Università del Piemonte Orientale"Amedeo Avogadro"

Department of Health Sciences

PhD in Molecular Medicine XXVI cycle

PhD thesis

Role of chocolate consumption on monocytes inflammatory pathways: a proteomic approach

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1. Introduction

1. NUTRITION

1.1 Definition

Food is one of the basic human needs. The importance of food goes beyond its material value and the physiological need to eat. In every culture and for all individuals, it takes on a symbolic value. From birth, food conceptualizes the idea of giving nourishment and creates the first fundamental social relationship between mother and son. Many aspects of different cultures and traditions are transmitted during and through the sharing of food. The meal itself is an important moment of socialization through which relationships are built and hierarchies established, being an integral part of the education for each. Finally, the choice of food itself characterizes each culture in a specific way. For example food expresses aspects of the habits of a people based on geographical location, access to raw materials and the religious beliefs and history of each country[1].

1.2 Nutrition and diseases

Nutrition is the intake of food, considered in relation to the body's dietary needs. Good nutrition is considered an adequate, well balanced diet combined with regular physical activity, the cornerstone of good health. Poor nutrition can lead to reduced immunity, increased susceptibility to disease, impaired physical and mental development and reduced productivity. According to the World Health Organization (WHO), about one-third of all cardiovascular diseases and cancers could be prevented through a healthy and balanced diet, thus preventing three million deaths each year simply by consuming a sufficient quantity of fresh fruits and vegetables[2]. The human body needs all types of nutrients to function properly. Some are essential to meet energy needs, to sustain the continuous replacement of cells and elements of the body, to perform physiological processes and to have protective functions. For this reason, a healthy diet should be maintained, being both varied and balanced. In spite of the fact that life expectancy has increased markedly during the last few centuries, worldwide populations may benefit from optimizing their nutrition to reduce the incidence of obesity, type 2 diabetes mellitus (T2D), cardiovascular diseases as well as several types of cancer and infectious diseases. Nutritional science should be focused on preventing the development of these diseases, as well as supporting the repair processes important for curing diseases that are already fully developed[3]. Research of traditional nutrition has contributed significantly to modern biomedicine and has promoted a prolonged life expectancy. However, there is still a large space for improving diet and health for many

groups in economically developing as well as developed countries. This potential can be exploited through the design of good studies and the application of new and advanced techniques, particularly those based on molecular methods and advanced biostatistics[4].

1.3 How it is changing the concept of nutrition

Good nutrition is of fundamental importance for normal growth and development and in the maintenance of health throughout life. Nutrition is highly complex. It is a concert of multitudes of essential nutrients both known and unknown, chemical compounds without known biological functions, an array of different cell types and the extensive microbiological activity in the intestine, combined with components of genetic and epigenetic variation. All these variables allow for an extensive variation between individuals as well as between different physiological states such as fasted, fed, cold, warm, rested, exercised, exhausted, male and female, menstrual cycle, pregnant, lactating and age, ranging from newborn to veteran. This extensive complexity of nutritional science demands advanced approaches to unravel the relations between diet and health for different ages, sexes, environmental conditions and so forth[5,6]. Nutrients have a global role (fig. 1). They can influence gene expression, either directly such as ligands for nuclear receptors or by inducing epigenetic modifications. However, nutrients are also essential building blocks (essential amino acids), they may act as coenzymes in chemical reactions (vitamins), can be converted into bioactive products (fatty acids), inhibit oxidation of other molecules (antioxidants) or serve as energy sources[7].

New and advanced molecular techniques provide opportunities in nutritional science. These technologies are often based on the different "omics" such as: genomics, epigenomics, transcriptomics, proteomics and metabolomics. With respect to these molecular methods, which can be applied to nutrition research, proteomics will be well explained in section 1.5[8].

Nutrigenetics and nutrigenomics are nascent areas that are evolving quickly and riding on the wave of "personalized medicine" that is providing opportunities in the discovery and development of nutraceutical compounds[9,10].

The human genome sequence and sequences of model organisms, provide the equivalent of comprehensive blueprints and lists of parts that describe dynamic networks and the basis for understanding their responses to external and internal perturbations. Unfolding the interrelationships between genes, gene products and dietary habits is fundamental for identifying individuals who will benefit most from, or be placed at risk by intervention strategies[11].

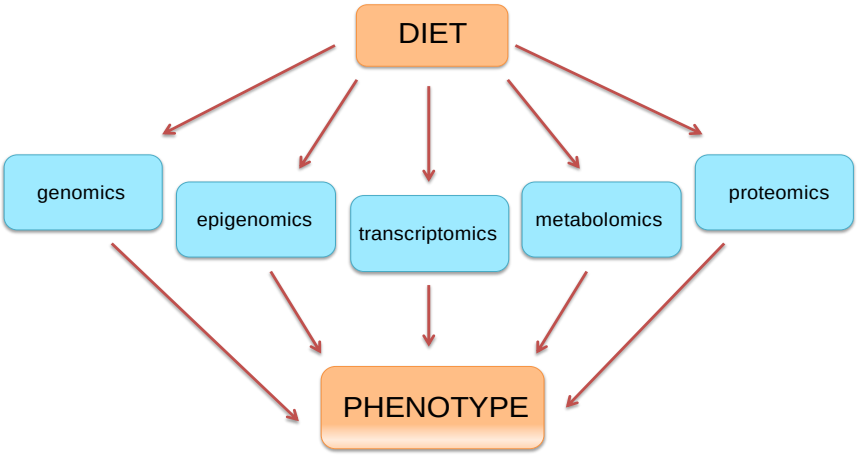


Figure 1. Dietary factors may interact with multiple biological processes. Nutrients interact with genes and alter functional outcomes (genomics); nutrients may induce epigenetic changes (epigenomics); nutrients may influence gene expression (transcriptomics); nutrients may post-translationally modify proteins (proteomics) and nutrients may change the metabolomic signature in the blood (metabolomics). Modified by Frode Norheim *et al.*[4]

More accurate assessment of the inputs to human health and the consequences of those inputs measured by transcriptomics, proteomics and metabolomics analyses, would bring personalized health/diet to practice far faster than waiting for predictions from genetic variations. It is widely recognized that systems and network biology has the potential to increase our understanding of how nutrition influences metabolic pathways and homeostasis, how this regulation is disrupted in a diet-related disease and to what extent individual genotypes contribute to such diseases[12].

Biochemical and physiologic associations among hypertension, diabetes and cardiovascular diseases have grown steadily, supported by basic, clinical and epidemiologic research. The possibilities of treating these pathologies include pharmacologic approaches, lifestyle adjustment and diet modification. The identification of foods that have cardiovascular health benefits has become a major public health objective. There is now a large body of epidemiologic evidence that supports the concept that diets rich in fruit and vegetables attenuate or delay the onset of certain chronic diseases, including cardiovascular and related diseases[13].

However, the physiologic and molecular mechanisms by which fruit and vegetables act to reduce the risk of vascular disease remain elusive[14].

The existence of data showing that the health benefits of fruit and vegetables are causally linked to their flavonoid content, is a starting point from which mechanistic uncertainties can be addressed. With respect to cardiovascular health, one class of flavonoids, the flavanols, is receiving increasing attention[15].

Cacao, tea, grapes and grapefruit are examples of edible plants that are rich in flavanols. Translational research that relates the consumption of these foods to cardiovascular health is of particular interest [16]. Numerous epidemiologic studies support the concept that regular consumption of foods and beverages rich in antioxidant vitamins and flavonoids is associated with a decreased risk of CVD mortality[17]. Foods that have been identified as having a high polyphenolic flavonoid content include many fruits and vegetables such as apples and onions, teas (green and black), red wines and specific types of chocolate[18,19,20,21].

1.4 New era "omics"

The exponential growth of technological sciences in recent decades has led researchers to have an increasing number of investigative techniques, such as gene cloning, the genome and proteome sequencing, methods of gene expression analysis, optimization of electrophoretic separation techniques and the evolution of mass spectrometry. These investigative techniques have provided researchers with the tools for large scale studies and in this context the concept "-omics" was born[7]. "Omics" are very convenient handles for describing the holistic approach for looking at complex systems and it is a general term for a broad discipline of science and engineering concerned with analyzing the interactions of biological molecular components in various 'omes'[22].

The development of these 'omics' has depended on, and has also been driven by, advances in chromatography and electrophoresis, as well as highly sensitive and specific analytical techniques to permit the handling of large numbers of samples with high selectivity and sensitivity. The classical methods of molecular biology (Northern blot analysis, polymerase chain reaction in real time, in situ hybridization, etc..) have the enormous limitation of being able to quantify the expression of only one or a few genes at one and the same time. The sequencing of the genome has opened the way to new avenues of research including the study of the expression of all genes of a cell at the transcriptional and translational level and the analysis of interactions among all proteins. The development and design of novel stationary phases for selective enrichment and separation is one of the key points for establishing a successfully running 'omics' platform[23,24]. Technologies of genomics, transcriptomics, proteomics, metabolomics and other-omics have the potential to provide an overview of the pathogenesis of complex diseases, especially if they are applied with an approach for the integration of different technologies. Genomics deals with the genetic mapping and sequencing of entire genomes (structural genomics) and seeks to understand the ways in which the genes that make up our genome direct the development and functioning of our

bodies and how their failure induces a pathological state (functional genomics). Transcriptomics is concerned with the transcriptome, the set of transcripts, in particular mRNA, which carry the information for protein synthesis. With the sequencing of the genome, biology has made great strides to understand the complexity of an organism from the point of view of the content of its information and as often happens in the scientific world, the information obtained immediately demonstrates that there are limits[25,26].

The huge amount of information contained in the genome, provides a list of proteins that can be expressed by the genome, but does not provide any information about the proteins that are actually expressed or made active at a given time or cellular compartment. Moreover the knowledge of the nucleotide sequence of a gene does not allow the prediction of the possible post-translational modifications that may occur following protein synthesis. Even an approach based only on the analysis of messenger RNA, does not indicate the presence and levels of protein expression, as there is a linear correlation between amount of mRNA and protein quantity[27]. The axiom of biologists "one gene = one protein" has now been overcome by a reality which is much more complex. The proteins represent the results of transduction and therefore their study is the key step to a realistic understanding of the biological events at the molecular level. Currently proteomics is one of the most fruitful technologies of post-genomic research, essential for highlighting the role of proteins expressed in a tissue and the protein-protein interactions for studies of cell function[28].

1.5 Proteomics

The term proteome was introduced by Wilkins et al. (1996) to identify proteins (pro-) expressed by a genome (-oma)[29]. Proteomics is the discipline that seeks to catalog the proteins of an organism (proteome) determining the sequence and analyzing the overall set of proteins in different cell types at different stages of development and in different pathophysiological stages (fig. 2).

At present, proteome means all proteins expressed by a genome, from a tissue, or a cell type, at a particular moment of the life of the cell and as such can be seen as the end product of the genome in a given moment of cellular life[30]. For the first time the concept of "time" has been introduced.

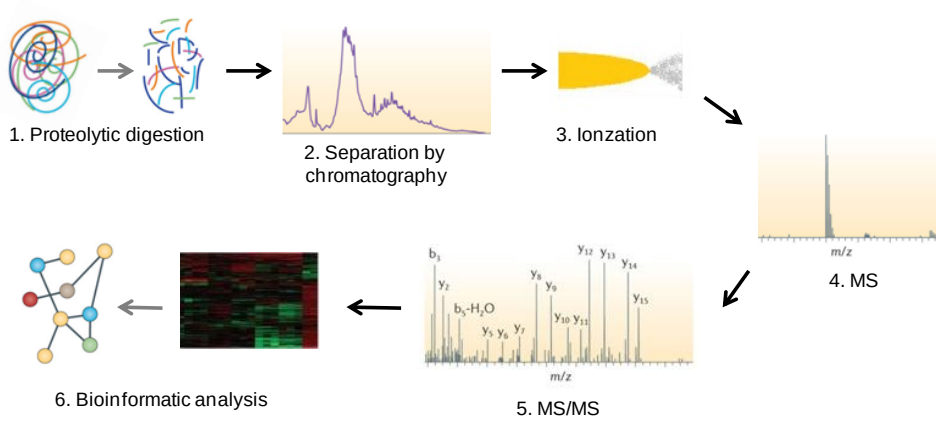


Figure 2. A common workflow in a proteomic experiment. Protein samples can be derived from tissues, plasma, cultured cells. Proteins are digested (1) and the resulting peptide (peptide mass fingerprint-PMF) are separated by chromatography (2), ionized (3) and the mass-to-charge ratio (m/z) is measured (4). To identify the amino acid sequence, peptides are selected for fragmentation and subjected to MMS/MS (5). Finally, bioinformatics tools are used to identified the proteins in the samples (6). Modified by A. F. Maarten Altelaar *et al.*[34].

In fact, while the genome remains constant inside the cell, the protein level is complex, where biological mechanisms may undergo many variations such as glycosylations, phosphorylations, etc. which are further linked to factors associated to time, physiological or pathological conditions, reproductive status, growth or death. The identification and sequencing of all the proteins of a cell are much more complex than mapping and sequencing a genome. If genomics on the one hand can help in the identification of mutations effecting phenotypic traits, proteomics can shed light on the protein kit of some post-translational modifications. There are in fact many potential variability factors between a gene and its corresponding finished product, the protein. These include deletions, amplifications and modifications, co-splicing and post-translational modifications. All this underlines the importance of the study of the proteome as a real science and as such in recent years it has been considered equal to the study of the genome. The challenge of proteomics goes well beyond that posed by functional genomics, being that for 30,000 genes there are about 500,000 proteins, but also the result of changes in gene expression, alternative splicing and post-translational modifications[31,32,33].

Proteomics can be divided into expression proteomics, which allows for a quantitative study of protein expression between different samples; structural proteomics, whose main objective is to map the entire cellular proteome; and functional proteomics, which in time includes the study of the interactions between proteins (interattomica), the study of the interactions between a protein and its substrates (metabolomics) and the study of specific functions of the

protein (enzyme genomics, genomics biochemistry). Proteomics is the comprehensive approach for the comparative analysis of the whole proteomic profile and large-scale protein expressed by a cell, tissue or organism under certain environmental conditions. Proteomics takes advantage of the combination of different technological analyses both, biochemical and computational, allowing for one to simultaneously study thousands of proteins which, when taken together, can decipher the structure, interpret the interactions and analyze the function. Therefore the purpose of proteomics is not only to identify all cellular proteins and their function, but also to create a map of the cell, in which is indicated the localization of each protein. Proteomics is the indispensable complement to genomics and, in an attempt to overcome the limitations related to the static nature of the genome, addresses the difficulties related to the enormous variety and variability of a set of proteins that are constantly changing in response to any type of and intra-extra-cellular event. For now, the main approach of clinical proteomics is directed at diagnosis and the identification of new biomarkers, but may in the future identify new therapeutic targets, new drugs and new vaccines, to improve the treatment and prevention of numerous diseases. Making use of advanced technologies such as mass spectrometry (Mass Spectrometry MS) is currently possible to determine which post-translational modifications were made, information to date which is poorly represented in the literature[33,34,35,36].

The basic concept of two-dimensional-electrophoresis (2D) is schematized in figure 3.

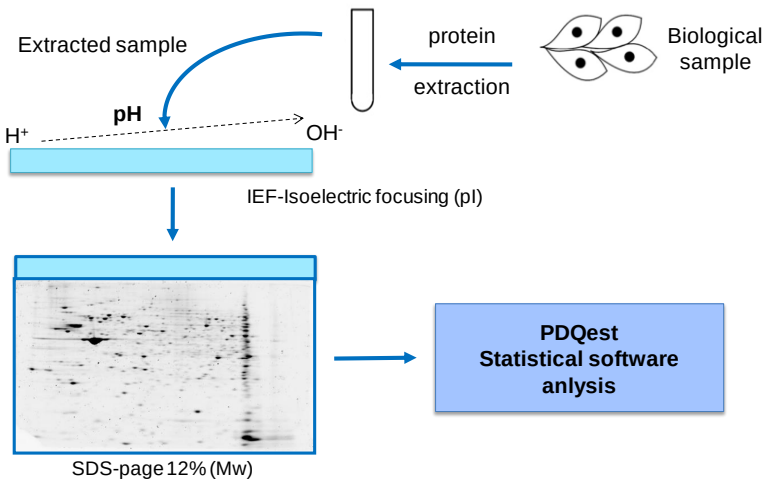


Figure 3. Schematic principle of two-dimensional electrophoresis. Modified by Rabilloud *et al.*[30].

The 2D analysis is a technique that exploits the double separation of complex mixtures of proteins. Firstly the proteins are separated by isoelctric focusing (IEF; the first dimension),

where they are separated on the basis of isoelectric point (pI). In this step the proteins have reached their pI and thus have no remaining electrical charge. Secondly the IEF-gel is then loaded on top of an SDS PAGE gel and the proteins are separated according to their molecular masses (SDS-PAGE; second dimension). The result is a map of "protein spots", which can be further identified and characterized. In order to make an analysis of differential expression, it is essential the digitization of two-dimensional maps and their comparison using appropriate image analysis software, which allows the detection and measurement of the spots on the gel after reducing the noise and removing artifacts.

The identification of the proteins is carried out by combining the use of two-dimensional electrophoresis and mass spectrometry, which allows the analysis of peptides obtained by hydrolyzing proteins with specific proteolytic enzymes[30].

In the recent decades, the remarkable technological advances of mass spectrometry have greatly contributed to the development of proteomics, due to its ability to accurately measure the molecular weight, charge and determine the amino acid sequence of the protein, allowing for a rapid identification of proteins[28,37].

2. CACAO AND CHOCOLATE

2.1 Definition

Cacao (*Theobroma cacao* L.; *Sterculiaceae*), referring to the unprocessed cocoa bean from the plant *Theobroma cacao*, originated deep in the equatorial rain forests of South America[38].

Figure 4. *Theobroma cacao* pods

Chocolate and cocoa are two different terms and are not interchangeable. Cocoa is the non-fat component of cocoa liquor (finely ground cocoa beans) which is used in the processing of chocolate, or as cocoa powder (commonly 12% fat) for cooking and drinks. Cocoa liquor contains approximately 55% cocoa butter and together this comprises cocoa solids, often cited on chocolate packaging. Chocolate refers to the combination of cocoa, cocoa butter, sugar and so forth into a solid food product[39]. A recent survey found that in Europe, 58% of people ate milk chocolate, closely followed by dark chocolate (43 %). For the UK, these figures were 61 and 35 %, respectively. In the USA, milk chocolate is also considered the most popular, but the majority of their confectionery consumption (87 %) is not as pure chocolate but rather it is embellished with nuts, wafer, fruit, etc.[40]. Cocoa taken as a beverage is also popular in some countries like Spain and should also be taken into account when surveying the intake of chocolate and cocoa products[39].

The consumption of cocoa products and chocolate contributes to human nutrition through provision of lipids, sugars, minerals (potassium, magnesium, copper and iron) and antioxidants, principally polyphenols. Tea and red wine have long been known for their high content of polyphenolic substances, but it is not well known that cocoa bean as well as cocoa derived products, also present a rich source of polyphenols, which exhibit an equal or even higher antioxidant capacity than some fruit or vegetables[41].

For the past 10 years, a number of human studies have been conducted utilizing different cocoa products[42,43,44].

However, questions arise as to which of these products could deliver the best polyphenol content and antioxidant effects. Cocoa-derived foods (cacao powders, chocolate, cocoa-related products) are phenolic-rich foods derived from the fermented, roasted and milled seeds of *Theobroma cacao*[45].

These products, consumed all over the world, are largely studied because of the antioxidant and antiradical in vitro properties of some phenolic constituents (phenolic acids, procyanidins, flavonoids)[46].

Phenolics of cocoa (as well as those of other plant species) have been reported in many studies as bio-active compounds (antioxidant, antiradical, anticarcinogenic properties)[47,48,49].

Further, the anti-microbial properties of cocoa phenolics against some food bacterial pathogens have been shown. The anti-microbial activity is directly correlated to the penetrance of the bacterial cell wall[50].

The in vivo bio-activity of cocoa phenolics (as well as phenolics from other foods like coffee and vegetables) have been well studied and are strictly correlated with absorption and metabolism[51].

2.2 Historical overview

The word cacao is derived from Olmec and the subsequent Mayan languages (kakaw). The chocolate-related term cacahuatl is Nahuatl (Aztec language), derived from Olmec/Mayan etymology. The scientific name *Theobroma cacao* was given to the species by the Swedish botanist Carl Linnaeus in 1753, which he published it in his famous book Species Plantarum, *Theobroma cacao* (food of the gods), a combination that blended Greek with Mayan etymology[52].

Over the centuries, the use of cacao has evolved to what we now know as chocolate (processed bean in solid or liquid form containing varying percentages of cocoa liquor, cocoa butter, sugar and milk) and cultivation has extended globally to include the equatorial regions of Africa and Asia. According to the Mayan and Mexican religions, cacao had divine origins. Cacao was discovered by the Gods in a mountain that also contained other delectable foods to be used by the Mayan. Historically speaking, cacao was viewed as magical and mystical. It played an important role in early meso-American cultures and had many uses ranging from a medication to a currency[38].

The medicinal use of cacao or chocolate, both as a primary remedy and as a vehicle to deliver other medicines, originated in the New World and diffused to Europe in the mid 1500s. These practices originated among the Olmec, Mayan and Mexican (Aztec) populations. Although

today chocolate is often viewed as a food with minimal nutritional value, from the middle of 17th century to the 20th century, Europeans praised the dietary and healing properties of cacao. It was believed that chocolate "..comforted the liver, aided in digestion and made one happy and strong." Additional uses for chocolate included stimulating the kidney and treatment of anemia, tuberculosis, fever and gout. Important for this study, during the 17th century, chocolate was also viewed as being of value for strengthening the heart and for the treatment of heart pain. The concept that chocolate (or the beverage cocoa) had health benefits was accepted throughout much of Europe until the 20th century, after which it tended to be viewed with a more neutral perspective. Early colonial era documents included instructions for the medicinal use of cacao[53].

Subsequent 16th to early 20th century manuscripts produced in Europe and New Spain, revealed more than 100 medicinal uses for cacao/chocolate. Three consistent roles can be identified: 1) to treat emaciated patients to gain weight; 2) to stimulate nervous systems of apathetic, exhausted or feeble patients; and 3) to improve digestion and elimination where cacao/chocolate countered the effects of stagnant or weak stomachs, stimulated kidneys and improved bowel function. Additional medical complaints treated with chocolate/cacao have included anemia, poor appetite, mental fatigue, poor breast milk production, consumption/tuberculosis, fever, gout, kidney stones, reduced longevity and poor sexual appetite/low virility. Chocolate paste was a medium used to administer drugs and to counter the taste of bitter pharmacological additives. In addition to cacao beans, preparations of cacao bark, oil (cacao butter), leaves and flowers have been used to treat burns, bowel dysfunction, cuts and skin irritations[52].

One of the first documents to mention cacao, or chocolate, in a Western language was penned by Hernando Cortés in his second dispatch to the Emperor of Spain in a letter dated October 30, 1520. The first Europeans to encounter cacao were Columbus and his crew in 1502, when they captured a canoe at Guanaja that contained a quantity of mysterious-looking "almonds," later identified as a source of currency in Mesoamerica. These "almonds" were cacao beans and Columbus remained unaware of their preparation as a beverage and of their importance in Mesoamerica, in fact, they were commonly used as money. In the centuries after the initial contact between the Spaniards and indigenous peoples of the New World, hundreds of descriptive accounts, monographs and treatises were published that contained information on the agricultural, botanical, economic, geographical, historical, medical and nutritional aspects of cacao/chocolate. Before initial European–Mexican contact in 1519, cacao was prepared only as a beverage and was a food reserved for adult males, specifically priests, highest

government officials, military officers, distinguished warriors and occasionally sacrificial victims for ritual purposes[52,54].

2.3 Production

Of the 22 known species of the genus *Theobroma*, only *T. cacao* is widely cultivated outside of its native habitat. Since the middle of 20th century, *T. cacao* varieties have been classified into three groups: "Criollo", "Forasetero" and "Trinitario", hybrid of the two previous varieties. The harvest of pods occurs about twice per year. In addition to the varietal differences, fermentation of cocoa beans is an important step for obtaining a proper flavor and characteristics. The fermentation step involves some changes in the phenolic content of cocoa nibs: a strong decrease of total soluble phenolic content and the polymerisation of certain constituents, above all (-)epicatechin with one other (-)epicatechin or with anthocyanidins, to form high molecular weight (tannins)[54].

After fermentation, there is the drying phase, following which the beans are cleaned and roasted. Once the beans are roasted, they are crushed to obtain the grain (cocoa nibs), which is then sent to a mill where it is further minced and then melted at 80°C to obtain a mass, the described *cocoa liquor*. The liquor is then hot pressed, where occurs the separation of the cocoa butter from the dry matter, the panel, which is then broken to obtain cocoa powder. Free low molecular weight polyphenols still present in chocolate are responsible for the astringent and bitter taste. Some polyphenolic compounds are clearly involved in color development of *T. cacao* beans, as well as other molecular classes (mainly proteins and reducing sugars involved in Maillard reactions) that act during the fermentation step and during the roasting process[51].

The next stage is the conching where further grinding and mixing takes place at a temperature of 80 ° C, in which the cocoa particles reach a size of 15-20 μM and to which are added cocoa butter and lecithin, to get a better homogenization and optionally other ingredients dependent on the type of chocolate being prepared. The step of tempering is very important. During this operation, the product is cyclically cooled to 18-28 °C to promote the crystallization of cocoa butter in the more stable polymorph and finally heated to 32 °C to melt the less stable crystalline forms. The last phase of the production of chocolate is the molding that occurs inside molds subjected to vibration and cooling to a temperature of 10 °C[40,49,55,56].

Unfermented Forastero cocoa beans have been reported to contain 120–180 g/kg polyphenolic compounds.

The total amount of soluble polyphenols in the dried fat-free mass of fresh cocoa beans is 15 to 20% (about 6% in air dried cocoa beans which contain 54% fat and 6% water) and about 5% in fermented beans. Wollgast and Anklam[46] report on the change in composition and quantity during fermentation, drying and the manufacturing of chocolate.

2.4 Bioactive substance of cocoa

Theobroma cacao L. and cocoa-derived products are phenolic-rich foods. These products are studied largely because of their antioxidant and antiradical in vitro properties due to their phenolic constituents[51]. Furthermore, cocoa can be defined as a functional food due to its high content of monomeric (epicatechin and catechin) and oligomeric (procyanidins) flavanols (fig.5)[45].

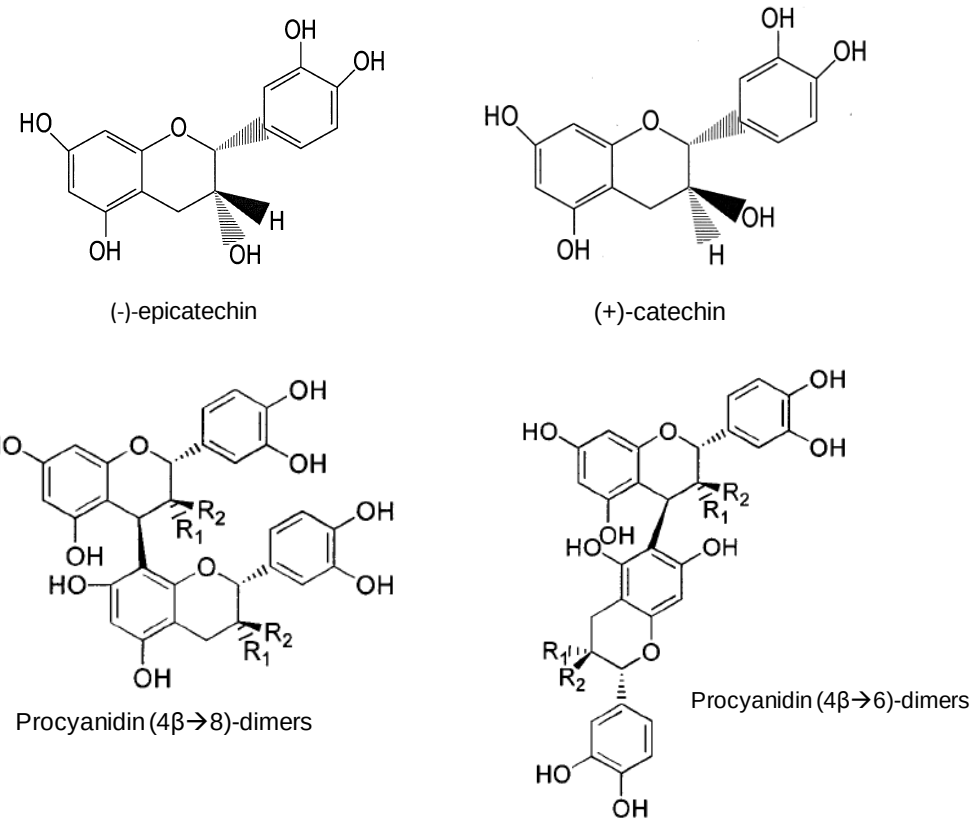


Figure 5. Chemical structure of the major cocoa monomeric flavanols, (-)-epicatechin and (+)-catechin. Two or more monomeric forms are oligomeric procyanidins like procyanidin (4β→8)-dimers and procyanidin (4β→6)-dimers the common procyanidins found in cocoa and cocoa products.

Polyphenols in cocoa and cocoa products can be classified into three main groups: catechins or flavan-3-ols (about 37%), anthocyanins (about 4%; cyanidin glycosides) and

proanthocyanidins (about 58%) [45,49]. According to Kim and Keeney and co-workers[58], the unfermented cocoa bean contains about 120–180 g/kg of polyphenolic compounds, with (-)-epicatechin being quantitatively the main compound (approximately 35%)[49]. Traces of (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin-3-gallate[59], and numerous procyanidins [19,60], as well as small quantities of quercetin, quercetin glycosides, naringenin, luteolin, apigenin, clovamide and phenolic acids such as caffeic, ferulic, gallic and p-coumaric acid have also been found in cocoa products[61,62].

For cocoa, the terms that are used to describe the particular compounds of interest are flavanols (also known as flavan-3-ols or catechins). Flavanols are a subclass of flavonoids which are, in turn, a subclass of polyphenols. Flavanols can be monomeric and those found in cocoa beans are (-)-epicatechin and (+)-catechin (their isomers may also be present in small quantities), dimeric (the most common in cocoa are B2 and B5, both made of two units of epicatechin with differing linkages) or they can be polymeric combinations of these monomers[63].

Procyanidins (PCs) are oligomers of flavan-3-ol monomer units formed from the association of several monomeric units: 2–10 units in (epi)catechin oligomers and over 10 units in (epi)catechin polymers. The PCs differ in the position and configuration of their monomeric linkages, whereby C4→C8 and / or C4→C6 bonds are the predominant types [57].

Their inclination to form complex compounds with proteins, polysaccharides and alkaloids enhances the complexity of cocoa products [45,64].

Cooper *et al.*[39] showed that epicatechin concentrations can be used to predict the content of other polyphenols, especially B2 and C1 and total polyphenol content. Flavonol glycosides such as quercetin-3-O-arabinose, isoquercitrin,quercetin-3-O-glucuronide and quercetin have also been reported in cocoa samples. Another minor phenolic cocoa constituents, such as clovamide, should be considered and more investigated for their beneficial properties[46,57,62, 65].

3. POLYPHENOLS

3.1 Polyphenols

Polyphenols are products of the secondary metabolism of plants. This is an extremely wide and complex group of plant substances, which can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. They constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets[66].

