Department of Physiology and Pharmacology "V. Erspamer"

A Dissertation in fulfillment of the Requirements for the Degree of Doctor of Philosophy in Toxicology

"Peroxidation of leukocytes index ratio and a functional mathematical index: their potential application in screening of non-communicable diseases"

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INTRODUCTION

1. OXIDATIVE BURST AND THE ROLE OF FLAVONOIDS

Oxidative burst (OB) can be used as a reliable measure of the innate immune response of a host, which constitutes the first line of defense against invading pathogens. An increased OB is a common feature during sepsis [Martins PS et al.], bacterial acute exacerbation of chronic obstructive pulmonary disease [Vaitkus M et al.] and in children with the periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome [Sundqvist M et al.]. On the other hand, the evaluation of OB is particularly relevant in conditions associated with an increased risk of infection such as X-linked chronic granulomatous disease [Yamada M et al.], autoimmune neutropenia [Macey MG et al.], human immunodeficiency virus type 1 (HIV-1) infection [Shalekoff S et al.], as well as in preeclamptic neutropenic neonates [Ahmad M et al.]. The OB of leukocytes has been also studied in autoimmune diseases [Ferretti G et al.], inflammatory condition [Bertelli R et al.] and cancer [Mikirova NA et al.]. Recently, interest has grown on the modulation of OB in healthy subjects, due to the involvement of leukocytes-induced oxidation of low density lipoprotein (LDL) in the pathologic process of atherosclerosis: Oxidative burst is an innate immune response to infection, the latter being associated also with marked changes in lipid and lipoprotein metabolism, aimed to neutralize endotoxin toxic effects. On the other hand, lipid overload may increase lipopolysaccharide circulating levels and oxidative stress. Whilst these changes may be beneficial from the perspective of host defense, if they become chronic, they likely increase the risk of atherosclerosis. In particular, oxidation of lipoproteins, resulting from an imbalance of the pro- and anti-oxidant equilibrium, is involved in the pathologic process of atherosclerosis, changing cellular functions. Lipid oxidation, induced by leukocytes derived reactive oxygen species, can amplify foam cell formation through oxidized LDL (oxLDL) formation and uptake [Peluso I, Morabito G et al.]. In this context, human studies provide strong evidence that adherence to a dietary regime high in polyphenol-rich foods induces an increase of the plasma antioxidant defences: dietary intervention studies have shown that consumption of plant foods is able to modulate plasma Non-Enzymatic Antioxidant Capacity (NEAC) in human subjects. However, mainly due to the wide number of phytochemicals potentially involved, the identification of the exogenous molecules able to tune antioxidant defences is far to be obtained. In recent years, epidemiological and experimental evidence has mounted on previously unrecognized properties of a large group of phytochemicals, such as PolyPhenols (PP) [Serafini M et al. 2011]. Besides, flavonoids, the most common group of plant polyphenols present in fruits, vegetables and beverages derived from plants, have been also suggested as anti-inflammatory and immune-modulating compounds: the antiinflammatory actions of flavonoids in vitro or in cellular models involve the inhibition of the synthesis and activities of different pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules and C-reactive protein. Molecular activities of flavonoids include inhibition of transcription factors such as NF-kB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) [Serafini M et al. 2010]. However, in vitro and in vivo findings concerning the effects of these bioactive compounds on OB are conflicting [Peluso I, Miglio C et al.]. Granulocytes have been used in a variety of detection methods developed to measure OB [Freitas M et al.]. In this context, flow cytometry represents an interesting methodologic approach to assess the functional status of leukocytes and has provided many new insights into the relationships among cell surface features and intracellular processes, such as cytokine and reactive oxygen species (ROS) production [Elbim C et al.]: Flow cytometric analysis provides a rapid screen for abnormalities of

polymorphonuclear neutrophil (PMN) function and reflect their behavior in vivo more accurately. PMN are key components of the first line of defense against bacterial and fungal pathogens. They contribute to the early innate response by rapidly migrating to inflamed tissues, where their activation triggers microbicidal mechanisms such as the release of proteolytic enzymes and antimicrobial peptides as well as rapid production of ROS in what is called the OB. ROS are essential for bacterial killing and also potentiate inflammatory reactions [Babior BM.]. PMNs are usually short-lived cells, which die spontaneously by necrosis or apoptosis. Apoptotic PMNs are recognized and phagocytosed by macrophages, a process that is essential to resolve inflammation [Greenberg S et al.]. In fact, this phagocytic removal of intact, apoptotic neutrophils prevents them from releasing their cytotoxic content into the extracellular environment, which would occur if the cells died by necrosis. Fine tuning of PMN responses to inflammatory stimuli is necessary for appropriate functional activity. In this context, the major advantage of flow cytometry (FCM) is that the majority of neutrophil functions can be measured in whole blood, which reduces artifactual changes in function caused by purification procedures [Macey MG et al.]. Elbim C. et al. demonstrated the utility of whole-blood analysis using FCM for a better understanding of PMN functionality, i.e., tuning PMN responses to inflammatory stimuli. Finally, FCM permits a simultaneous analysis of phenotypic, functional and morphometric parameters assessing whole-blood PMN apoptosis, in particular in response to Toll-like receptor agonists and during simian immunodeficiency virus infection [Elbim C et al.]. In particular, the intracellular dihydrorhodamine 123 (DHR123) is one of the major probes used to evaluate the OB of leukocytes: so, a laser dye for ROS production studies is dihydrorhodamine 123 [Bitzinger DI et al.]. DHR123 freely enters the cell membrane, and after oxidation by ROS to rhodamine 123, it emits a bright fluorescent signal. Since rhodamine 123 is known to bind to cellular and mitochondrial membranes, the fluorescent signal is mainly localized inside the cell. DHR123 is specifically responsive to hydrogen peroxide (H_2O_2) accumulation [Walrand S et al.]. It has been known for over ten years that its oxidized derivate rhodamine 123 (Rho123) is extruded by the multidrug resistance transport proteins (MDR) [Nelson EJ et al.]. Therefore, also other fluorescent intracellular probes could be substrate of the MDR, due to their molecular mass and structure [Kimura Y et al.]. In humans, common metabolism and MDR-mediated transport of flavonoids and xenobiotics may raise concern about bioavailability and absorption of therapeutic drugs [Cermak R.]: polyphenols are substrates of enzymes like cytochrome P450 enzymes and phase II conjugation enzymes, as well as of drug transporters involved in drug excretion. Thus, they share the same metabolic pathways with many therapeutic drugs. A number of studies have demonstrated inhibition of various cytochrome P450 monooxygenases and drug transporters by flavonoids. Flavonoid-induced effects on drug bioavailability were also shown.

On the other hand, the effects of flavonoid ingestion on xenobiotic transporters expression and activity can contribute to the extrusion of undesired molecules such as carcinogens [Brand W et al.]: the transcellular transport of ingested food ingredients across the intestinal epithelial barrier is an important factor determining bioavailability upon oral intake. This transcellular transport of many chemicals, food ingredients, drugs or toxic compounds over the intestinal epithelium can be highly dependent on the activity of membrane bound ATP binding cassette (ABC) transport proteins, able to export the compounds from the intestinal cells; the flavonoid-mediated interactions at the level of the intestinal ABC transport proteins may be an important mechanism for unexpected food-drug, food-toxin or food-food interactions. Therefore, flavonoids could modulate the MDR mediated transport of fluorescent probes. Moreover, flavonoids could interfere also with intracellular esterase activity needed for the staining of fluorescent probes [Li P et al.], and their antioxidant properties in humans [Serafini M et al. 2011] could inhibit the oxidation of probes without affecting the OB of leukocytes. In this context, an unexplored topic is the interaction of flavonoids with fluorescent intracellular probes and the methodological implications in the evaluation of OB by flow cytometry.

Therefore, we aimed to point out this aspect in this review article, particularly relevant in nutritional intervention studies.

1.1. FLAVONOIDS BIOAVILABILITY AND INTERACTION WITH TRANSPORTERS

Flavonoids are a wide group of almost 5,000 secondary plant metabolites, sharing a common carbon skeleton of two benzene rings, joined by a 3-carbon bridge [*Peluso I, Manafikhi H, **Reggi R**, Palmery M. 2014*]. The distribution in food, the structural differences and the metabolism of flavonoids have been previously reviewed by several authors [Del Rio D et al.]: Flavonoids are polyphenolic compounds comprising 15 carbons with two aromatic rings connected by a three-carbon bridge (Fig. 1.1). The main subclasses of these C6–C3–C6 compounds are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. Other flavonoid groups that are more minor dietary components are the chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones. The basic flavonoid skeleton can have numerous substituents. The majority of flavonoids occur naturally as glycosides rather than aglycones.

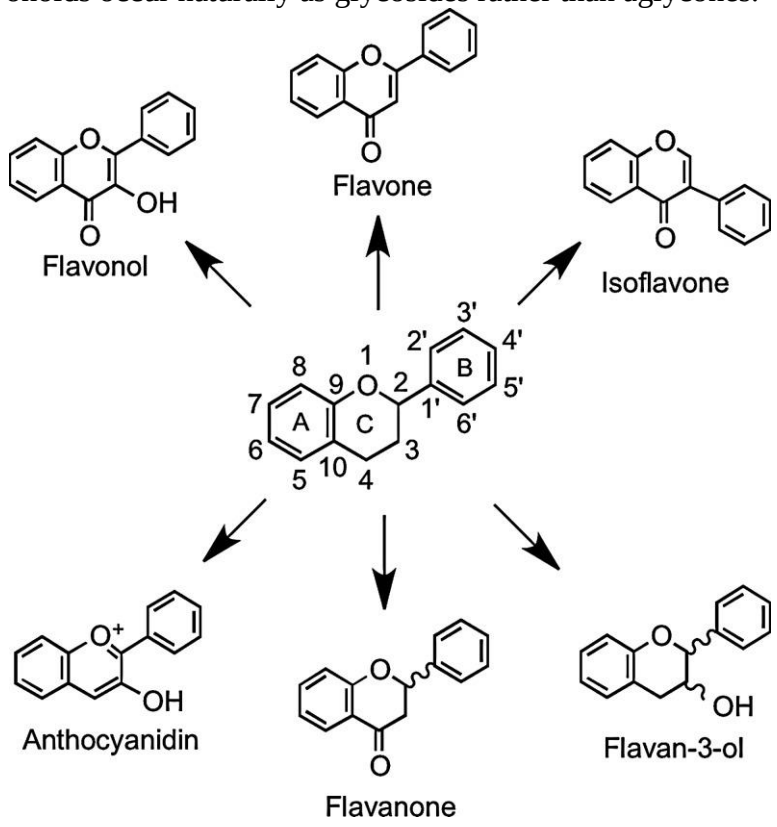

Figure 1.1 Structure of the flavonoid skeleton. [Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 2013;18:1818-1892]

After the acute ingestion, absorption of some, but by no means all, components into the circulatory system occurs in the small intestine. Typically, the absorption of flavonoid glycosides, as illustrated in Figure 1.2, is associated with cleavage and release of the aglycone as a result of the action of lactase phloridzin hydrolase (LPH) in the brush border of the small intestine epithelial cells. LPH exhibits broad substrate specificity for flavonoid-O-b-D-glucosides, and the released aglycone may

then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane [Day AJ et al.]. An alternative hydrolytic step is mediated by a cytosolic b-glucosidase (CBG) within the epithelial cells. For CBG-catalyzed hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter 1 (SGLT1) [Gee JM et al.]. Thus, there are two possible routes by which the glycoside conjugates are hydrolyzed, and the resultant aglycones appear in the epithelial cells, namely LPH/diffusion and transport/CBG (Fig. 1.2). However, an investigation in which SGLT1 was expressed in Xenopus laevis oocytes has shown that at least in this model system, SLGT1 does not transport flavonoids, and that glycosylated flavonoids and some aglycones have the capability to inhibit the glucose transporter [Del Rio D et al.]. Using Caco-2 cells, Johnson et al. [Johnson K et al.] found that glucose uptake into cells under sodium-dependent conditions was inhibited by flavonoid glycosides and non-glycosylated polyphenols, whereas aglycones and phenolic acids were without effect. Before passage into the blood stream, the aglycones undergo some degree of phase II metabolism forming sulfate, glucuronide, and/or methylated metabolites through the respective action of sulfotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyltransferases (COMTs). There is also efflux of some of the metabolites back into the lumen of the small intestine, and this is thought to involve members of the adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein (Pgp) (Fig. 1.2). MRP-3 and the glucose transporter GLUT2 have also been implicated in the efflux of metabolites from the basolateral membrane of the enterocytes. Once in the portal bloodstream, metabolites rapidly reach the liver, where they can be subjected to further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion [Del Rio D et al.].

