17.4 ± 0.9c
Tartary buckwheat and chia bread	7.7 ± 0.5cd	3.6 ± 0.1a	36.2 ± 2.4a	47.0 ± 2.1a	67.4 ± 2.6a	10.1	5.7 ± 2.3a	267.9 ± 14.3a	384.1 ± 19.3a

Data are means ± SD ($n = 3$). Means with different letters within a column of the same group (raw material samples and experimental bread samples) are significantly different ($p < 0.05$).

^a g/kg of total fatty acids on DW.

^b Polyunsaturated fatty acids/ saturated fatty acids.

^c g/100 g of edible portion.

^d mg/100 g of edible portion.

^e Not detected.

4.2.4. Total phenol and flavonoid content

The total phenol contents of the four flour types and of the six experimental breads were expressed as mg gallic acid equivalents per gram of DW (Table 4). Tartary buckwheat flour had the highest phenolic content (72.8 mg GAE/g), followed by common buckwheat flour, chia flour and wheat flour, with the lowest phenolic content. In accordance with the above results, the highest phenolic content in the bread samples was found in tartary buckwheat bread (59.3 mg GAE/g). In this case, the addition of 10% of chia flour led to a slight increase in the total phenolic content of tartary buckwheat bread 90:10, although this was not statistically significant. On the contrary, there was a decrease in the total phenol content after the chia flour's inclusion in common buckwheat bread samples. This decrease was also not significantly different. A reversed trend was observed in wheat flour bread samples, as the addition of chia flour led to an increase in the total phenolic content; this difference was significant. Therefore, if the flour used is already rich in polyphenols, as in tartary and common buckwheat flour, the addition of chia flour to the experimental bread does not change the phenolic content. Even if there is a decrease, this change is not so negative as to enact significant changes. Instead, chia flour can increase the total polyphenol content in bread made from wheat flour, improving not only its nutritional profile, as seen previously (see sections 4.2.2. and 4.2.3.), but also increasing the amounts of non-nutritional healthy compounds.

Concerning the total flavonoid content in the four flour types, expressed as mg rutin equivalents per gram of DW, the highest content was found in the tartary buckwheat flour, with a value of 22.2 mg RE/g. Regarding the other three flours, the differences were not statistically significant. The flavonoid contents of the two buckwheat flour types were in the range found by Qin et al. (2010) for different buckwheat cultivars. Conforming to the results found for the flour, among the six types of bread, the two samples of tartary buckwheat bread had the highest flavonoid content and, as expected, the addition of chia flour entailed a significant decrease in the flavonoid value (16.8 mg RE/g in 100:0 bread in comparison to 16.1 mg RE/g in 90:10 tartary buckwheat bread formulations). As for the raw materials, the flavonoid contents of the other types of bread were not statistically different. The inclusion of chia flour led to a slight increase in the total flavonoid content of 90:10 wheat bread; still, this increase was not statistically significant.

From these data, it can be determined that tartary buckwheat breads have the highest phenol and flavonoid contents, probably due to this flour's high rutin content. Further studies

are needed to understand the actual rutin and quercetin contents. However, this raw material appears to be the best suited to the prevention of possible lipid peroxidation of the high omega-3 content during processing and *in vivo* after the ingestion.

4.2.5. Total antioxidant capacity

The antioxidant properties in flour and bread samples were investigated through two assays: the FRAP and the ORAC. FRAP does not measure thiol-based antioxidants, such as glutathione (Prior *et al.*, 2005). For this reason, the ORAC assay was also performed. Both analyses were performed to make relative comparisons among the total antioxidant capacity of the four flours and of the six experimental breads. The FRAP results were expressed as mmol of ferrous ion (Fe^{2+}) equivalents per gram of DW, whereas the ORAC results were expressed as mmol of gallic acid equivalents per gram of DW. The data showed similar differences among the different flour types, as seen in both the FRAP and the ORAC values (Table 4). Indeed, tartary buckwheat flour had the highest antioxidant capacity (40.5 mmol Fe^{2+} /g and 450.3 mmol GAE/g), whereas wheat flour had the lowest antioxidant capacity (0.30 mmol Fe^{2+} /g and 2.6 mmol GAE/g). Regarding common buckwheat and chia flours, no significant differences were found. As for the types of flour, the FRAP and ORAC methods suggested similar differences among the bread samples. The lowest antioxidant capacity among all the bread samples was the one made from wheat flour, whereas those with the highest antioxidant capacity were made from tartary buckwheat flour 100:0 (30.0 mmol Fe^{2+} /g and 124.6 mmol GAE/g) and 90:10 (32.0 mmol Fe^{2+} /g and 128.6 mmol GAE/g). Thus, the addition of chia flour to the different bread formulations generated an increase in the total antioxidant capacities which was significant in the ORAC results between the 100:0 and 90:10 wheat bread samples and in the FRAP results between the 100:0 and 90:10 common buckwheat bread samples. In all other samples, even if there was an increase between the 100:0 and 90:10 samples, it was not statistically significant.