For decades, plant polyphenols have interested scientists because they are essential to plant physiology for their contribution to plant morphology (ie., pigmentation). They are also involved in growth and reproduction and provide plants with resistance to pathogens and predators (by acting as phytoalexins or by increasing food astringency, thus making food unpalatable), they protect crops from plague and pre harvest seed germination, amongst numerous other roles. The polyphenolic profiles of plants differ between varieties of the same species [66,67,68].

As such, polyphenols, have been studied for taxonomic purposes or to determine the adulteration of food products. They have several industrial applications, such as in the production of paints, paper and cosmetics, as tanning agents and in the food industry as additives (natural colorants and preservatives)[66].

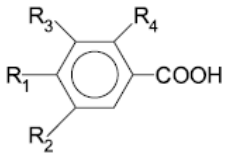
In addition, some phenolic compounds, the flavonoids, have applications as adjuvant therapeutic treatments and anti-inflammatory agents, as well as in the treatment of diseases such as hypertension, vascular fragility, allergies, hypercholesterolemia amongst numerous others[68]. An interest in food phenolics has greatly increased recently, due to the antioxidant and free radical-scavenging abilities associated with some phenolics and their potential effects on human health[69].

The chief reason for this interest is the recognition of the antioxidant properties of polyphenols, their great abundance in our diet and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases. Much of the literature refers to a single group of plant phenolics, the flavonoids[70].

3.2 Chemical characteristics

Phenolic compounds as food components are represented by more than 6000 identified substances, the largest group of secondary metabolites in plant foods [57].

They are characterized by a large range of structures and functions, but generally possess an aromatic ring bearing one or more hydroxy substituents. Kroll *et al.* [63] summarized the structure, classification and distribution of phenols in plant foods, intake and their physiological effects. Polyphenols are the most abundant antioxidants in a diet and the daily intakes of total polyphenols may range from less than 100 mg to in excess of 2 g [57]. According to Harborne *et al.*[71], polyphenols can be divided into at least 10 different classes depending on their basic chemical structure. With regard to their structure, the major groups of phenolic compounds are distinguished by a number of constitutive carbon atoms in conjunction with the basic phenolic skeleton. The food relevant groupings include firstly, the hydroxybenzoic acid derivatives (HBAs) with a general structure C6-C1 (e.g. salicylic and gallic acids) (fig. 6)[72].



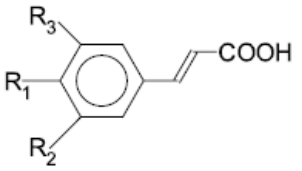
hydroxybenzoic acid	R_1	R_2	R_3	R_4
benzoic acid	H	H	H	H
salicylic acid	H	H	H	OH
para-hydroxybenzoic acid	OH	H	H	H
protocatechuic acid	OH	OH	H	H
gallic acid	OH	OH	OH	H

Figure 6. The general structure of hydroxybenzoic acid derivatives with a general structure C6→C1

Variations in their basic structure being hydroxylations and methoxylations of the aromatic ring. As a general rule, HBAs are present as conjugates, although they can also be detected as free acids in some fruits. The main source of gallic acid is tea, particularly green tea, where it occurs also esterified as (–)-epigallocatechin- 3-gallate (EGCG)[72].

The second group are the phenolic compounds having the general formula C6-C3 (hydroxycinnamic acid derivatives, fig. 7), representing a series of trans-phenyl-3-propenoic acids differing in their ring substitution. These compounds are widely distributed as

conjugates in plant material including many foods and beverages where the most common are the caffeic acid (3,4-hydroxycinnamic acid)[73].



hydroxycinnamic acid	R_1	R_2	R_3
cinnamic acid	H	H	H
para-coumaric acid	OH	H	H
caffeic acid	OH	OH	H
ferulic acid	OH	OCH_3	H
sinapinic acid	OH	OCH_3	OCH_3

Figure 7. The general structure of hydroxcinnamic acid derivatives with a general structure C6→C3

The third and the largest group are represented by the flavonoids, which can be further subdivided into 13 classes, with more than 4000 compounds having been characterized[63].

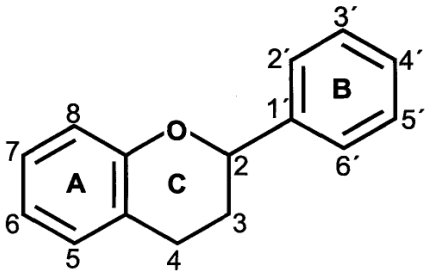


Figure 8. The structure of the flavonoids with a C6-C3-C6 flavone skeleton

They are also the most widespread and diverse, with their common structure being diphenylpropanes (C_6-C_3-C_6) consisting of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle[65]. Figure 8 adapted from Bravo *et al.*[66] represents the basic structure and the system used for carbon numbering of the flavonoid nucleus. Flavonoids occasionally occur in plants as aglycones, although they are most commonly found as glycoside derivatives[66].

The common classes and food sources of flavonoids include: flavanols (quercetin, kaempferol, myricetin in onions, apples, tea and red wine), isoflavones (daidzein, genistein in soy), flavan-3-ols or flavanols (catechin, epicatechin in tea, chocolate, red wine), flavanones

(naringenin, hesperitin in citrus fruits), flavones (apigenin in celery, luteolin in red pepper) and anthocyanins (in the pigments of red fruits such as berries and red grapes) (fig. 9)[69].

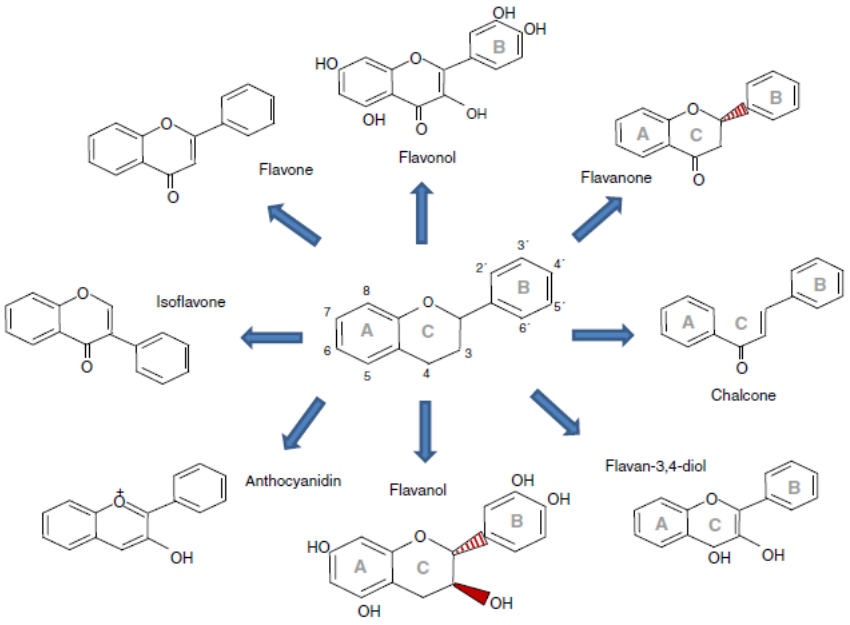
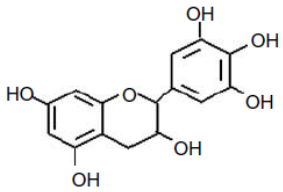


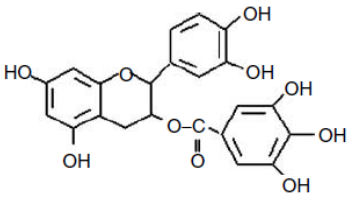
Figure 9. Chemical structures of the main flavonoid families present in plants that are part of human diets. In the center: the basic generic flavonoid structure showing A, B, and C rings and the numbers for the various positions in the flavan structure. For the flavanol family, the structure of (−)-epicatechin is shown.

As a subclass of the flavonoid family, flavan-3-ols can be subdivided based upon the degree of polymerization, oxidative state and the substitution pattern of the B- and C-rings[74].

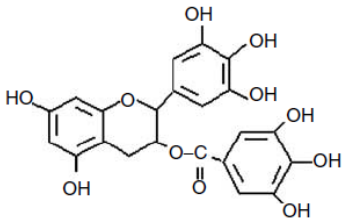
Five major monomeric flavan-3-ols, referred to as catechins, are found in the diet: (+)-catechin (C), (−)-epicatechin (EC) (fig. 5), (-)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG) (fig. 10). Structurally, gallocatechins (EGC and EGCG) differ from catechins (C, EC, and ECG) by having a third B-ring hydroxyl group at C5. Catechin gallates (EGCG and ECG) have a gallic acid residue esterified to the C3 hydroxyl[75,76].



(-)-Epigallocatechin (EGC)



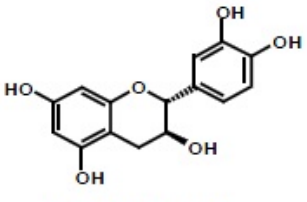
(-)-Epicatechin-3-gallate(ECG)



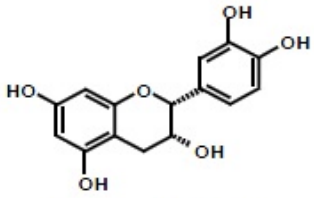
(-)-Epigallocatechin-3-gallate (EGCG)

Figure 10. Chemical structure of the major tea monomeric flavanols.

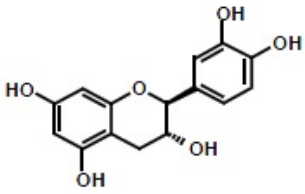
Due to the two chiral carbons in the C-ring (C2 and C3), multiple stereoisomers exist for each catechin (fig. 11)[39,77].



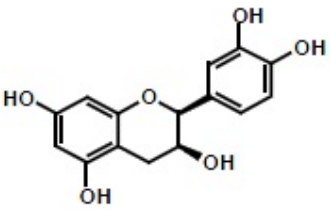
(+)-Catechin (2R, 3S)



(-)-Epicatechin (2S, 3R)



(-)-Catechin (2S, 3R)



(+)-Epicatechin (2S, 3S)

Figure 11. Structure of the catechin/epicatechin enantiomers

(-)Epicatechin is quantitatively the main compound of cocoa phenolics (approximately 35% of polyphenols content of unfermented Forastero cocoa beans). The total soluble phenolics of good fermented dried cocoa beans ranges from 2 to 6%, strictly depending on the variety as well as the geographic origin. The typical content for Forastero is about 6%, while the soluble phenolic content in Criollo cacao is about 2/3 of Forastero. A high content are often

an index of bad fermentation. In addition to monomers, more complex flavan-3-ols exist, including the proantocyanidins (PAs). The PAs are dimers (2 monomer residues), oligomers (3–7), and polymers (≥8) of flavan-3-ol monomers. Monomers are bonded by interflavan linkages between the C-ring of the first monomer and either the A- or C-ring of the next. Proanthocyanidins (PAs) are subdivided in procyanidins (PCs), prodelphinidins (PDs) e Propelargonidins (PGs). The PAs consisting exclusively of (epi)catechin are PCs[78,19].

A-type PAs have monomers joined by two interflavan linkages: the C4→C8 bond plus a C2→O→C7 ether bond. The B-type PAs have only one interflavan linkage (typically aC4→C8 or C4→C6 carbon-carbon bond) and finally the C-type condensed tannins are trimeric B-type condensed tannins[70,79,80]

3.3 Bioavailability

It is important to know not only a person's daily intake of dietary polyphenols but also the bioavailability of those ingested polyphenols, since their nutritional significance and potential systemic effects will greatly depend on their behavior in the digestive tract[81].

Only some 5% of dietary polyphenols are absorbed in the duodenum, and of this only 5%, mainly flavanols, reach the plasma unchanged. Over 95% of the intake passes to the colon and is fermented by the gut microflora[57].

This is not a straightforward process, with little is known about the absorption of polyphenols in the gastrointestinal tract, such as whether they are retained in the body after absorption and what their biologic significance might be[82].

The enormous variability of this group of substances, as well as their occurrence in plant materials as a complex mixture of phenolic compounds, creates great difficulties in the study of their bioavailability and their physiologic and nutritional effects. Direct evidence of bioavailability can be gained from plasma concentrations of the flavonoid or its metabolites and indirect evidence can be obtained from biomarkers such as plasma antioxidant capacity, LDL oxidation susceptibility, platelet function, vascular tone and immune responsiveness [38,83,84,85,86].

The first human bioavailability trial of polyphenols from chocolate found that with 40 g of black chocolate, epicatechin was indeed absorbed into the blood. Epicatechin was present in plasma as metabolites conjugated with glucuronide and sulphate groups. These compounds exhibited a Tmax of 2 h in the plasma and Cmax of over 100 ng/ml[39].

Chocolate (particularly dark chocolate) can be seen as a relevant source of phenolic antioxidants. With respect to the bioavailability of polyphenols, in particular flavanols, reliable data on the real content of polyphenols in food are still scarce[51].

Despite this, the bioavailability of flavanols (as well as all phenolics) has been discussed. For example, independently of the doses of chocolate and cacao ingested, only 0.5% of catechin was recovered in the free unbound form in the plasma and in the urine[51,49,54,87].

Estimates from human studies indicate that dietary flavanols, including those found in cocoa, display variable oral absorption, with reported values varying from less than 1 to greater than 50 percent of the ingested dose, as cited by Rein *et al*[88], Richelle *et al.*[44] and Schramm *et al.*[89] but more realistic values of the observed average absorption should be around 5%. The variable absorption of flavanols between subjects probably results from the complexity of the in vivo system, a large inter-individual variability, as well as variability between differing compounds such as quercetin, epicatechin and soy isoflavones. Chemical structure plays a large role in determining the absorption, distribution, metabolism and excretion and ultimately the biologic properties of these phenolic[87].

Significant losses of bioactive substances are also attributed to gastric pH-induced degradation[90], enterocyte metabolism of flavanols, first pass metabolism and the type and activity of intestinal microflora[89].

The plasma half-life of all flavonoids is relatively short (24 hours) and consistent with this, plasma epicatechin concentrations typically return to baseline values within six to eight hours after the consumption of a flavonoid-rich chocolate meal[38].

Another issue to take into consideration when talking about bioavailability, is the chiral nature of polyphenols and the effect of chirality. For instance, the (+) form of catechin tends to dominate in cocoa beans and the (-) form in chocolate. Schroeter *et al.*[91] found that chocolate tended to contain predominantly (-)-epicatechin and (-)-catechin, with only small amounts of (+)-catechin and negligible (+)-epicatechin. The same research indicated that the (+) form of catechin was almost 10 times more absorbed than the (-) form using a rat perfusion model, which may explain why catechin from cocoa is not as well absorbed as from other foods[87].

Flavonoids circulating in plasma, including those derived from cocoa, occur primarily as glucuronide, methyl and sulfate conjugates, or combinations thereof as a result of phase I and II enzyme action in the small intestine and liver[92].

Orally administered epicatechin in mammals is thought to largely undergo glucuronidation at the level of the intestinal mucosa[93].

Thus, the rise in epicatechin in plasma observed after the proantocyanidinis-rich chocolate intake may be due in part to epicatechin metabolites (in particular, unmethylated glucuronide, glucuronide sulfate and unmethylated sulfate conjugates), as well as to free epicatechin[57,88].

Epicatechin is rapidly absorbed in humans. Indeed, in healthy human adults, plasma concentrations can approach 1umol/L within two hours after the consumption of flavonoid-rich chocolate. Plasma epicatechin concentrations from dark chocolate are in the same range have been observed following consumption of comparable flavonoid quantities from onions, apples and tea[75].

After absorption in the intestinal tract, (-)-epicatechin is rapidly metabolized by both enterocytes and hepatocytes. In both cell types, (-)-epicatechin undergoes transformation by uridine-50-diphosphate glucuronosyl-transferases, sulfo-transferases, and catechol-O-methyltransferases. The net result of the extensive first-pass metabolism is the presence of glucuronides, sulfates, and/or methyl conjugates in the bloodstream. The metabolic fate of (-)-epicatechin after the ingestion of cocoa powder, chocolate and teas In humans has been previously investigated. The majority of those reports have analyzed (-)-epicatechin after the exposing the biological samples to glucuronidase or sulfatase enzymes[92].

Recently, Ottaviani *et al.*[94] identified two main metabolites, however, they could not completely describe the entire conjugate forms because the analytical methodologies employed were semi-quantitative due to the lack of purified and isolated (-)-epicatechin conjugates for use as standards. Therefore, despite several investigations, the complete (-)-epicatechin conjugates in biological samples are still unclear and it will be a crucial step in elucidating the molecular mechanism of action[92].

Experiments reported in the literature have used extracts of different plant materials that contain a mixture of soluble phenolic compounds or pure standards used as supplements in complex foods administered either to laboratory animals or human volunteers. When plant extracts are used, it is possible to gain information on the effect of their constituent polyphenols as a group, but not on the digestive fate and specific effects of individual polyphenols. Conversely, differences in the absorption, metabolism and physiologic effects of food phenolics administered as supplements, compared with polyphenols that are part of a complex food matrix, cannot be ruled out. Finally, the extrapolation of animal data to humans is not clear[66].

Bioavailability can also be affected by the matrix in which the cocoa polyphenols are delivered. It is possible that different matrices affect the release of polyphenols from food, making them more or less available for absorption[39].

This is a question that is often asked when considering health effects of dark or milk chocolate? Many countries around the world predominantly consume cocoa as part of milk chocolate rather than dark. The polyphenols in chocolate come from the cocoa liquor. Hence, as milk chocolate generally contains less cocoa liquor than dark chocolate, it will contain less polyphenols. White chocolate contains no cocoa liquor and hence no polyphenols at all. However, this is complicated by the fact that polyphenols can be destroyed during the processing of the raw cocoa depending on the manufacturing methods used. The concentration of flavanols in any chocolate depends on both the flavanol content of the cacao plant and the procedures used for transforming the cocoa into chocolate[16].

Interestingly, Serafini *et al.* [95] demonstrated an increase in total antioxidant capacity and plasma epicatechin concentrations after consumption of dark chocolate (100 g) in 12 healthy volunteers; however, these effects were reduced by the presence of milk. It was also shown that the absorption of epicatechin from chocolate was significantly less when consumed with milk or as milk chocolate. Thus, the hypothesis is that milk proteins bind to cocoa polyphenols, which in turn prevents their absorption in the gastrointestinal tract. However, this study generated much controversy in the literature. Schroeter *et al.*[96] reported that the presence of milk in cocoa products did not influence the absorption or biological activity of epicatechin from the cocoa product. Matrix effects are becoming increasingly important for food due to a new EU legislation Directive 2000/13/EC that came into effect in January 2007 which makes it essential that any food labeling a high content of a beneficial compound must be able to demonstrate that it is bioavailable from that food product and has its implied beneficial effect. However, it is felt that this issue with respect to chocolate, has not been resolved, as there has been no definitive study confirming the bioavailability with solid milk chocolate. It is important to resolve this issue as milk chocolate is much more popular in many countries[39].

All of these limitations represent difficulties to overcome in studying the bioavailability of polyphenolic compounds and their nutritional significance and as such caution in interpreting results is necessary. Both in vivo and in vitro studies using polyphenolic compounds with different chemical structures and solubility illustrate varying susceptibility to digestion, fermentation and absorption within the gastrointestinal tract[66].

3.4 Polyphenols effect

Experimental studies support a role for polyphenols in the prevention of cardiovascular diseases[97], cancers[98], neurodegenerative diseases[99], diabetes and osteoporosis[100].

However, it is very difficult to predict from these results the effects of polyphenol intake on disease prevention in humans[65].

Firstly, estimation of dietary intake of polyphenols is difficult, due to limited availability of food composition data and the inherent bias of dietary assessment methods, thus identifying a need for good biomarkers[101].

Secondly, most of the observed physiological effects are based on either in vitro models, cell/tissue culture studies, animal experiments or epidemiological data. The results thus obtained are generally projected to show if a strong association or correlation of activity in humans is possible or not, since data from human investigations are relatively rare. Thirdly, these studies have often been conducted at doses or concentrations far beyond those documented in humans. More human studies are therefore needed to provide clear evidence of their health protective effects and to better evaluate the risks possibly resulting from too high polyphenols consumption[65].

Recent human studies have proven that chocolate has beneficial effects on some pathogenic mechanisms of heart disease such as endothelial function and blood pressure[38,102].

Flavanol-rich foods can positively affect hemostasis, through mechanisms that either directly affect platelet function or increase certain endothelium-derived factors that maintain platelet acquiescence or increase fibrinolysis. Holt *et al.*[103] found that after the consumption of 25 g chocolate, supplying 220 mg flavanols and procyanidins, there was a decrease in primary hemostasis, measured by the time to occlude an aperture in a collagen membrane, being observed 2 h after consumption. Further, Murphy *et al.*[104] demonstrated that an acute dose of flavanols and oligomeric procyanidins from cocoa powder inhibits platelet activation and function over 6 h in humans. The inverse association of a high fruit and vegetable intake with the risk of CVD mortality in numerous epidemiologic studies has led to many hypotheses regarding the physiologic role of flavonoids. Cocoa, tea and wine are increasingly viewed as sources of dietary components with potentially beneficial functional activities. It should be noted, however, that there are no epidemiologic studies specifically evaluating the relationship of chocolate intake and CVD risk. The chemical structure of flavonoids suggests that they have antioxidant capacity, with the ability to scavenge free radicals and chelate redox active metal ions. It has been postulated that these bioactive compounds can contribute

to the maintenance of the integrated network of cellular and plasma oxidant defense mechanisms, to vascular wall tone and to a reduction in platelet reactivity with a subsequent reduction in the risk of clot formation. The key to determining the physiologic significance of dietary flavonoids is to develop an understanding of their metabolism and mechanisms of action[105]. In the past 10 years, there has been increased interest in the potential health-related benefits of antioxidant- and phytochemical-rich dark chocolate and cocoa[106].

It has been becoming clear that there is a relationship between the effect of antioxidants in a diet and immune functions. Recently it was suggested that chocolate contains large amounts of polyphenols that have antioxidant activities[48]. All polyphenols exert an antioxidant action *in vitro*; however, this does not mean that all polyphenols have an antioxidant effect *in vivo*[45].

It is apparent in most of the studies, which examined the antioxidant properties of chocolate or cocoa beverage, that the major cocoa flavanol, epicatechin, is readily absorbed and peaks in the plasma concentration at 2 hours. The antioxidant properties of cocoa or chocolate consumption are associated with an increase in plasma epicatechin concentrations[69].

The flavan-3-ols have been identified as the major antioxidant components of different cocoa ingredients and chocolate preparations. Oxygen radical absorbance capacity (ORAC) data show that chocolate, as a whole food, has a potent antioxidant capacity when compared with other phytochemical-rich foods such as garlic, blueberries and strawberries[38].

Commercial chocolate products were found to decrease lipid oxidation when added to LDL preparations in vitro. That the amount of epicatechin absorbed from a dose of chocolate can be physiologically important is suggested by the observation that significant increases in plasma antioxidant capacity and decreases in plasma lipid oxidation products, correlate with the changes in plasma epicatechin concentrations. Examination of the literature regarding CVD risk reduction and plasma antioxidant effects of catechins from dietary sources other than chocolate reveals mixed results, but, overall, a trend toward beneficial health effects is apparent. A recent meta-analysis of epidemiologic studies on tea consumption concluded that 3 cups a day may reduce the population incidence rate of myocardial infarction[107].

Comparison of individual clinical trials must take into account variations in design, methods, and outcome measures of the studies. For example, two studies reported that consumption of six cups of green or black tea, or a green tea infusion containing about 400 mg of catechin, resulted in a significant increase in the total plasma antioxidant capacity in humans. In contrast to this, consumption of green or black tea was not shown to increase the resistance of LDL to ex vivo oxidation, despite observations that tea catechins significantly increased LDL resistance when added to the assay in vitro[38].

The antioxidant properties of cocoa were largely studied in recent years by means of different approaches: chemical characterization of involved antioxidant species (HPLC, HPLC-MS)[108], in vitro chemical studies (in order to study the ability to scavenging some stable radicals like DPPH, ABTS+), in vitro biological studies and nutrigenomic-based studies (bioavailability, in vivo interaction with cellular/molecular species)[109,26] .

Some bio-active properties of cocoa are strictly related to phenolic content as well as to some compounds from the Maillard reactions (non-enzymatic brown pigments)[51].

In conclusion, the citation: "It is yet to early to give an answer to the question, whether chocolate and / or other sources rich in catechins and procyanidins are beneficial to human health and thereby becoming functional in their nature", still explains the current status. Factors like food matrix (e.g. consumption of milk at the same time as dark chocolate reduce the bioavailability of polyphenols) still need to be considered[46].

3.5 Bioactive action of cocoa polyphenols

It is an appealing idea that a food commonly consumed for pure pleasure could also bring tangible benefits for health. Cocoa is rich in polyphenols, similar to those found in green tea, and as polyphenols have been shown to have beneficial effects on CVD, it has resulted in heart health being the most common target for research on cocoa[39]. Richelle *et al.*[44] first demonstrated the appearance of epicatechin in blood after consumption of black (dark) chocolate and 3 years later a study demonstrated the presence of a dimer in the plasma within 30 min post-consumption of flavanol-rich cocoa. Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage. Manach and co-workers[110] reviewed 97 studies of various classes of polyphenols. They have compiled the data from the most relevant studies, those using well-described polyphenol sources and accurate methods of analysis, to calculate mean values for several bioavailability measures, including the maximal plasma concentration (Cmax), time to reach Cmax, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion. The results clearly showed wide variability in the bioavailability of the different polyphenols and the metabolites present in blood, resulting from digestive and hepatic activity, usually differ from the native compounds. Cocoa polyphenols have been reported in many studies as bioactive compounds, with antioxidant, antiradical and anticarcinogenic properties[111]. Oligomeric procyanidins

isolated from cocoa have been shown to possess biological activities potentially relevant to oxidant defences and immune function[112].

Further, it is well-known that methylxanthines have physiological effects on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems[45]. Flavanols are attractive candidates for natural and alternative medicine due to their high abundance in green tea, red wine, cocoa and chocolates. Interestingly, it was found that a relatively small community of Kuna Indians residing in a Panamanian archipelago exhibit low blood pressure and a reduced incidence of cardiovascular disease and stroke. After a comprehensive assessment of genetic and environmental factors, epidemiological studies concluded that these health benefits may result not necessarily from genetic predisposition but likely from a high intake of cocoa extract that was found to be especially enriched in flavanols[113]. The research is predominantly focused on effects on the vascular system: however, there are other areas of research on man in vivo which are not so extensively investigated, such as those concerned with cognition, cancer, diabetes and inflammation. Multiple approaches have been used to investigate the mechanism of action of cocoa polyphenols including clinical, preclinical and in vitro studies[39].

3.5.1 Anti-hypertensive effect

Hypertension is a well-established risk factor for the development and acceleration of atherosclerosis. Oxidative stress and the inactivation of NO by vascular superoxide anion ($O2^{\bullet-}$) play critical roles in the pathogenesis of vascular disease, including hypertension[101].
Gómez-Guzmán *et al.* [114] studied the effects of chronic treatment with epicatechin on blood pressure, endothelial function and oxidative status in deoxycorticosterone acetate (DOCA)-salt-induced hypertension. Rats were treated for 5 weeks with (−)-epicatechin at 2 or 10 mg kg^{-1} day^{-1}. The high dose of epicatechin prevented both the increase in systolic blood pressure and the proteinuria induced by DOCA-salt. They found that epicatechin increased the transcription of nuclear factor-E2-related factor-2 (Nrf2) and Nrf2 target genes in aortas from control rats and also improved the impaired endothelium-dependent relaxation response to acetylcholine and increased the phosphorylation of both Akt and eNOS in aortic rings[115].
In the recent years, regarding the great interest about this topic, many meta-analyses have been published. Hooper and co-worker[116] performed a meta-analysis, a total of 1637 potentially relevant titles and abstracts were identified from searches on Medline, EMBASE, and the Cochrane Library, together with other reference sources and information provided by

relevant experts. Ninety-eight articles were collected as full text and assessed for inclusion, and 42 acute and short-term chronic studies were identified as being randomize controlled trials (RCTs) of chocolate, cocoa, or cocoa flavan-3-ols.

They observed a reductions in diastolic blood pressure and mean arterial pressure after cocoa chronic intake but no effects on systolic blood pressure. Their results suggest that epicatechin dose may be a key contributor to the effects observed. Doses of >50 mg epicatechin/d reduced systolic and diastolic blood pressure, whereas doses <50 mg/d did not. These findings support that an oral administration of pure (-)-epicatechin mimics acute vascular effects of flavan-3-ol–rich cocoa. This suggests that lower-dose studies may dilute the "true" response of chocolate or cocoa, thus reducing the apparent effectiveness within meta-analyses.

The identification of healthy foods and the understanding of how food components influence normal physiology will help to improve the health of the population. This could be especially relevant with regard to untreated pathologic states[16]. Several open-label studies described by Egan *et al.*[115] reported that dark chocolate lowered blood pressure among volunteers with a wide range of ages and baseline blood pressure values. In the 13 reports, dark chocolate lowered BP in 6 of 7 open-label studies but diastolic BP only in 1 of 6 double-blind studies. Collectively, these reports raise questions on the BP-lowering efficacy of dark chocolate/cocoa, including the possibility that BP effects are negligible. These studies suggest that dark chocolate could be useful for the prevention and management of hypertension in a broad segment of the population. However, the published studies on chocolate and blood pressure include a relatively small number of subjects, with conflicting result, such that a clear answer on the antihypertensive effects of dark chocolate may not yet be given.

3.5.2 Effect on lipid oxidation

Intervention trials and prospective studies have shown that hypercholesterolemia, especially increased concentrations of LDL cholesterol, leads to the development of atherosclerosis. Epicatechin, the major polyphenol in chocolate and chocolate extracts, is a powerful inhibitor of plasma lipid oxidation due to polyphenols ability to bind to lower density lipoproteins[38]. There is also evidence that oxidized LDL has a pathogenic role in the development of atherosclerosis. Uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks, a key event in early atherosclerosis[85]. Waterhouse and colleagues[117] wrote a letter, which was published in 1996, that described an in vitro

experiment that was to open up a whole new area for nutrition and health. Polyphenols were extracted from commercial cocoa and chocolate, with the polyphenol content and antioxidant activity against LDL oxidation assessed. They found a potent inhibition by the cocoa polyphenols. At 5mmol/L total polyphenols (expressed as gallic acid equivalents), LDL oxidation was inhibited by 75 %, compared to red wine at 37–65%. This was the first publication to state that the action and content of polyphenols from cocoa meant that it could be considered as a dietary source of antioxidants[39]. Studies carried out in healthy human subjects also showed a decrease in LDL cholesterol concentrations, increases in HDL cholesterol concentrations and a resistance of LDL to oxidation following the intake of dairy cocoa powder. An example is the study conducted by Baba *et al.*[85] where they evaluated plasma LDL cholesterol and oxidized LDL concentrations following the intake of different levels of cocoa powder (13, 19.5, and 26 g/d) in normocholesterolemic and mildly hypercholesterolemic humans. In their comparative, double-blind study, 160 subjects who ingested either cocoa powder containing low-polyphenolic compounds (placebo-cocoa group) or 3 levels of cocoa powder containing high-polyphenolic compounds (13, 19.5, and 26 g/d for low-, middle-, and high-cocoa groups, respectively) for 4 wk. A stratified analysis was performed on 131 subjects who had a LDL cholesterol concentrations of ≥ 3.23 mmol/L at baseline. In these subjects, plasma LDL cholesterol, oxidized LDL, and apo B concentrations decreased, and the plasma HDL cholesterol concentration increased, relative to baseline in all the cocoa groups. The results suggest that polyphenolic substances derived from cocoa powder may contribute to a reduction in LDL cholesterol, an elevation in HDL cholesterol, and the suppression of oxidized LDL. However, this finding was not stable to sensitivity analysis in which lower-quality studies were removed or in which data from different study durations were used.