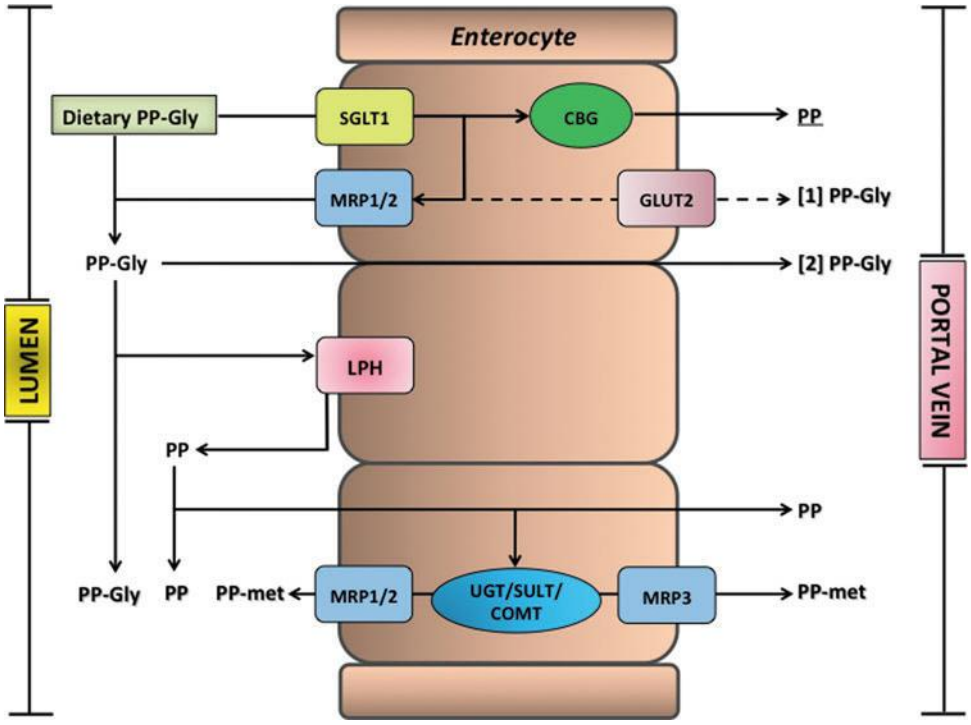

Figure 1.2 Proposed mechanisms for the absorption and metabolism of (poly)phenolic compounds in the small intestine. CBG, cytosolic bglucosidase; COMT, catechol-O-methyl transferase; GLUT2, glucose transporter; LPH, lactase phloridzin hydrolase; MRP1-2–3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenol sulfate/glucuronide/methyl metabolites; SGLT1, sodiumdependent glucose transporter;

SULT, sulfotransferase; UGT, uridine-5'-diphosphate glucuronosyltransferase [Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 2013;18:1818-1892]

Table 1.1 describes the main dietary sources and the circulating concentrations in humans of the six major subclasses of flavonoids. Although data on bioavailability indicate a limited absorption of flavonoids, great metabolic variability among different compounds has been observed, depending upon chemical structures [Simons AL et al.]. Their metabolism generally starts in the lumen of the small intestine, but evidence suggests that absorption at gastric level for the aglycone forms is also possible [Crespy V et al.]. Metabolic activities have been observed also in the oral cavity, such as degalloylation of flavanol gallate esters previous to absorption through the oral mucosa [Spencer JP. 2003].

Subclass:	**Food or beverage content (mg/100 g or 100 ml):**	**Plasma levels:**	**Plasma metabolite levels:**
Flavonols: isorhamnetin, kaempferol, morin, myricetin, rutin, quercetin	Spinach, Yellow and red onions	10^{-7} to 10^{-6} M	10^{-8} to 10^{-7} M
Flavanones: hesperetin, naringenin	Citrus and grape juices	10^{-8} to 10^{-6} M	n.d. to 10^{-7} M
Flavanols: catechins, procyanidins	Black and green tea, Dark chocolate and cocoa (1,500–3,400)	10^{-8} to 10^{-7} M	10^{-8} to 10^{-4} M
Isoflavones: daidzein, genistein	Soy products (200-400)	10^{-8} to 10^{-6} M	10^{-8} to 10^{-7} M
Anthocyanins: cyaniding, pelargonidin	Black grape and strawberry, Black berry fruits (595–1,316)	10^{-9} to 10^{-8} M	n.d. to 10^{-7} M

Table 1.1 Food content and bioavilability of flavonoids. [Peluso I, Manafikhi H, Reggi R, Palmery M. Interference of flavonoids with fluorescent intracellular probes: methodological implications in the evaluation of the oxidative burst by flow cytometry. Cytometry A. 2014 Aug;85(8):663-77. doi: 10.1002/cyto.a.22490. Epub 2014 May 28. Review. PubMed PMID: 24889089]

Once absorbed, flavonoids are targeted to as xenobiotics and metabolized by Phase I [(cytochrome P450 (CYP450)] and phase II (conjugation) drug metabolism enzymes so as to be eliminated. Metabolism of flavonoids by CYP450 isoemzimes, such as CYP1, CYP2, and CYP3, has been reported [Del Rio D et al.]. Besides, flavonoids undergo an extensive phase II metabolism during transfer from the intestinal lumen to the circulatory stream, resulting in sulfate, glucuronide and/or methylated conjugates [Spencer JP et al. 1999]. Phase II metabolism enzymes, comprising SULT, UGT and COMT, operate conjugation of flavonoids [O'Leary KA et al.].

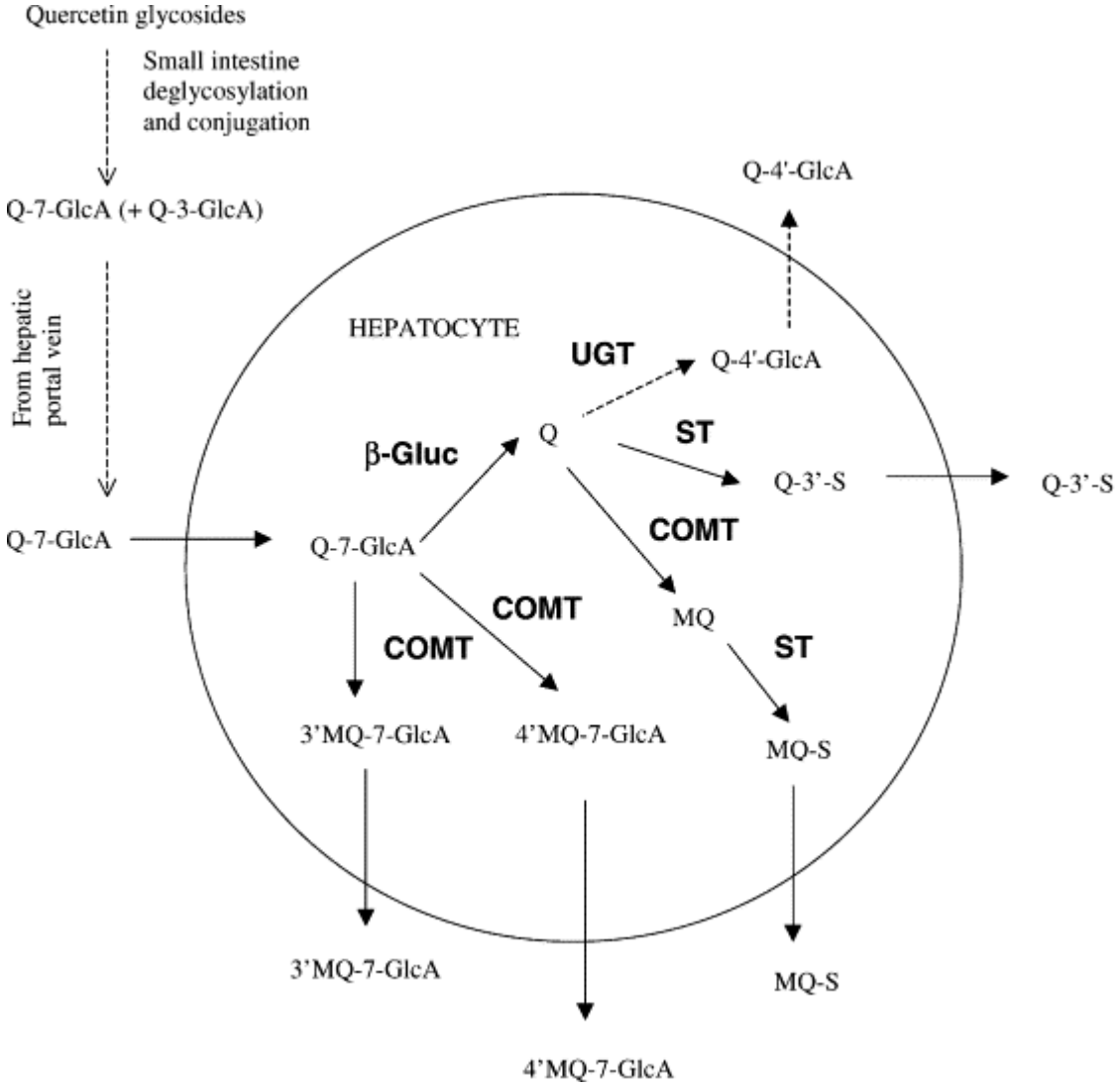

Figure 1.3 Proposed mechanism of quercetin-7-glucuronide uptake and metabolism in mammalian liver. Quercetin-7-glucuronide enters into the hepatocyte by an unidentified transporter. Metabolism of the glucuronide involves deglucuronidation by β-glucuronidase, methylation by the enzyme COMT, possible re-glucuronidation by the enzyme UGT and sulfation by sulfotransferase (ST). Quercetin (derived from deglucuronidation of quercetin-7-glucuronide) can be additionally glucuronidated at different positions but this reaction was only seen for quercetin glucuronides when they were incubated with a COMT inhibitor in HepG2 cells. A comparable metabolic pathway for quercetin-3-glucuronide is also proposed [O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G. Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol 2003;65:479-491]

Figure 1.3 illustrates the possible reactions of quercetin-7-glucuronide that could occur *in vivo*, illustrating a route for deconjugation by β-glucuronidase, methylation, glucuronidation and sulfation. O'Leary KA et al. proposed that β-glucuronidase present in liver cells will hydrolyse the quercetin glucuronide to the free aglycone and that this deglucuronidation reaction does not occur extracellularly. Under some conditions, quercetin-7-glucuronide can be deglucuronidated and may be re-glucuronidated at the 4′-position, and this reaction also occurs with quercetin. Methylation is likely to be in the 3′- or 4′-position in the liver, with the position of sulfation in the 3′-position for quercetin. So, O'Leary KA et al. concluded that β-glucuronidase present in human liver cells is capable of hydrolysing quercetin glucuronides that enter the liver cell [O'Leary KA et al.].