Comparing the flour types before the baking process to the bread samples, it is worth noting that the breadmaking process created a loss of the total antioxidant capacity and of the total contents of polyphenols and flavonoids (Table 4). Furthermore, it is also possible that the real decrease is greater than found in this study, since the thermal processing of cereals and pseudocereals, such as during baking, can also result in the synthesis of substances with antioxidant properties, including certain Maillard reaction products that occur in the bread crust. These syntheses may mask the real decrease of total phenolic and flavonoid content

(being able to react with the Folin–Ciocalteu reagent), as well as any loss in total antioxidant capacity (Zhang *et al.*, 2010). On the other hand, Gawlik-Dziki *et al.* (2009) showed that digestive enzymatic extracts after the simulated *in vitro* digestion of wheat bread enriched with tartary buckwheat flavones had a significantly higher amount of polyphenols and antioxidant capacity than those of chemical extracts. This may be due to the fact that enzymatic digestion liberates compounds, such as proteins and fibres, that are able to associate with flavonoids. Indeed, the extraction solvents in common use do not allow for a complete release of these antioxidant molecules.

In any case, it is worth noting that the sole inclusion of chia flour had allowed a consistent increase in the total antioxidant capacity of the common white bread. Indeed, the 64% increase in the total antioxidant capacity in 90:10 wheat bread compared to 100:0 wheat bread has been found. Such an increase reached 75% of the total antioxidant capacity in 90:10 tartary buckwheat bread, emphasising that this composition is the best, not only to avoid the oxidation of large amounts of omega-3 fatty acids stemming from the chia seed addition, but also to formulate foods with a high antioxidant potential. These increases in the percentages of antioxidant capacity are consistent with the results of Vogrinčič *et al.* (2010), who compared wheat bread and tartary buckwheat bread.

Table 4. Polyphenols, flavonoid and total antioxidant capacity contents of raw material and experimental bread samples.

	Total phenolic content (mg GAE ^b /g ^a)	Total flavonoid content (mg RE ^c /g ^a)	Total antioxidant capacity	ORAC ^e assay (mmol GAE ^b /g ^a)
			FRAP ^d assay (mmol Fe ²⁺ E/g ^a)	
Raw material samples				
Wheat flour	9.6 ± 0.1d	0.80 ± 0.05b	0.30 ± 0.01c	2.6 ± 1.1c
Common buckwheat flour	29.3 ± 0.5b	1.0 ± 0.2b	9.1 ± 1.6b	139.3 ± 33.4b
Tartary buckwheat flour	72.8 ± 0.5a	22.2 ± 0.3a	40.5 ± 4.4a	450.3 ± 57.7a
Chia flour	16.4 ± 0.9c	1.1 ± 0.2b	11.0 ± 1.4b	131.0 ± 13.3b
Experimental bread samples				
Wheat bread	3.3 ± 0.2d	0.6 ± 0.2c	0.6 ± 0.2d	31.4 ± 2.1c
Wheat and chia bread	4.5 ± 0.3c	0.70 ± 0.09c	2.0 ± 0.5cd	86.7 ± 17.3b
Common buckwheat bread	16.5 ± 1.1b	0.60 ± 0.04c	4.3 ± 0.2c	96.9 ± 0.5b
Common buckwheat and chia bread	14.8 ± 0.6b	0.60 ± 0.02c	8.0 ± 1.0b	100.8 ± 5.1b
Tartary buckwheat bread	53.3 ± 7.1a	16.8 ± 0.3a	30.0 ± 2.4a	124.6 ± 5.6a
Tartary buckwheat and chia bread	59.3 ± 0.2a	16.1 ± 0.1b	32.0 ± 2.6a	128.6 ± 4.2a

Data are means ± SD (n = 3). Means with different letters within a column of the same group (raw material samples and experimental bread samples) are significantly different ($p < 0.05$).

^a Of DW samples.

^b GAE: gallic acid equivalents.

^c RE: rutin equivalents.

^d FRAP: ferric reducing antioxidant power.

^e ORAC: oxygen radical absorption capacity.

5. Conclusions and future objectives

The results obtained in this study indicated that the combination of chia flour with tartary buckwheat flour allowed the creation of bread with enhanced functional properties without adversely affecting the investigated technological parameters in comparison to corresponding control. Chia flour-supplemented breads contained significantly greater amounts of proteins, insoluble dietary fibres, ash and especially omega-3 fatty acids, with an omega-6/ omega-3 ratio shifted in favour of the latter that was 12 to 13 times higher in 90:10 buckwheat bread samples than the 100:0 counterparts. Moreover, tartary buckwheat flour may be suitable for preventing the oxidation of polyunsaturated fatty acid amount in bread fortified with 10% of chia flour, providing a high amount of flavonoids and a 75% increase in the total antioxidant capacity. In light of these results this food might be functional not only for the following patient categories: hypertensive and patients with micro- and macro-inflammatory diseases for the higher total antioxidant capacity and omega-3 content; diabetics and obese for the low-carbohydrates and higher protein and fiber contents; but also to maintain the health status of healthy people. In addition, both chia flour and tartary buckwheat flour contain no gluten and can be consumed by patients with celiac disease, thus making a first step in formulating foods suitable for celiacs and at the same time nutritionally valid to avoid CVD risk associated with current GFD (see 1.3 section).