3.5.3 Effects on glucose metabolism and insulin resistance

Numerous studies indicate that polyphenol-rich chocolate reduces fasting blood glucose, blood pressure and total cholesterol in healthy and hypertensive individuals, with or without glucose intolerance. Grassi and co-worker[118] reported that the consumption of dark chocolate improves glucose metabolism and decreases blood pressure. They studied 15 healthy young adults with typical Italian diets that were supplemented daily with 100 g dark chocolate or 90 g white chocolate, each of which provided 480 kcal. The authors found that the dark

chocolate supplement was associated with decreased insulin resistance and increased insulin sensitivity.

In another study published by Almoosawi *et al.*[119] they demonstrated that polyphenol-rich dark chocolate reduces fasting blood glucose levels and blood pressure in overweight and obese individuals. Moreover Hopper and co-worker[116], mentioned in the section 3.5.1 above, in the same meta-analysis suggested significant reductions in fasting serum insulin concentrations, serum insulin after glucose challenge after chocolate or cocoa interventions.

Their results support the reciprocal relation between insulin resistance and endothelial function and suggest that the effect of cocoa/chocolate interventions on fasting insulin concentrations may be associated with endothelial function as suggested by significant studies on insulin and vascular diseases or VCAM regulation[120,121]. These data are further supported by in vitro studies showing effects of flavan-3-ols and their metabolites on glucose transport, bioavailability and bioactivity of nitric oxide, inflammation, platelet function, and angiotensin-converting enzyme activity[116].

3.5.4 Effect on the inflammatory system

During the past years, a growing number of studies, mostly conducted in vitro or ex vivo, have demonstrated that flavanols share the capacity to modulate inflammation as well as other major metabolic and immunological pathway. It has been suggested that cocoa-derived flavonoids may affect the production of pro-inflammatory mediators. In general, flavonoids are often considered as potential anti-inflammatory molecules. However, in some studies, procyanidins, as present in cocoa, partly increased the production of tumor necrosis factor alpha and NF-kB dependent pro-inflammatory gene expression. Among the myriad of health promoting effects suspected for antioxidants, anti-inflammatory actions seem to be promising. In fact, flavanols inhibit lipid peroxidation and affect production of lipid or lipid-derived molecules regulating the immune response and, recently, dietary cacao has been shown to ameliorate obesity-related inflammation in high fat-fed mice[122]. Data from numerous studies suggest that cocoa-derived flavanols can effectively modify the inflammatory process[45]. It has been observed that cocoa products influence specifically endothelium-derived NO synthesis and metabolism, cytokines production and eicosanoids metabolism through a specific action on peripheral blood mononuclear cells (PBMC)[123].

Furthermore, preliminary in vitro investigations have suggested that cocoa flavanols or procyanidins may possess immunoregulatory effects and may help to modulate immune responses[124].

Sanbongi *et al.*[125] demonstrated that cacao liquor polyphenols inhibit reactive oxygen species and reduce the expression of interleukin 4-2 mRNA in human lymphocytes. Polyphenols from other plant sources also inhibit the cellular expression of IL-8 and monocyte chemoattractant-1 when induced by the proinflammatory cytokine tumor necrosis factor-α Several clinical studies have shown improved endothelial function after cocoa consumption, but it is not known if these improvements are due to a subtle combination of mild effects rather than a single targeted effect[39].

Dietary intervention trials investigating the effect of flavonoids on markers of inflammation in human subjects are scarce and are focused on a limited number of foods of plant origin such as black and green teas, fruit juices, grape extract and red wine. The experimental evidence in human subjects suggests a direct role for plant foods in modulating the inflammatory response in vivo. However, the mechanism and the molecules responsible for this effect have not been identified[126]. Murphy *et al.*[104] showed an effect on P-selectin plasma levels together with an increase of plasma catechins after 4 weeks of cocoa tablet administration. Widlansky *et al.*[127] observed, in patients with coronary artery disease, an increase in plasma catechin concentrations after 4 weeks of daily ingestion of 900 ml black tea, without any effect on CRP. Steptoe *et al.*[128] showed a reduction in CRP levels after 6 weeks of black tea consumption, however, no evidence was provided regarding the extent of flavonoid absorption. Studies with alternative sources of dietary flavonoids such as grape juice and red wine were also contradictory. Watzl *et al.*[129] showed that both acute and chronic administration of red wine, de-alcoholized red wine and red grape juice had no effect on cytokine production, phagocytic activity of neutrophils and monocytes, lymphocyte proliferation and lytic activity of natural killer cells[130]. However, Estruch and co-workers[131] found reduced plasma levels of fibrinogen, IL-1α, CRP, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and an increase in plasma levels of epigallocatechin, after 4 weeks of red wine consumption. Although existing evidence indicates that flavonoids potentially display a multitargeting anti-inflammatory action, a clear conclusion on their effects in human subjects cannot be drawn.

3.5.5 Effect on endothelium

Cocoa polyphenols have been investigated predominantly for their effect on the vascular system, with NO concentrations being a central target. One of these effects is on endothelial function, which is an extremely promising biomarker to calculate heart attack risk[45].

NO is produced from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS). This enzyme is present in mammals in different isoforms: endothelial (eNOS), neuronal (nNOS), inducible (iNOS), which is expressed in response to different stimuli as well as mitochondrial NOS (mtNOS). NO reacts with several metal centers, molecular oxygen, thiol groups and some oxygen radicals[80].

The beneficial cardiovascular effects of flavonoids are attributed to their ability to improve endothelial function, by activation of the NO synthase system. In particular cocoa polyphenols may activate endothelial nitric oxide synthase (eNOS) thereby increasing the concentration of vasodilative nitric oxide. L-arginine is the substrate of eNOS and arginase competes with eNOS for L-arginine as the common substrate. Thus, the concentration of this aminoacid is an important determinant for cellular NO production by eNOS[132,133].

It is hypothesized that the potential beneficial effects of chocolate on vascular health are at least partly mediated by cocoa polyphenols including procyanidins. Based on cell culture studies, molecular targets of chocolate polyphenols are endothelial nitric oxide synthetase as well as arginase[69]. There is also some evidence suggesting that platelet aggregation may be modulated through a flavan-3-ols. Platelet function is related to the risk of developing atherothrombosis, the principal cause of heart attack and stroke. Indeed, activated blood platelets play a central role in this chronic inflammatory condition as they are major components of thrombi that occlude arteries[134].

Platelet function can be affected by various dietary components, including a wide range of plant-based products, however, the mechanisms by which those compounds affect platelet function are not yet fully understood [135].

The experimental evidence for beneficial vascular effects of chocolate in human interventions studies is yet not fully convincing. Some human intervention studies on chocolate and its polyphenols lack a stringent study design. They are sometimes underpowered and are not always placebo controlled. Dietary chocolate intake in many of these human studies was up to 100 g per day. Since chocolate is a rich source of sugar and saturated fat, it is questionable as to whether chocolate could be recommended as part of a nutrition strategy to promote vascular health.[133,136].

3.5.6 Other effects

Gut microbiota plays a key role in host physiology and metabolism. Indeed, the relevance of a well-balanced gut microbiota composition to an individual´s health status is essential for the person's well-being. The percentage of polyphenol absorption is very low and as much as 90% of these compounds persist into the colon. There, they are metabolized via esterase, glucosidase, demethylation, dehydroxylation and decarboxylation activities of bacteria, resulting in smaller metabolites, some of which can be absorbed across the intestinal mucosa[137]. Hayek *et al.*[138] has recently revisited data showing that cacao and/or chocolate modifies intestinal flora in the same way that prebiotics and probiotics do. As the functional interactions between gut microbiota and host metabolism are indeed shown in several studies and result in health maintenance, some of the benefits of cacao may be due to this indirect mechanism. Potential health-promoting benefits of flavanols in cacao require more experimental effort.

3.6 Other bioactive substance of cocoa

Although most of the studies indicate that the health benefits of cocoa or cocoa products are attributable to polyphenols, it should be noted that cocoa and cocoa products are not only rich in polyphenols, but are also rich in xanthines, which account for about 3.2% of defatted unsweetened chocolate composition. Xanthines are a variety of compounds produced by plants and animals that have not been studied as frequently as other substances of metabolism, such as ATP or GTP. The active compounds in cacao that are structurally similar to xanthine are methylxanthines, of which the action on human physiology is quite remarkable. The main methylxanthines of cocoa are theobromine (3.7% on a fat-free basis) and caffeine (about 0.2%). The possible synergistic interactions between flavonoids and methylxanthines are also unclear and need further study, so the contribution of theobromine in cocoa products towards health benefits should be considered[139].

It is clear that the cocoa flavonoids represent an exciting new area of nutritional research with significant implications for cardiovascular protection. Further experimental studies with cocoa flavonoids are needed to define the specific mechanisms of action. Furthermore, the plasma concentration of chocolate polyphenols is relatively low, often in the nanomolar range, and the flavonoid concentrations, as used in cultured cells, may sometimes be higher than physiological levels. In addition to epicatechin, cocoa appears to be an important source of other dietary polyphenols, including proanthocyanidins and quercetin. Until now, the

antioxidant capacity of cocoa has been attributed only to the epicatechin content. However, other compounds might also be important contributors to the total polyphenols present in cocoa and convey potential health benefits. It is clear that cocoa is a complex plant food that, in its finished products may well contain sufficient amounts of these interesting phytochemical antioxidants to favorably influence in vivo oxidant defense. Data as described herein in the present thesis, may support this hypothesis.

4. INFLAMMATION AND DIET

4.1 Inflammation: a new perspective

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection and chemical irritation. This biological process also involves the innate and adaptive immune systems. At a damaged site, inflammation is initiated by migration of immune cells from blood vessels and release of mediators, followed by recruitment of inflammatory cells and release of reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines to eliminate foreign pathogens, resolving infection and repairing injured tissues[140].

Thus, the main function of inflammation is beneficial for a host's defense. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation causes various chronic disorders. In this respect, in recent years an idea has been developing that chronic inflammation can inflict more serious damage to a host tissue than bacterial infections. Inflammatory chemicals produced by inflamed and immune cells also attack normal tissues surrounding the infected tissue, causing oxidative damage and extensive tissue inflammation. Studies show that chronic inflammation is linked to a wide range of progressive diseases, including cancer, neurological disease, metabolic disorder and cardiovascular disease. This suggests that the elimination of chronic inflammation is a major way to prevent various chronic diseases[48]. Immune cells, especially macrophages, are crucial for directing the host's foreign body reactions and producing various proinflammatory mediators[141]. It has long been recognized that macrophages produce oxygen- and nitrogen-reactive metabolites during phagocytosis or when stimulated by a variety of agents[142].

ROS such as superoxide anion (O2 −) are widely investigated as signaling mediators of both protection and destruction in macrophages[143].

Diverse Reactiv Oxygen Species and Reactive Nitrogen Species such as O_2 (superoxide anion), OH (hydroxyl radical), H_2O_2 (hydrogen peroxide), nitric oxide (NO) and $^1O_2$ (singlet oxygen) generated by inflammatory cells, injure cellular biomolecules including nucleic acids, proteins and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation[144].

The majority of dietary antioxidants are derived from the intake of vegetables, fruits, tea and wine. Epidemiological studies provide convincing evidence that natural dietary compounds consumed by humans as food, possess many biological activities. Among these natural bioactive compounds, flavonoids are widely recognized for their biological and

pharmacological effects, including antiviral, anti-carcinogenic, antioxidant, antimicrobial, anti-inflammatory, anti-angiogenic and anti-thrombogenic properties[126,144].

4.2 Oxidative stress

For the past 40 years, oxidative stress has been increasingly recognized as a contributing factor in aging and in various pathophysiologies which are generally associated with aging[144]. The term ‘‘oxidative stress’’ began to be used frequently in the 1970s, but its conceptual origins can be traced back to the 1950s to researchers pondering the toxic effects of ionizing radiation, free radicals and the similar toxic effects of molecular oxygen [145], and the potential contribution of such processes to the phenomenon of aging[144]. ROSs are constantly produced in aerobic organisms as by-products of normal oxygen metabolism and include free radicals such as superoxide anion (O_2^-) and hydroxyl radical (OH^-), and non-radical hydrogen peroxide (H_2O_2) [146].

At low concentrations, ROSs serve as an important second messenger in cell signaling, both for endogenous and exogenous reactions/pathways. However, at higher concentrations and long-term exposure, ROSs can damage cellular macromolecules such as DNA, proteins and lipids, which leads to necrosis and apoptotic cell death[147].

ROSs are natural by-products of metabolism and these molecules play important roles in cell signaling, but excessive levels of ROSs can be toxic to cells[148].

Much of the research on ROSs has been centered on the damaging effects of oxidative stress. However, it is now apparent that ROSs activate a battery of cellular enzymes that either prevent the generation of ROSs or detoxify ROSs and thereby protect the cell against damage caused by oxidative stress[147], including superoxide dismutases (SODs), heme oxygenase-1 (HO-1), NAD(P)H quinine oxidoreductase-1 (NQO-1), catalase and thioredoxin. However, they are not sufficient to control ROSs and minimize ROS-induced damage. A compromised anti-oxidant defense system can lead to excessive oxidative stress and ultimately result in cell damage[146]. Therefore, it is clear that all cells must continuously strive to keep the levels of ROSs in check. ROSs attack DNA and other cellular macromolecules causing oxidative stress and many other physiological and pathological conditions. These conditions include aging, neurodegenerative diseases, arthritis, arteriosclerosis, inflammatory responses and tumor induction and promotion[149].

The acceptance of free radical biology was remarkably slow, probably due to the largely theoretical and hypothetical nature of its beginnings, the evanescent nature of free radicals, and the lack of experimental tools to study them[144].

4.3 Implication of oxidative stress in diseases

There has been a recent explosion of interest in the notion that chronic low grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of type 2 diabetes. It was demonstrated that markers of inflammation predict and/or are associated with type 2 diabetes and that inflammation is involved in the pathogenesis of atherosclerosis, a common feature of type 2 diabetes[140,151].

Obesity is regarded as a hyper-oxidative and chronic inflammatory status, the significant interrelationships among obesity-related indices, oxidative stress, inflammatory markers and adipokines have been well documented[152].

It is reported by Furukawa et al., that in cultured adipocytes, increased oxidative stress may cause deregulated production of adipokines, including leptin, adiponectin, PAI-1 and monocyte chemotactic protein-1 (MCP-1) thus lead to metabolic derangements[153].

Antioxidant components are microconstituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation of oxidizing chain reactions, and are also involved in scavenging free radicals. Food such as fruits, vegetables and grains are reported to contain a wide variety of antioxidant components, including phenolic compounds, such as polyphenols. These compounds are found to be well correlated with antioxidant potential. As previously described, cocoa beans have a high phenolic content and these compounds were reported to be a potential candidate to combat free radicals, which are harmful to our body and food systems[48].

In vitro studies demonstrated that these compounds have several biological activities, such as the ability to scavenge superoxide radicals and hydroxyl radicals, reduce lipid peroxyl radicals and inhibit lipid peroxidation[40,153].

4.4 NRF2 pathway

4.4.1 The transcriptional factor: NRF2

The cellular defense system protects against oxidative stress caused by a vast range of xenobiotics, inflammation and ionizing radiation[154].

Therefore, it is obvious that cells must constantly labor to control levels of ROSs, preventing them from accumulating. Cells have mechanisms to activate over two hundred defensive genes that protect against ROSs and the diseases to which they contribute[155].

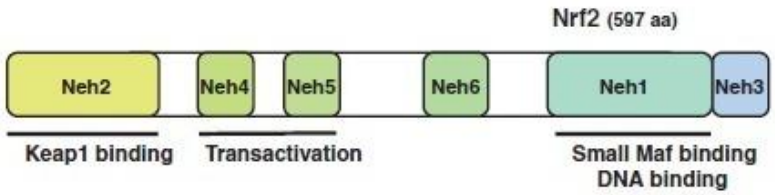


Figure 12. Schematic representation of NRF2 domains.

In the early 1990s, the antioxidant response element (ARE) was first identified as cis-element in the upstream regulatory region of the GSTA2 gene and was found in the promoters of detoxifying enzyme genes such as glutathione S-transferases, NAD(P)H:quinine oxidoreductases, gastrointestinal glutathione peroxidase and peroxiredoxin[154].

As more and more AREs were identified, it became apparent that the ARE sequences of different genes, and even of the same gene from different species, varied significantly. John Hayes and colleagues have carefully compared AREs and categorized them into four classes[155]. ARE is recognized by the family of Cap'n'Collar containing basic leucine zipper proteins including Nrf2 (fig. 12). The NF-E2-related factor-2 (Nrf2) is a transcription factor that is ubiquitously expressed at low levels in all human organs. It was cloned independently in 1996 by Kant and coworkers and it was identified as a regulator of the expression of the beta-globin genes, and as such was named Nrf2[156].

It belongs to the cap 'n' collar (CNC) subfamily, a family of basic leucine zipper transcription factors conserved in worms, insects, fish, birds and mammals, including humans, but absent in plants and fungi. They are defined by the presence of a conserved 43 amino acid CNC domain located N-terminally to the DNA binding domain[157].

In spite of the high homology in their DNA binding and leucine zipper domains, six members in this family have been identified: NF-F2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2 each with distinct biological roles. NF-E2 expression is erythroid-specific-1, Nrf1, is expressed in all tissues with its absence lethal effecting embryonic development. Nrf2 is also ubiquitously expressed, but it is dispensable for normal development. Nrf3 is preferentially expressed in the placenta[158,159].

Several homologue domains were identified when Nrf2 from different species, such as human, mouse and chicken, were aligned. They are designated as Neh 1–6 (NRF2-ECH

homology: Neh). The Neh1 domain contains a CNC-type basic leucine zipper, which is necessary for DNA binding, dimerization with other transcription and it is also a functional nuclear localization signal (NLS)[160].

The N-terminal Neh2 domain contains seven lysine residues for ubiquitin conjugation, so it confers negative regulation of the Nrf2 activity through proteasome-mediated degradation of Nrf2[155]. It also binds to the Kelch domain of Keap1[161]. The Neh4 and Neh5 are two independent transactivation domains that are rich in acidic residues and interact with CREB-binding protein (CBP)[162].

The function of Neh6 remains largely unknown, although it is known to have a high content of serine residues. The C-terminal Neh3 is indispensable for transcriptional activity of Nrf2 by recruiting CHD6, a coactivator with both helicase domain and chromodomain[155].

Among the family, Nrf2 is the most potent transcription factor in regulating the basal and inducible expression of antioxidant enzyme genes[154].

The most convincing data demonstrating that Nrf2 is a critical regulator of AREs came from Tom Kensler's laboratory. This group showed that Nrf2-knockout mice had reduced levels and impaired induction of detoxifying enzymes and redox balancing proteins, rendering the knockout mice more susceptible to carcinogen-induced cancers. Nrf2 has emerged as the master regulator of a cellular defense mechanism that elicits an adaptive response and promotes cell survival under stress[163].

4.4.2 Keap1

In the 1999, Ken Itoh and colleagues[161], identified Keap1 (fig. 13), kelch-like ECH-associated protein1, the negative regulator of Nrf2.

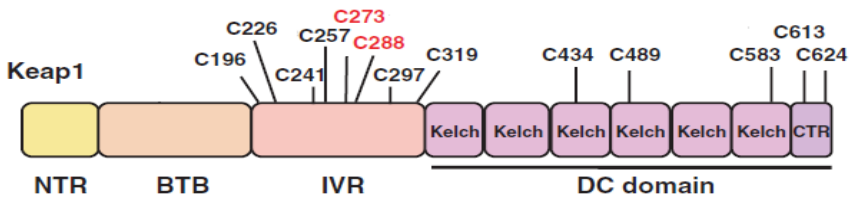


Figure 13. Schematic representation of KEAP1 domains.

This protein is essential for the regulation of activity of NRF2. Keap1 exist as dimers inside the cell with the complex consisting of two Keap1 molecules and one Nrf2 molecule. These proteins interact with each other through the double glycine-rich domain ok keap1 and the hydrophilic region in the neh2 domain of NRF2[164]. Under normal conditions, Nrf2 resides within the cytoplasm of the cells interacting with an actin-bound cytosolic protein, Keap1, being constantly degraded via ubiquitin-proteasome pathway in a keap1-dependent manner[165]. The inhibitor Keap1 is a cysteine-rich protein with human Keap1 containing 27 cysteine residues with C257, C273, C288, C297 and C151 acting as sensors of oxidative stress. Moreover Keap1 functions as a substrate adaptor protein for a Cullin 3, (Cul3)-dependent ubiquitin-protein ligase, complex to maintain the steady-state levels of Nrf2. Covalent conjugation of proteins by ubiquitin usually involve three enzymatic activities for activating (E1), conjugating (E2), and ligating (E3) ubiquitin to a substrate. Keap1 constantly targets Nrf2 for ubiquitination and subsequent degradation by the 26S proteasome. Under induced conditions, the activity of the Keap1-Cul3 E3 ligase is inhibited due to modification of cysteine residues in Keap1, resulting in stabilization of Nrf2 and activation of the Nrf2 pathway. However the precise mechanism by which the modification of cysteine residues in the repressor leads to a conformational change remains elusive[161.165,166].

4.4.3 NRF2/KEAP1/ARE pathway

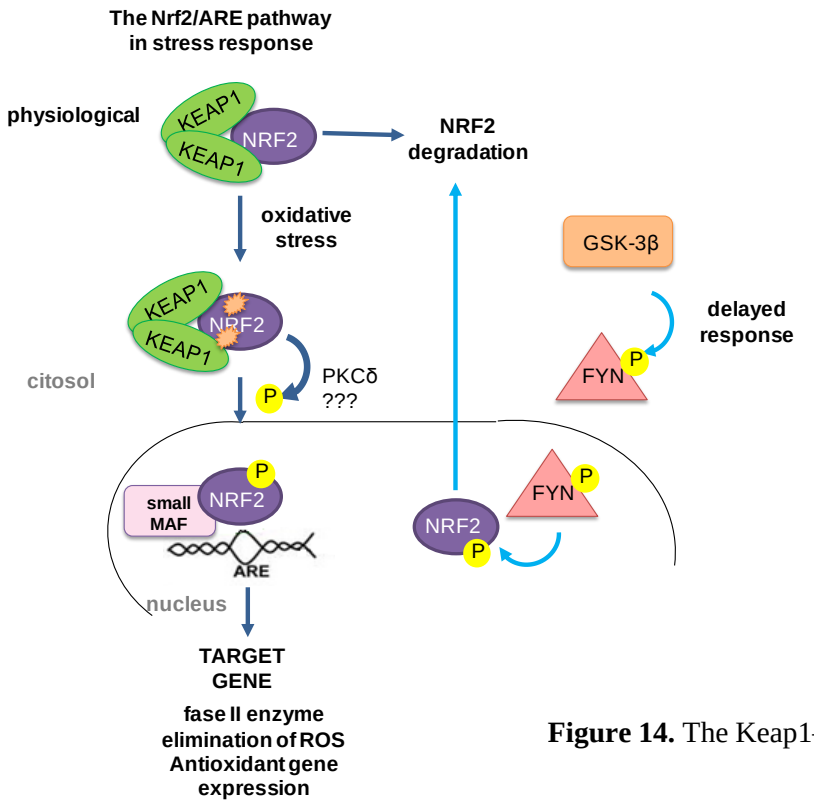


Figure 14. The Keap1–Nrf2 pathway.

When a cell encounters stress, Nrf2 dissociates from Keap1 and translocates into the nucleus (fig. 14). Oxidative/electrophilic stress induced modification of keap1-Cysteine151 results in the release of NRF2 from the inhibitor. In addition, to disrupt the binding between NRF2 and its repressor, there is another step control: the phosphorylation of NRF2 at Serine (S40) and at threonine residues by kinases such as phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), c-Jun NH2-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) or transmembrane protein kinase PERK[165,167,168].

Nrf2 is stabilized and translocates into the nucleus, where it heterodimerizes with small Maf, Jun or yet unknown proteins and in turn binds to the ARE that leads to the coordinated the activation of a myriad of genes that protect cells against oxidative/electrophilic stress and neoplasia such as phase II detoxifying enzymes and antioxidant-responsive genes[169,170].

In addition to these mechanisms, Nrf2 appears to be an important regulator of the peripheral inflammatory responses and thus may also contribute to the neuro-inflammatory component of ischemic brain injury[171].

The abundance of Nrf2 inside the nucleus is tightly regulated by positive and negative factors that control nuclear import, binding to ARE, export and degradation of Nrf2. A delayed response of oxidative/electrophilic stress activates GSK-3β that phosphorylates Fyn at unknown threonine residue(s). Phosphorylated Fyn translocates to the nucleus and phosphorylates Nrf2 Tyrosine 568, which is essential for nuclear export of the transcriptional factor[172,173].

The degradation of Nrf2, both in the cytosol and nuclear compartments, rapidly brings down its levels to normal resulting in suppression of Nrf2 downstream gene expression. An auto-regulatory loop between Nrf2 and keap1 controls their cellular abundance. Nrf2 regulates keap1 by controlling its transcription, and keap1 controls Nrf2 by degrading it. In conclusion, switching on and off of Nrf2 combined with promoting an auto-regulatory loop between them regulates activation/ deactivation of defensive genes leading to protection of cells against adverse effects of oxidative and electrophilic stress thus promoting cell survival[154].

4.4.4 NRF2 and diseases

Indeed, accumulating evidence has been provided recently indicating a protective role of Nrf2 against many human pathological conditions, such as Alzheimer's disease[171], Parkinson's disease[174] and cardiovascular disease[175].

For this reason, many researchers have focused on identifying potent Nrf2 inducers with low toxicity to enhance the Nrf2- mediated adaptive response for disease prevention. For

example, human clinical trials, pioneered by Tom Kensler [176], were conducted to evaluate the efficacies of Nrf2 inducers such as oltipraz and broccoli sprouts. Mechanistic studies indicate that the chemopreventive activities of Nrf2 inducers lie in their ability to modulate absorption, distribution, metabolism and excretion of carcinogens, as well as the anti-inflammatory response. As chronic inflammation has been recognized as a risk factor for cancer development, the anti-inflammatory activity of Nrf2 is considered a contributing factor to its role in chemoprevention. Paradoxically, the role of Nrf2 in cancer promotion and in cancer resistance to therapeutic treatment, the negative side of Nrf2, has recently been revealed. Constitutive up-regulation of Nrf2 is associated with many types of cancers, including skin, breast, prostate, lung, neck and endometrium[177,178].

Studies using Nrf2 deficient mice have demonstrated that this transcription factor regulates immune cell responsiveness, prostanoids, cytokines and chemokines expression in response to various insults. For example, mice deficient in Nrf2 showed up-regulated gene expression of pro-inflammatory cytokines, interferon-inducible transcripts and nuclear factor kappa B (NFκB)-binding activity in peritoneal macrophages after administration of bacterial endotoxin or lipopolysaccharide[179]. A wide variety of dietary polyphenols and other classes of phytochemicals have been reported to induce the expression of enzymes involved in both cellular antioxidant defenses and elimination/inactivation of electrophilic carcinogens. Induction of such cytoprotective enzymes by edible phytochemicals is recognized as one of the highly effective strategies for preventing cancer in the human population[180].

Detailed investigations into the specific dietary components of these foods have revealed that many polyphenolic constituents exert anti-oxidant effects on key substrates involved in the pathogenesis and progression of ischemic injury. These data have perpetuated the belief that the protective effects of flavonoids result from direct anti-oxidant actions and the transcriptional factor NRF2 has emerged as a critical regulator of flavonoid-mediated protection through the induction of various cytoprotective genes[181].

For example the oxidation of Keap1 cysteine thiols can be mediated by some edible polyphenols. In this context, it is noticeable that among flavonoids, the higher their intrinsic potential to generate oxidative stress and redox cycling, the stronger their potency as inducers of ARE-mediated gene expression[168].

For instance, the green tea polyphenol (–)-epigallocatechin gallate (EGCG) was found to produce substantial amount of H_2O_2 under cell culture conditions. Therefore, it is paradoxical that the activation of NRF2-ARE signaling by antioxidant polyphenols to induce cytoprotective enzymes is attributable, at least in part, to their pro oxidant activity[168,182].

Yu *et al.*[183] reported that exposure of HepG2 cells to green tea polyphenols stimulates transcription of phase II enzymes through ARE pathway, which seems to be regulated by MAPK. During extensive screening of vegetable extracts for GST-inducing activity in cultured rat liver epithelial RL-34 cells, Morimitsu[184] and colleagues have identified a sulphoraphane analogue, 6-methylsulphinylhexyl isothiocyanate (6-HITC), as a key GST-inducer present in Japanese horseradish, wasabi. The compound potently stimulating nuclear translocation of NRF2 and subsequent activation of ARE pathway. In another study, Dinkova-kostova *et al.*[164] provides evidence that sulphoraphane directly reacts with critical cycteine residues oh KEAP1 stoichiometrically and in 2003 Dikinson *et al.* [186] showed that curcumin increased nuclear translocation of NRF2.

Several nutrients and non-nutritive phytochemicals are being evaluated in intervention trials for their potential as cancer chemopreventive, anti-inflammatory agents. Despite significant advances in our understanding of multistage oxididative stress little is known about the mechanism of action of most antioxidant agents. The antioxidant effects that most dietary phytochemicals exert are likely to be the sum of several distinct mechanisms.

5. Experimental section I

AIMS OF THE STUDY I

Diet is one of the key lifestyle factors involved in the genesis, prevention and control of many pathologies. Dietary components such as polyphenols present in cocoa (*Theobroma cacao* L.), show important functional properties. The polyphenolic profile of cocoa is well described within the literature, including monomeric and polymeric polyphenols, which show antioxidant and anti-inflammatory capacities. Chocolate consumption (particularly dark chocolate, as shown in many *in vivo* recent studies), triggers a positive influence on human health, as well elucidated in the introduction. Monocytes are key to the inflammatory status and offer an important model to investigate the role of dietary components and their metabolites.

Thus, the aim of this research was to study the proteomic profile of human circulating monocytes, before and after dark/white chocolate administration, through a proteomic approach, to figure out whether an interaction between the flavonoids found in diet and the human circulating monocytes is possible.

In particular a bioavailability on (-)-epicatechin (and its glucuronide metabolite) was performed by liquid chromatography-mass spectrometry (LC-MS) in the plasma of five healthy volunteers, before and after chocolate administration. Cocoa was administered in the form of chocolate bars with and without flavonoids (dark vs white chocolate), characterized for their chemical composition. Human circulating monocytes were isolated from the blood, before and after the administration of chocolate bars at the peak of metabolites as indicated by the bioavailability study. Cells were lysed and analyzed by 2D-electrophoresis with IPG strips pH 5-8, with the respective proteomic profiles compared using PDQuest statistical software.

The data obtained from the bioavailability experiments and proteomic analyses represent the initial phase of a translational ongoing study that will contribute to understanding how chocolate flavonoids act on inflammation and what biomarkers could be used in future clinical studies.

5.1 MATERIALS AND METHODS

5.1.1 Characterization of chocolate bar

All dark chocolate bars used to carry out the experiments came from a single batch of Nigerian chocolate containing 80% cocoa and produced by a leading company in the field. The sample of white chocolate, used as a control, was produced by a Piedmont chocolate lab.