It has been observed that, while sulfation mainly occurs in the liver, glucuronides and methylated conjugates are primarily generated in the enterocytes, prior to further conjugation in the hepatocytes [Manach C et al.].
Not all the flavonoids and related compounds are absorbed in the small intestine, but some of them reach the large intestine, where undergo metabolism by the colonic micro-flora, including hydrolysis, dehydroxylation, demethylation, ring cleavage, decarboxylation and deconjugation [Stockley C et al.]. Microbial metabolism includes also the breakdown of the flavonoid backbone to form simple phenolic acids and hydroxycinnamates, which can be absorbed in the circulatory system [Spencer JP. 2003]. Once in the blood stream, flavonoid metabolites circulate bound to proteins, in particular to albumin, possibly affecting the rate of clearance of metabolites and their delivery to cells and tissues [Manach C et al.]. Most of the absorbed metabolites are subject to urinary excretion, but it is possible that part of them is recycled back into the small intestine through the enterohepatic transport. It is believed that extensively conjugated metabolites are eliminated through the bile, while the smaller ones are preferentially eliminated in urine [D'Archivio M et al.].
Flavonoids interact with transporters of the phase III drug detoxifing system, mainly Pgp [Passamonti S et al.], the MRP [Nait Chabane M et al.], the Breast Cancer Resistance Protein (BCRP) [Brand W et al.], but also with the Organic Anion-Transporters (OAT) [Nait Chabane M et al.] and the monocarboxylate transporter 1 (MCT-1) [Shim CK et al.]. These transporters are characterized by low substrate specificity and have been suggested to be responsible for the extrusion of flavonoids [Nait Chabane M et al.], glucosides [Walgren RA et al.] and glucuronidate- and sulfateconjugates [O'Leary KA et al.].
However, the efflux and influx of flavonoids, mediated by these transporters, depend on the polyphenols' structure. In Caco-2 cells, MRP2 was involved in the efflux of epicatechin gallate [Vaidyanathan JB et al.], naringenin, quercetin [Nait Chabane M et al.], and quercetin 40-beta-glucoside [Walgren RA et al.]. Naringenin, together with its glycosidic forms, is a flavanone abundant in grapefruit and orange; and it was secreted via Pgp and absorbed by MRP1, whereas quercetin was not a MRP1 and Pgp substrate but was absorbed by the OAT-B [Nait Chabane M et al.]. Inter-individual variations in bioavailability have been observed, probably due to drug metabolism/transport systems genetic polymorphisms [Ingelman-Sundberg M et al.], but also differences in the colonic micro-flora could affect flavonoids metabolism [Setchell KD et al.].
On the other hand, the effects of flavonoids on drug metabolism/transport system cause various food-drug interactions [Brand W et al.].
Transporter-mediated active efflux of cytotoxic agents is one of the best characterized mechanisms by which cancer cells develop MDR. Pgp, MRP1 and BCRP have been shown to confer resistance to a number of anti-cancer agents. However, potent and nontoxic inhibitors remain to be identified for clinical use in MDR reversal. Quercetin being a class of integral flavonoids in our common diet should have the advantage of low toxicity: infact has been suggested as a potential chemosensitizer, due to its MDR modulating properties [Chen C et al.]. In particular, aglycone flavonoids reduced the permeability of cyclosporine to a greater extent than glycosylated flavonoids across Caco-2 and MDCKII-MDR1 cells: in the study of Rodriguez-Proteau R et al., the hypothesis tested was that specific flavonoids such as epicatechin gallate, epigallocatechin gallate, genistein, genistin, naringenin, naringin, quercetin and xanthohumol will modulate cellular uptake and permeability of multidrug-resistant substrates, cyclosporin A (CSA) and digoxin, across Caco-2 and MDCKII-MDR1 cell transport models. 3H-CSA/3H-digoxin transport and uptake experiments were performed with and without co-exposure of the flavonoids. Aglycone flavonoids reduced the permeability of CSA to a greater extent than glycosylated flavonoids with 30 mM xanthohumol producing the greatest effect ($7.2x10^{-6}$ to $6.6x10^{-7}$ and $17.9x10^{-6}$ to $4.02x10^{-6}$ cm s–1 in Caco-2 and MDCKII-MDR1 cells, respectively); while no measurable effects were seen with digoxin.
Xanthohumol significantly demonstrated:
(1) saturable efflux

(2) increased uptake of 3H-digoxin
(3) decreased uptake of 3H-CSA in the Caco-2 cells [Rodriguez-Proteau R et al.].
It has been proposed that quercetin effects on oral bioavailability of cyclosporine are due to its intestinal glucuronidate- and sulphate-conjugates [Hsiu SL et al.].
In rats, administration of epigallocatechin-3-gallate [Li C et al.], naringin [Ali MM et al.] and quercetin [Choi JS et al.] increased the bioavailability of various drugs, due to the inhibition of Pgp.
On the other hand, the OAT-mediated transport of atorvastatin was efficiently inhibited by apigenin (IC(50) for OAT1A2: 9.3 μM, OATPB1: 13.9 μM), kaempferol (IC(50) for OAT1A2: 37.3 μM, OAT2B1: 20.7 μM) and quercetin (IC(50) for OAT1A2: 13.5 μM, OAT2B1: 14.1 μM) [Mandery K et al.]. To the contrary, Wang et al. reported that rutin, but not its aglicone quercetin, stimulated the uptake of [3H]-dehydroepiandrosterone sulfate (DHEAS) in OATP1B1-expressing HeLa cells, while genistein, but not its glycoside genistin, inhibited DHEAS uptake [Wang X et al.].
These conflicting results could be due to the differential interactions with the cytosolic and the transmembrane domains of MDR. It has been suggested that flavonoids interact with the substrate-binding sites and with the nucleotide-binding domains of the transporters [Conseil G et al.].
Furthermore, in plasma membrane vesicles prepared from the MRP-overexpressing cell line GLC4/ADR, genistein and kaempferol stimulated the ATPase activity of the transporters [Hooijberg JH et al.].
Finally, in addition to being substrates of phase I, phase II, and phase III drug metabolism/transport systems, flavonoids are also able to modulate their expression through:
- the activation protein-1 (AP-1) [Bark & Choi]
- the nuclear factor kB (NF-kB) [Zhou S et al.]
- the NF-E2 related factor 2 (Nrf2) [Maher et al.]
- the aryl hydrocarbon receptor (AhR) [Ramadass et al.]
- the pregnane X receptor (PXR) [Dong et al.].
Therefore, they could also self-regulate their own and drugs bioavailability after long-term consumption.

1.2. MEASUREMENT OF REACTIVE SPECIES IN LEUKOCYTES AND PLATELETS BY FLOW CYTOMETRY

Oxidative stress is the result of the imbalance between ROS formation and enzymatic and non-enzymatic antioxidants. Biomarkers of oxidative stress are relevant in the evaluation of the disease status and of the health-enhancing effects of antioxidants.
Marrocco et al. aim to discuss the major methodological bias of methods used for the evaluation of oxidative stress in humans [Marrocco I et al.]. There is a lack of consensus concerning the validation, standardization, and reproducibility of methods for the measurement of the following:

(1) ROS in leukocytes and platelets by flow cytometry
(2) markers based on ROS-induced modifications of lipids, DNA, and proteins
(3) enzymatic players of redox status
(4) total antioxidant capacity of human body fluids.

It has been suggested that the bias of each method could be overcome by using indexes of oxidative stress that include more than one marker. However, the choice of the markers considered in the global index should be dictated by the aim of the study and its design, as well as by the clinical relevance in the selected subjects. In conclusion, the clinical significance of biomarkers of oxidative stress in humans must come from a critical analysis of the markers that should give an overall index of redox status in particular conditions.

In humans, under physiological conditions, ROS and Reactive nitrogen species (RNS) generated by leukocytes, through NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS), have a role in the innate immune response to infection [El-Benna J et al.].
Polymorphonuclear neutrophils comprise more than 60% of the circulating white blood cells in humans. They have a central role in innate immunity and they regulate adaptive immunity. Their vital importance in host defense against bacteria and fungi has been known for decades as several functional defects result in life-threatening infections. When neutrophils encounter the pathogen, they first recognize evolutionary conserved motifs or 'pathogen associated molecular patterns' (PAMP), found in a wide range of microbes, through cell surface pattern recognition receptors (PRR), such as Toll-like receptors (TLR). Neutrophils then firmly bind to the pathogen and engulf it; binding and engulfment of the pathogen are promoted by opsonins bound to the pathogens, such as immunoglobulins G (IgG) and the C3b and C3bi proteins, resulting from antibody production and activation of the complement system respectively. Following recognition, the plasma membrane surrounds the pathogen to enclose it within a phagosome. Phagocytosis triggers the activation program of the neutrophils, leading to the intra-phagosomal release of antibacterial peptides, proteases, myeloperoxidase, and superoxide anion ($O_2^{-\bullet}$), which is produced via the activation of the NADPH oxidase. Superoxide anion is the initiating 'spark' leading to the generation of ROS, i.e., hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl), all contributing to the death and destruction of the bacteria within the phagosome (Fig. 1.4). ROS production by the phagocytes was first referred as the 'respiratory burst' or 'oxidative burst' due to the rapid and cyanide-insensitive increase in oxygen uptake, increase in glucose consumption, and immediate ROS release. The enzyme system dedicated to $O_2^{-\bullet}$ production, i.e., the phagocyte NADPH oxidase, a member of the NOX family of proteins also referred as NOX_2, is composed of six proteins, two transmembrane proteins (p22phox and gp91phox) that form the flavocytochrome b558, and four soluble proteins (p47phox, p67phox, and p40phox and the small G-proteins, Rac1/2). The membrane and cytosolic components of the NOX_2 are segregated in resting cells but assemble at the membrane upon activation (Fig. 1.4 Left).