Further studies are needed regarding the actual rutin contents, the sensory acceptability and the *in vivo* effects of these experimental breads. Moreover, these future goals will be planned: the design and the implementation of other types of foods containing chia seeds and tartary buckwheat or different antioxidant ingredients in order to determine the best among these to prevent omega-3 lipid peroxidation, and the *in vivo* evaluation of the functionality of these foods in pathological animal models or human patients.

5.1. Preliminary data: tartary buckwheat and chia gluten-free cracker with pumpkin seeds

Preliminary data are shown about the formulation of gluten-free cracker based on chia and tartary buckwheat with the addition of pumpkin seeds. The idea to use pumpkin seeds comes from the necessity to mask the bitter taste of tartary buckwheat. The recipe had the following percentages for the dried ingredients: 71% tartary flour, 7% chia flour, 1% salt, 8% olive oil, 13% pumpkin seeds (placed only in the surface, see figure 33). A control-sample

was made without pumpkin seeds, but with the other ingredients in the same proportions. The dough was obtained after the addition of about 2 L of water, after that, the dough in the shape of breadsticks (control-cracker without pumpkin seeds) or of a small disks (cracker with pumpkin seeds), was poured on an oven plate, the pumpkin seeds were added on the surface and they were baked at 250 °C (Fig. 33).



Figure 33. Experimental tartary buckwheat and chia gluten-free crackers: control cracker without pumpkin seeds, A; cracker with pumpkin seeds, B.

The first results shown that there are no differences between the experimental tartary buckwheat and chia bread and cracker for protein contents. Instead, the lipid amounts were increased for the presence of olive oil and pumpkin seeds at the expense of carbohydrate contents, which are decreased (Table 5).

The results for phenolic and flavonoid contents, and total antioxidant capacity are comparable to those found for breads. It is noteworthy that the pumpkin seed addition does not cause a significant decrease of phenolic and flavonoid contents, and of total antioxidant capacity (Table 6). Anyway, these are preliminary results, and as such they require further investigations.

Table 5. Chemical composition (g/100g of edible portion) and energetic value of raw material and experimental cracker samples.

	Moisture	Carbohydrates	Proteins	Lipids	Ash	Kcal ^a	Kjoules ^a
Raw materials							
Tartary buckwheat flour	13.2	65.58	12.00	5.27	3.95	358	1498
Chia flour	4.40	37.06	22.35	32.00	4.19	526	2201
Pumpkin seed	5.00	28.84	30.82	30.20	5.14	510	2134
Experimental crackers							
Cracker without pumpkin seeds	6.10	56.23	12.25	20.10	5.32	455	1904
Cracker with pumpkin seeds	7.50	54.69	14.88	18.16	4.77	442	1849

^a values of 100 g of edible portion.

Table 6. Polyphenols, flavonoid and total antioxidant capacity contents of raw material and experimental cracker samples.

	Total phenolic content (mg GAE ^b /g ^a)	Total flavonoid content (mg RE ^c /g ^a)	Total antioxidant capacity	
			FRAP ^d assay (mmol Fe ²⁺ ·E/g ^a)	ORAC ^e assay (mmol GAE ^b /g ^a)
Raw materials				
Tartary buckwheat flour	72.32 ± 1.98 a	24.63 ± 0.28 a	202.96 ± 4.79 a	932.55 ± 45.67 a
Chia flour	11.97 ± 0.44 b	0.79 ± 0.17 b	45.33 ± 0.42 b	89.26 ± 3.74 b
Pumpkin seed	5.43 ± 0.47 c	1.08 ± 0.13 b	6.4 ± 0.09 c	72.46 ± 14.51 b
Experimental crackers				
Cracker without pumpkin seeds	43.41 ± 0.80 a	12.00 ± 0.61 a	168.78 ± 8.26 a	283.50 ± 13.95 a
Cracker with pumpkin seeds	40.68 ± 1.81 a	11.18 ± 0.39 a	146.32 ± 9.83 b	262.64 ± 9.14 a
^a of DW samples				
^b GAE: gallic acid equivalents; ^c RE: rutin equivalents; ^d FRAP: ferric reducing antioxidant power; ^e ORAC: oxygen radical absorption capacity.				
Data are means ± SD (n=3). Means with different letters within a column of the same group (raw material samples and experimental cracker samples) are significantly different (<i>p</i> < 0.05).				

^a of DW samples

^b GAE: gallic acid equivalents; ^c RE: rutin equivalents; ^d FRAP: ferric reducing antioxidant power; ^e ORAC: oxygen radical absorption capacity.

Data are means ± SD (n=3). Means with different letters within a column of the same group (raw material samples and experimental cracker samples) are significantly different (*p* < 0.05).

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