The overall composition was analyzed directly using the chocolate bars, where moisture, total protein, lipids and ash levels were determined. The moisture content, determined in order to express the results on a dry weight (dw) basis, was obtained using an oven at 103 ° C (until constant weight). The total nitrogen content and total protein content (conversion factor: 6,25) were obtained according to the Kjeldahl method, using Kjeltec system I (Foss Tecator AB, Höganäs, Sweden). The ash content was determined in a muffle furnace according to the AOAC (1990) procedure. The quantification of lipid component in the samples was performed by continuous extraction in semiautomatic Soxhlet (BUCHI EXTRACTION SYSTEM B-811) using dichloromethane (Sigma-Aldrich) as the solvent. The defatted powder was subsequently used to obtain chocolate phenolic extract used for subsequent quantifications. Total phenol was determination by the classic Folin-Ciocalteu assay, using the protocol described by Arlorio *et al.*[187]. Results were expressed as catechin equivalents, through the calibration curve of (±)-catechin monohydrate. Using the Vanillin assay described by Travaglia *et al.*[188] the total proanthocyanidins were measured and expressed as catechin equivalents, like the previous analysis. The DPPH radical scavenging assay was performed according to the method reported by Locatelli *et al.*[189]. This analysis was conducted to verify the antiradical activity of the chocolate phenolic fraction; the results, expressed as DPPH radical inhibition percentage, are referred to 200 µg/µL of extract. For the identification and quantification of polyphenolic compounds a chromatographic method (reversed phase HPLC) was optimized. The qualitative identification of the major polyphenols (and eventual co-extracted methylxanthines) in the extracts was obtained by comparison with the retention times and the UV spectra of the individual standard molecules. The identified compounds were quantified by interpolation on the corresponding calibration lines. The analysis of the white chocolate are in process. White chocolate was used as a control because it contains the same ingredients as dark chocolate but lacks cocoa and therefore the bioactive substances present in dark chocolate.

5.1.2 Study design

The study was approved by the Local Ethical Committee. The participants, five healthy normal weight young volunteers (age, mean±SD: 29.2±3.4 years; BMI: 22.1±1.0 Kg/m^2), followed a cocoa washout period before each intervention, were instructed to abstain from alcoholic beverages and any polyphenol-rich foods 48 hours before the study. The same diet indications were followed during the testing sessions. Subjects underwent two sub-studies: 1) bioavailability study; 2) monocytes study.

Bioavailability study. Firstly, a blood sampling was carried out before and after eating dark chocolate, every hour for 4 hours following administration (daily administration). Secondly, the volunteers were subjected to a weekly study, in which it was taken a sample of blood before and 4 hours following administration of dark chocolate for seven consecutive days.

Monocytes study. Monocytes study was designed after the bioavailability analysis. After an overnight fasting for at least 12 h, all subjects randomly underwent the following interventions: i) fasting (B); ii) assumption of 70g of dark chocolate bars (DC), iii) assumption of 70 g of white chocolate bars (WC), and iv) assumption of 70 g/day of DC for a week. Subsequently to all the interventions were performed the collection of blood, firstly, for the bioavailability study and secondly, understood the correct timing, the collection of blood has been made for the isolation of monocytes for subsequent proteomic analysis (fig. 15).

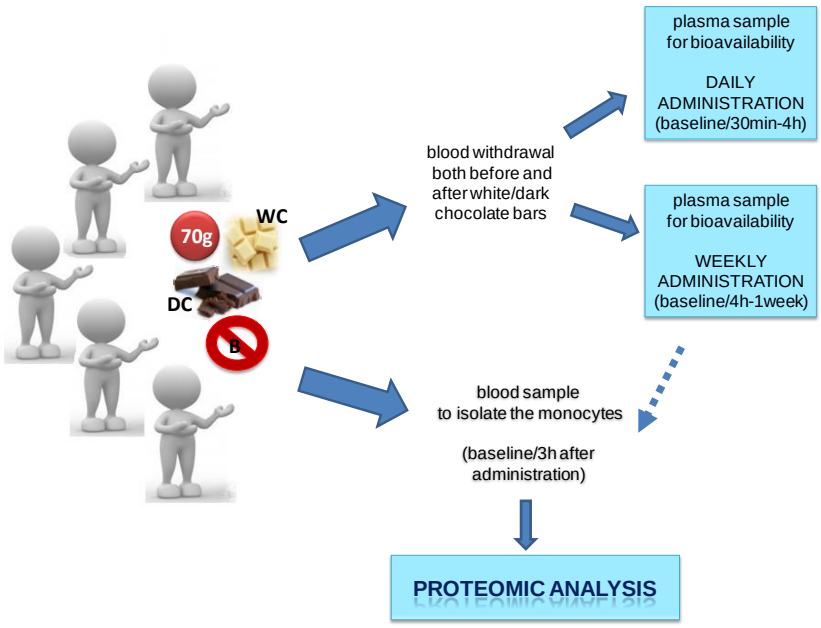


Figure 15. Schematic representation of the study design

5.1.3 Sample preparation and LC-MS/MS Conditions

Plasma samples were obtained following the centrifugation of whole blood and stored at -80 ° C until analysis. The plasma samples were purified and concentrated by the use of cartridges suitable for the removal of phospholipids (Phenomex i-Phree™).

Following the precipitation of plasma proteins, each sample and the internal standard IS 4-metil-umbelliferil-β-D-glucuronide (UG, 22 µg/L in acetonitrile), were analyzed by LC-ESI-MS, as described below, SRM mode by monitoring the transitions: 351→175 for the IS and in MRM mode 465→175 / 465→289 for the (-)-epicatechin-3'-O-glucuronide (Ec-G2). The objective was to identify the metabolites in the plasma and, according to the results obtained, to plan the time to perform the withdrawals of blood samples for the isolation of monocytes and the study of the protein profile. To date, no other epicatechin metabolites have been investigated.

5.1.4 Catechin (C) and Epicatechin (Ec) incubation with human liver microsomes (HLM)

The incubation mixture was constituted with 2 mM UDPGA in a TRIS•HCl buffer, (50 mM, pH 7.4), containing 5 µl of methanol (1% of total volume) and C or Ec (0.5 mM), brought to a final volume of 0.5 ml. After pre-equilibration of the mixture at 37 °C, an appropriate volume of microsomal suspension, previously activated by alamethicin (50 µg/mg protein) at 4 °C for 15 min, was added to give a final protein concentration of 1.5 mg/ml. The incubation was shaken for 60 minutes at 37 °C. A control incubation was carried out in the absence of UDPGA. Each incubation was stopped by addition of 500 µl ice-cold acetonitrile, vortexed and centrifuged at 12,500 rpm for 10 min. The supernatants were directly analyzed by LC-ESI-MS.

5.1.5 LC-ESI-MS analyses

A Thermo Finningan LCQ Deca XP plus system equipped with a quaternary pump, a Surveyor AS autosampler, and a vacuum degasser was used for LC/MS analyses (Thermo Electron Corporation, Waltham, MA). All chromatographic separations were performed on a Kinetex C18 (100 x2.1 mm; 2.6 µm d.p.) core-shell column (kept at a 40 °C) preceded by a 0.5 µm KrudKatcher depth filter (Phenomenex srl, Castel Bolognese, Italy). Aliquots (10 µL) of supernatants obtained from incubations were injected into the system and eluted with a mobile phase (flow rate 0.2 mL/min) consisting of A: 0.2% aqueous formic acid solution, B: methanol.

The following gradient program was used: 0 min [B=15%], 7.00 min [B=30%], 7.50 min [B=85%], 9.00 min [B=85%], 9.50 min [B=15%], and 14.00 min [B=15%].

The eluants were filtered through a 0.45-µm pore size polyvinylidene difluoride membrane filter before use. The eluate was injected into the electrospray ion source (ESI) and an MS spectra was acquired and processed using Xcalibur® software. The operating conditions on the ion trap mass spectrometer were as follows: spray voltage, 3.30 kV; source current, 80 µA; capillary temperature, 300 °C; capillary voltage, -34.00 V; tube lens offset, -35.00 V; multipole 1 offset, 10.50 V; multipole 2 offset, 14.00 V; sheath gas flow (N2), 50 Auxiliary Units. Data were acquired in negative full-scan, product ion scan (MS2) and single reaction monitoring (SRM) modes using mass scan range m/z 150-550. The collision energy was optimized to a range of 28-32%.

5.1.6 Isolation of human monocytes

Human monocytes were isolated from heparinized venous blood (30–40 mL) of the same healthy donors before and after administration of chocolate bars. The samples were taken at the time point where metabolites were identified LC-ESI-MS in plasma with the highest peak of (-)-epicatechin-3'-β-D-glucuronide (EC-3'-G) used as the marker[91]. The isolation was carried out via the standard technique of dextran sedimentation with a Hystopaque (density = 1.077 g/cm^3) gradient centrifugation (400×g, 30 min, room temperature), as described by Cermak and coworkers[190]. Cells were re-suspended in RPMI 1640 medium, supplemented with 10 mM Hepes, 2 mM of L-glutammine and 100 U/mL of penicillin and streptomycin (Sigma-Aldrich). Purified monocytes populations were obtained by adhesion (60 min, 37 °C, 5% CO2), followed which fresh medium supplemented with 5% heat-inactivated fetal bovine serum (FBS). Cell viability was determined by trypan blue exclusion, which was generally > 98% (data not shown).

5.1.7 Preparation of plasma extract

As described by Koga and co-workers[191], plasma samples were collected prior to and following the administration of the same quantity of dark and white chocolate. A total of 10 mL of pooled plasma was mixed with 40 mL of acetone containing 1% acetic acid. The mixture was shaken continuously at 4 °C for 2 h and then centrifuged for 30 min at 5000 xg at 4 °C. The supernatant fluid was separated from the precipitate and evaporated in a rotary evaporator. The remaining water phase was further lyophilized. The residue was washed 2 times with 10 mL

chloroform, dried under nitrogen gas and dissolved in 1 mL dimethylsulfoxide (DMSO):water (1:9 v/v). The plasma extracts were stored at - 80 °C until the LC-MS/MS analysis and in vitro treatment of human monocytes.

5.1.8 Two dimensional gel electrophoresis (2-DE)

Following the isolation of monocytes, total protein was immediately extracted[192] and the protein samples diluted in rehydration solution (350 uL final volume) containing 7 M urea, 2 M Thiourea, 4% CHAPS, 120 mM dithiothreitol (DTT), 40 mM Tris-HCl and 2% ampholyte pH 3-10 (Sigma-Aldrich).

Duplicate gels were run for each sample and conditions. For the first dimension of 2-DE (isoelectric focusing, IEF), immobilized pH gradient strips (IPGs) pH 5-8 (Bio-Rad, Hercule, CA) where run using protean IEF cell system (Bio-Rad). At completion, the strips were then equilibrated twice for 15 min with gentle shaking in the equilibration buffer containing DTT and second buffer containing iodoacetamide. The second dimension was performed by applying the equilibrated strips (IPGs) to a 12% polyacrylamide SDS-gel (7 cm). Following electrophoresis, the proteins were fixed in 10% (v/v) methanol and 7% (v/v) acetic acid and stained with Sybro Ruby total protein Stain (Bio-Rad). The stained 2-DE gels were digitized using using a ChemiDoc Imager with a 615-645 nM filter (630BP30; Bio-Rad). The bioinformatic analysis was performed with PDQuest software (version 8.0; Bio-Rad).

5.1.9 Gel analysis

The analysis of protein spots on the gels was performed with the statistical software PDQuest. All RAW data files of scanned gel were opened with PDQuest, with all images cropped to the same size and aligned to include only the resolving area of the gels, excluding for this purpose the dye front. Each image was analyzed with the PDQuest spot detection software to identify valid spots. Errors of omission and commission were corrected manually on each gel after a careful visual inspection of the gel images magnified on the computer monitor. Replicates of each experimental condition were compared to a master gel, which represents the spots that were reproducibly present. The gels for various conditions were compared to find differences in spot intensity that resulted from treatment. Spots identified by PDQuest as differentially expressed were also examined manually to ensure that the analysis was correctly carried out. To compensate for any variation in protein loading and development level of Sybro Ruby,

spots quantities were normalized based on the total staining density of the image. Differences between groups and treatment were compared using Student's t-test, *p* values set to <0.05 were considered statistical significant.

5.1.10 In-gel trypsin digestion

The two-dimensional gels were stained overnight with Bio-Safe Coomassie G-250 Stain and after washes the protein spots of interest were excised manually. The gel pieces were then destained with destaining buffer (100 mM ammonium bicarbonate in 50% acetonitrile) prior to reduction with 10 mM DTT in 0,1 M ammonium bicarbonate and alkylation with 55 mM iodoacetamide in 0,1 mM ammonium bicarbonate. The gel pieces were dried and a modified porcine trypsin (sequencing grade; Promega, Madison, WI) was add to a final concentration of 20ug/mL with the digestion performed at 37 °C overnight. The reaction was stopped by adding 0.1% trifluoroacetic acid (TFA) followed by washing in extraction buffer (50% acetonitrile, 5% TFA in water). Following a centrifugation at maximum speed, the samples were dried in a SpeedVac and resuspended in 0,05% of TFA in water and stored at -80 °C until further use. The analyzes were conducted using an LC-MS (Thermo Scientific) system that includes a chromatographic pump for mass spectrometry (Surveyor MS Pump Plus) with an autosampler for the injection of samples (Surveyor Autosampler Plus), all connected to a mass spectrometer LTQ linear ion trap equipped with an ESI source that ionizes in positive polarity. The samples are injected into the system and separated by means of a chromatographic column for reverse phase HPLC (Zorbax SB-CN 2.1x150x5).
The proteins were identified by comparing the masses of their tryptic peptides with those of all known proteins and the MASCOT database (www.matrixscience.com).

5.1.11 Western Immunoblotting

To confirm the proteomic results, two protein spots were analyzed by western immunoblot. The analysis was performed directly on the bidimensional gel (12% SDS-PAGE) and transferred to Immuno-Blot PVDF membrane (Bio-Rad Loboratories, Inc). The membrane was blocked with 5% Blotting-Grade Blocker (nonfat milk; Bio-Rad Laboratories, Inc) in TBST (0.1% Tween-20 in TBS) for 1 h at 37 °C. Thereafter, the membranes were incubated at 4 °C with mouse monoclonal anti-Rho GDP-dissociation inhibitors (Ly-GDI E-7; sc-374525) or mouse monoclonal anti-macrophage capping protein (CapG D-5; sc-365472)(Santa Cruz

Biotechnology, CA) at a concentration of 1:500 in TBST. After an overnight incubation and subsequent washes, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at 1:5000 in TBST for 1 h at 37 °C. Immunoreactive protein spots were visualized by the enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology; Rockford, IL) according to the manufacturer's instructions and images were detected using the Quantity One 1D image analysis software program.

5.2 RESULTS

5.2.1 Characterization of chocolate bars

The tests to evaluate the composition of cocoa and its derivatives were carried out as described in materials and methods, section I. The results of these analyses are reported in table 1.

5.2.2 Identification of (-)-epicatechin metabolites (EC-G2) in human plasma

Because of the lack of a commercially available EC glucuronide standard, we identified these metabolites by incubating EC with human liver microsomes (HLM) in the presence of the UDPGA cofactor. The LC-MS chromatograms of the incubation samples revealed both the presence of the EC m/z 289 and two peaks m/z 465 EC-G1 (Rt= 3.71 min) and EC-G2 (Rt= 6,35 min), showing the same fragmentation pathways of the EC glucuronides reported in the literature (fig. 16, 17).

Due to the relative abundance of the EC-G2 metabolite with respect to EC-G1, in accordance with the finding of Goretta and co-workers[92], we can speculate the metabolite EC-G2, corresponds to epicatechin-3'-O-glucuronide, being that this metabolite is the EC glucuronide described the most in the literature.

The plasma samples collected after the intake of chocolate, were analyzed by LC-MS monitoring the ion at m/z 465 corresponding to the epicatechin-3'-O- glucuronide, the most described EC metabolite[92]. Plasma samples were purified and concentrated by protein precipitation using acetonitrile as the precipitating agent, followed by phospholipid removal using Phenomenex Phree® phospholipid removal cartridges. Samples were injected into LC-MS system and analyzed in negative multi reaction monitoring (MRM) mode. 4-metil-umbelliferil-β-D-glucuronide (UG) was added as an internal standard. The chromatograms depicted in figure 18, show the presence of the peak labeled as EC-G2 (Rt = 6.4 min, m/z 465) in the samples T1-T4 and its absence in T0 traces. All the results performed after a weekly dark chocolate administration show the same results of the daily consumption, but after a week was not detected an accumulation of epicatechin (data not shown). For all analyses performed on plasma following the administration of white chocolate, the EC-G2 peak is absent (fig. 19)

5.2.3 Protein expression profile in monocytes/macrophages before and after chocolate consumption

According to the previous results on the bioavailability, blood samples for the monocyte isolation, were taken from healthy donors at baseline (T0) and 3 hours after the consumption of dark chocolate containing 80% cocoa (T3 dark) or white chocolate (T3 white).The proteins were extracted from the isolated monocytes and duplicate 2D gels were run for T0 and T3 dark and white. After a careful visual inspection to remove background interference including bubbles or Sybro Ruby speackles from the gel images, an average of 746±52 protein spots were detected on each gel by PDQuest software. The density for each spot was normalized against the total spot quantity of valid spots on each gel. A representative example of the typical 2-D gel profile of monocytes is shown in figure 20.

A 2D analysis in the pH range 5 to 8 was chosen in order to have a higher resolution and as such a clearer separation of protein spots. A total of 131±17 ($P< 0.05$) protein spots were found to be significantly different between the two groups, with 69 of these spots having a significance of $P<0.01$. Only 11 significant protein spots were chosen for further analysis (Table 2), because the remaining 58 protein spots had a lower intensity and were not visible with coomassie staining, necessary for the subsequent cutting and isolation of the spots.

As delineated by the colored rectangles, there are a clusters of spots, which appear to have an alteration/modification of their intensity, with respect to the baseline profile. Representative gel images for each cluster of spots are shown in figure 21 .

Regarding cluster A (fig. 22), there are only one protein spot, SSP1106 ($p<0.01$), which is expressed exclusively after the administration of dark chocolate. This protein was chosen for further analysis. The remaining three spots are present in all conditions, in particular spot SSP1104 which appears to be more highly expressed after white chocolate consumption.

Nine spots were included in cluster B (fig. 23). As observed in the image and in the table, there are less abundant spots, SSP3104 and SSP3105, which are each expressed after dark chocolate administration.

Cluster C (fig. 24) includes 5 protein spots, which are absent following white chocolate consumption but present in the other two conditions. In particular the SSP4808 and 4809 ($p<0.05$) are observed only after the administration of dark chocolate, while the other two, while present at baseline, are less expressed.

Cluster D (fig. 25), comprised of 3 spots, is the only example of irreproducibility of the spot profile between subjects, with a variability in the expression of one of the 2 spots, SSP5403

and SSP5401 ($p<0.05$). Understanding this irreproducibility is a limitation of the proteomic technique, however possible explanations for this variability could be due to post-translational modifications, the absence or presence of a specific isoform of proteins or a change in the amino acid charge.

5.2.4 Peptide Mass Fingerprint

Selected protein spots (table 3) showing a differential expression, were excised from the gels, and following an in situ digestion with trypsin, the resulting peptide mass fingerprint (PMF) was determined by mass spectrometry performed by Dr Donatella Caruso, Department of Pharmacology, University of Milan. The data obtained from the mass were subjected to proteomic investigation program on-line MASCOT. The results showed a score greater than or equal to 56 are statistically significant ($p<0.05$). The analysis by mass spectrometry identified MED19 (Mediator of RNA polymerase II transcription subunit 19; A0JLT2; table 3a), RM34 (Belongs to the ribosomal protein L34P family; table 3 a). KIF2a (Kinesin-like protein; O00139; table 3 d), NRL (Transcription factor which regulates the expression of several rod-specific genes; table 3 e), PAGE3 (Belongs to the GAGE family; table 3 e) and H1FNT (Testis-specific H1 histone; Q75WM6; table 3 e). While the results remain to be confirmed, they have not led to the identification of proteins of interest for the present study.

5.2.5 Identification and confirmation of RHO-GDP-dissociation inhibitor and macrophage-capping protein with specific antibodies

To further confirm the proteomic results, different protein spots were analyzed by 2D immunoblots. Amongst the spots predicted to show an hypothetical alteration in expression between T0 and T3 dark and white, were SSP5403 and 5401 in cluster D which showed intersubject variability,y and the SSP1106 in cluster A, observed only after the administration of dark chocolate.

According to the results published by Jin and co-workers[193], the first antibody tested was directed to the protein Rho GDP-dissociation inhibitors (P52566) . The antibody recognized a protein of 5.0 pI and 23-27 KDa in monocytes after dark chocolate consumption, but was not detected at baseline or after the administration of white chocolate (fig. 26). These results are in accordance with a review published by Loirand *et al.*[194] where they suggest that the activators of Rho proteins, Rho GEFs expressed in cardiovascular cells, are potential targets for the

treatment of cardiovascular disorders. Since Rho GDP-dissociation inhibitors, negatively regulate the Rho family of GTPases, it is possible that its expression is regulated by bioactive substances such as polyphenols present in dark chocolate.

The other two proteins (SSP 5401 and SSP 5403) with the same molecular weight but different isoelectric point, were predicted to be macrophage capping protein (P40121). Based on the study published by Burillo *et al.*[193], the difference in the isoelectric point was due to the presence of an amino acid change: Arg335His. This amino acid change observed was due to a SNP present in the gene, rs6886 (G/A). Thus this could be an explanation for the difference in the expression between the volunteers (Fig. 27).

5.2.6 Effect of EC-G2 and the plasma metabolites on human monocytes

Based on the results of Koga *et al.*[191], in order to understand if the protein changes observed were caused specifically by the bioactive substances present in chocolate, an analysis on human monocytes treated with concentrated plasma samples before and after the administration of dark and white chocolate, was performed. Even in this case, the presence of glucuronides of epicatechin was confirmed only after the consumption of dark chocolate (Fig. 28).

As you can see in the figure 29, on the basis of these results, a correlation between previous observations using the in vivo model, cannot be made.

The only potential correlation to previous results, is the spot identified as Rho GDP dissociation inhibitor, which was present only after the administration of dark chocolate.

%		%		%		%	
humidity		proteins		ashes		lipids	
average	sd	average	sd	average	sd	average	sd
1,91	0,14	12,07	0,05	2,91	0,30	43,34	1,40

mg/g catechin equivalent		mg/g catechin equivalent		% DPPH radical	
total polyphenols		total proanthocyanidins		antioxidant activity	
average	sd	average	sd	average	sd
9,44	1,12	17,99	0,98	64,23	0,51

ug/g		ug/g		ug/g		ug/g		ug/g		ug/g	
gallic acid		protocatechuic acid		acid pOH Benzoic		Catechin		Epicatechin		procyanidin B2	
average	sd	average	sd	average	sd	average	sd	average	sd	average	sd
15,68	0,01	14,84	0,09	4,32	0,15	7,14	0,52	834,64	8,90	236,46	0,88

mg/g		mg/g	
Theobromin (mg/g)		Caffeine (mg/g)	
average	sd	average	sd
26,65	0,29	1,88	0,03

ug/g		ug/g		ug/g		ug/g		ug/g	
Quercetin		Querc-glucoside		Querc-galattoside		kaempferol-glucoside		Kampf- rutinoside	
average	sd	average	sd	average	sd	average	sd	average	sd
4,64	0,68	10,70	0,21	4,51	0,15	9,00	0,17	21,88	0,18

Table 1. Characterization of dark chocolate bars. Quantification data of the compounds identified in the extracts of cocoa used in the study. The values given are mean ± standard deviation.

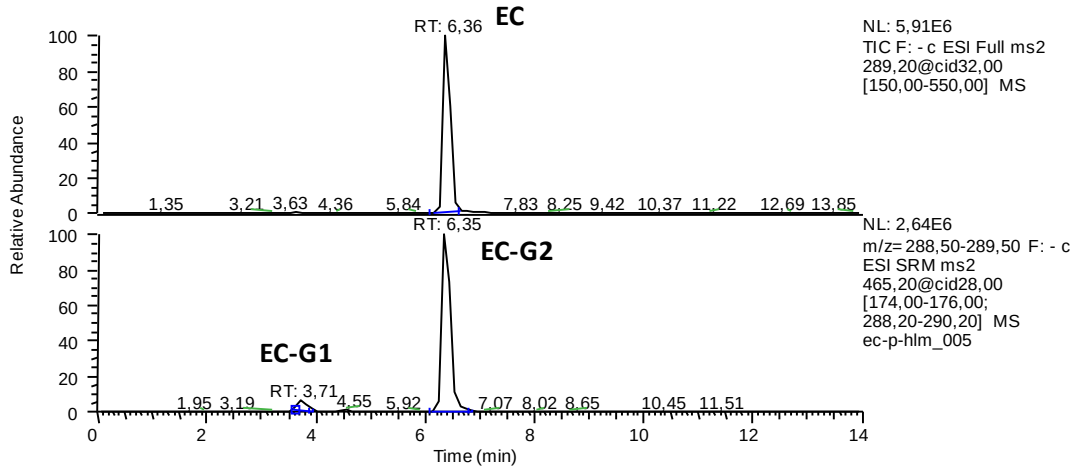


Figure 16. LC-MS^2 chromatograms of EC incubation sample in the presence of HLM and UDPGA cofactor.

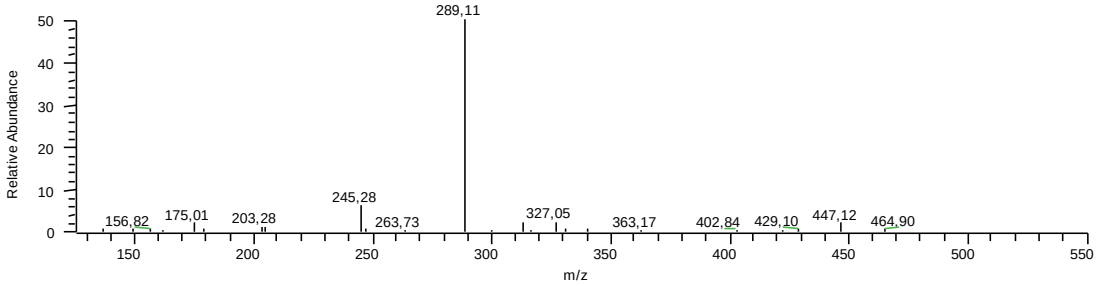


Figure 17. Structure and negative product ion spectrum of the 3 'glucuronide of epicatechin (EC-G2)

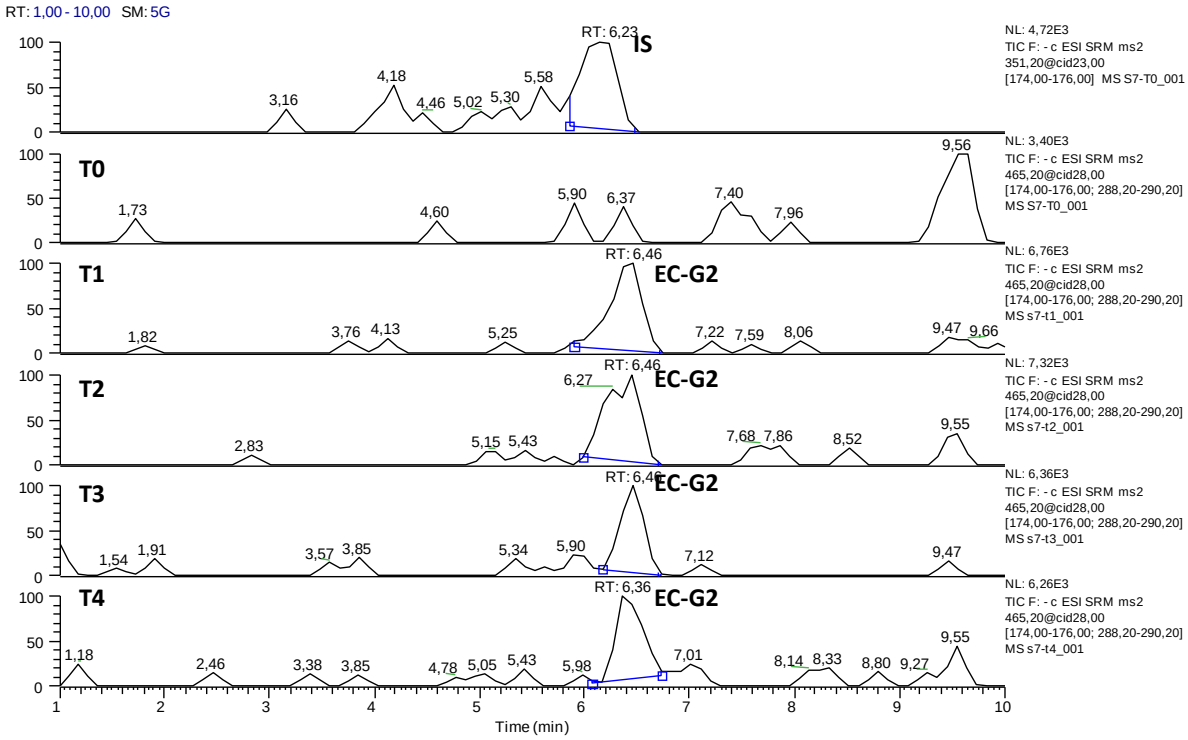


Figure 18. Chromatograms of plasma samples collected after chocolate bars intake. (-)-EC glucuronide (EC-G2): 465→289/175, R_t = 6.4 min. IS: 351→175, R_t = 6.2 min.

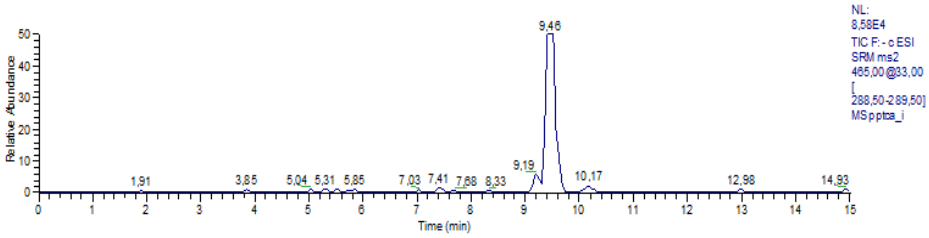


Figure 19. Chromatogram performed on plasma samples 4 hours following administration with white chocolate (7 chocolate bars). The peak with a retention time equal to 6.6 minutes is absent.