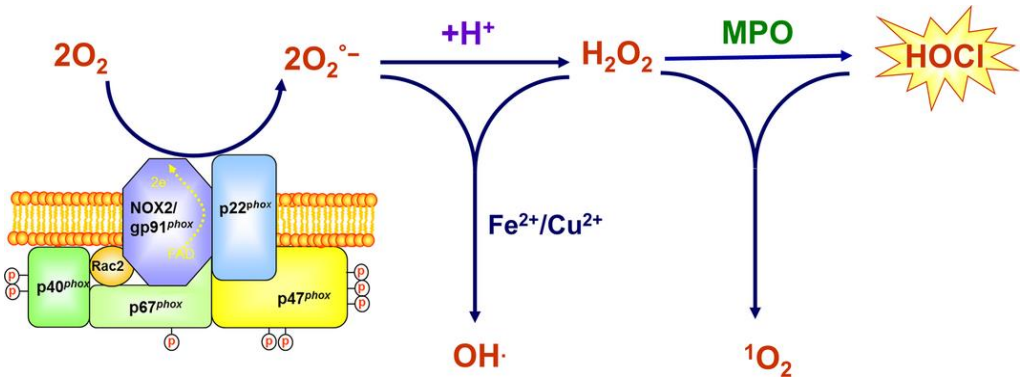

Figure 1.4 The active NADPH oxidase complex and ROS generation.
(Left) The active NADPH oxidase is composed of the membrane-bound flavocytochrome b558 (gp91phox/NOX2 and p22phox), which binds several cytosolic proteins (p67phox, p47phox, p40phox, Rac2). (P) denotes phosphorylation. The activated NADPH oxidase uses cytosolic NADPH to transfer electrons (e-) via FAD onto oxygen to form superoxide anion ($O_2^{-\bullet}$); **(Right)** ROS produced by the active NADPH oxidase complex: Superoxide anion produced by the NADPH oxidase can react with protons to generate hydrogen peroxide (H_2O_2), which is used by the myeloperoxidase enzyme (MPO) to produce hypochlorous acid (HOCl). Superoxide can react with H_2O_2 to produce hydroxyl radical ($OH^\bullet$). This reaction can occur in the presence of H_2O_2 and metals such as Fe^{2+} or Cu^{2+}. phox, phagocyte oxidase; ROS, reactive oxygen species.
[El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PM. Priming of the neutrophil respiratory burst: role in host defense and inflammation. Immunol Rev. 2016 Sep;273(1):180-93. doi: 10.1111/imr.12447.]

However, ROS and RNS can induce lipid peroxidation of polyunsaturated fatty acids (PUFA), which propagate via peroxyl radicals (ROO•) within the membrane, as well as in the LDL [A. Negre-Salvayre et al.]. Lipids, cholesterol, PUFA are a main target of oxidative attack and this leads to the formation and the accumulation of lipid oxidation products, in particular oxysterols, hydroperoxides and endoperoxides.

In the context of metabolic syndrome and chronic inflammation, the oxLDL activate leukocytes and/or platelets to produce ROS and RNS [Peluso I, Morabito G et al]. Lipid oxidation, induced by leukocytes derived reactive oxygen species, can amplify foam cell formation through oxLDL formation and uptake. The main enzymes, operating during oxidative burst, involved in LDL oxidation are NADPH oxidase and myeloperoxidase. In vitro studies have shown that oxLDL are able to induce many proatherogenic processes, including modulation of oxidative burst. OxLDL may also induce maturation of dendritic cells and regulate the shift from classical (M1) to alternative (M2) macrophage activation and from T helper 1 to T helper 2 response, suggesting that these could act as a bridge between innate and adaptative immunity, both involved in plaque development.

The direct quantification of ROS/RNS is a valuable and promising biomarker that can reflect the disease process. However, given the short half-life of these species, their measurement in biological systems is a complex task. Approaches include electron spin resonance, fluorescence magnetic resonance, and mass spectrometry techniques [C. C. Winterbourn], but their use has been limited to cell cultures and other in vitro applications.

The strategy for detecting reactive oxidants differs depending on whether they are inside or outside the cell. Intracellular detection normally involves flow cytometry or fluorescence microscopy, whereas released oxidants can be monitored in real time by fluorescence or UV/visible spectrophotometry, or by sampling the medium at intervals, for example by HPLC or mass spectrometry. Although some of the complications with probes are the same in each case, extracellular detection is more straightforward as conditions are much more amenable to manipulation to optimise detection.

Although free radicals' production can be measured by spectrophotometric or luminescence methods, all extracellular free radicals' measurements are deeply affected by cell count and viability. On the other hand, flow cytometry is one of the most powerful tools for single-cell analysis of the immune system and it is routinely used in the diagnosis and progression evaluation of blood cancers and human immunodeficiency virus (HIV) infection [Marrocco I et al.].

In addition to the role of oxidative burst evaluation by flow cytometry in the diagnosis of chronic granulomatous disease, this instrumentation has been used for many years to evaluate oxidative burst in many conditions, such as autoimmune neutropenia and asymptomatic HIV+ individuals. Many fluorescent probes for the detection of reactive species have been developed in the last years, with a different degree of specificity and sensitivity [Gomes A et al.].

The scientific research in the field of ROS associated biological functions and/or deleterious effects is continuously requiring new sensitive and specific tools in order to enable a deeper insight on its action mechanisms. However, reactive species present some characteristics that make them difficult to detect, namely their very short lifetime and the variety of antioxidants existing in vivo, capable of capturing these reactive species. It is, therefore, essential to develop methodologies capable of overcoming this type of obstacles. Fluorescent probes are excellent sensors of ROS due to their high sensitivity, simplicity in data collection, and high spatial resolution in microscopic imaging techniques.

The fluorescent probes used for the detection of reactive species in blood cells via flow cytometry are summarized in Table 1.2 [Marrocco I et al.].

Probe (localization)	ROS/RNS	Fluorescence	Leukocytes	Platelets	Limitations and confoundings
DCFH-DA (intracellular)	$HO^{\bullet}$ $ONOO^{-}$ $ROO^{\bullet}$ $NO_2^{\bullet}$ Indirect H_2O_2	↑ green (DCF)	Yes	Yes	- Hemolysis - Self-propagation of DCF radicals - MDR substrates or inducers - Esterase inhibitors - Plasma esterase in whole blood or PRP - EDTA and citrate - Antioxidants
AF-2 DA/DAF-FM DA (intracellular)	$NO^{\bullet}$	↑ green (DAF-Ts)	Yes	No	- MDR substrates or inducers - Esterase inhibitors - Plasma esterase in whole blood
DHR123 (intracellular)	HClO H_2O_2 $ONOO^{-}$	↑ green (Rho123)	Yes	No	- Self-propagation of DHR radicals - MDR substrates or inducers - Antioxidants
HE (intracellular)	$O_2^{\bullet-}$	↑ red (ethidium)	Yes	No	- Intercalating agents
C11-BODIPY$^{581/591}$ (membrane)	$HO^{\bullet}$ $ROO^{\bullet}$	Shift from red to green	Yes	Yes	- Hemolysis - Antioxidants

Table 1.2 Fluorescent probes used for the measurements of reactive oxygen and nitrogen species by flow cytometry.
C11-BODIPY$^{581/591}$: 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DAF-2 DA: 4,5-diaminofluorescein diacetate; DAF-FM DA: 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DAF-Ts: triazolofluoresceins; DCF: 2′,7′-dichlorofluorescein; DCFH-DA: dihydrochlorofluorescein diacetate; DHR123: dihydrorhodamine 123; EDTA: ethylenediaminetetraacetic acid, H_2O_2: hydrogen peroxide; HClO: hypochlorous acid; HE: hydroethidine; MDR: multidrug resistance; $NO^{\bullet}$: nitrogen monoxide; $NO_2^{\bullet}$: nitrogen dioxide; $O_2^{\bullet-}$: superoxide radical; $HO^{\bullet}$: hydroxyl radical; $ONOO^{-}$: peroxynitrite; PRP: platelet-rich plasma; Rho123: rhodamine 123; $ROO^{\bullet}$: peroxyl radicals [Marrocco I, Altieri F, Peluso I. Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans. Oxid Med Cell Longev. 2017;2017:6501046. doi: 10.1155/2017/6501046.].

For instance, intracellularly converted diacetate derivatives of probes such as dihydrochlorofluorescein diacetate (DCFH-DA), 4,5-diaminofluorescein diacetate (DAF-2 DA), and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FMDA) have widely been used for ROS/RNS detection. Once taken up by cells, these probes are hydrolyzed by intracellular esterases, generating the non fluorescent and membrane-impermeable DCFH, DAF-2, or DAF-FM. Subsequent oxidation by ROS/RNS results in the formation of the fluorescent 2′,7′-dichlorofluorescein (DCF) and triazolofluoresceins (DAF-Ts), respectively. DCFH, the more commonly used probe, does not directly react with H_2O_2 to form the fluorescent product. DCFH can be instead oxidized to DCF by several one-electron-oxidizing species including HO• radicals, products formed from peroxidase or heme proteins reacting with H_2O_2, HClO, and nitrogen dioxide ($NO_2^{-\bullet}$) generated by myeloperoxidase and peroxynitrite decomposition. DCFH oxidation can also be promoted by Fe2+ in the presence of O_2 or H_2O_2. The 1electron oxidation of DCFH generates the DCF semiquinone anion radical ($DCF^{-\bullet}$) (Fig. 1.5). This intermediate can rapidly react with O_2 to form $O_2^{-\bullet}$, which in turn can dismutate yielding additional H_2O_2 and establishing a redox-cycling mechanism that leads to an artificial amplification of the fluorescence signal [Kalyanaraman B et al.].

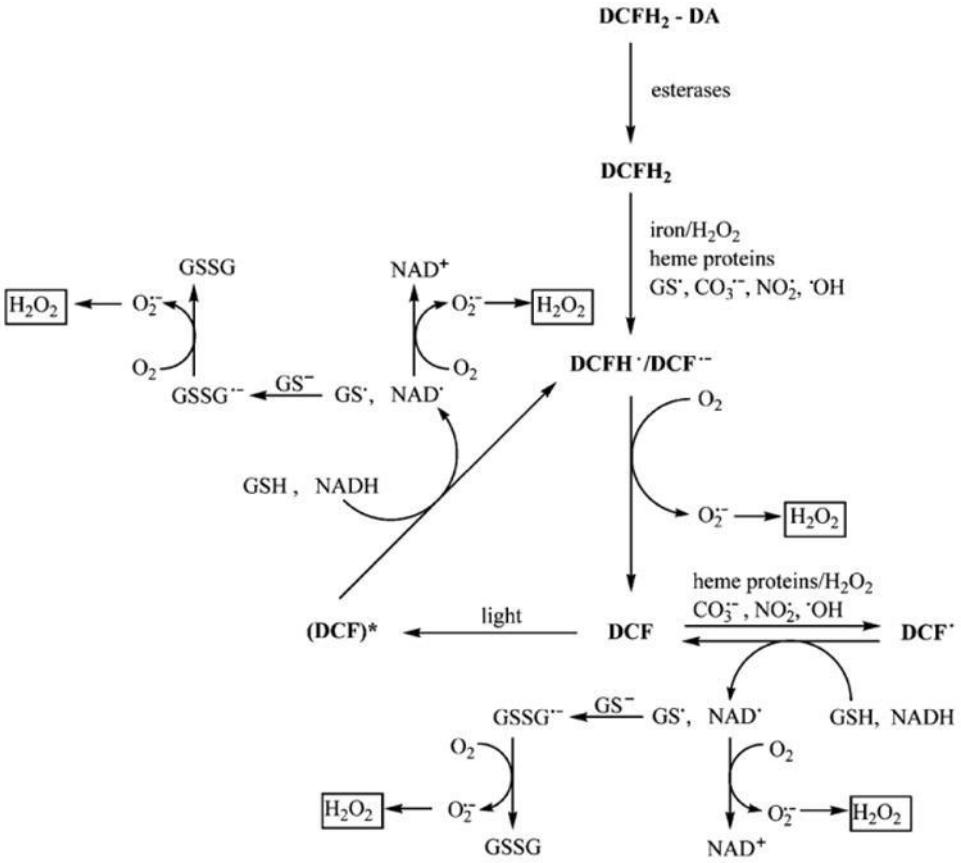

Figure 1.5 Oxidative reactions mediated by the dichlorodihydrofluorescein radical (DCF$^{\bullet-}$) in the presence of oxygen, heme proteins, and glutathione. [Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ 2nd, Ischiropoulos H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic Biol Med. 2012 Jan 1;52(1):1-6.]