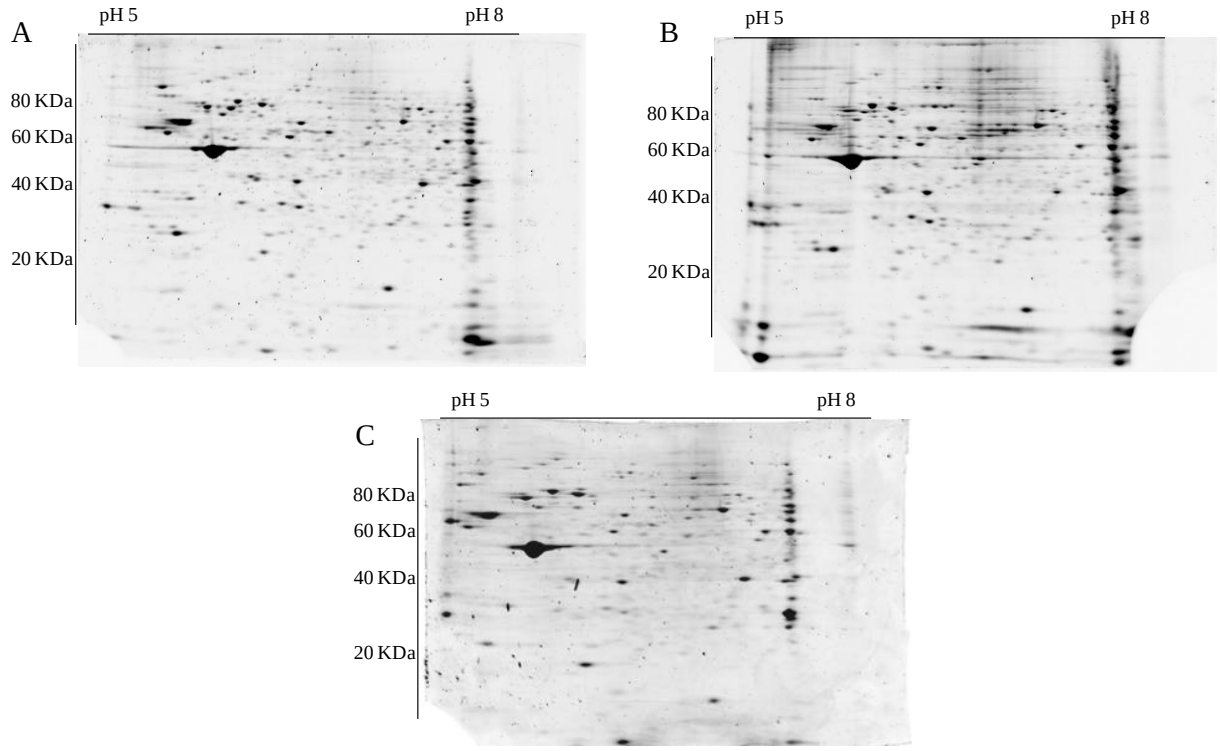


Figure 20. Representative image of two-dimensional analysis of proteins (IEF: pH 5-8, SDS-page 12% polyacrylamide) of extracted human monocytes at baseline (fig. A) and after 3 hours following the consumption of 7 dark (fig. B) and white (fig. C) chocolate bars.

	PDQuest ID	p-value	MW (KDa)	pI	preliminary identification	Accession No. Swiss-prot	References
cluster A	104	p< 0,05	26.8	4.9	Grancalcin	P28676	M. Jin *et al., 2006*
	1101	p< 0,01	27.5	4.9	Grancalcin	P28676	M. Jin *et al.*, 2006
	1102	p< 0,05	26.8	5	N/D	-	
	1104	-	26.8	5	Rho GDP-dissociation inhibitor 2	P52566	M. Jin *et al.*, 2006
	1106	p< 0,01	26.7	5.1	Rho GDP-dissociation inhibitor 2	P52566	M. Jin *et al.*, 2006
cluster B	2202	p< 0,01	32.4	5.4	N/D	-	
	3101	-	30.2	5.4	N/D	-	
	3104	-	32.3	5.5	N/D	-	
	3105	-	30.1	5.5	N/D	-	
	4201	-	32.4	5.5	N/D	-	
	4204	p< 0,05	32.2	5.6	N/D	-	
	4104	-	32.1	5.6	N/D	-	
	4107	-	30.1	5.6	N/D	-	
	3203	-	32.5	5.5	N/D	-	
cluster C	3807	p< 0,05	75	5.4	N/D	-	
	4808	-	75	5.5	N/D	-	
	4809	p< 0,05	75	5.5	N/D	-	
	4805	-	75	5.6	N/D	-	
	4807	-	75.1	5.6	N/D		
cluster D	5401	p< 0,05	42.3	5.7	Macrophage capping protein	P40121	M. Jin *et al.*, 2006; H.S. Gadgil *et al.*, 2003
	5403	p< 0,05	42.3	5.7	Macrophage capping protein	P40121	M. Jin *et al.*, 2006; H.S. Gadgil *et al.*, 2003
	5404	p< 0,01	43.5	5.7	Leukocyte elastase inhibitor	P30740	M.J. Pabst *et al*., 2008

Table 2. Some significantly modulated human monocyte proteins after dark chocolate administration .

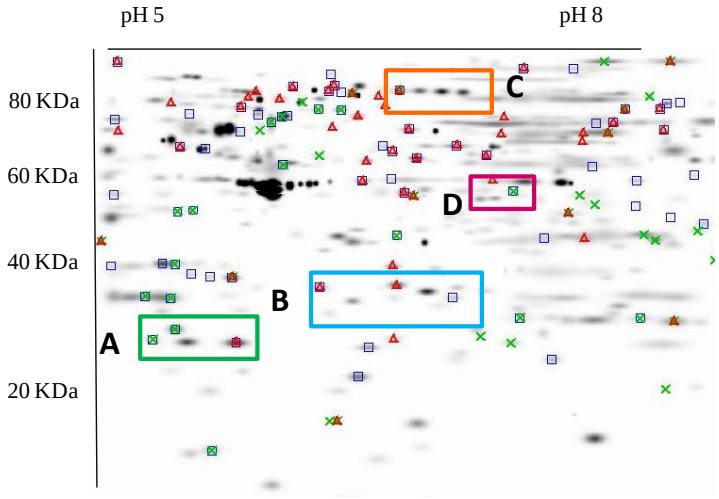


Figure 21. Statistical analysis of the protein spots between the three different condition; the blue squares (T0 versus T3 dark), the green cross (T0 versus T3 white), and the red triangle (T3 dark versus T3 white) indicate the spots with significant differences (p<0,01). the colored rectangle indicate the cluster of protein with the protein spots that change between before and after the chocolate administration (A, B, C and D).

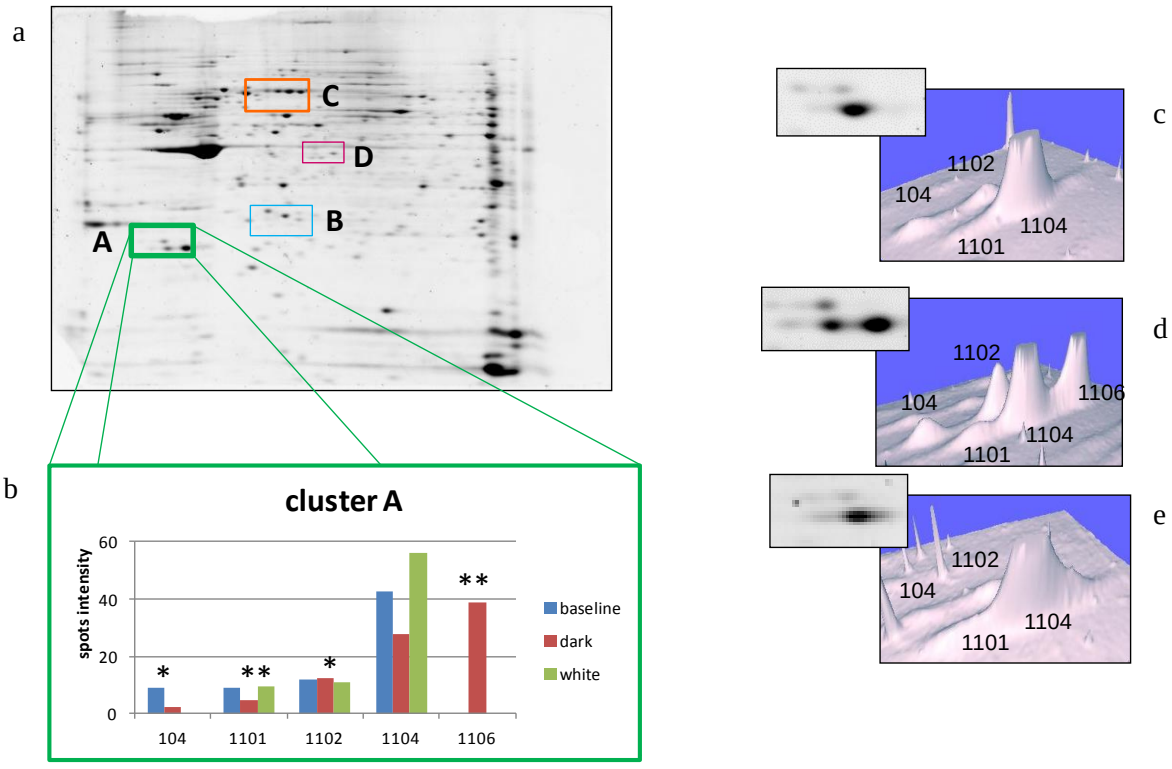


Figure 22. Schematic representation of the protein cluster A. a) two-dimensional gel (IEF: pH 5-8, SDS-page 12% polyacrylamide) of extracted human monocyte proteome; the colored rectangle indicate the protein spots that change between baseline and after the chocolate administration. b) density analysis of the protein spots, (* = p<0,05; ** = p<0,01; PDQuest estimation). Enlargement and the three-dimensional reconstruction of the area of the 2D-gel which includes the protein spots at baseline (c) and 3 hours following the consumption of 7 dark (d) and white (e) chocolate bars.

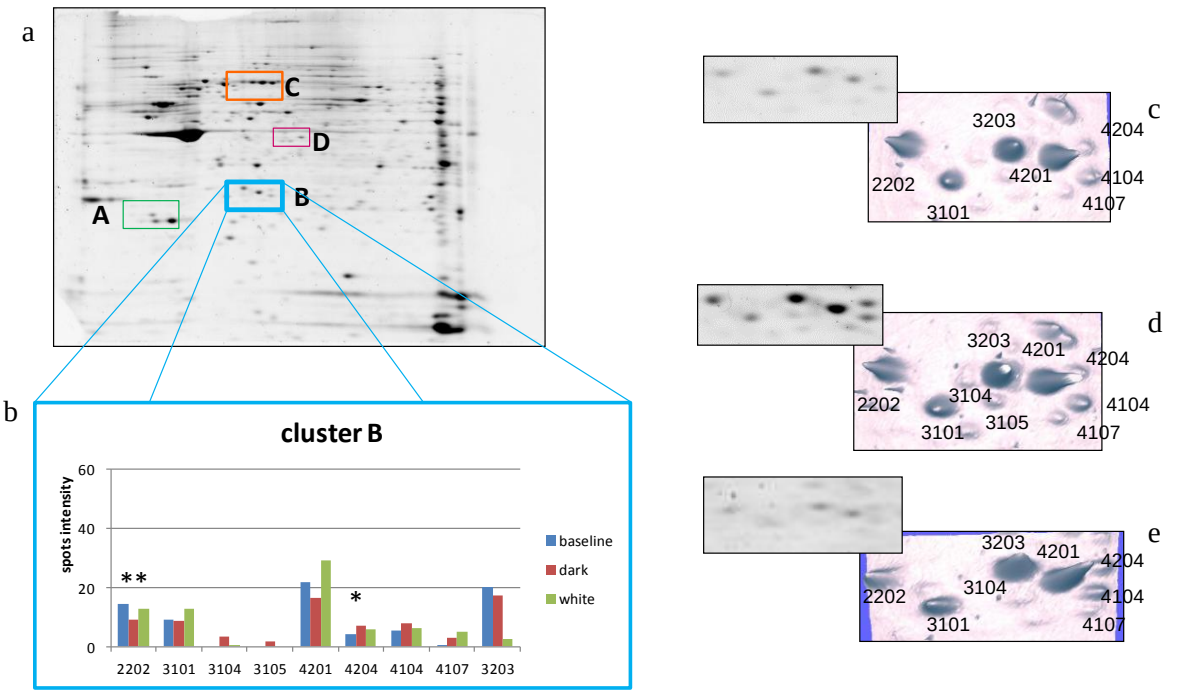


Figure 23. Schematic representation of the protein cluster B. a) two-dimensional gel (IEF: pH 5-8, SDS-page 12% polyacrylamide) of extracted human monocytes proteome; the colored rectangle indicate the protein spots that change between baseline and after the chocolate administration. b) density analysis of the protein spots, (* = p<0,05; ** = p<0,01; PDQuest estimation). Enlargement and the three-dimensional reconstruction of the area of the 2D-gel which includes the protein spots at baseline (c) and after 3 hours following the consumption of 7 dark (d) and white (e) chocolate bars.

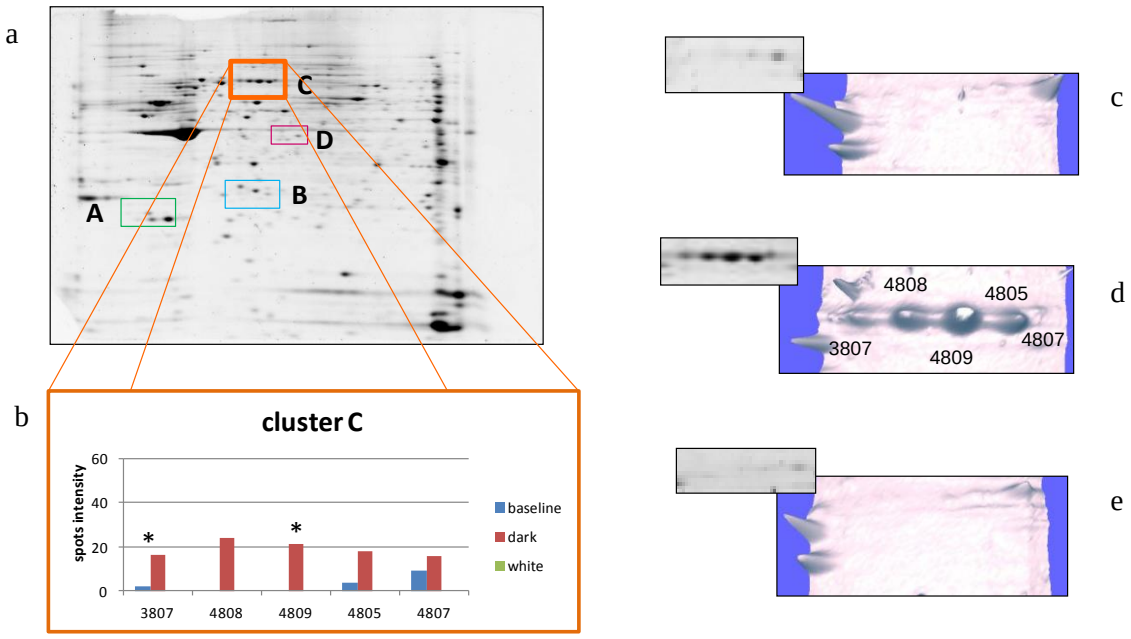


Figure 24. Schematic representation of the protein cluster C. a) two-dimensional gel (IEF: pH 5-8, SDS-page 12% polyacrylamide) of extracted human monocytes proteome; the colored rectangle indicate the protein spots that change between baseline and after the chocolate administration. b) density analysis of the protein spots, (* = p<0,05; PDQuest estimation). Enlargement and the three-dimensional reconstruction of the area of the 2D-gel which includes the protein spots at baseline (c) and after 3 hours following the consumption of 7 dark (d) and white (e) chocolate bars.

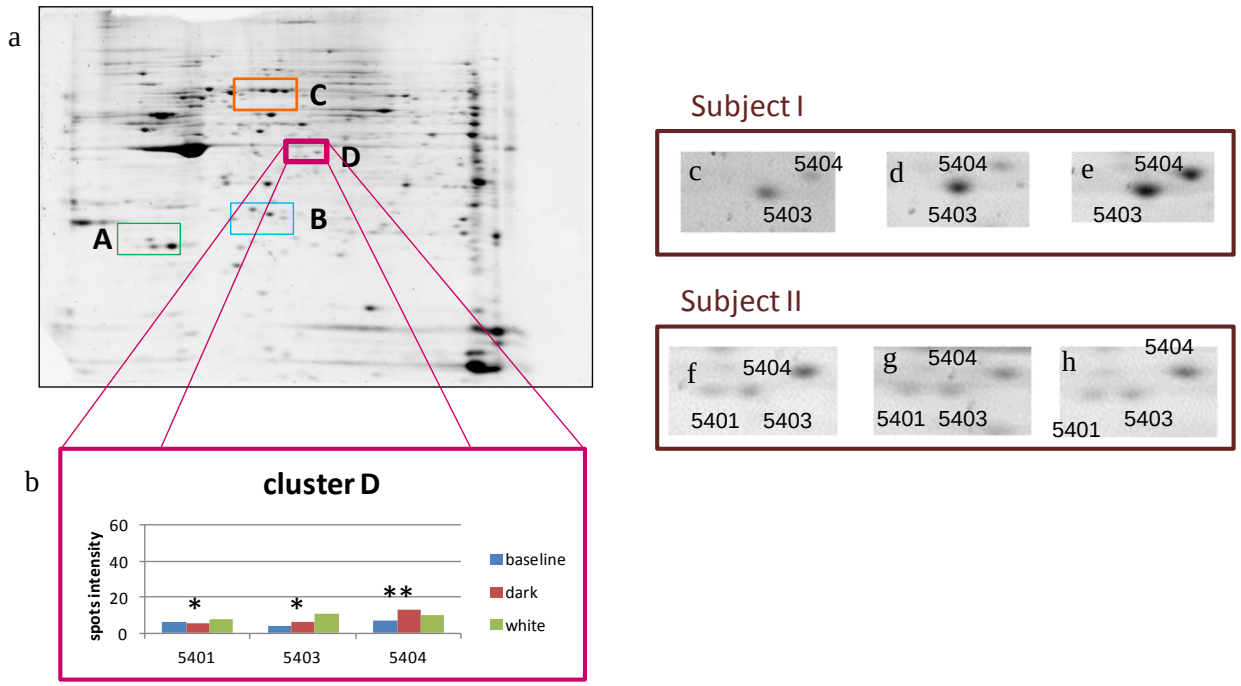


Figure 25. Schematic representation of the protein cluster D. a) two-dimensional gel (IEF: pH 5-8, SDS-page 12% polyacrylamide) of extracted human monocytes. b) density analysis of the protein spots, (* = p<0,05; ** = p<0,01; PDQuest estimation). Enlargement of the 2D-gel area which includes the protein spots at baseline (c,f) and after 3 hours following the consumption of 7 dark (d,g) and white (e,h) chocolate bars. The spot SSP 5401 is presented only in the subject II.

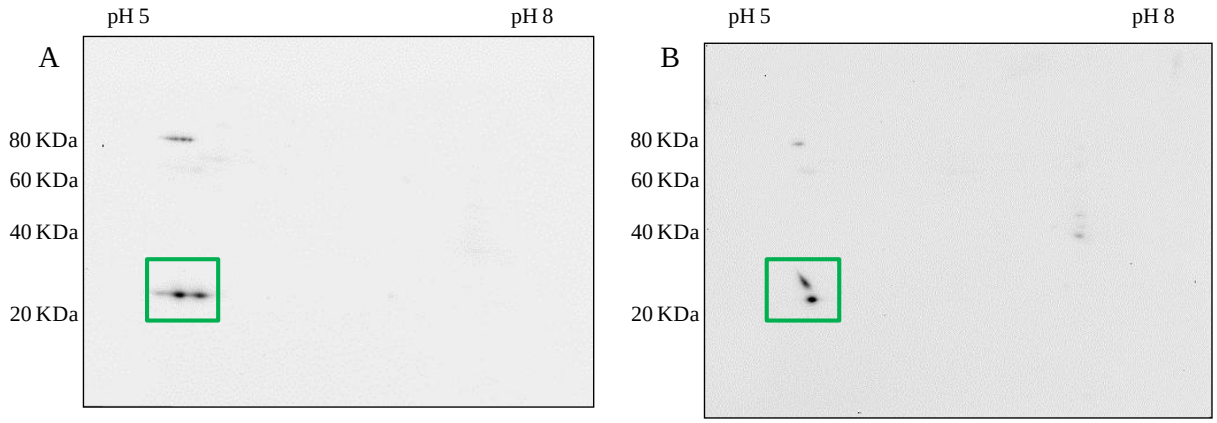


Figure 26. Western Immunoblot with Rho GDP-dissociation inhibitors antibody on 2D-gel before (A) and after (B) dark chocolate administration.

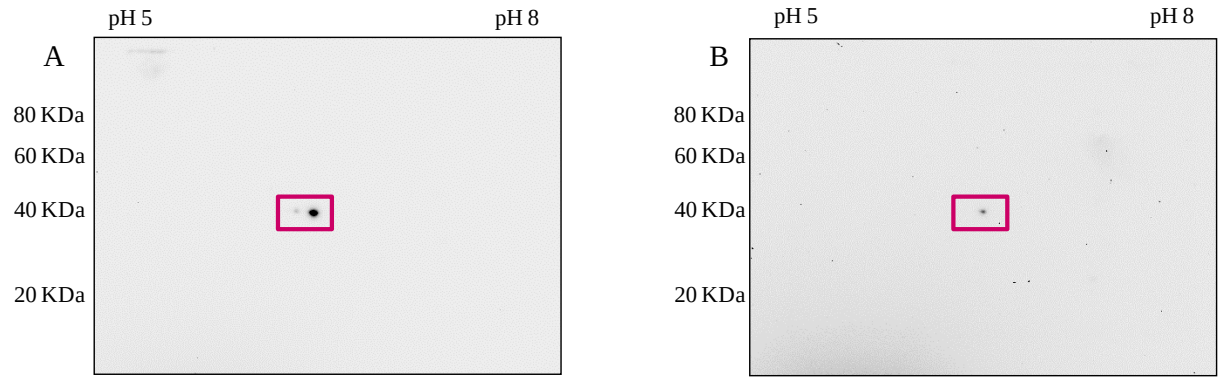


Figure 27. Western immunoblot with macrophage capping protein antibody on 2D-gel. Variability of the expression between subject I (A) and subject II (B).

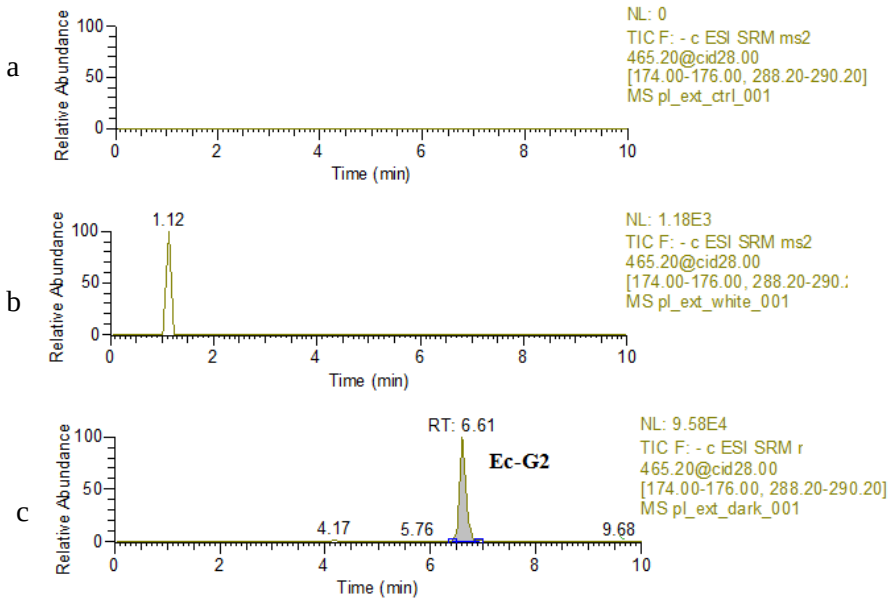


Figure 28. Chromatograms performed on concentrated plasma samples before (a) and following the administration with white (b) and dark (c) chocolate bars (7 chocolate bars). The peak with a retention time equal to 6.6 minutes only after dark chocolate consumption may be the peak of the EC-G2.

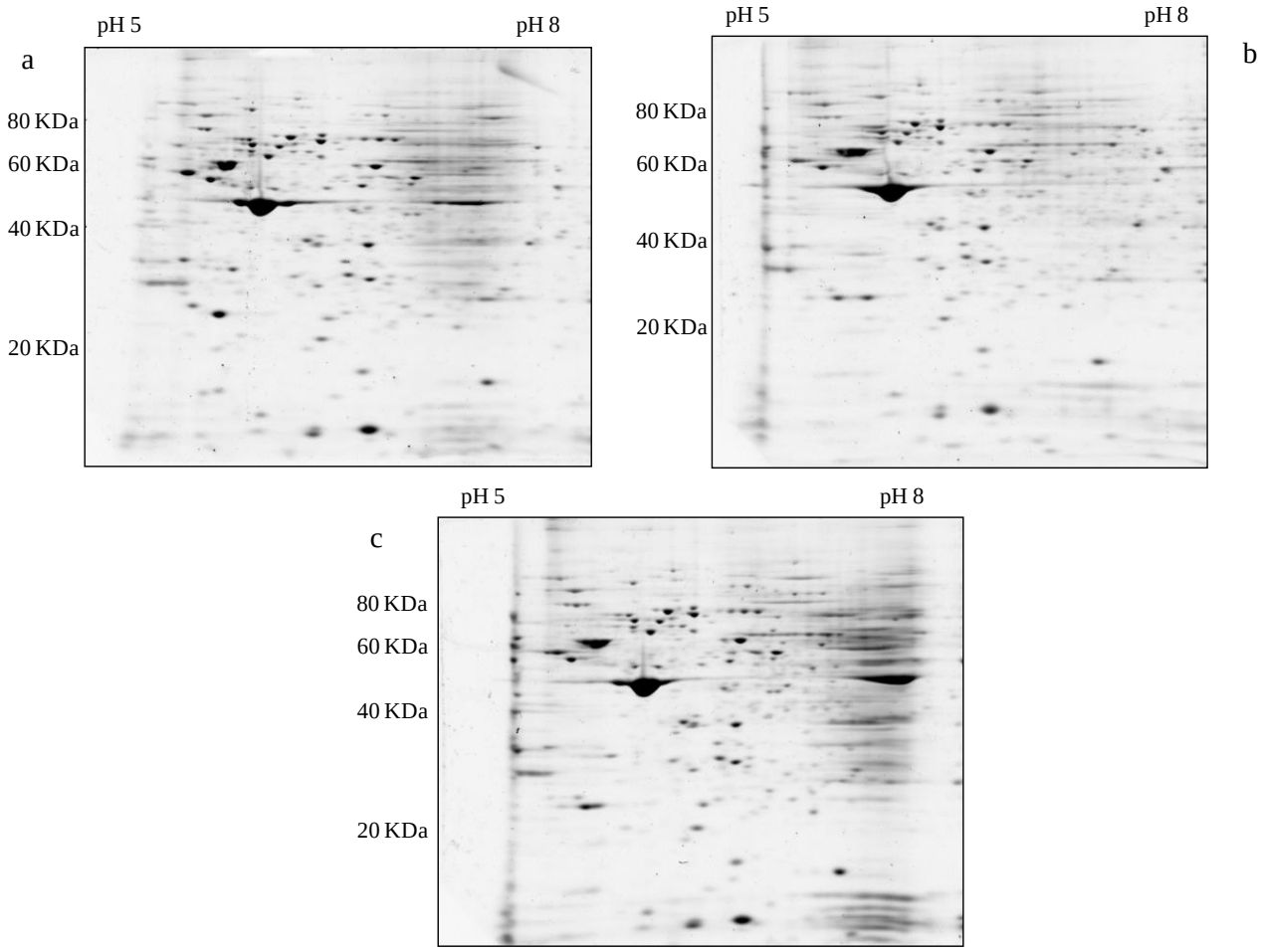


Figure 29. Representative image of two-dimensional analysis of proteins (IEF: pH 5-8, SDS-page 12% poly-acrylamide) of isolated human monocytes at baseline (a) and after an incubation of plasma extracted at 3 hours, following the consumption of 7 white (fig. b) and dark (fig. c) chocolate bars.

Table 3. The results of peptide mass fingerprint (PMF) obtained from the mass were subjected to proteomic investigation program on-line MASCOT. The results showed a score greater than or equal to 56 are statistically significant ($p<0.05$).

a

SSP 3203	PROT	SCORE	MASS Mr	COVERAGE	scor+cover
883	RND3	49	27351	19	68
931	YH009	42	34143	50	92
1297A	RM34	64	10159	43	107
1297B	MED19	61	25257	14	75
1297C	VEGFA	52	27024	16	68
1923	SMYD5	48	47310	10	58
2317	MSGN1	40	20765	28	68
4973	EDN1	54	24409	58	112

b

SSP 3101	PROT	SCORE	MASS Mr	COVERAGE	scor+cover
883	PHF13	50	33560	33	83
931	PHF13	46	33560	34	80
1301	LCE3E	53	9500	20	73
1450	IGF1	39	21827	45	84
2325	KCNE3	32	11703	28	60
2637	HAND1	46	23612	23	69
3277	TFPI2	40	26916	27	67
4976	U2AF1	52	27854	37	89

c

SSP 1102	PROT	SCORE	MASS Mr	COVERAGE	scor+cover
881	PRR13	46	15375	27	73
1292	VEGFA	52	27024	20	72
2323	SPIC	39	29162	20	59
4969	FUND2	53	20663	46	99

d

SSP 4201	PROT	SCORE	MASS Mr	COVERAGE	scor+cover
883	CE047	44	19194	85	129
934	CE047	48	19194	78	126
1294A	KIF2A	58	79905	19	77
1294B	LCE3E	45	9500	26	71
1450	RAB13	48	22760	44	92
2325	RSLAB	42	23214	25	67
4961	CCD84	48	37950	46	94
5018	FMR1N	53	29221	33	86

e

SSP 2202	PROT	SCORE	MASS Mr	COVERAGE	scor+cover
885A	NRL	80	25924	40	120
885B	H1FNT	56	28099	72	128
1257	RM34	55	10159	42	97
1450	PHF13	49	33560	25	74
1565	RBM8A	44	19877	21	65
1967	PTTG	51	20310	37	88
2931A	PAGE3	60	12472	24	84
2931B	PRR15	49	13707	18	67
3282	UB2D2	48	16724	31	79
4713	CT455	45	21132	40	85

5.3 DISCUSSION

Diet is a key environmental factor affecting health and the incidence of many chronic diseases[26]. Food and beverages are the only physical matter we take into our body, if we disregard the air we inhale and the drugs we may have to utilize. It is therefore logical and natural that nutrition exerts the strongest life-long environmental impact on human health. In fact, this interplay between nutrition and health has been known for centuries[6]. Understanding how our diet and nutritional status influence the composition and dynamic operations of our gut microbial population, and the innate and adaptive arms of our immune system, represents an area of scientific need, an opportunity and a challenge[8]. The concept that food components can affect physiological functions by interacting and modulating molecular mechanisms has revolutionized the field of nutrition. The concept of developing functional foods to prevent disease and maintain good health, requires the identification of the biologically active molecules and a demonstration of their efficacy. The disciplines "nutrigenetics" and "nutrigenomics" have evolved to address these issue. Nutrigenomics attempts to study the genome-wide influences of nutrition. From a nutrigenomic perspective, nutrients are dietary signals that are detected by the cellular sensor systems that influence gene and protein expression and, subsequently, metabolite production. It is an appealing idea that a food commonly consumed for pure pleasure could also bring tangible benefits for health. Olive oil, green tea and red wine have been commonly researched in the past, but now there is growing interest in cocoa[39,45]. Over the centuries, the use of cacao has evolved to what we now know as chocolate[45,57]. Chocolate can be defined as a functional food due to its high content of monomeric (epicatechin and catechin) and oligomeric (procyanidins) flavanols, a sub-class of polyphenols, which are the most abundant antioxidants in the diet[100]. Epicatechin, the major polyphenol in chocolate and chocolate extracts, is a powerful inhibitor of plasma lipid oxidation due to polyphenol's ability to bind to low density lipoproteins[40]. Although most of the studies indicate that the health benefits of cocoa or cocoa products are attributable to polyphenols, it should be noted that cocoa and cocoa products are not only rich in polyphenols, but are also rich in methylxanthines and theobromine. The possible synergistic interactions between flavonoids and methylxanthines are also unclear and need further study, thus the contribution of theobromine in cocoa products towards health benefits, should be considered[64].