While DCFH is used in both platelets and leukocytes, dihydrorhodamine 123 (DHR123) and hydroethidine (HE) are used only in the evaluation of the oxidative burst by polymorphonuclear leukocytes (PMN) (Table 1.2). DHR123 is an uncharged nonfluorescent probe that passively diffuses across cell membranes and is converted upon oxidation to the fluorescent membrane-impermeant rhodamine 123 (Rho123), which predominantly localizes in the mitochondria [Freitas M et al.]. Dihydrorhodamine 123 (DHR), a structural analog of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), was introduced in the late 1980s. This probe is lipophilic, enabling it to readily permeate cell membranes. Upon oxidation to fluorescent rhodamine 123 ($\lambda_{excitation}$ = 505 nm; $\lambda_{emission}$ = 529 nm), one of the two equivalent amino groups tautomerizes to a charged imino, effectively trapping rhodamine 123 within cells [Freitas M et al.].

HE passively diffuses into cells and is preferentially oxidized by $O_2^{-\bullet}$ to ethidium, which results in intercalation in DNA and consequently a significant enhancement of its red fluorescence intensity.

HE is a cell permeable compound, capable to diffuse into the neutrophils. Inside the cell it is readily oxidized by $O_2^{-\bullet}$ to form ethidium cation (E+), a highly fluorescent compound ($\lambda_{excitation}$ = 520 nm; $\lambda_{emission}$ = 610 nm). The produced E+ is trapped in the nucleus by intercalation into DNA, which results in an increase of cellular red fluorescence. Due to its DNA intercalation property, E+ is also used in techniques to differentiate necrotic from apoptotic cell death [Freitas M et al.].

C11-BODIPY$^{581/591}$ is an oxidation-sensitive fluorescent fatty acid analogue that shifts its fluorescence from red to green when challenged with oxidizing species. C11-BODIPY$^{581/591}$ is sensitive to a variety of oxidizing species [Drummen GP et al.]. The 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) probe is the only lipophilic probe used to evaluate ROS in leukocytes and platelets. C11BODIPY581/591 is a derivatized 11-carbon fatty acid in which the boron dipyrromethene difluoride (BODIPY) core is

substituted by a phenyl group via a conjugated diene [Marrocco I et al.]. This conjugated diene interconnection is oxidation sensitive, and when oxidized by $HO^{\bullet}$ or $ROO^{\bullet}$, disruption and shortening of the conjugated electron resonance structures between the phenyl group and the BODIPY core shifts C11-BODIPY$^{581/591}$'s fluorescence from red to green. Conversely, $ONOO^{-}$ induces not only oxidation but also nitration of BODIPY, reducing red fluorescence but not necessarily increasing green fluorescence. Although excimers of the oxidized form are red fluorescent, labelling conditions up to 30 μM provides sufficient staining of the plasma and organelle membranes well below the range in which self-quenching or excimer formation occurs [Drummen GP et al.]. Therefore, excimers do not interfere with the fluorescence of BODIPY and the measured red signal depends only on the reduced form of the probe. Furthermore, neither C11-BODIPY$^{581/591}$ nor its oxidation products are able to spontaneously leak from the lipid bilayer and the ratio of oxidized to nonoxidized C11-BODIPY$^{581/591}$ can be used to normalize probe incorporation in cells of different size (lymphocytes, monocytes, and granulocytes) [Peluso I et al. The effect of sample storage on the Peroxidation of Leukocytes Index Ratio (PLIR) measure].

Only hemolysis and antioxidants, in particular the end-product of purine metabolism, uric acid (UA), could bias the measurement of ROS generation. On the contrary, when analyzing the results of intracellular probes, many factors must be taken into account (Table1.2). Ethidium displacement by molecules, such as chemotherapeutics or flavonoids, could decrease the ethidium fluorescence signal, making the interpretation of data difficult. Artefactual amplification of the fluorescence intensity has been suggested to occur via intermediate radicals for both DCF and DHR, whereas the presence of quenching and reducing antioxidants could either decrease or increase the oxidation of probes without affecting ROS production. Heme proteins and reduced iron (Fe^{2+}) have been shown to oxidize DCFH, and the suitability of DCFHDA for measuring intracellular ROS is increasingly being questioned [Kalyanaraman B et al.]. There are numerous examples in the literature that support the role of oxidant-induced iron signaling in DCFH oxidation. The limitations and caveats associated with DCF assay apply to DHR assay as well. The roles of $^{\bullet}NO_2$ and $O2^{\bullet-}$ or iron in intracellular DHR oxidation should be independently confirmed with appropriate inhibitors (e.g., L-NAME, PEG–SOD, desferrioxamine). Thus, DHR can be used only as a nonspecific indicator of intracellular $ONOO^{-}$ and HOCl formation [Wardman P.].

The combination of fluorescently labeled antibodies against targets such as the pan-leukocyte marker CD45 and the platelet marker CD61 and/or physical properties such as size (FS: forward scatter) and internal complexity (SS: side scatter) can identify different leukocyte populations and platelets (Figure 1.6) [Peluso I. et al. The effect of sample storage on the Peroxidation of Leukocytes Index Ratio (PLIR) measure].

In activated samples, platelet microparticles, platelet aggregates, and leukocyte-platelet aggregates are formed (Figure 1.6). In particular, platelet activation in whole blood induces the formation of platelet conjugates with granulocytes or monocytes and leukocyte aggregates with platelets are more prone to apoptosis after in vitro activation (Figure 1.6) [Marrocco I et al.].

Regarding the normalization strategies, stimulation indexes calculated from the mean intensity fluorescence (MIF) values and expressed as fold change relative to unstimulated samples have been suggested for evaluating the production of ROS in both granulocytes and platelets [N. Carrim et al.]. However, these methods do not take into account probe leakage nor autofluorescence differences. While it is well known that autofluorescence generates false-positive monocytes [Li F et al.], this aspect is neglected in platelet assays.

Despite controversy regarding the relationship between CVD and platelet size, measured as mean platelet volume (MPV) or FS, it is well known that FS increases after platelet activation and that large and small platelet subpopulations have different auto fluorescence profiles [Frojmovic M et al.] (Figure 1.6). Consequently, differences in autofluorescence in unstimulated and stimulated samples imply that stimulation indexes do not necessarily measure ROS production. Therefore, it must always be taken into account that the fluorescence signals and not the radicals are measured

and that the oxidation of the probe is not always related to ROS production. Overall, the reviewed potential bias and confounding factors suggest that accurate gating and normalization strategies must be applied in order to avoid misinterpretation of the results [Marrocco I et al.].

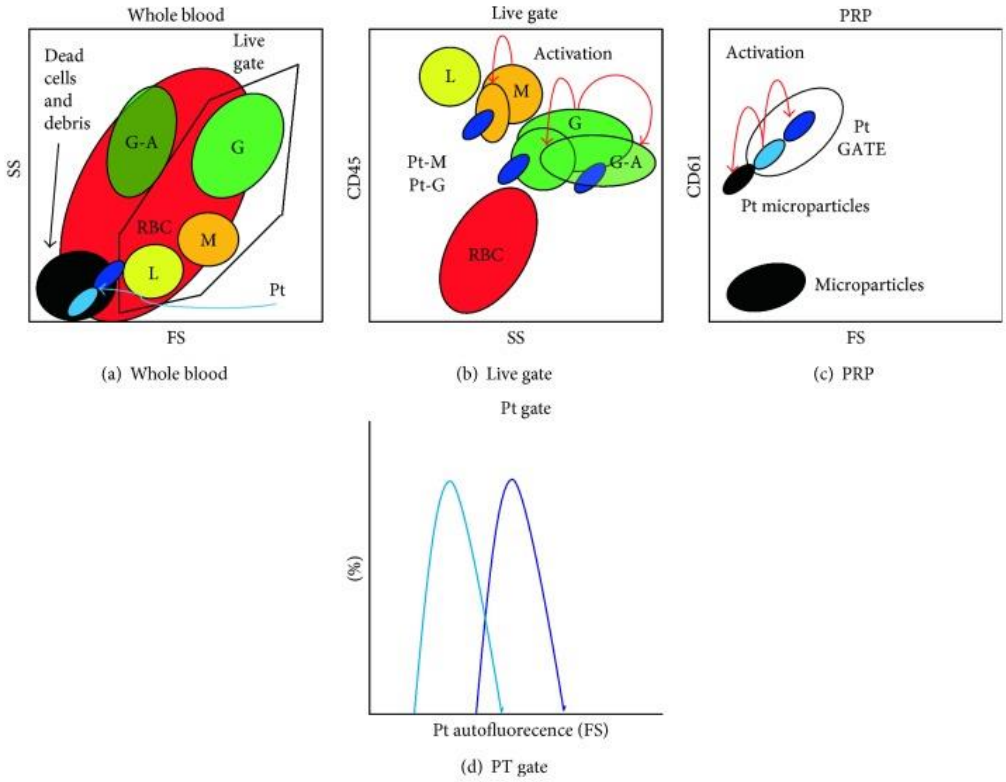

Figure 1.6 Gating strategies in the measure of free-radical production by flow cytometry. Different leukocytes populations (lymphocytes: L, monocytes: M, and granulocytes: G) in whole blood can be identified by CD45 (b) in the live gate assigned in the forward scatter (FS) and side scatter (SS) dot plot (a) by excluding dead cells and debris. Red blood cells (RBC) can be excluded as CD45 negative (b). Platelets (Pt) can be identified by CD61 in platelet-rich plasma (PRP) (c). In activated samples, platelet microparticles (c) and leukocyte-platelet aggregates (b: Pt-G and Pt-M) are formed and Pt-G are more prone to apoptosis (G-A). After platelet activation, FS increases due to platelet aggregation inducing an increase in autofluorescence (d). [Marrocco I, Altieri F, Peluso I. Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans. Oxid Med Cell Longev. 2017;2017:6501046. doi: 10.1155/2017/6501046]

1.3. EFFECTS OF FLAVONOIDS ON OXIDATIVE BURST

1.3.1. IN VITRO EVIDENCES

Flavonoids have been suggested to modulate the activity of different cell types involved in both innate and acquired immunity [Peluso I, Miglio C et al.].