Flavonoids are partly absorbed from the gastrointestinal tract in animals and humans[87]. Recent studies have shown that metabolites such as glucuronide or sulfate conjugates and

methylated conjugates accumulate in plasma, whereas the unconjugated compounds are detected in very low concentrations[79]. Conjugated metabolites have been suggested to play greater roles in the biological activity of flavonoids than their parent compounds. Nevertheless, little is known about the biological activities of the metabolites of flavonoids that are present in the blood. The precise circulating cocoa metabolites present after consumption, are only partially understood, as such the aim of the study was to understand if after a dark chocolate ingestion, there was an accumulation or a time-dependent presence of polyphenol metabolites. Based on a recent study by Goretta and co-workers[92], an LC-MS analysis on plasma samples was performed using as a marker for the presence of cocoa metabolites in the circulating plasma, an (-)-epicatechin glucuronide, specifically the most abundant, 3'-glucuronide[92]. Although our analytical method for the bioavailability study is not sufficiently sensitive due to the small quantities of EC-G2, the results obtained confirmed the presence of epicatechin glucuronide in all plasma samples from 30 minutes to 4 hours following the ingestion of dark chocolate. To confirm that the pick at Rt= 6.6 min is equivalent to epicatechin glucuronide, we also used an internal standard (IS) with the same Rt. Furthermore, as with the previous analysis, the glucuronide product detected by the HLM, confirmed the results. At present, it is not possible to quantify the plasma concentration of EC-G2, due to the lack of commercially available standards as well as the cost of production, with those in possession not willing to make them available.

It is very difficult to predict the effects of polyphenol intake on disease prevention in humans, because most polyphenols exert an antioxidant action *in vitro*, however, this does not mean that all polyphenols have an antioxidant effect *in vivo*[39]. The results thus obtained were expected to show if a strong association or correlation of activity in humans is possible or not, since data from human investigations are rare. Of these limited studies, the data support a role for polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases and diabetes[100]. However, from these studies it is very difficult to predict the long term effects of polyphenol intake on disease prevention in humans[65]. Experimental data is now accumulating regarding phenolic compounds as natural phytochemical antioxidants that possess anti-inflammatory effects[123]. Dietary components appear in complex mixtures and hence not only the concentrations of single compounds but also the interactions between them have an impact on the final bioavailable ingredients and the bio-efficacy[28,69]. Thus, the precise mechanism/s by which flavonoids exert positive effects have yet to be elucidated. Studies on health effects of polyphenols from cocoa or chocolate and/or other cocoa products

are few. We need biomarkers of the function of dietary components to elucidate how they function in acute and in chronic conditions.

Proteomics has logically developed as a central platform in nutrigenomics, which attempts to holistically understand how our genome is expressed in response to diet. Monocytes and macrophages are key to the inflammatory status and offer an important model in investigating the role of dietary components and their metabolites because they are involved in a several physiological processes. Another reason for this choice of cell model was determined by the simplicity and non-invasive sampling from human subjects. In the present study, a proteomic analysis of monocytes isolated from peripheral human blood was performed to examine the mechanisms by which chocolate consumption may modulate the monocyte/macrophage proteome in healthy subjects in the order to plan future functional studies. We analyzed the effects of cocoa polyphenols on monocyte proteomic profile to identify proteins that alter their expression following the consumption of chocolate. According to the LC-MS results, the time collection selected was between 3 and 4 hour following the ingestion of chocolate. We speculated that if there were metabolites in the plasma, they could act as a stimulator or inhibitor of protein expression in circulating cells, including monocytes.

A clear relationship between the nutrition-related metabolic changes and inflammatory gene expression profiles has been found[198]. Despite their biological importance, limited information exists regarding the proteins expressed in peripheral blood mononuclear cells (PBMCs) by a 2-DE approach. In fact, a proteomic database of monocytes exists only for pig. The use of this technique for the separation of proteins presents a valid tool due its high degree of resolution and its ability to examine multiple changes at one and the same time. This method was used to identify proteins that could be used as potential therapeutic marker. Following their extraction, proteins were separated by 2D electrophoresis and their protein profiles analyzed by PDQuest. The analysis of statistical data has allowed us to detect differences in the protein profiles following consumption of dark chocolate and led to the identification of 11 different spots. These spots were excised and proteolytically digested with trypsin and analyzed through mass spectrometry. Some spots were not considered to be identifiable by MS due to their spot intensity. Mass spectrometry requires that the protein spot must be visible by coomassie staining, rather than Sybro Ruby stain which has a 100-fold higher level of sensitivity.

To overcome this problem are ongoing further analysis in order to optimize the method of proteolytic digestion, modifying the times and temperatures in which the enzymatic reaction happens.

Proteomics is a very useful technique which allows researchers to study the actual output of the cells rather than just the DNA blueprints but as every technique has its disadvantages such as: only the detection of proteins more expressed, long times of work and the 2D gels el may not be reproducible.

Due to the lack of a current protein database on monocytes, to identify the single spots while waiting for the mass spectrometry data, reference was made to results published in literature to date. In particular Gadgil et al.[197], Pabst et al.[198] and Jin et al.[193] ,offer the most detailed current monocyte proteomic database and provide new perspectives into the study of monocyte biology. By examining the layout of the spots on the 2D-gels, the estimated isoelectric point and molecular weight, predictions were made as to the identify of significantly variable proteins pre- and post-chocolate consumption. With antibodies specific to these predicted proteins, a WIB analysis of 2D-gels was able to identify these proteins. The first protein positively identified was the Rho GDP-dissociation inhibitors (GDIs), an endogenous inhibitor of Rho GTPases, which play an important role in regulating the biological activities of rho GTPases. GDIs form a complex with the GDP-bound form of RHO family of small G proteins and inhibits their activation.

There are three regulators of Rho GTPase activity. First, the conversion from the inactive GDP-bound form to the active GTP-bound form, is catalyzed by upstream guanine nucleotide exchange factors, GEFs. Second, the intrinsic GTPase activity of Rho GTPases is stimulated by GTPase-activating proteins (GAPs) and results in the hydrolysis of the bound GTP to GDP and consequent inactivation. The third type of regulators of Rho GTPases, the guanine nucleotide dissociation inhibitors (GDIs), interact and stabilize the GDP-bound form to prevent spontaneous activation. The interaction of GDIs with the prenylated form of Rho GTPases plays a critical role in the regulation of the cytosolic versus membrane distribution of Rho-GDP and also protects them from degradation. Rho GDIs possess at least two biochemical functions. First, they preferentially interact with the inactive, GDP-bound form of the Rho family and prevent them from being converted to the active form; second, after the inactivation of GTP-bond form, Rho GDIs form a complex with the inactive form and translocate it from the membrane to the cytosol. The small G proteins of the Rho family consisting of Rho, Rac and Cdc42 subfamiles, are implicated in various cell functions, such as cell shape change, cell motility and cytokinesis, through the reorganization of actin filaments[194].

There are three isoforms: Rho GDIα, β and ϒ. The first one is ubiquitously expressed, GDIβ is expressed exclusively in hematopoietic tissues and the last one is preferentially expressed

in brain. Studies available in the literature agree that it is possible that it is involved as a common component for the pathogenesis of several cardiovascular disorders including hypertension, coronary and cerebral vasospasm, atherosclerosis and diabetes[194,199].

Another protein spot from the proteomic analysis that drew attention with a differential protein expression in the studied subjects, was predicted and identified to be macrophage capping protein (CapG). It belongs to the gelsolin family of proteins binding to actin in a calcium-dependent manner. CapG is a specific capping protein for macrophage and macrophage-like cells. One of their roles is to block the barbed ends of actin filaments without severing preformed actin filaments, a step that is critical for regulating actin-based motility in non-muscle cells. CapG null macrophages show impaired receptor-mediated ruffling, phagocytosis and vesicular rocketing[197]. These processes play critical roles in the function of macrophages. CapG also binds to phosphatidylinositol-4,5- bisphosphate with a strong affinity and it is thought to regulate receptor-mediated phosphoinositide turnover and calcium signaling[197]. Dupont and co-workers[200] showed a correlation between the oxidation of LDL (oxLDL) and CapG. After oxLDL stimulation, expression of cytoskeletal and related proteins increased. These include, for example, gelsolin (3.3-fold up-regulation over control cells), which is involved in cell growth and maintenance and is an essential component of the cytoskeletal machinery.

There is another protein probably identified by comparison with the published proteomic data, the grancalcin, which may play a role in the formation of focal adhesions. Because the significance was detected after a reworked analysis of our data, it was decided to confirm the spot without WIB but through the mass spectrometry[201].

Overall, with this analysis it can be hypothesized that after administration of dark chocolate there is a component/s in the plasma that promotes the expression of specific proteins involved in the inflammatory pathway. To test whether the protein spots examined change their expression following administration of dark chocolate will proceed with an analysis of *in vitro,* by treating human monocytes, and monocyte-derived macrophages (MDMs) with (-)-epicatechin; and *in vivo* by assessing whether a chronic administration, at lower doses of chocolate, it will be able observe the same changes in the protein spots.

For the moment, in the present study all of the attention has been given to intracellular proteins of monocytes. It will be very interesting to also evaluate what the cell releases into the culture medium following treatment with the metabolites of epicatechin. In response to various pathological and physiological signals, the cells produce a wide range of biological products, known collectively as the "secretome", including cytokines, coagulation factors,

growth factors, adhesion and binding proteins, enzymes, stress proteins, and signaling molecules. Research evaluating these secreted proteins could be a valid method in to find new biomarkers on which to study new therapeutic strategies.

It is important to recognize that diet cannot replace drug treatments but offer an important aspect to consider for long term health benefits. This has been demonstrated by the Kuna Indian population, who live on islands off the coast of Panama and consume by tradition large amounts of cocoa daily, have lower blood pressure values and a reduced cardiovascular risk as compared with other Pan-American populations. As such, information regarding the bioactive substances present in foods such as cocoa that are normally conveyed throughout the body, to which tissues and at which level they act within the cellular systems are needed. The current study using a proteomic approach, approaches this question and holds promise for revealing more about how bioactive substances present in food, in this case (-)-epicatechin, may affect the activity of human monocytes and as such the inflammatory pathway/s. Even though its nutraceutical value and its possible beneficial effects have been sufficiently described, a clear conclusion regarding the direct effect in humans has not yet been clarified. Hence, studies such as the present are essential for designing future treatment plans, particularly with respect to chronic disease such as obesity and cancer.

6. Experimental section II

AIM OF THE STUDY II

Modern nutrition science explores the health-related aspects of bioactive food components, thereby promoting health, preventing or delaying the onset of disease, optimizing performance and assessing the benefits and risks in individuals and in subpopulations. In recent years, a growing number of studies, mostly conducted *in vitro* or *ex vivo,* have demonstrated that flavanols share the capacity to modulate inflammation as well as other major metabolic and immunological pathways. To date, most of the work in vitro with flavonoids have been carried out on neuronal and epithelial cells, but very little is known about the cytoprotective activity of flavonoids on immune cells.

The main target of this research is to study the key molecular mechanisms through which the bioactive components of cocoa may modulate the inflammatory state. One of the principal bioactive components of cocoa, (-)-epicatechin (EC), was tested in time and dose-dependent experiments, using the U937 cell line which has been widely used as a powerful *in vitro* model to analyze the modulatory effect of substances on the inflammation. The U937 cells were preincubated with 100 µM of epicatechin for 30 min to 3 hour followed by the presence of lipopolysaccharide (LPS, 1 µg/mL) for the last 6 hour, following which nuclear and cytoplamatic fractions were isolated. Aliquots of each protein extract were evaluated by western immunoblot using anti-NRF2, anti-p NRF2, anti-NRF1, anti-HO-1 and internal controls to investigated a possible involvement of the transcriptional factor NRF2 in the molecular mechanisms activated by flavonoids in a monocytic-like cells.

6.1 MATERIALS AND METHODS

6.1.1 Cells culture condition

The human monocyte cell line, U-937, were purchased from the American Type Cell Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 supplemented with 10% of heat-inactivating Fetal Bovine Serum (FBS), 100 U/mL of penicillin/100 µg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO2 at 37 °C. The U-937 cell line was chosen because it is a well-established human cell line with numerous monocytic characteristics and has been used extensively as an in vitro model for macrophage stimulation. Besides has, unlike other cell lines of monocyte-macrophages, an available proteomics database. Monocyte-like cell line U-937 activated by lipopolysaccharide (LPS), is a model often used to analyze the modulatory effect of substances on inflammation.

6.1.2 MTT cell survival assay

U937 cells were plated at density of $1x10^5$ cells/well in 96-well plates and treated with or without increasing concentrations of (-)-epicatechin (0, 5, 10, 50, 100, 200, 500 µM). Cells were incubated with MTT solution 1mg/mL for 3 hours and after solubilization in DMSO, the absorbance at 570nM was measured. Experiments were performed in duplicate with each data point representing the mean ± S.D normalized to the value of control cells.

6.1.3 U937 experimental set up

To study the time-course effects of epicatechin, cells were seeded in T75cm^2 flasks in presence of RPMI-1640 serum-free medium for 24 h before the assay, to exclude the effect of factors found in the fetal bovine serum which may influence the results. Subsequently, cells were pre-incubated with 100µM of epicatechin, according to the results of MTT assay, at different incubation times (0, 30, 60, 120, 180 min), followed by a stimulation with 1µg/mL of LPS (from E. Coli 055:B5) for 6h. Lipopolysaccharide, the main cell wall component of gram negative bacteria, strongly induces the production and release of various cytokines and inflammatory factors that initiate the inflammation process. The primary inflammatory response induced by gram-negative bacteria involves activation of the innate immune system. A minimum of three independent experiments were performed. Each condition was tested in duplicate.

6.1.4 Western Immunoblotting

To study the activation of NRF2 pathway, U937 cells were incubated as described previously. Nuclear and cytoplasmic proteins were extracted by a Paris Kit (Ambion). Protein concentration was determined using BCA reagent (Pierce, USA) according to the manufacturer's instructions. As described previously, membranes were incubated at 4 °C with control antibodies, anti-lamin B (LamB C-5: sc-365962; Santa Cruz, CA, USA) and anti-α-tubulin and β-actin (Sigma-Aldrich), or for proteins of interest including anti-NRF2 (C-20, sc-722), anti-NRF2 (H-300, sc-13032), anti- Nrf1 (NRF1 H-4: sc-28379), anti-Heme Oxygenase 1 (HO-1 23: sc-136256) and anti-NRF2 (phospho S40) antibody (EP1809Y; Abcam). After an overnight incubation and subsequent washes, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:5000 in TBST for 1 h at 37 °C. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology; Rockford, IL) according to the manufacturer's instructions using Quantity One 1D image analysis software.

6.1.5 Measurement of free radical scavenging activity

ROS scavenging activity was determined using DCFDA Cellular ROS Detection Assay (ab113851; Abcam). This assay uses the florescent 2',7'-dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7'-dichlorofluorescin (DCF). Detection involved fluorescent spectroscopy with excitation and emission of 485 nm and 520 nm respectively. U937 cells were plated at density of 1x106 cells in T25cm2 flasks and treated with or without 100 µM of (-)-epicatechin, followed by 1µg/mL of LPS. Experiments were performed in triplicate with each data point representing the mean ± S.D normalized to control cells.

6.1.6 Measurement of cytochines and chemokines

The multi analyte ELISArray Kit are designed to simultaneously profile the level of multiple cytokines and/or chemokines using the conventional and simple Sandwich-Based Enzyme-Linked Immunosorbant Assay (ELISA) technique according to the manufacturer's instructions. The 96-well ELISA microplate has been coated with a panel of target-specific

capture antibodies giving a qualitative profile for up to six samples. The cytokines and chemokines represented by these array are IL-1α, IL-1β, IL-2, IL-4, IL-6 IL-10, IL-12, IL-17α,IFNϒ, TNFα and GM-CSF. The serum-free medium of the control group (U937 cells alone, with LPS 1µg/µL, the vehicle and the bioactive substance) and after the incubation with both epicatechin (100 µM) and LPS (1µg/mL) for 6h and 24h were collected and used for the analysis. Absorbance was read at 450 and 570 nm. Experiments were performed in duplicate with each data point representing the mean ± S.D normalized to control cells.

6.1.7 Statistical analysis

Data are expressed as mean ± SD. Skewed variables were logarithmically transformed before analyses when necessary. Differences between groups, treatments and *in vitro* studies were compared using Mann-Whitney U or Wilcoxon test, Student's t test and χ^2 test when necessary. Statistical significance was assumed for $p<0.05$. The statistical analyses were performed with GraphPad Prism for Windows.

6.2. RESULTS

6.2.1 MTT cell survival assay

To assess the cytotoxic effect of various concentrations of epicatechin on the cell line U937, an MTT assay was performed (fig. 30). As shown, increasing concentrations of epicatechin reduced the cell viability to between 79 and 38%, but only with the three highest concentrations (200, 400 and 500 µM). With respect to the lower concentrations, cell viability was always greater than 80%. As such, all subsequent experiments on U937 cells were done with a 100 µM concentration of epicatechin.

6.2.2 Western Immunoblotting

To explore the possible intracellular mechanism of (-)-epicatechin (EC), cellular stress on U937 cells was induced with LPS and we investigated the expression of a profile of proteins involved in oxidative stress. In particular, focus was given to the expression of the transcription factor NRF2, demonstrated to be a critical regulator of flavonoid-mediated protection.

As expected, NRF2 was expressed exclusively in the cytosol of untreated cells and did not undergo nuclear translocation with LPS or EC alone (fig. 31 a). Alternatively, when treated with both LPS and EC, there was a time dependent increase in whole cell NRF2 levels with respect to controls, as shown in figure 32 a, b. Further, analysis of both the cytosolic and nuclear compartments showed that pre-treatment with epicatechin and a subsequent 6h of stimulation with LPS, increased in time dependent manner the nuclear translocation of p-NRF2 (S40) in figure 33. To further investigated the potential role of NRF2 from the perspective of a defense mechanism induced by oxidative stress, the expression of Heme oxygenases-1 protein downstream of NRF2 was evaluated[202]. While the expression levels are very low, as shown in figure 34, the preliminary results show a 30% up-regulation of HO-1 in nuclear compartment after EC and LPS incubation, with respect to the control cells.

The involvement of NRF1 was also investigated, being that NRF1 has been shown to bind to ARE and regulate the expression of a number genes involved in oxidative stress. In figure 35, despite the fact that LPS alone and EC express a consistent level of Nrf1 mainly in the cytosolic compartment, it can be seen that LPS in conjunction with EC increases the total expression and the possible translocation of NRF1 from the cytoplasm to the nucleus. What is difficult to explain at the present moment and is awaiting further experimentation, is how (-)-

epicatechin could be able to modulate Nrf1 and if the two transcriptional factor are interdependent.

6.2.3 Measurement of free radical scavenging activity

To establish whether ROS generation was involved in contributing to cell death after EC and LPS treatment, ROS levels were determined according to the fluorescence levels of 2' ,7'-dichlorofluorescein (DCF), in the U937 cells (fig. 36). The results demonstrate that the EC treatment significantly decreased ROS levels. Despite this, the amount of ROS also remained high even in untreated cells, when compared with LPS-treated cells. Taken together, these observations suggest that ROS was elevated after the LPS incubation but these levels were decreased following EC treatment ($P< 0.05$).

6.2.4 Measurement of cytokines and chemokines

To further explore the anti- or pro- inflammatory effect of EC on U937, we measured 11 common chemokines and cytokines known to be involved in the inflammatory pathways (fig. 37 a, b). As expected, the expression levels of all chemokines and cytokines were low in the control group (U937 and DMSO). These levels remained low in the EC alone treated cells as opposed to the LPS treatment, where a clear increase in IL-6 and TNF-α were observed. Treatment with both EC and LPS showed an increase in the levels of IL-2, IL-4, Il-17α and IFNγ, with respect to EC alone, with levels of these cytokines tending to rise with an increasing time of incubation with EC and LPS combined (24h). What was unusual and unexpected was IL-17α, which while absent in LPS and EC alone, showed a significant up-regulation exclusively at 24h in the presence of both.

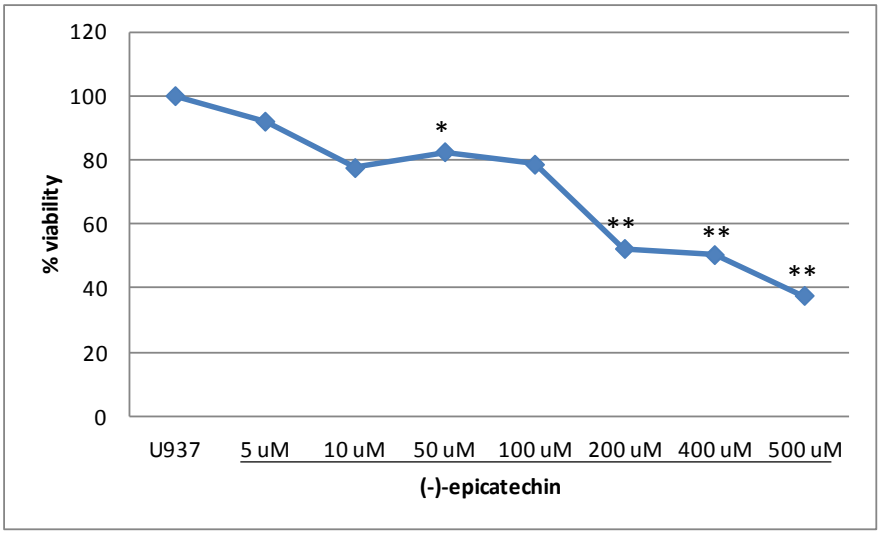


Figure 30. U937 cells were incubated with increasing concentrations of EC for 24 h. Cell viability was tested by the conventional MTT assay. (* = p<0,05; ** = p<0,01).

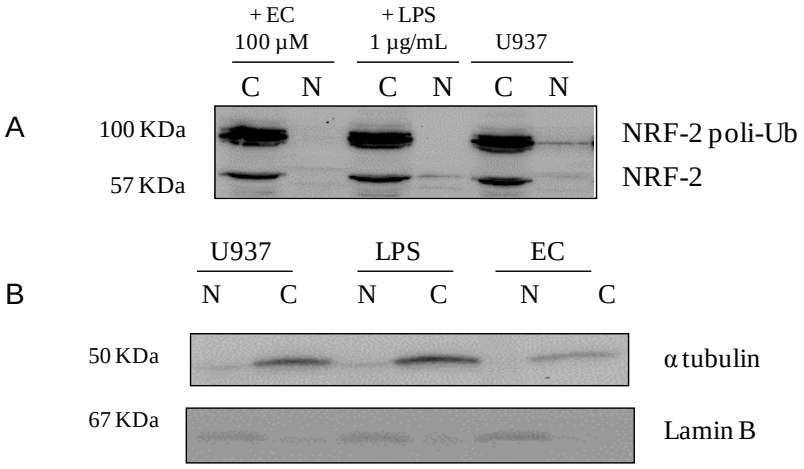


Figure 31. Incubation (24 h) with EC or LPS alone (6 h) do not induce the translocation of NRF2. A) Representative example of western immunoblot of NRF2 protein expression. B) α-tubulin and lamin-β was used to verify the protein concentration.

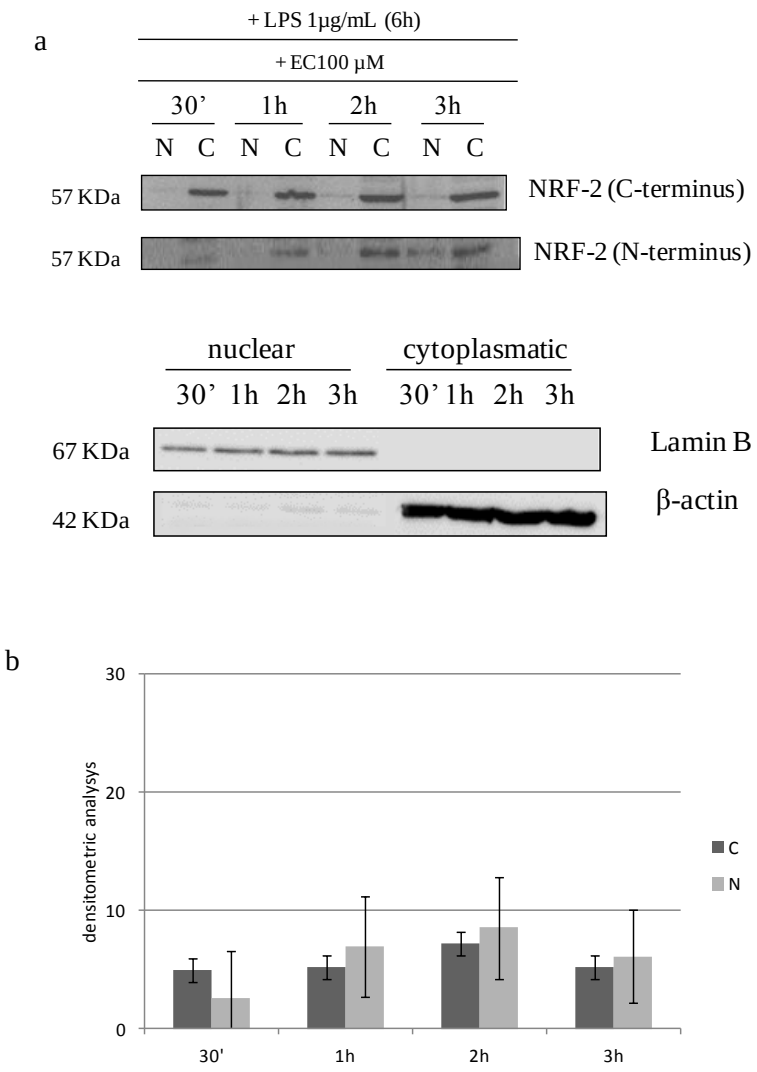


Figure 32. a) Cytoplasmic (C) and nuclear (N) extracts from U937 cells incubated with 100 uM of epicatechin (EC) for 30 min, 1, 2, and 3 hours, and then stimulated with LPS 1 µg / mL for the last 6 hour of incubation. The proteins were subjected to western immunoblotting to detect NRF-2 (β-actin and Lamin β are used as a control). b) Densitometric quantification of the bands detected (* = $p<0,05$; ** = $p<0,01$).

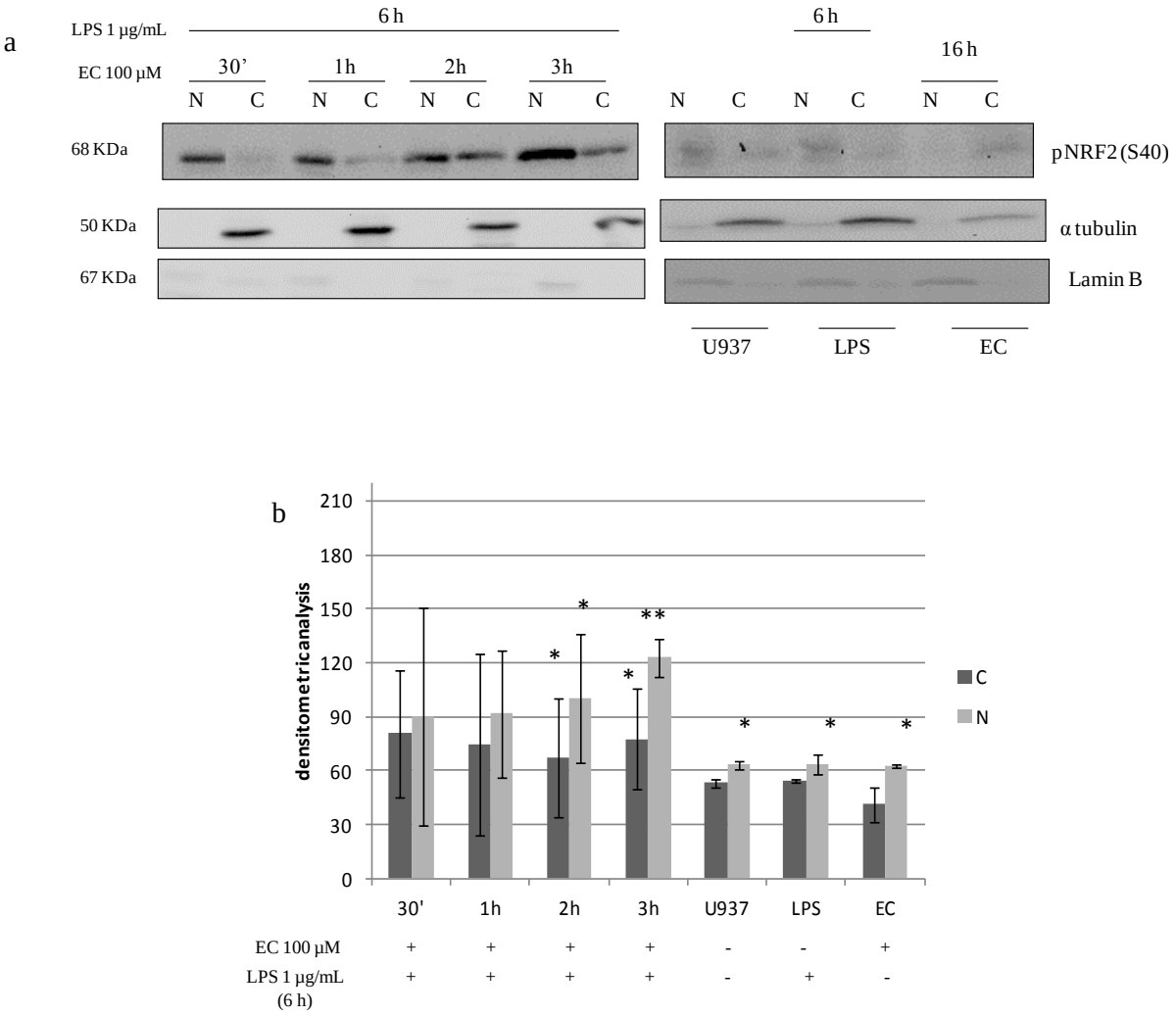
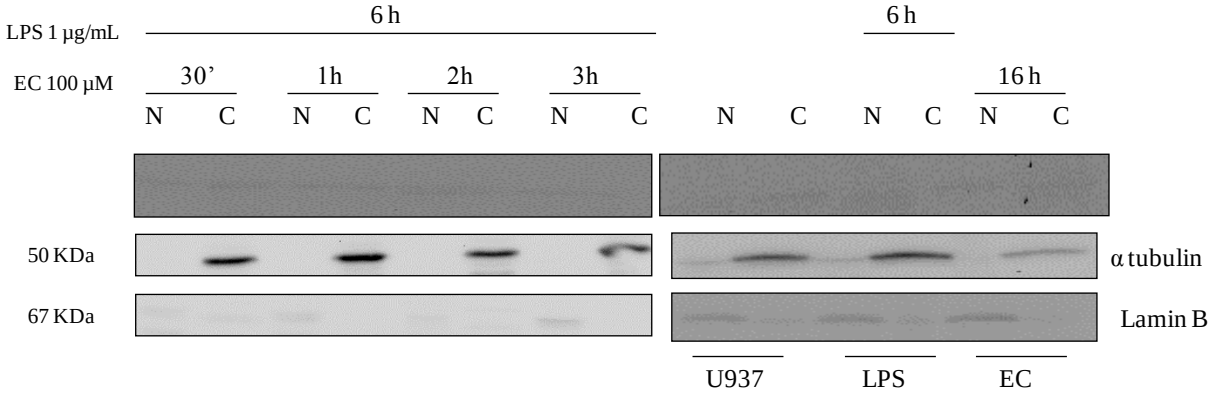


Figure 33. a) Cytoplasmic proteins (C) and nuclear (N) extracts from U937 cells incubated with 100 uM of epicatechin (EC) for 30 min, 1, 2, and 3 hours, and then stimulated with LPS 1 µg / mL for the last 6 hour of incubation. The proteins were subjected to western immunoblotting to detect pNRF-2 phosphorylated (α-tubulin and Lamin β are used as a control). b) Densitometric quantification of the bands detected (mean±SD; n=3;* = $p<0,05$; ** = $p<0,01$).

a



b

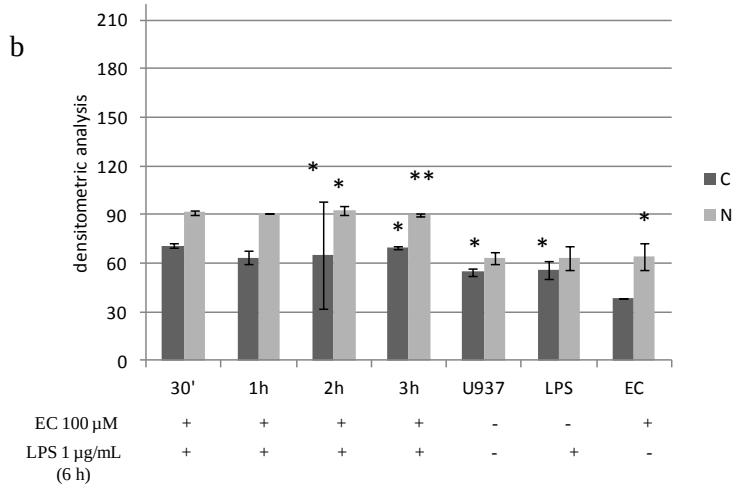


Figure 34. a) Cytoplasmic (C) and nuclear (N) extracts from U937 cells incubated with 100 µM of epicatechin (EC) for 30 min, 1, 2, and 3 hours, and then stimulated with LPS 1 µg/mL for the last 6 hour of incubation. The proteins were subjected to western immunoblotting to detect HO-1 (α-tubulin and Lamin β are used as a control). b) Densitometric quantification of the bands detected (mean±SD;* = $p<0,05$; ** = $p<0,01$).