Interesting results were obtained when studies involving healthy subjects, healthy subjects with risk factors or subjects affected by diseases were considered separately as described in Figure 1.7. While no great difference was observed for what concern the effect on interleukin-6 (IL-6) between the two groups, the effect on tumor necrosis factor-α (TNF-α) resulted to be completely different in relation to subject's health status. None of the intervention studies (0/21) conducted in healthy subjects was effective in reducing levels of TNF-α after ingestion of flavonoid-rich foods or

supplements (Figure 1.7). On the other hand, in cases of subjects characterized by risk factors for cardiovascular diseases (CVD), flavonoids decreased TNF-α in almost 30% of the interventions (5/17) (Figure 1.7) [Peluso I, Miglio C et al]. The effect is more pronounced if we restrict the field to patient affected by different diseases: despite the scarce number of available studies, the 60% of the interventions (4/7) were effective in reducing TNF-α values after supplementation with either a papaya preparation [Marotta, F. et al.], soy products [Azadbakht L. et al.], green tea extracts [Hsu, S. P. et al.], and 1 year adherence to Mediterranean diet [Marfella R et al.] (Figure 1.7).

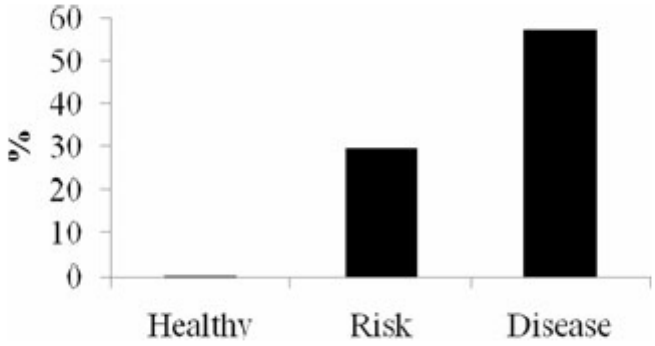

Figure 1.7 Dietary interventions trials with flavonoid-rich foods or quercetin and TNF-α levels in healthy subjects (*n* = 21), subjects characterized by risk factors for CVD (*n* = 17) and subjects with disease (*n* = 7).
Data are expressed as percentage (%) of successful studies.
[Peluso I, Miglio C, Morabito G, Ioannone F, Serafini M. Flavonoids and immune function in human: A systematic review. Crit Rev Food Sci Nutr]

The molecular mechanisms involved in the anti-inflammatory activities of flavonoids include: the induction of Nrf2, the inhibition of NF-kB and AP-1 and the modulation of the protein kinase C (PKC) and of the mitogen activate protein kinase (MAPK) [Serafini et al 2010]. These pathways are involved in the regulation of the oxidative modification of LDL by the OB of leukocytes [Peluso I, Morabito G, Urban L et al.].
In vitro evidence using fluorescent probes, suggested that flavonoids suppress ROS production in macrophage and granulocytes [Sanbongi C et al.], with IC50 values ranging between 1025 M and 1024 M depending on the polyphenol structure: Sanbongi C et al. studied the effects of antioxidants from chocolate, cacao liquor polyphenol (CLP), on human immune functions *in vitro.* CLP is an enriched polyphenol fraction purified from cacao liquor that is a major component of chocolate. It has been shown that polyphenols have antioxidant activity, and reactive oxygen species (ROS) are involved in immune responses. CLP inhibited both hydrogen peroxide and superoxide anion, typical ROS, production by phorbol myristate acetate-activated granulocytes. CLP also inhibited menadione-induced production of both hydrogen peroxide and superoxide anion in normal human peripheral blood lymphocytes (PBL). In a review Ciz et al. concluded that the effects of flavonoids on the OB of neutrophils are complex, and that there are several sites of action depending upon the flavonoid structure and subcellular distribution. Schematic diagram showing the possible mechanisms underlying the inhibition of ROS production by neutrophils using flavonoids is shown in Figure 1.8.

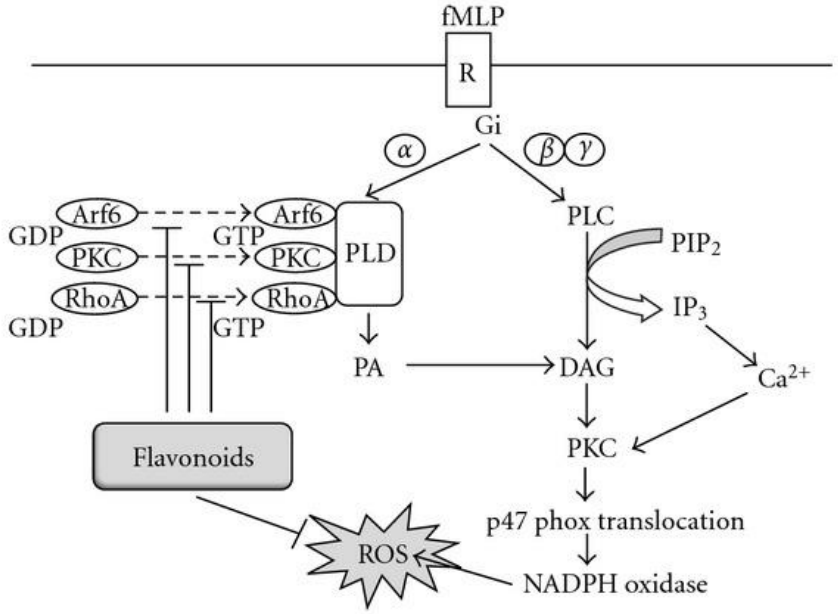

Figure 1.8 Schematic diagram showing the possible mechanisms underlying the inhibition of ROS production by neutrophils using flavonoids. The signaling molecules generally employed in mediating the activation of phospholipase D (PLD) are shown (some additional signaling molecules in these pathways are omitted). Blunt lines indicate the possible sites of the action of flavonoids (the blockade of PKC, Arf, and RhoA, as well as the scavenging of ROS generated by neutrophils) [Ciz M, Denev P, Kratchanova M, Vasicek O, Ambrozova G, Lojek A. Flavonoids inhibit the respiratory burst of neutrophils in mammals. Oxid Med Cell Longev 2012;2012:181295]

Besides, Ciz M. et al. pointed out that many of the in vitro studies did not take bioavailability and metabolism factors into consideration, and that the effects reported in those studies did not necessarily occur in vivo. However, in vitro data indicated that flavonoids metabolites are more potent inhibitors of OB than parental compounds. In human neutrophils, the metabolite quercetin 3-glucuronide, but not quercetin itself, caused a significant reduction (35%) in the N-formyl-methionyl-leucylphenylalanine (fMLP)-evoked calcium influx [Suri S et al.], the first signal after cell activation. In RAW cells the IC50 values of nitrite release were 1.25 mM and 1.25 μM for morin and morin sulfates/glucuronides, respectively [Fang SH et al.]. Furthermore, the microbial metabolite 3,4-dihydroxyphenylacetic acid, from quercetin glycosides, at 1 μM showed, with luminolchemiluminescence assay, an inhibition (84%) of ROS production by polymorphonuclear leucocytes [Merfort I et al.]. However, recently results suggest that naringenin and its phase II metabolites are able to perturb macrophage gene expression in directions that are not always consistent with antiinflammatory effects [Dall'asta M et al.]. Flavanones represent one of the six main sub-groups of flavonoids and occur as hydroxyl, glycosylated, and O-methylated derivatives, in high amounts especially in citrus fruits. The main exponents of this class are hesperetin and naringenin, the latter being abundant, in particular, in grapefruit and orange juice, together with its glycosidic forms [Perez-Jimenez J et al.]. Dall'asta M. et al. evaluated the effect of naringenin, naringenin-40-O-glucuronide and naringenin-7O-glucuronide (600 nM) on the expression of specific genes in human classical (M1) and alternatively (M2) polarised macrophages. Naringenin-40-glucuronide was able to increase tumor necrosis factor alpha (TNF-a) expression in M1 macrophages and, similar to the aglycone, to reduce its downregulation in M2 cells. On the other hand, naringenin and naringenin-40-glucuronide increased Nrf2 expression in M1 and M2 macrophages, whereas a reduction of Nrf2 expression was found in M1 cells treated with naringenin-7glucuronide. The latter metabolite and naringenin both increased the expression of the ATP-binding cassette human transporter 1. Therefore, the modulating effects of flavonoids and

metabolites on inflammatory cytokines and transporters could affect the evaluation of OB after flavonoid consumption in humans [Dall'asta M et al.].

1.3.2. HUMAN INTERVENTION TRIALS

Of the reviewed human intervention trials, eight studies measured ex vivo OB after flavonoid consumption by fluorescence experiments, among which five used DHR123, two HE, and one DCF (Table 1.3) [*Peluso I, Manafikhi H,* ***Reggi R****, Palmery M. 2014]*. Between the different flavonoids, the flavonol quercetin is the one with the largest information available and four out of five studies reported also data on bioavailability. However, the absorption of quercetin in the mainstream was not related to any effect on OB (Table 1.3). A supplementation of 3 or 12 weeks with quercetin at 500 or 1,000 mg/ day did not affect ex vivo OB of granulocyte, using HE or DHR123 as probe. These effects were consistent with other immunological or inflammatory markers, such as phagocytosis, NK activity, lymphocyte proliferation, plasma levels of MPO, interleukin (IL)-6 and TNF-a (Table 1.3) [*Peluso I, Manafikhi H,* ***Reggi R****, Palmery M. 2014*].
Accordingly, in a meta-analysis Peluso I. et al. concluded that studies did not reveal any significant effect for quercetin on TNF-α and IL-6 plasma levels, while other sources such as red wine and tea extracts significantly reduced inflammatory cytokines [Peluso I et al. Effect of flavonoids on circulating levels of TNFa and IL-6 in humans: a systematic review and meta-analysis]. Red wine (RW) consumption is considered to be protective against diseases associated with oxidative stress. This effect seems to be caused by the antioxidant polyphenols (PP) contained in RW, in particular flavonoids that are present at concentrations from 750 to 1060 mg/l [Covas et al.]. On the other hand, alcohol consumption is associated with oxidative stress, in particular with liver oxidative damage [Lieber CS].

Study (Ref.) subjects	**Treatment**	**Effect (method)**	**Other markers**
Castilla et al., 2008; hemodialysis patients	14 days red grape juice; (100 ml; 640 mg polyphenols)	↓ Neutrophils (DHR123, whole blood); ↓ neutrophils (DCFH, isolated neutrophils)	↓ LDLox; ↔ CRP
Ellinger et al., 2008; Healthy subjects	a) 42 Days dealcoholized red wine (175 ml; 272 mg polyphenols) b) 42 Days red wine (200 ml; 293 mg polyphenols) c) Bolus dealcoholized red wine (175 ml; 272 mg polyphenols) d) bolus red wine (200 ml; 293 mg polyphenols)	a) ↔ Granulocytes; monocytes (DHR123, whole blood) b) ↔ Granulocytes; ↓ monocytes (DHR123, whole blood) c) ↔ Granulocytes; ↑ monocytes (DHR123, whole blood) d) ↔ Granulocytes; ↑ monocytes (DHR123, whole blood)	a) ↔ Phagocytosis b) ↔ Phagocytosis c) ↔ Phagocytosis d) ↓ Granulocytes phagocytosis; ↑ monocytes phagocytosis
Heinz et al., 2010; healthy subjects	84 days quercetin; (500–1,000 mg)	↔ Granulocytes (HE, whole blood)	↑ Plasma quercetin; ↔ Phagocytosis, NK activity, IL-6, TNF-α
Henson et al., 2008; healthy subjects	21 Days quercetin (1,000 mg)	↔ Granulocytes (DHR123, whole blood)	↔ Plasma myeloperoxidase
Konrad et al., 2011; healthy subjects	Bolus quercetin (1,000 mg) + epigallocatechin 3-gallate (120 mg) + isoquercetin (400 mg)	↔ Granulocyte; ↔ monocytes (HE, whole blood)	↑ Plasma quercetin; ↔ phagocytosis, CRP, IL-1β, TNFα
Nieman et al., 2007; Healthy subjects	21 Days quercetin (1,000 mg)	↔ Granulocytes; (DHR123, whole blood)	↑ Plasma quercetin; ↔ NK activity, lymphocyte proliferation, plasma myeloperoxidase

Nieman et al., 2009; Healthy subjects	14 Days quercetin (1,000 mg) +epigallocatechin 3-gallate (120 mg) + isoquercetin (400 mg)	↑ Granulocyte (DHR123, whole blood)	↑ Plasma quercetin; ↔ plasma myeloperoxidase, TNF-α; ↓ IL-6, CRP
Perche et al., 2014; Healthy subjects	4 Weeks orange juice (500 ml; 292 mg hesperidin)	↔ Neutrophils (DHR123, white blood cells after ammonium chloride hemolysis)	↔ NK activity, IL-2 and IL-4

Table 1.3 Effect of flavonoids on oxidative burst in humans. ↑, Increase; ↓, decrease; ↔, no change; DHR123, dihydrorhodamine 123; DCFH, dihydrochlorofluorescein; HE, hydroethidine; CRP, C reactive protein; IL, interleukin; LDLox, oxidized low density lipoprotein cholesterol (LDL); NK, natural killer cells; TNF-α, tumor necrosis factor alpha.[Peluso I, Manafikhi H, Reggi R, Palmery M. Interference of flavonoids with fluorescent intracellular probes: methodological implications in the evaluation of the oxidative burst by flow cytometry. Cytometry A. 2014 Aug;85(8):663-77. doi: 10.1002/cyto.a.22490.]

We aimed to review the available evidence that have investigated the effects of RW on the postprandial-induced metabolic and oxidative stress in humans [*Peluso I, Manafikhi H, **Reggi R**, Palmery M. 2015*]. This review includes a description of the findings of the effects of RW, RW-PP, and Ethanol (EtOH) on postprandial metabolic and oxidative stress. The reviewed results suggest that RW, but not dealcoholized red wine (DRW), increased Triglycerides (TG) and UA. The latter contributes to the antioxidant effect of RW. In fact, NEAC increased in 80 % of the interventions with RW and in 66.6 % of the studies that investigated the effect of RW-PP. Therefore, despite the improvement in NEAC and lipoperoxidation markers after RW consumption with meal, the influence of confounding factors such as UA should be taken into account. The increase in UA and lipemia induced by EtOH could induce liver damage.

A decrease in inflammatory markers and an improvement of OB of granulocyte was observed, after 3 days of heavy exertion in trained cyclists, when quercetin was administered with the tea flavanol epigallocatechin 3-gallate, for 2 weeks before and 1 week after exercise [Nieman DC et al. 2009]. Surprisingly the post exercise-induced decrease in OB was unaffected after bolus consumption of the same supplement [Konrad M et al.]. Konrad et al. suggested that flavonoids may require a week or longer to be incorporated into tissues and influence the post-exercise inflammation. However, it must be taken into account that these studies used two different fluorescence probe and supplement increased DHR123 fluorescence in granulocytes after long-term intervention [Nieman DC et al. 2009] but not HE fluorescence after bolus consumption [Konrad M et al.]. Therefore, considering that flavonoids long term exposure could modulate the expression of transporters, the increase in DHR123 could be due to a major intracellular retention of the probe. Plasma levels of total quercetin (aglicone and metabolites) after each intervention ranged from 300 μg/l to 700 μg/l (10^{-7} M to 10^{-6} M) after long-term consumption [Nieman DC et al. 2009] and were about 6,300 μg/l (10^{-5} M) after bolus ingestion [Konrad M et al.]. Opposite effects have been observed after acute or chronic intervention also by Ellinger et al.: chronic (14 days) intake of red wine (200 ml), containing flavanols (26.5 mg of catechin and 14.4 mg of epicatechin) and anthocyanins (8.5 mg of malvidin and 1.0 mg of peonidin), but not of 175 ml of dealcoholized red wine (10.8 mg of catechin, 8.4 mg of epicatechin, 4.7 mg of malvidin, and 0.5 mg of peonidin), reduced the OB of monocytes. On the contrary after bolus consumption both red wine and dealcoholized red wine increased the OB of monocytes. These finding are in contrast with the results of Ghanim et al. who observed, using luminol method, that orange juice intake prevents the highfat-high-carbohydrate meal-induced ROS production in both mononuclear and polymorphonuclear cells. Citrus and grape juices are rich in flavanones such as hesperidin and naringenin (Table 1.1). Therefore, the differential effect on acute postprandial or exercise induced stress, could be due to the different flavonoid composition. In hemodialysis patients' consumption of red grape juice (50 ml twice/day)

for two weeks decreased the ex vivo OB of neutrophils with both DHR123 in whole blood and DCFH in isolated neutrophils [Castilla P et al.]. However, this study has been conducted not on healthy subject and it is well known that inflammatory states increased Pgp expression in peripheral blood mononuclear cells (PBMC) [Liptrott NJ et al.]. Accordingly, in a recent work Perche et al., using DHR123 assay, did not observe changes of OB in healthy subjects after 4 weeks of orange juice consumption. Finally, also biomolecules involved in metabolism (CYP450) of flavonoids are characterized by high interindividual variability and influenced by inflammatory state [Liptrott NJ et al.], suggesting that it is difficult to compare data from healthy and disease subjects.

AIMS

This PhD thesis has been conducted in the context of a project aimed to evaluate the possible use of the PLIR, a test that measures both the resistance of leukocytes to an exogenous oxidative stress and the leukocytes functional capacity of oxidative burst upon activation, as redox marker in humans.
The clinical relevance of a biomarker must be critically evaluated before the use in large trials. For the above reason we have planned pilot studies.
In order to evaluate the redox status in particular conditions (smoking habit, disease states), ex vivo free-radical production and oxidative stress in body fluids are measured. These methods are used also in human intervention studies to associate the levels of ingested antioxidants (by foods or supplements) with improvement of the body antioxidant status. Despite the fact that it has been suggested that nutraceuticals are capable of improving health, significant methodological bias must be taken into account in the interpretation of data from the measurement of reactive species in leukocytes and platelets by flow cytometry, from the evaluation of markers based on ROS-induced modifications, from the assay of the enzymatic players of redox status, and from the measurement of the total antioxidant capacity of human body fluids.
It has been suggested that the bias of each method could be overcome by the evaluation of oxidative stress by using more than one criterion and indexes of redox status have been proposed [Marrocco et al. 2017]. The OXY-SCORE was computed by subtracting the protection score (GSH, alpha- and gamma-tocopherol levels, and antioxidant capacity) from the damage score (plasma free and total malondialdehyde, GSSG/GSH ratio, and urine F2-IsoPs). In some diseases, the choice of the markers that must be considered in the global index should dictate the clinical relevance in the subjects selected. In patients with chronic venous insufficiency (CVI) was used for OXyVen index calculation the normalized and standardized plasma parameters which showed a significant statistical difference between CVI patients and controls (SH, MDA-bound protein, protein carbonyls, and CAT activity).
However, the major bias of these index is the use of markers that do not have at the moment normal values. The oxidative-INDEX was calculated by subtracting the OXY (the antioxidant capacity measured with the OXY adsorbent test) standardized variable from the ROM (the reactive oxygen metabolites measured with the d-ROM) standardized variable. Although normal values have been proposed for these variables, the OXY adsorbent test quantifies the ability of the plasma non-enzymatic antioxidant compounds to cope with the in vitro oxidant action of hypochlorous acid (HOCl; an oxidant endogenously produced). This type of approach does not consider the important role of free radicals in the innate response and in the resistance to infection, that declines in some conditions, such as overtraining. Within the total antioxidant capacity assays the FRAP, exploits the same principle of biological antioxidant potential (BAP) (i. e. the reduction of ferric to ferrous ions), matches the antioxidant capacity to the reducing ability. It is well known that reduced iron is critical in the onset of oxidative stress due to the Fenton reaction, that generates the hydroxyl radical initiator of lipid peroxidation. Furthermore, the antioxidant capacity is strongly influenced by UA. The latter is a well known pathogenic factor when at high concentrations.
In a previous postprandial study we observed that a functional food covered by dark chocolate and containing glucomannan, inulin, fructooligosaccharides, and Bacillus coagulans strain GanedenBC30 significantly improved postprandial metabolic stress (insulin, glucose, and triglycerides), reduced the postprandial increase of UA, and improved PLIR of lymphocytes, but not of monocytes and granulocytes. We suggested that, although PLIR is a functional index that is independent of baseline levels of oxidation, measuring the ratio between the resistance to

exogenous and the resistance to endogenous ROS injury, this ratio calculation could mask the effect of foods that inhibit both the exogenous ROS injury and the oxidative burst.

From that the aims of this thesis were:

1. Evaluate the relationships between PLIR and FRAP, its major endogenous determinant UA and FRAP-UA, by using a GTE due to its reported UA-lowering and potential pro-oxidant effects.

2. Study the relationships between PLIR and a mathematical index that considers health-related habits and UA plasma levels.

2. AIM 1:
THE PEROXIDATION OF LEUKOCYTES INDEX RATIO REVEALS THE PROOXIDANT EFFECT OF GREEN TEA EXTRACT

2.1. INTRODUCTION

The Supplement Information Expert Committee (DSI EC) indicated that consumption of green tea extract (GTE) could induce liver damage [Sarma D. N. et al.,]. Infact, there are an increasing number of case reports of hepatotoxicity in humans associated within take of green tea (GT) dietary supplements.

In a research, nineteen cases of hepatotoxicity related to the consumption of herbal products containing green tea were identified. The hepatic reactions involved mostly females (16/19 = 84 %), between 24 and 63 years old; males (3/19 = 16 %) were between 16 and 76 years old [G. Mazzanti et al.]. In this study the authors concluded that use of green tea infusion can be considered as safe when consumed as a beverage in normal quantity (1–2 cups/day), provided that quality concerns and other risk factors do not exist; nevertheless, an idiosyncratic reaction remains always possible. Furthermore, use of multicomponent preparations appears much more dangerous with respect to green tea alone. In the following, a panel of rather distinct Dietary Supplement preparations associated with liver injury are described (Table 2.1) [F. Stickel et al.].