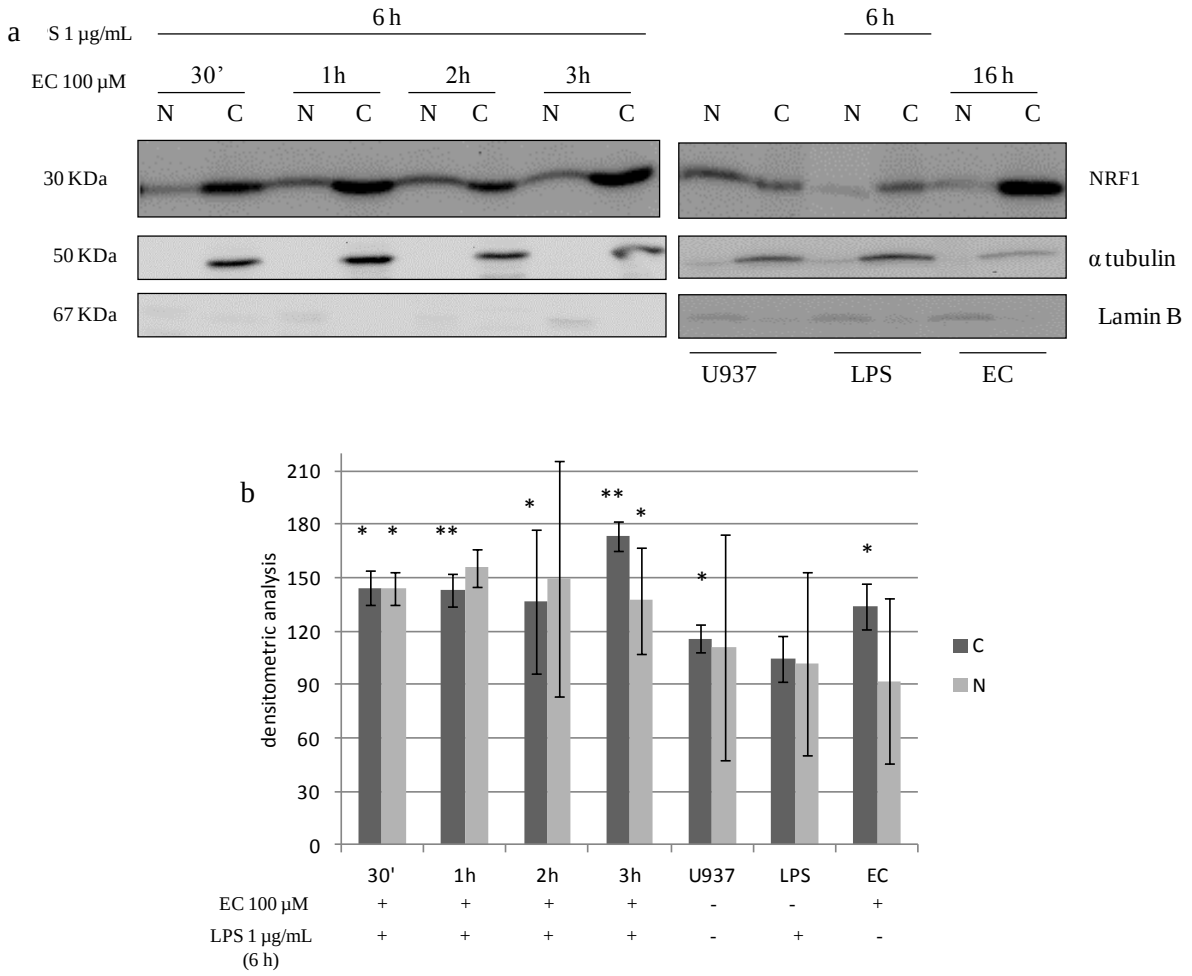


Figure 35. a) Cytoplasmic (C) and nuclear (N) extracts from U937 cells incubated with 100 µM of epicatechin (EC) for 30 min, 1, 2 and 3 hours, and then stimulated with LPS 1 µg/mL for the last 6 hour of incubation. The proteins were subjected to western immunoblotting to detect NRF1 (α-tubulin and Lamin β are used as a control. b) Densitometric quantification of the bands detected (mean±SD;* = $p<0,05$; ** = $p<0,01$).

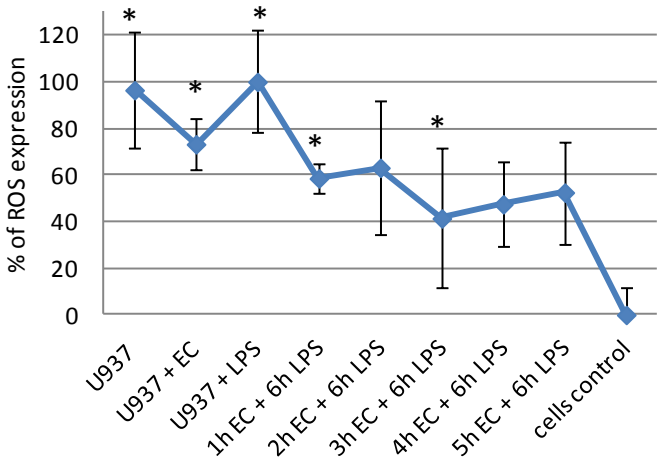
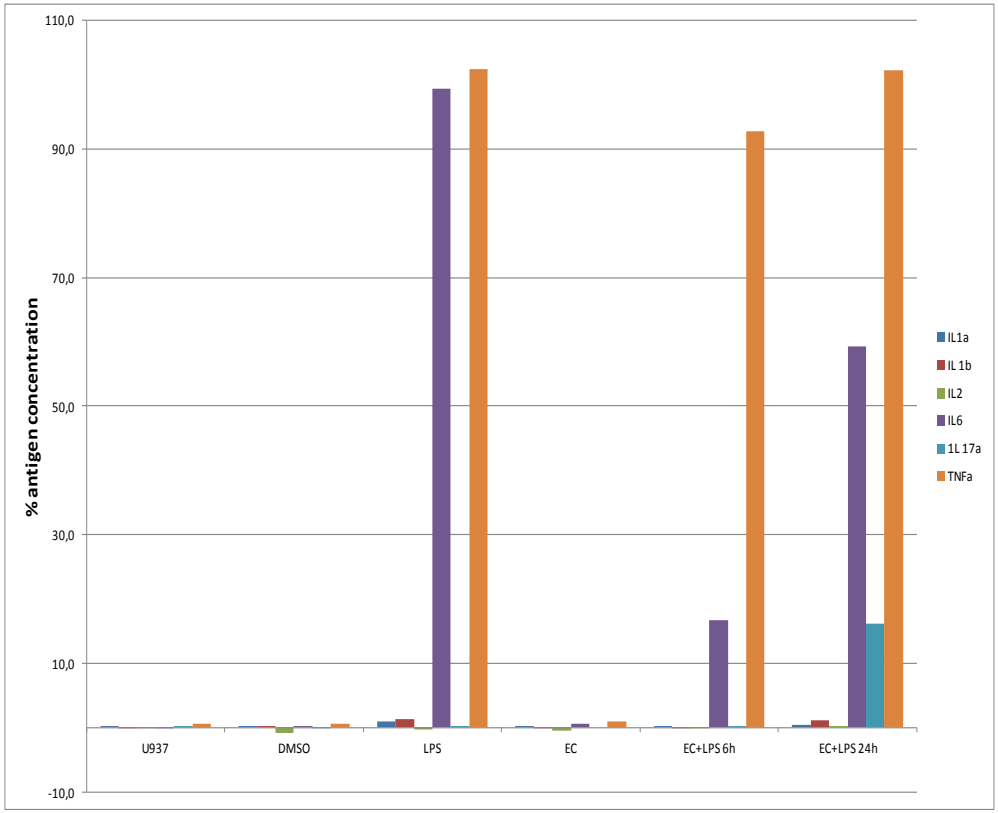


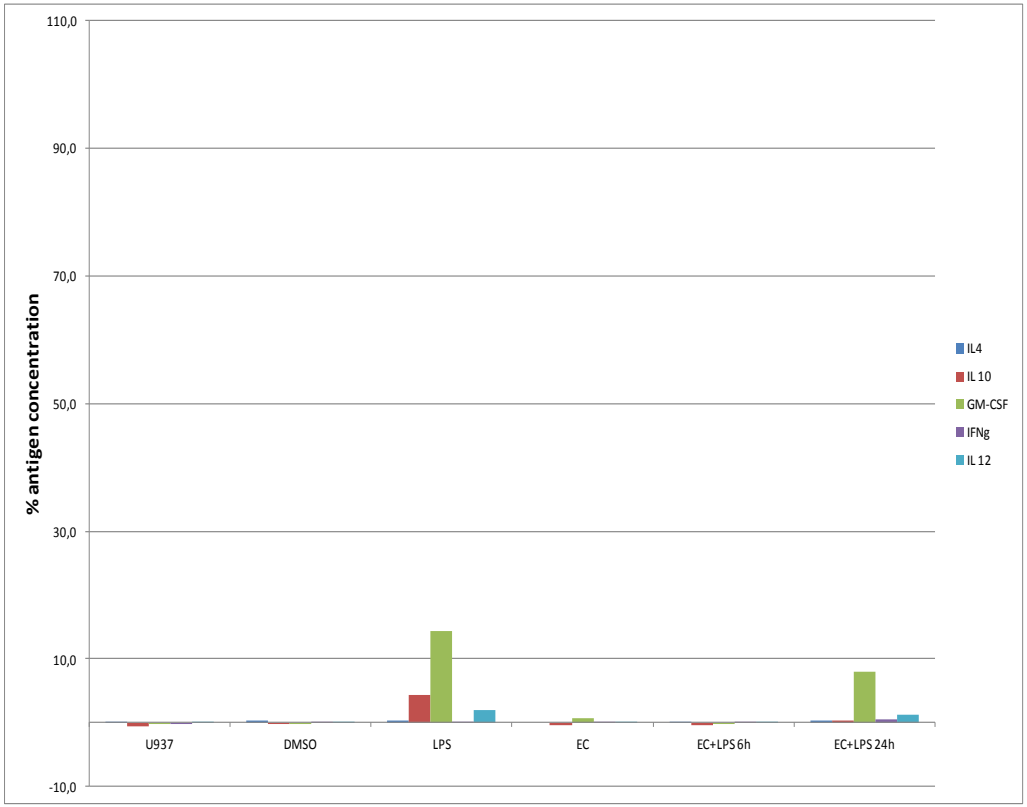
Figure 36. Labeled U937 cells were treated with 100 µM of EC from 1 to 5h and than incubated with 1µg/mL of LPS for 6h. Results are expressed as the % ROS with respect to the control group (mean±SD; n=2; * = p<0,05; ** = p<0,01).

Figure 37. The qualitative expression of cytochines and chemokines following treatment of U937 cells with LPS and EC alone and in combination: a) IL-1α, IL-1β, IL-2, IL-6, IL-17α, TNFα and b) IL-4, IL-10, IL-12, IFNγ, GM-CSF. Serum-free medium of control groups (U937 cell alone, with LPS 1µg/uL, the vehicle and the bioactive substance, DMSO) and after the incubation with either epicatechin (EC 100µM) and LPS (1µg/mL) alone or in combination for 6h and 24h, were assayed (n=2).

a



b



6.3 DISCUSSION

During this second experimental part, the attention focused on the potential involvement of the transcription factor NRF2 in the cellular pathway/s activated by epicatechin. A number of transcription factors have been connected to oxidative stress, such as NF-kB, activator protein-1 (AP-1) and importantly NRF2, which also modulates signaling pathways involved in the regulation of cell detoxification, proliferation, survival, death and differentiation.

The nuclear factor erythroid 2-related factor 2, NRF2, is an emerging regulator of cellular resistance to oxidants. NRF2 controls the basal and induced expression of an array of antioxidant response element-dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure.

From these hypotheses, *in vitro* studies were performed to show a correlation between the activation of NRF2 and the EC treatment on the human monocytic cell line, U937. Based on the cytotoxicity studies, in this preliminary study, an elevated concentration of EC at 100 µM, was used to try to decipher if the expression of NRF2 could be observed with a limit concentration of epicatechin. After increasing times of pre-incubation, from 30 minutes to 3 hours with EC, a subsequent treatment with LPS was set for the last 6 hours[203], which is a sufficient enough time to allow intracellular expression of NRF2 targeted proteins such as HO-1[204]. The results showed a time-dependent translocation of activated NRF2, corresponding to phosphorylation at Ser40. This is in agreement with previously studies which hypothesized that the phosphorylation of Ser-40 with oxidative stress, can be regulated by PKCδ, resulting in the dissociation of NRF2 from its cytoplasmic inhibitor KEAP1, thus promoting its translocation into the nucleus[205]. Therefore in addition to trialing lower concentrations of EC which are more realistic to a diet containing this component, an investigation of PKCδ, one of the enzymes responsible for the phosphorylation of NRF2, and KEAP1 will be performed to understand if they have in fact an active role. Known downstream targets of NRF2 including HO-1 were investigated. However the results of the activation of the enzyme downstream were unsatisfactory. Because, therefore it is an inducible stress protein, it was detected also in untreated cells. This is probably due the type of inflammatory cells chosen, which have always a certain type of latent activation, at the moment there are no data in literature about this observation. Thus, to obtain more satisfactory results, it may be necessary to perform an immunoprecipitation to isolate the enzymes prior to the WIB analysis. In addition, other enzymes expressed followed activation of NRF2, such as phase I and II metabolizing enzymes and detoxifying proteins, such as

NAD(P)H:quinone oxidoreductase 1 (NQO1) and Glutathione S-transferases (GST) should also be investigated. To verify if it was actually epicatechin to modulate the expression of these proteins will be performed the same experiments using the EC or LPS treatments alone, and not at the same time as it has been presented in this second experimental part. Moreover, as reported by the team of Biswal, to demonstrate whether NRF2 accumulated in the nucleus by EC actually binds to the ARE, the ARE-binding complex with NRF2 antibody using an electrophoretic mobility shift assay supershift (EMSA) should be performed[206]. It has also been suggested that NRF2 responds to inducible oxidative stimuli and that NRF1 regulates more constitutive forms of oxidative stress, however both are responsible for regulating the expression of many antioxidant genes[207]. Currently, the mechanisms controlling NRF2 activity have been studied, while studies examining Nrf1 regulation are lacking. For this reason it was decided to check whether following incubation with LPS and EC there was also the activation of NRF1. As for NRF2, there was increase in the translocation of NRF1 from the cytoplasm to the nucleus, paralleled by an overall increase in its total protein expression. Recent studies have established NRF1 as a pivotal transcriptional regulator of the genes of the proteasome subunits and the synergistically combining activities of NRF1 and NRF2 may protect cells during cellular stresses and may enable both normal and cancer cells to survive in toxic environments[208]. Then after observing the activation of NRF2, we would like to check the reduction of ROS following incubation with the flavonoid. We demonstrated that LPS-induced ROS expression was down-regulated by EC treatments in a time-dependent manner. Alternatively, in the presence of LPS alone and even untreated cells, ROS levels were sustained. ROSs are produced normally during the respiratory burst of phagocytes as a defense mechanism against pathogens. However, as showed by Irani[209], ROSs also in non-stressed condition regulate multiple cellular functions such as growth, differentiation, proliferation, apoptosis and gene expression. These results support the antioxidant role of the bioactive substance. Considering that ROS are regulators of apoptosis and that there is a network of communication between the systems of regulation anti-oxidants and those pro-oxidants[209], it would be interesting to evaluate this signaling pathway to understand if EC could have a potential role in this pathway. Further, to understand whether the results can be attributed to the activation of NRF2, it will be necessary to block the activation of the transcription factor or use RNA interference to determine whether the investigated pathways are activated in response to stress stimuli or incubation with the flavonoid.

The preliminary results presented in the current study support the concept of the anti-inflammatory activity of EC. Thus, we evaluated also the inhibition of the pro-inflammatory

cytokines and chemokines. The results obtained support this hypothesis, although there is a progressive increase in inflammatory cytokines with increasing time of incubation with LPS, which evidently shows a dominant effect with respect to the action of epicatechin. In fact it is wildly recognized that LPS activated the tool-like receptor (TLRs) which are now implicated in a number of acute and chronic inflammatory diseases[210]. Additional analyzes will be needed using traditional ELISA kits to evaluate the single cytokines that show changes, in particular IL-17α, present exclusively and repeatedly in the treatments with EC and LPS at 24h. This observation are in accordance with the research performed by Zizzo *et al.*[211], where they reported that the presence of IL-17 prevents monocyte/macrophage apoptosis and the restoration of anti-inflammatory conditions[211].

These preliminary results provide attractive details on how flavonoids could modulate the activation of NRF2, with the battery of down-streaming genes related to it, and their effect on the ROS level into monocytes. Understanding how the bioactive substances present in the diet are able to modulate the inflammatory state at the cellular level require further investigations. Consequently, further efforts are needed to elucidate the benefits and the risks of flavonoids since they are used increasingly in dietary supplements.

References

1. Food and Agricultural Organization of the United States; www.fao.org
2. World Health Organization; www.who.int/en/
3. B. Van Ommen, J. Bouwman, L.O. Dragsted, C.A. Drevon, R. Elliott, P. De Groot, J. Kaput, J.C. Mathers, M. Muller, F. Pepping, *et al.* Challenges of molecular nutrition research: The nutritional phenotype database to store, share and evaluate nutritional systems biology studies. *Genes Nutr*. (2010) 5, 189–203.
4. F. Norheim, I.M.F. Gjelstad, M. Hjorth, K.J. Vinknes, T.M. Langleite, T.Holen, J. Jensen, K.T. Dalen, A.S. Karlsen, A. Kielland, A.C. Rustan and C.A. Drevon. Molecular Nutrition Research—The Modern Way Of Performing Nutritional Science. *Nutrients* (2012) 4, 1898-1944.
5. D.J Raiten, S. Namaste´, B. Brabin, G. Combs Jr, M.R. L'Abbe, E. Wasantwisut and I. Darnton-Hill. Executive summary—Biomarkers of Nutrition for Development: Building a Consensus. *Am J Clin Nutr* (2011) 94: 633S–50.
6. M. Kussmann and P.J. Van Bladeren. The extended nutrigenomics–understanding the interplay between the genomes of food, gut microbes, and human host. *Frontiers in genetics* (2011) 2: 1-13.
7. D.M. Mutch, W. Wahli, and G. Williamson. Nutrigenomics and nutrigenetics: the emerging faces of nutrition. *FASEB J*. (2005) 19, 1602–1616.
8. E. Trujillo, C. Davis, J. Milner. Nutrigenomics, Proteomics, Metabolomics, and the Practice of Dietetics. *J Am Diet Assoc*. (2006) 106:403-413.
9. Chávez, M. Muñoz de Chávez. Nutrigenomics in public health nutrition: short-term perspectives. *Eur J Clin Nutr*. (2003) 1:S97-100.
10. Muller M., Kersten S. Nutrigenomics: goal and strategies. *Nature Review Genetics* (2003) 4: 315-322.
11. M.T. Subbiah. Understanding the nutrigenomic definitions and concepts at the food-genome junction. *OMICS*. (2008) 12(4):229-35.
12. G. Panagiotou1 and J. Nielsen. Nutritional Systems Biology: Definitions and Approaches *Annual Review of Nutrition* (2009)Vol. 29: 329-339.
13. H.C. Hung, K.J. Joshipura, R. Jian *et al.* Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst* (2004) 96:1577–1584.
14. F.L. Crowe, A.W. Roddam1, T.J. Key. Fruit and vegetable intake and mortality from ischaemic heart disease: results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heart study. *European Heart Journal* (2011).
15. C.L. Keen, R.R. Holt, P.I. Oteiza, C.G Fraga, and H.H. Schmitz. Cocoa antioxidants and cardiovascular health. *Am J Clin Nutr* (2005) 8: 298S–303S.
16. C.G Fraga. Cocoa, diabetes, and hypertension: should we eat more chocolate? *Am J Clin Nutr* (2005) 81:541–2.
17. S. Eilat-Adar, T. Sinai, C. Yosefy and Y.Henkin. Nutritional Recommendations for Cardiovascular Disease Prevention. *Nutrients* (2013), 5: 3646-3683.
18. P.C.H. Hollman, A. Geelen and D. Kromhout. Dietary Flavonol Intake May Lower Stroke Risk in Men and Women. *American Society for Nutrition* (2010) 599-604.
19. J.F. Hammerstone, S.A. Lazarus, A.E. Mitchell, R. Rucker and H.H. Schmitz. Identification of flavan 3-ol s in cocoa (Theobroma cacao) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem*. (1999) 47: 490-496.
20. P.C.H. Hollman, and I.C.W. Arts, Flavonols, flavones and flavanols: nature, occurrence and dietary burden. *J. Sci. Food Agric*. (2000) 80: 1081–1093.

21. I.C.W Arts, P.C.H Hollman, D. Kromhout. Chocolate as a source of tea flavonoids. *Lancet.* (1999) 354:488.
22. F.L. Kiechle, X. Zhang, C.A. Holland-Staley. The -omics era and its impact. *Arch Pathol Lab Med*. (2004) 128:1337–1345.
23. R. Bakry, M. Rainer, C.W. Huck and G.K. Bonn. New stationary phases for enrichment and separation in the 'omics' era. *Bioanalysis* (2009) 1: 151-1.
24. D.C. Ward and D.C. White. The new 'omics era. *Current Opinion in Biotechnology* (2002) 13:11–13
25. M. Wilkins, K. Williams, D. Hochstrasser. Proteome research: new frontiers in functional genomics. *Springer*, Berlin Heideberg (1997).
26. García-Ca˜nas, C. Simó, C. León, A. Cifuentes. Advances in Nutrigenomics research: Novel and future analytical approaches to investigate the biological activity of natural compounds and food functions. *Journal of Pharmaceutical and Biomedical Analysis* (2010) 51: 290–304.
27. S.P. Gygi, Y. Rochon, B.R. Franza, R. Aebersold. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol*. (1999) 3: 1720-30.
28. M. Kussmann , F. Raymond, M. Affolter. OMICS-driven biomarker discovery in nutrition and health. *Journal of Biotechnology* (2006) 124:758–787.
29. M.R. Wilkins, R.D. Appel, K.L. Williams, D.F. Hochstrasser. Proteome Research: Concepts, Technology and Application. (2008) *Springer* Berlin Heidelberg.
30. T. Rabillouda, C. Lelong. Two-dimensional gel electrophoresis in proteomics: A tutorial. *J Proteomics*. (2011) 10:1829-41.
31. D.W. Speicher. Proteome Analysis: Interpreting the Genome. *Elsevier* (2004).
32. Mallick P. Kuster B. Proteomics: a pragmatic perspective. *Nature biotechnology* (2010) 28: 695-709.
33. K. Chandramouli and Pei-Yuan Qian. Proteomics: Challenges, Techniques and Possibilities to Overcome Biological Sample Complexity. *Human Genomics and Proteomics* (2009) 1-22.
34. A.F. Maarten Altelaar, J. Munoz and A.J.R. Heck. Next-generation proteomics: towards an integrative view of proteome dynamics. *Nature Reviews Genetics* (2013) 14: 35-48.
35. P.R. Graves, and T.A. Haystead. Molecular biologist's guide to proteomics. *Microbiol Mol Biol Rev* (2002) 66: 39-63.
36. H. Mischak, et al. Clinical proteomics: A need to define the field and to begin to set adequate standards. *Proteomics Clin Appl* (2007) 1:148-56 .
37. W.S. Hancock, S.L. Wu, P. Shieh, The challenges of developing a sound proteomics strategy. *Proteomics*. (2002) 2:352-9.
38. F.M. Steinberg, M.M. Bearden, C.L. Keen. Cocoa and chocolate flavonoids: Implications for cardiovascular health. *J Am Diet Assoc.* (2003) 103:215-223.
39. K.A. Cooper, J.L. Donovan, A.L. Waterhouse and G. Williamson. Cocoa and health: a decade of research. *British Journal of Nutrition* (2008) 99:1–11.
40. J.A. Vinson, J.Proch, P. Bose, S. Muchler, P. Taffera, D. Shuta, N. Samman and G.A. Agbor. Chocolate is a powerful ex vivo and in vivo antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J Agric Food Chem (2006) 54: 8071–8076).
41. K.W. Lee, Y.J. Kim, H.J. Lee, C.Y. Lee. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem. (2003) 51: 7292–7295.
42. A.T. Borchers, C.L. Keen, S.M. Hannum, M.E. Gershwin. Cocoa and chocolate: composition, bioavailability, and health implications. J Med Food (2000) 3: 77–105.

43. D. Ramljak, L.J. Romanczyk, L.J. Metheny-Barlow, N. Thompson, et al., Pentameric procyanidin from Theobroma cacao selectively inhibits growth of human breast cancer cells, Mol. Cancer Therap. (2005) 4:537 –546.
44. M. Richelle, I. Tavazzi, M. Enslen, E.A. Offord. Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr (1999)53: 22–6.).
45. C. Selmi, C.A. Cocchi, M. Lanfredini, C.L. Keen and M.E. Gershwin. Chocolate at heart: The anti-inflammatory impact of cocoa flavanols. *Mol. Nutr. Food Res*. (2008) 52: 1340 – 1348)
46. J. Wollgast and E. Anklam.(a). Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International* (2000) 33: 423–447.
47. W. Ren, Z. Qiao, H. Wang, L. Zhu, and L. Zhang. Flavonoids: promising anticancer agents. *Medical Research Review*. (2003) 23:519–534.
48. C. Sanbongi, N. Osakabe, M. Natsume, T. Takizawa, S. Gomi, and T. Osawa. Antioxidative polyphenols isolated from Theobroma cacao. *Journal of Agricultural and Food Chemistry* (1998) 46:454–457.
49. b. J. Wollgast and E. Anklam. Polyphenols in chocolate: is there a contribution to human health? *Food Research International,* (2000) 33:449–459).
50. M. Arlorio, J.D. Coı¨sson, A. Turri, and A. Martelli. Effetto anti-batterico di estratti fenolici in Theobroma cacao. *La Rivista di Scienza dell'Alimentazione.* (2000) 30:251–260.
51. M. Arlorio, J.D. Coı¨sson , F. Travaglia, F. Varsaldi, G. Miglio, G. Lombardi, A. Martelli. Antioxidant and biological activity of phenolic pigments from Theobroma cacao hulls extracted with supercritical CO2. *Food Research International* (2005) 38: 1009–1014.
52. T.L. Dillinger, P. Barriga, S. Escarcega, M. Jimenez, D. Salazar Lowe,L.E. Grivetti. Food of the gods: Cure for humanity? A cultural history of the medicinal and ritual use of chocolate. *J. Nutr*. (2000) 130, 2057S–2072S.
53. D.L. Pucciarelli and L.E. Grivetti. The Medicinal Use of Chocolate in Early North America. Mol. Nutr. Food Res. 2008, 52, 000 – 000.
54. M. Rusconi, A. Conti. Theobroma cacao L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacological Research* (2010) 61: 5–13
55. P. Cabras, A. Martelli. Chimica degli alimenti. Editore Piccin-Nuova Libraria s (2004).
56. S.J. Misnawi, J. Selamat, J. Bakar, and N. Saari. Oxidation of polyphenols in unfermented and partly fermented cocoa beans by cocoa polyphenol oxidase and tyrosinase. *J Sci Food Agr* (2002) 82: 559– 566.
57. H.M. Rawel and S.E. Kulling. Nutritional contribution of coffee, cacao and tea phenolics to human health. J. Verbr. Lebensm. (2007) 2: 399 – 406)
58. H. Kim and P.G. Keeney. Epicatechin content in fermented and unfermented cocoa beans. *Journal of Food Science*. (1984) 49: 1090–1091.
59. W.G.C Forsyth. Cacao polyphenolic substances II. Changes during fermentation. *Biochem J* (1952)51: 516–20.
60. G.E. Adamson, S.A. Lazarus, A.E. Mitchell, R.L. Prior, *et al.*, HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* (1999) 47:4184 –4188.
61. A.T. Borchers, C.L. Keen, S.M. Hannum, M.E. Gershwin. Cocoa and chocolate: composition, bioavailability, and health implications. *J Med Food* (2000) 3: 77–105.
62. F. Sanchez-Rabaneda, O. Jaurequi, I. Casals, C. Andres-Lacueva, M. Izquierdo-Pulido, R.M. Lamuela-Raventos. Liquid chromatographic/electron spray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao). *J Mass Spectrom* (2003) 38: 35–42).

63. J. Kroll, H.M. Rawel and S. Rohn. Reactions of Plant Phenolics with Food Proteins and Enzymes under Special Consideration of Covalent Bonds. *Food Sci. Technol. Res.* (2003) 9:205–218
64. Belšcˇak, D. Komes, D. Horzˇic´ , K. K. Ganic´ , D. Karlovic. Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Research Internatio*nal (2009) 42: 707–716.
65. Scalbert, I.T. Johnson, and M. Saltmarsh. Polyphenols: antioxidants and beyond. *Am J Clin Nutr* (2005) 81: 215S–7S.
66. L. Bravo. Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. *Nutrition Reviews*, (1998) 56: 31 7-333
67. D.O. Kennedy and E.L. Wightman. Herbal Extracts and Phytochemicals: Plant Secondary Metabolites and the Enhancement of Human Brain Function. *Adv. Nutr*. (2011) 2: 32–50.
68. X.Han, T.Shen and H.Lou. Dietary Polyphenols and Their Biological Significance. *Int. J. Mol. Sci*. (2007), 8, 950-988.
69. M.B. Engler, M.M. Engler. The vasculoprotective effects of flavonoid-rich cocoa and chocolate *Nutrition Research* (2004) 24: 695–706
70. A. Manach, A. Scalbert, C. Morand, C- Rémésy, and L. Jiménez. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* (2004) 79: 727–47.
71. J.B. Harborne. Methods in plant biochemistry, I: plant phenolics. *London: Academic Press*. (1989).
72. F.A. Tomás-Barberán and M.N. Clifford Dietary hydroxybenzoic acid derivatives: nature, occurrence and dietary burden. *J. Sci. Food Agric.* (2000) 80: 1024–1032.
73. M.N. Clifford. Chlorogenic acids and other cinnamates: nature, occurrence, dietary burden, absorption and metabolism. *J.Sci. Food Agric.* (2000) 80, 1033–1043.
74. G.R. Beecher. Overview of dietary flavonoids: nomenclature, occurrence and intake. *J. Nutr*. (2003)133: S3248–54.
75. A.P. Neilson and M.G. Ferruzzi. Influence of formulation and processing on absorption and metabolism of flavan-3-ols from tea and cocoa. *Annu. Rev. Food Sci. Technol*. (2011) 2: 125–51
76. R.L.H. Bigelow and J.A. Cardelli. The green tea catechins, (_)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells. *Oncogene* (2006) 25: 1922–1930.
77. M. Kofink, M. Papagiannopoulos and R. Galensa. (-)-Catechin in Cocoa and Chocolate: Occurence and Analysis of an Atypical Flavan-3-ol Enantiomer. *Molecules* 2007, *12*, 1274-1288.
78. L. Gu, M.A. Kelm, J.F. Hammerstone, G. Beecher, J. Holden, D. Haytowitz, S. Gebhardt, and R.L. Prior. Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. *J. Nutr*. (2004) 134: 613–617.
79. M. Natsume, N. Osakabe, M. Oyama,M. Sasaki, S. Baba, Y. Nakamura, T. Osawa and J. Terao. Structures of (-)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (_)-epicatechin: differences between human and rat. Free *Radical Biology & Medicine*. (2003) 34: 840–849.
80. C.G. Fraga, P.I. Oteiza Dietary flavonoids: Role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radical Biology & Medicine* (2011) 51: 813–823.
81. F.A. Tomas-Barberan, E. Cienfuegos-Jovellanos, A. Marın, B. Muguerza, A. Gil-Izquierdo, B. Cerdaä, P. Zafrilla, J. Morillas, J. Mulero, A. Ibarra,M. Pasamar, D. Ramoän and J.C. Espìn. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J. Agric. Food Chem.* (2007) 55: 3926-3935.
82. C. Kwik-Uribe and R.M. Bektash. Cocoa flavanols: measurement, bioavailability and bioactivity. *Asia Pac J Clin Nutr* (2008) 17: 280-283.

83. S. Baba, N. Osakabe, A. Yasuda, M. Natsume, T. Takizawa, T. Nakamura, and J. Terao. Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radical Res.* (2000), *33*: 635-641.
84. A. Rein, T.G. Paglieroni, T. Wun, D.A. Pearson, *et al.*, Cocoa inhibits platelet activation and function. *Am. J. Clin. Nutr*. (2000) 72: 30–35.
85. S. Baba, N. Osakabe, Y. Kato, M. Natsume, A. Yasuda, T. Kido, K. Fukuda, Y. Muto, and K. Kondo. Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. *Am J Clin Nutr* (2007) 85: 709 –17.
86. E.L. Ding, S.M. Hutfless, X. Ding, S. Girotra. Chocolate and prevention of cardiovascular disease: a systematic review. *Nutr. Metab*. (2006) 3: 1-12.
87. J.L. Donovan, V. Crespy, M. Oliveria, K.A. Cooper, B.B. Gibson, G. Williamson. (+)-catechin is more bioavailable than (−)-catechin: relevance to the bioavailability of catechin from cocoa. *Free Radic. Res*. (2006) 40: 1029–34.
88. A. Rein, S. Lotito, R.R. Holt, C.L. Keen, H.H. Schmitz and C.G. Fraga. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *J Nutrit* (2000) 130: 2109S-2114S.
89. D.D. Schramm, M. Karim, H.R. Schrader, R.R. Holt, N.J. Kirkpatrick, J.A. Polagruto, J.L. Ensuna, H.H. Schmitz. and C.L Keen. Food effects on the absorption and pharmacokinetics of cocoa flavanols. *Life Sci* (2003) 73: 857–869.
90. Q.Y. Zhu, D.D. Schramm, H.B. Gross, R.R. Holt, S.H. Kim, T. Yamaguchi, C.L. Kwik-Uribe and C.L. Keen. Influence of cocoa flavanols and procyanidins on free radical-induced human erythrocyte hemolysis. *Clinical & Developmental Immunology*. (2005) 12: 27–34.
91. H. Schroeter, C. Heiss, J. Balzer, P. Kleinbongard, Carl L. Keen, N.K. Hollenberg, H. Sies, C. Kwik-Uribe, H.H. Schmitz and M. Kelm. ()-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *PNAS* (2006) 103: 1024–1029.
92. L. Actis-Goretta, A. Lévèques, F. Giuffrida , F. Romanov-Michailidis, F. Viton, D. Barron, M. Duenas-Paton, S. Gonzalez-Manzano, C. Santos-Buelga, G. Williamsonc, F. Dionisi. Elucidation of (-)-epicatechin metabolites after ingestion of chocolate by healthy humans. *Free RadicalBiologyandMedicine* (2012) 53: 787–795.
93. L. Zhang, Z. Zuo and G. Lin. Intestinal and Hepatic Glucuronidation of Flavonoids. *Molecular Pharmaceutics* (2007) 6: 833–845.
94. J.I. Ottaviani, T.Y. Momma, G.K. Kuhnle, C.L. Keen, H. Schroeter. Structurally related (−)-epicatechin metabolites in humans: Assessment using de novo chemically synthesized authentic standards. *Free Radical Biology & Medicine* (2012) 52: 1403–1412.
95. M. Serafini, R. Bugianesi, G. Maiani, S. Valtuena, S. De Santis, A. Crozier. Plasma antioxidants from chocolate. *Nature* (2003) 424: 1013.
96. H. Schroeter, R.R. Holt, T.J. Orozco, H.H. Schmitz and C.L.Keen. Milk and absorption of dietary flavanols. *Nature* (2003) 426: 787-788.
97. Buitrago-Lopez, J. Sanderson, L. Johnson, S. Warnakula, A. Wood, E. Di Angelantonio, O.H. Franco. Chocolate consumption and cardiometabolic disorders: systematic review and meta-analysis. *BMJ* (2011) 343.
98. P. Batra, A. K. Sharma. Anti-cancer potential of flavonoids: recent trends and future perspectives. Biotech (2013) 3: 439-459.
99. J.P.E. Spencer. Flavonoids and brain health: multiple effects underpinned by common mechanisms. *Genes Nutr.* (2009) 4: 243-250.
100. M.N Clifford. Diet-derived phenols in plasma and tissues and their implications for health. Planta med. (2004) 70: 1103-1114.

101. L.I. Mennen, D. Sapinho, A. de Bree, N. Arnault, S. Bertrais, P. Galan, and S. Hercberg Consumption of foods rich in flavonoids is related to a decreased cardiovascular risk in apparently healthy french women. J. Nutr. 134: 923–926, 2004.D.
102. Taubert, R. Roesen, C. Lehmann, N. Jung, E. Schömig. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide. JAMA. (2007) 298: 49-60.
103. R.R Holt, S.A. Lazarus, M.C. Sullards, Q.Y. Zhu, D.D. Schramm, J.F. Hammerstone. C.G. Fraga, H.H. Schmitz and C.L Keen. Procyanidin dimer B2 [epicatechin-(4_-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am J Clin Nutr* (2002) 76: 798–804.
104. K.J. Murphy, A.K. Chronopoulos, I. Singh, M.A. Francis, H. Moriarty, M.J. Pike, A.H. Turner, N.J. Mann and A.J. Sinclair. Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. *Am J Clin Nutr* (2003)77:1466–73.
105. G. Williamson and C. Manach. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* (2005) 81: 243S–55S.
106. W.D. Crews, D.W. Harrison and J.W. Wright. A double-blind, placebo-controlled, randomized trial of the effects of dark chocolate and cocoa on variables associated with neuropsychological functioning and cardiovascular health: clinical findings from a sample of healthy, cognitively intact older adults. *Am J Clin Nutr* (2008) 987: 872– 80.
107. U. Peters, C. Poole, L. Arab. Does tea affect cardiovascular disease? A meta analysis. *Am J Epidemiol.* (2001)154: 495-503.
108. G.E. Adamson, S. A. Lazarus, A.E. Mitchell, R.L. Prior, G. Cao, P.H. Jacobs, B.G. Kremers, J.F. Hammerstone, R.B. Rucker, K.A. Ritter and H.H. Schmitz. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* (1999) 47: 4184-4188.
109. N. Motohashi, M. Kawase, T. Kurihara, Y. Shirataki, K. Kamata, H. Nakashima. Relationship between radical intensity and biological activity of cacao husks extracts. *Anticancer Research*, (1999) 19: 1125–1130.
110. C. Manach, G. Williamson, C. Morand, A. Scalbert and Christian Rémésy. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* (2005) 81: 230-42.
111. D. Vauzour, A. Rodriguez-Mateos, G. Corona, M.j. Oruna-Concha and J.P.E. Spencer. Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients* (2010) 2: 1106-1131
112. G.E. Arteel, H. Sies. Protection against peroxynitrite by cocoa polyphenol oligomers, *FEBS Lett.* (1999) 462: 167 –170.
113. V. Bayard, F. Chamorro, J. Motta and N.K. Hollenberg. Does flavanol intake influence mortality from nitric oxide-dependent processes? ischemic heart disease, stroke, diabetes mellitus, and cancer in panama. *Int. J. Med. Sci*. (2007) 4: 53-58.
114. M. Gómez-Guzmán, R. Jiménez, M. Sánchez, M.J. Zarzuelo, P. Galindo, A.M. Quintela, R. López-Sepúlveda, M. Romero, J. Tamargo, F. Vargas, F. Pérez-Vizcaíno, J. Duarte. Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension. *Free Radic Biol Med*. (2012) 52: 70-9.
115. B.M. Egan, M.A. Laken, J.L. Donovan, R.F. Woolson. Does Dark Chocolate Have a Role in the Prevention and Management of Hypertension? Commentary on the Evidence. *Hypertension* (2010) 55: 1289-1295.

116. L. Hooper, P.A Kroon, E.B Rimm, J.S. Cohn, I. Harvey, K.A. Le Cornu, J.J. Ryder, W.L. Hall and A. Cassidy. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* (2008) 88: 38 –50.
117. A.L. Waterhouse, J.R. Shirley, J.L. Donovan. Antioxidants in chocolate. *The Lancet.* (1996) 348: 384.
118. D. Grassi, G. Desideri, S. Necozione, C. Lippi, R. Casale, G. Properzi, J.B. Blumberg and C. Ferri. Blood Pressure Is Reduced and Insulin Sensitivity Increased in Glucose-Intolerant, Hypertensive Subjects after 15 Days of Consuming High-Polyphenol Dark Chocolate. *J. Nutr*. (2008) 138: 1671–1676.
119. S. Almoosawi, C. Tsang, L. M. Ostertag, L. Fyfed and E.A.S. Al-Dujailid. Differential effect of polyphenol-rich dark chocolate on biomarkers of glucose metabolism and cardiovascular risk factors in healthy, overweight and obese subjects: a randomized clinical trial. *Food Funct*. (2012) 3: 1035-43.
120. J.A. Kim, M. Montagnani, K.K. Koh, M.J. Quon. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation* (2006) 113: 1888–904.
121. R. Madonna, A. Pandolfi, M. Massaro, A. Consoli, R. De Caterina. Insulin enhances vascular cell adhesion molecule-1 expression in human cultured endothelial cells through a pro-atherogenic pathway mediated by p38 mitogen-activated protein-kinase. *Diabetologia* (2004)47:532–6
122. Y. Gu and J.D. Lambert. Modulation of metabolic syndrome-related inflammation by cocoa. *Mol. Nutr. Food Res*. (2013) 57: 948–961.
123. T.P. Kenny, C.L. Keen, H.H. Schmitz and M.E. Gershwin. Immune Effects of Cocoa Procyanidin Oligomers on Peripheral Blood Mononuclear Cells. *Exp Biol Med* (2007) 232: 293–300.
124. T.K. Mao, J. Van De Water, C.L. Keen, H.H. Schmitz And M.E. Gershwin. Modulation of TNF-a Secretion in Peripheral Blood Mononuclear Cells by Cocoa Flavanols and Procyanidins. *Developmental Immunology*. (2002) 9: 135–141.
125. C. Sanbongi, N. Suzuki and T. Sakane. Polyphenols in Chocolate, Which Have Antioxidant Activity, Modulate Immune Functions in Humans *in Vitro. Cellular Immunology* (1997) 177: 129–136.
126. G.S. Hotamisligil and E. Erbay. Nutrient sensing and inflammation in metabolic diseases. *Nature Reviews Immunology.* (2008) 923-934.
127. M.E. Widlansky, S.J. Duffy, N.M. Hamburg et al. Effects of black tea consumption on plasma catechins and markers of oxidative stress and inflammation in patients with coronary artery disease. *Free Radic Biol Med* (2005) 38, 499–506
128. E.L. Steptoe, R. Gibson, R. Vuononvirta et al. The effects of chronic tea intake on platelet activation and inflammation: a double-blind placebo controlled trial. *Atherosclerosis* (2007) 193: 277–282.
129. B. Watzl, A. Bub, K. Briviba et al. Acute intake of moderate amounts of red wine or alcohol has no effect on the immune system of healthy men. *Eur J Nutr* (2002) 41: 264–270.
130. B. Watzl, A. Bub, G. Pretzer et al. Daily moderate amounts of red wine or alcohol have no effect on the immune system of healthy men. *Eur J Clin Nutr* (2004) 58: 40–45.
131. R. Estruch, E. Sacanella, E. Badia *et al.* Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial. Effects of win on inflammatory markers. *Atherosclerosis* (2004) 175: 117–123.
132. R. Jiménez, J. Duarte and F. Perez-Vizcaino. Epicatechin: Endothelial Function and Blood Pressure. *J. Agric. Food Chem*. (2012) 60: 8823–8830.

133. I.Ramirez-Sanchez, L. Maya, G. Ceballos, F. Villarreal (-)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways. *Hypertension* (2010) 55: 1398-1405.
134. Sudano, A.J. Flammer, S. Roas, F. Enseleit, F. Ruschitzka, R. Corti, G. Noll. Cocoa, Blood Pressure, and Vascular Function. *Current Hypertension Reports* (2012) 14: 279-284.
135. L.M. Ostertag, N. O'Kennedy, P.A. Kroon, G.G. Duthie1 and B. de Roos. Impact of dietary polyphenols on human platelet function – A critical review of controlled dietary intervention studies. *Mol. Nutr. Food Res* (2010) 54: 60–81.
136. O. Khawaja, J.M. Graziano and L. Dioussé. Chocoalte and coronary heart disease: a systematic review. *Curr. Atheroscler. Rep.* (2011) 13: 447-452
137. U. Etxeberria, A. Fernandez-Quintela, F.I. Milagro, L. Aguirre, J.A. Martínez and M.P. Portillo. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *J. Agric. Food Chem.* (2013) 1-49.
138. N. Hayek. Chocolate, gut microbiota, and human health. *Frontiers in Pharmacology* (2013)
139. R. Franco, A. Onatibia.Astibia and E. Martinez.Pinilla. Health benefits of methylxanthines in cacao and chocoalte. *Nutrients* (2013) 5: 4159-4173.
140. R. Medzhitov and T. Horng. Transcriptional control of the inflammatory response. *Nature Reviews Immunology* (2009) 9: 692-703.
141. R. Tréhin, H.P. Merkle, Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur. J. Pharm. Biopharm*. (2004) 58: 209–233.
142. K.E. Iles, H.J. Forman. Macrophage signaling and respiratory burst. *Immunol. Res*. (2002) 26: 95–105.
143. J-H.S. Kuo, M-S Jan, Y-L. Lin, C. Lin. Interactions between octaarginine and U-937 human macrophages: Global gene expression profiling, superoxide anion content, and cytokine production. *Journal of Controlled Release* (2009) 139: 197–204.
144. B.M. Hybertson, B. Gao, S.K. Bose, J.M. McCord. Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation. *Molecular Aspects of Medicine* (2011) 32: 234–246.
145. R. Gerschman, D.L. Gilbert, S.W. Nye, P. Dwyer, W.O. Fenn. Oxygen poisoning and X-irradiation: a mechanism in common. *Science* (1954) 119: 623–626.
146. K-A Jung and M-K. Kwak. The Nrf2 System as a Potential Target for the Development of Indirect Antioxidants. *Molecules* (2010) 15: 7266-7291.
147. S.K. Niture, J.W. Kaspar, J. Shen and A.K. Jaiswal. Nrf2 Signaling and Cell Survival. *Toxicol Appl Pharmacol*. (2010) 244(1): 37–42.
148. S. Lee, Y. Park, M.Y. Zuidema, M. Hannink, C. Zhang. Effects of interventions on oxidative stress and inflammation of cardiovascular diseases. *World J Cardiol* (2011) 26: 18-24.
149. J. Kima, Y-N. Chad, Y-J Surh. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders.
150. J.C. Pickup. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* (2004) 27: 813–823.
151. K.E. Wellen, G.S. Hotamisligil. Inflammation, stress and diabetes, *The Journal of Clinical Investigation* (2005) 5: 1111–1119.
152. J.F. Keaney, M.G. Larson, R.S. Vasan, P.W. Wilson, I. Lipinska, D. Corey, J.M. Massaro, P. Sutherland, J.A. Vita, E.J. Benjamin. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. *Arterioscler Thromb Vasc Biol*. (2003) 23:434-439.
153. S. Furukawa, T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, O. Nakayama, M. Makishima, M. Matsuda, I. Shimomura. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* (2004) 114: 1752-1761.

154. J.W. Kaspar and A.K. Jaiswal1 An Autoregulatory Loop between Nrf2 and Cul3-Rbx1 Controls Their Cellular Abundance. *The Journal Of Biological Chemistry* (2010) 21349–21358.
155. D. D. Zhang. Mechanistic studies of the NRF2-KEAP1signaling pathway. Drug Metabolism Reviews. (2006) 38: 769–789.
156. P. Moi, K. Chant, I. Asunis, A. Cao and Y.W. Kant. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the f-globin locus control region. *Proc. Nati. Acad. Sci. USA* (1994) 91: 9926-9930.
157. G.P. Sykiotis, I.G. Habeos, A.V. Samuelson and D. Bohmann. The role of the antioxidant and longevity-promoting Nrf2 pathway in metabolic regulation. *Curr Opin Clin Nutr Metab Care*. (2011) 14(1): 41–48.
158. Kobayashi, E. Ito, T. Toki, K. Kogamei, S. Takahashi, K. Igarashi, N. Hayashi and M. Yamamoto. Molecular Cloning and Functional Characterization of a New Cap'n' Collar Family Transcription Factor Nrf3 *The Journal Of Biological Chemistry* (1998) 6443–6452.
159. K. Flick and P. Kaiser. Protein degradation and the stress response. *Seminars in Cell & Developmental Biology* (2012) 23: 515– 522
160. K. Itoh, M. Mochizuki, Y. Ishii, T. Ishii, T. Shibata, Y. Kawamoto, V. Kelly, K. Sekizawa, K. Uchid and M. Yamamoto. Transcription Factor Nrf2 Regulates Inflammation by Mediating the Effect of 15-Deoxy-_12,14-Prostaglandin J2 . *Molecular And Cellular Biology* (2004) 36–45.
161. K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel and M. Yamamoto. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & Development* (1998) 13:76–86.
162. T. Nguyen, P. Nioi, C.B. Pickett. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* (2009) 284: 13291–13295.
163. M. Ramos-Gomez, M.K. Kwak, P.M. Dolan, K. Itoh, M. Yamamoto, P. Talalay, T.W. Kensler. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* (2001) 98: 3410–3415.
164. A.T. Dinkova-Kostova, W. David Holtzclaw, R.N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto and P. Talalay. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Nat. Acad. Sci.* (2002) 99: 11908–11913.
165. S.B. Cullinan, J.D. Gordan, J. Jin, J. W. Harper and J.A. Diehl. The Keap1-BTB Protein Is an Adaptor That Bridges Nrf2 to a Cul3-Based E3 Ligase: Oxidative Stress Sensing by a Cul3-Keap1 Ligase. *Molecular And Cellular Biology*. (2004) 8477–8486.
166. S-C Lo, X. Li, M.T. Henzl, L.J. Beamer and M. Hannink. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *The EMBO Journal* (2006) 25: 3605–3617.
167. D. D. Zhang. The Nrf2-Keap1-ARE Signaling Pathway: The Regulation and Dual Function of Nrf2 in Cancer. *Antioxidants & Redox Signaling* (2010) 13: 1623-26
168. Y-J Surh, J.K. Kundu, H-K Na .Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals. *Planta Med* (2008) 74: 1526–1539.
169. K. Taguchi, H. Motohashi and M. Yamamoto. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes to Cells* (2011) 16: 123–140.
170. W. Li, T.O. Khor, C. Xu, G. Shen, W-S Jeong, S. Yu, A-N Kong. Activation of Nrf2-antioxidant signaling attenuates NFkB-inflammatory response and elicits apoptosis. *Biochemical Pharmacology* (2008) 76: 1485-1489.

171. M. Calkins, D.A. Johnson, J.A. Townsend, M.R. Vargas, J.A. Dowell, T.P. Williamson, A. D. Kraft, J-M Lee, J. Li and J.A. Johnson. The Nrf2/ARE Pathway as a Potential Therapeutic Target in Neurodegenerative Disease. *Antioxidants & Redox Signaling* (2009) 497-508.
172. A.K. Jain and A.K. Jaiswal. GSK-3 Acts Upstream of Fyn Kinase in Regulation of Nuclear Export and Degradation of NF-E2 Related Factor 2. *The Journal Of Biological Chemistry* (2007) 282: 16502–16510.
173. A.K. Jain and A.K. Jaiswal. Phosphorylation of Tyrosine 568 Controls Nuclear Export of Nrf2. (2006) 281: 12132–12142.
174. A. Cuadrado, P. Moreno-Murciano, J. Pedraza-Chaverri. The transcription factor Nrf2 as a new therapeutic target in Parkinson's disease. *Expert Opin Ther Targets*. (2009) 13(3): 319-29.
175. G.E Mann, B. Bonacasa, T. Ishii, R.C.M. Siow. Targeting the redox sensitive Nrf2–Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. *Current Opinion in Pharmacology* (2009) 139–145.
176. T.W. Kensler, N. Wakabayashi, S. Biswal. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. *Annu. Rev. Pharmacol. Toxicol*. (2007) 47: 89–116
177. M.B. Sporn and K.T. Liby. NRF2 and cancer: the good, the bad and the importance of context. *Nature Reviews Cancer* (2012) 12: 564-571..
178. X.J. Wang, Z. Sun, N.F. Villeneuve, S. Zhang, F. Zhao, Y. Li, W. Chen, X. Yi, W. Zheng, G.T. Wondrak, P.K. Wong, D.D. Zhang. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* (2008) 29(6): 1235-43.
179. R. K. Thimmulappa, H. Lee, T. Rangasamy, S.P. Reddy, M. Yamamoto, T.W. Kensler and Shyam Biswa. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J. Clin. Invest*. (2006)116: 984–995.
180. Y-Y Surh. Cancer chemoprevention with dietary phytochemicals. *Nature Review* (2003) 3: 768-780
181. C.C. Leonardo and S. Doré. Dietary flavonoids are neuroprotective through Nrf2-coordinated induction of endogenous cytoprotective proteins. *Nutr Neurosci*. (2011) 14(5): 226–236.
182. B. Granado-Serrano, M. A. Martìn, G. Haegeman, L. Goya, L. Bravo and S. Ramos. Epicatechin induces NF-kB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells. *British Journal of Nutrition* (2010) 103: 168–179.
183. R. Yu, J.J Jiao, J.L Duh, K. Gudehithlu, T.H. Tan, A.N. Kong. Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant-responsive element-mediated phase II enzyme gene expression. *Carcinogenesis* (1997) 18(2): 451-6..
184. Y. Morimitsu, Y. Nakagawa, K. Hayashi, H. Fujii, T. Kumagai, Y. Nakamura. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J Biol Chem*. (2002) 277: 3456–3463.
185. D.A. Dickinson, K.E. Iles, H. Zhang, V. Blank and H.J. Forman. Curcumin alters epre and ap-1 binding complexes and elevates glutamate-cysteine ligase gene expression1 showed that curcumin increased nuclear translocation of Nrf2. *The FASEB Journal* (2003) 17: 473-475.
186. D.A. Dickinson, K.E. Iles, H. Zhang, V. Blank and H.J. Forman. Curcumin alters epre and ap-1 binding complexes and elevates glutamate-cysteine ligase gene expression1 showed that curcumin increased nuclear translocation of Nrf2. *The FASEB Journal* (2003) 17: 473-475.
187. M. Arlorio, M. Locatelli, F. Travaglia, J-D Coı¨sson, E. Del Grosso, A. Minassi, G. Appendino, A. Martelli. Roasting impact on the contents of clovamide (N-caffeoyl-L-DOPA)

and the antioxidant activity of cocoa beans (Theobroma cacao L.). *Food Chemistry* (2008) 106: 967–975.

188. F. Travaglia, M. Bordiga, M. Locatelli, J. D. Co¨ısson and M. Arlorio. Polymeric Proanthocyanidins in Skins and Seeds of 37 *Vitis vinifera* L. Cultivars: A Methodological Comparative Study. *Journal of Food Science* (2011) 76: 742-749.
189. M. Locatelli , R. Gindro, F. Travaglia, J-D. Coïsson, M. Rinaldi, M. Arlorio Study of the DPPH-scavenging activity: Development of a free software for the correct interpretation of data. *Food Chemistry* (2009) 114: 889–897.
190. J. Cermak, N.S. Key, R.R. Bach, J. Balla, H.S. Jacob, G.M. Vercellotti. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood*. (1993) 82:513-520.
191. T. Koga and M. Meydani Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells. *Am J Clin Nutr* (2001) 73: 941–8.
192. N. Singhto, K. Sintiprungrat, S. Sinchaikul, S.T. Chen and V. Thongboonkerd Proteome Changes in Human Monocytes upon Interaction with Calcium Oxalate Monohydrate Crystals. *Journal of Proteome Research* (2010) 9: 3980–3988 .
193. M. Jin, P.T Diaz, T. Bourgeois, C. Eng, C.B Marsh and H. M Wu. Two-dimensional gel proteome reference map of blood monocytes. *Proteome Science* (2006) 4:16.
194. G. Loirand, E. Scalbert, A. Bril and P. Pacaud. Rho exchange factors in the cardiovascular system. *Current Opinion in Pharmacology* (2008) 8:174–180.
195. E. Burillo, D. Recalde, E. Jarauta, S. Fiddyment, A.L. Garcia-Otin, R. Mateo-Gallego, A. Cenarro, F. Civeira. Proteomic study of macrophages exposed to oxLDL identifies a CAPG polymorphism associated with carotid atherosclerosis. *Atherosclerosis* (2009) 207: 32–37.
196. C.N. Lumeng and A.R. Saltiel. Inflammatory links between obesity and metabolic disease. *J Clin Invest.* (2011) 121: 2111–2117
197. H.S. Gadgil, K.M. Pabst, F. Giorgianni, E.S. Umstot, D.M. Desiderio, S. Beranova-Giorgianni, I.C. Gerling, M. J. Pabst. Proteome of monocytes primed with lipopolysaccharide: Analysis of the abundant proteins. *Proteomics* (2003) 3: 1767–1780.
198. M.J Pabst, K. M Pabst, D.B Handsman, S. Beranova-Giorgianni and F. Giorgianni. Proteome of monocyte priming by lipopolysaccharide, including changes in interleukin-1beta and leukocyte elastase inhibitor. *Proteome Science* (2008) 6:13.
199. R.C. Nayak, K-H. Chang, N-S. Vaitinadin, J.A. Cancelas. Rho GTPases control specific cytoskeleton-dependent functions of hematopoietic stem cells. *Immunological Reviews* (2013) 255-268.
200. Dupont, M. Chwastyniak, O. Beseme, A-L. Guihot, H. Drobecq, P. Amouyel and F. Pinet. Application of Saturation Dye 2D-DIGE Proteomics to Characterize Proteins Modulated by Oxidized Low Density Lipoprotein Treatment of Human Macrophages. *The Journal of Proteome Research* (2008) 7: 3572–3582.
201. A. Boyhan, C.M. Casimir, J.K. French, C.G. Teahan and A.W. Segal. Molecular Cloning and Characterization of Grancalcin, a Novel EF-hand Calcium-binding Protein Abundant in Neutrophils and Monocytes. *The Journal Of Biological Chemistry* (1992) 267(5): 2926-2933.
202. Y.M. Lee, Q.S. Auh, D.W. Lee, J.Y. Kim, H.J. Jung, S.H. Lee and E.C. Kim. Involvement of Nrf2-Mediated Upregulation of Heme Oxygenase-1 in Mollugin-Induced Growth Inhibition and Apoptosis in Human Oral Cancer Cells. *BioMed Research International* (2013)
203. J.B. Lewis, R.L. Messer, V.V. McCloud, P.E. Lockwood, S.D. Hsu, J.C. Wataha. Ni(II) activates the Nrf2 signaling pathway in human monocytic cells. *Biomaterials* (2006) 27: 5348–5356
204. M. Tana, Y. Ouyangc, M. Jind, M. Chenb, P. Liub, X. Chaob, Z. Chenb, X. Chene, C. Ramassamyf, Y. Gaoa, R. Pi. Downregulation of Nrf2/HO-1 pathway and activation of JNK/c-

Jun pathway are involved in homocysteic acid-induced cytotoxicity in HT-22 cells. *Toxicology Letters* (2013) 223: 1–8.

205. S.K. Niture, A.K. Jain and A.K. Jaiswal. Antioxidant-induced modification of INrf2 cysteine 151 and PKC-δ-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. *J. Cell Sci.* (2010) 122: 4452-4464.
206. T. Rangasamy, C.Y. Cho, R.K. Thimmulappa, L. Zhen, S.S. Srisuma, T.W. Kensler, M.Yamamoto, I. Petrache, R. M. Tuder and S. Biswal. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke–induced emphysema in mice. *J. Clin. Invest*. (2004) 114: 1248–1259.
207. N.L. Chepelev, J.D. Bennitz, T. Huang, S. McBride, W.G. Willmore. The Nrf1 CNC-bZIP Protein Is Regulated by the Proteasome and Activated by Hypoxia. *PLoS ONE* (2011) 6(12): e29167.
208. M.A. Schultz, A.B. Abdel-Mageed and D. Mondal. The Nrf1 and Nrf2 Balance in Oxidative Stress Regulation and Androgen Signaling in Prostate Cancer Cells. *Cancers* (2010) 2: 1354-1378.
209. K. Irani. Oxidant Signaling in Vascular Cell Growth, Death, and Survival A Review of the Roles of Reactive Oxygen Species in Smooth Muscle and Endothelial Cell Mitogenic and Apoptotic Signaling. *Circ Res*. (2000) 87:179-183.
210. Regulation of lipopolysaccharide-induced inflammatory response and endotoxemia by β-arrestins. K.J. Porter, B. Gonipeta, S. Parvataneni, D. M. Appledorn, S. Patial, D. Sharma, V. Gangur, A. Amalfitano and N. Parameswaran. *J Cell Physiol*. (2010) 225(2): 406–416.
211. G. Zizzo, P.L. Cohen. IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids. *J Immunol*. (2013) 190(10): 5237-46.