Dietary supplement	**Indication for use**	**Liver lesion**	**Toxicity mechanisms**
Herbalife®	Various (weight loss, nutritional support, 'well-being')	Variable (acute and chronic hepatitis, cholestasis, cirrhosis, hepatic failure)	Unknown; autoimmunity? Bacterial contamination?
Camellia sinensis (green tea)	Weight loss	Acute epatiti	Oxidative stress from (−)-epigallocatechin gallate?
LipoKinetix®	Weight loss	Acute epatiti	Uncoupling of respiratory chain?
Hydroxycut	Weight loss	Acute and/or cholestatic hepatitis, liver failure	Unknown
Senna (*Cassia angustifolia*)	Constipation	Variable (acute hepatitis, granulomatous hepatitis, cirrhosis)	Drug idiosyncrasy (CYP2D6 variation)?; uncoupling of respiratory chain?
Noni juice (*Morinda citrifolia*)	'Immunostimulation', health tonic	Acute hepatitis, hepatic failure	Unknown; drug-induced autoimmunity?
Ma huang (*Ephedra sinica*)	Weight loss	Acute hepatitis, hepatic failure	Unknown; drug-induced autoimmunity? Oxidative stress?
Germander (*Teucrium chamaedrys*)	Weight loss	Acute, and fulminant hepatitis, chronic hepatitis with fibrosis/cirrosi	Hepatocyte apoptosis from toxic neoclerodane diterpenoids
'Onshidou-Genbi-Kounou', 'Chaso' (Japanese herbals)	Weight loss	Acute hepatitis, hepatic failure	Hepatocyte apoptosis due to *N*-nitroso-fenfluramine
Vitamin A	Disease prevention, immune function	Disease prevention, immune function	Fibrosis from HSC/MFB activation; formation of toxic polar retinoid metabolites with concomitant alcohol consumption
Anabolic steroids	Muscle build-up	Cholestasis, benign/malignant liver tumour	Dysfunction of biliary transporter

Table 2.1 Nutritional supplements associated with liver injury (*n*>1 case). HSC, hepatic stellate cells; MFB, portal myofibroblasts. [F. Stickel, K. Kessebohm, R.Weimann and H. K. Seitz,"Review of liver injury associated with dietary supplements," Liver International, vol. 31, no. 5, pp. 595–605, 2011]

As herbal products continue to be used widely around the World, herbal hepatotoxicity will continue to be observed. Such events are not necessarily unique to herbal medications as they can be seen with prescription medications such as antibiotics, anticonvulsants, etc. It is therefore imperative that the recognition and reporting of herbal hepatotoxicity be held to the same standards as prescription medications. The diagnosis of herbal hepatotoxicity depends on a proper knowledge of the available literature on hepatotoxicity with the spectrum of herbal preparations ingested and also on a heightened awareness for such hepatotoxic events. Other herb–drug interactions that may result in hepatotoxicity or significantly affect practice are summarised in Table 2.2 [C. Bunchorntavakul and K. R. Reddy].

Medications	**Herbs**	**Interactions and potential consequences**
Warfarin and aspirin	Danshen (*S. miltiorrhiza*)	Increased INR → bleeding risk
Dong quai (*A. sinensis*)	Increased INR → bleeding risk	
Garlic	Increased INR → bleeding risk	
Papaya	Increased INR → bleeding risk	
Tamarind	Increased aspirin level → bleeding risk	
Feverfew	Platelet dysfunction → bleeding risk	
Gingko biloba	Platelet dysfunction → bleeding risk	
Ginseng	Decreased INR → clotting risk	
St. John's wort	Decreased INR → clotting risk	
Devil's claw (*H. cumbens*)	Purpura	
CYP34A drugs	Pyrrolizidines	CYP3A4 induction → hepatotoxicity
Germander	CYP3A4 induction → hepatotoxicity	
Cyclosporine	St. John's wort	CYP3A4 induction → rejection risk
Grape fruit juice	CYP3A4 induction → rejection risk	
Methotrexate	St. John's wort	Increased methotrexate level and toxicity
Echinacea	Increased hepatotoxicity ?	
Prednisolone	Ginseng	Possible additive effect
Glycyrrhizin (licorice root)	Reduced clearance → hypokalemia	
Sho-saiko-to	Altered clearance → low prednisolone level	
Protease inhibitors	St. John's wort	CYP3A4 induction → suboptimal antiviral activity
Garlic	CYP3A4 induction → suboptimal antiviral activity	
Spironolactone	Glycyrrhizin (licorice root)	Mineralocorticoid → low spironolactone level
Benzodiazepines	Kava	Increased sedative effects

Table 2.2 Herb–drug interactions relevant to hepatology. CYP, cytochrome P450; INR, international ratio. [C. Bunchorntavakul and K. R. Reddy, "Review article: herbal and dietary supplement hepatotoxicity," Alimentary Pharmacology and Therapeutics, vol. 37, no. 1, pp. 3–17, 2013].

Adverse effects of Camellia sinensis seem to be modulated by various factors and, in particular, by the chemical composition and the type of herbal preparation. In fact, all preparations differ in their chemical composition, as follows:

(1) powdered leaves contain all the tea active components;
(2) infusions and aqueous extracts contain mostly hydrophilic compounds;
(3) hydroalcoholic extracts contain both hydrophilic and lipophilic components.

The components most frequently indicated as responsible for hepatotoxicity are catechins and their gallic esters. In particular, the role of EGCG (epigallocatechin-3-gallate) seems predominant, as shown also in experimental in vitro and in vivo assays; this conclusion could also be supported by its high concentration in green tea extracts. The association seems further confirmed by the lack of known adverse effects of fermented tea (black tea), in which the content of EGCG is significantly reduced [C. Di Lorenzo et al.]. Despite these apparently reassuring findings, it is important to direct the attention of clinicians to the possibility of rare but severe adverse effects from botanical preparations or ingredients of food supplements or traditional medicines. For example, the severe hepatotoxicity of Camellia sinensis (green tea) was unknown before the product Exolise, containing a hydroalcoholic extract, was marketed and which was found to be responsible for a number of cases of acute hepatitis in France and Belgium. Although very rare (considering the large number of green tea consumers in the world), the severity of these reactions needs information and vigilance [Sharma T et al.]. However, there are also cases reporting hepatotoxicity after GT infusion.
In particular, a case has been reported with features mimicking autoimmune hepatitis, with abnormal liver histology and elevated levels of aspartate aminotransferase, alanine aminotransferase, alkalinephosphatase, gammaglutamyl-transferaseandbilirubin, associated with hypergammaglobulinemia, and the transient presence of anti-smooth-muscle antibodies (ASMA) and anti-neutrophil cytoplasmic antibodies (ANCA) [S. Vanstraelen et al.]. GT withdrawal resulted in a slow and continuous improvement with a complete resolution after 7 months. Furthermore, the Food and Drug Administration (FDA) and the European Food Safety Administration (EFSA) have denied the proposed health claims for GT and decreased risk of non-communicable diseases [J. T. Dwyerand J. Peterson,]. In particular, despite GT increased plasma non-enzymatic antioxidant capacity (NEAC), the EFSA denied claims related to tea and protection of DNA and lipids from oxidative damage. The FDA denied proposed health claims for green tea and decreased risk of cardiovascular disease, gastric cancer, colorectal cancer, and esophageal, pancreatic, and other cancers. For green tea and cancer, the FDA concluded that it was highly unlikely that green tea decreased breast and prostate cancer risks. Similarly, EFSA concluded that the substantiation for the health claims related to tea did not suffice in several recent submissions the agency received [J. T. Dwyerand J. Peterson].
GT contains several flavonoids with antioxidant properties, in particular the flavanol monomers known as catechins, where epigallocatechin-3-gallate (EGCG) is the most effective antioxidant compound [J. D. Lambert et al.]. Green tea, which represents 20% of world consumption, is characterized by the presence of large amounts of flavan-3-ols also known as catechins (Fig. 2.1).

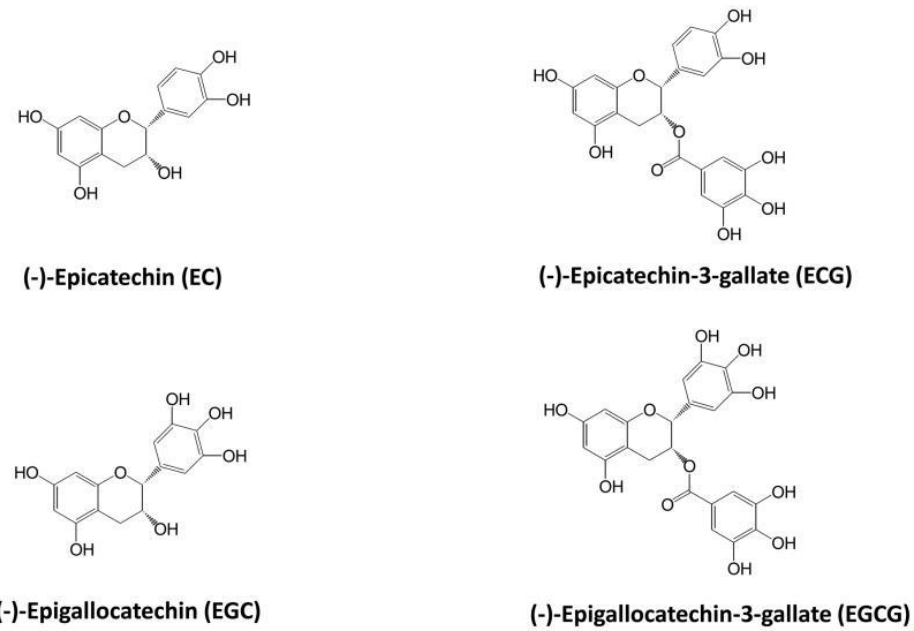

Figure 2.1 Structures of the major tea polyphenols - J. D. Lambert and R. J. Elias, "The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 65–72, 2010

Tea polyphenols are strong radical scavengers and metal chelators in model chemical systems, and these effects correlate with the presence of the dihydroxy and trihydroxy groups [Higdon JV, Frei B]. Research examining more direct measures of oxidative stress offers some support for the idea that tea catechins function as antioxidants in vivo. In humans, modest acute increases in plasma antioxidant capacity have been demonstrated consistently with an increased consumption of green tea, black tea, and green tea catechins. Studies in animal models have been more consistent in demonstrating an increase in the resistance of lipoproteins to ex vivo oxidation than studies in humans, as have studies of biomarkers of lipid peroxidation in vivo. Although a number of studies have examined the effect of high levels of tea or tea polyphenol consumption on the ex vivo oxidation of LDL isolated from plasma, few have examined ex vivo oxidation of plasma, a setting in which watersoluble green tea catechins may provide more protection from oxidation. An increasing number of studies have also demonstrated these antioxidative effects in vivo. For example, treatment of 24 month old rats with 100 mg/kg, i.g. EGCG decreased the hepatic levels of lipid peroxides (50% decrease) and protein carbonyls (39% decrease) [Senthil Kumaran V et al.].
EGCG treatment also increased the hepatic levels of both small moecule antioxidants and antioxidant enzymes compared to control rats. A second study by the same group found similar results using a much lower dose of EGCG (2 mg/kg, i.g.) over a relatively long period of time (30 d) [Srividhya R et al.]. However, tea catechins could have also prooxidant activity: Studies using higher doses of EGCG also show that pro-oxidant effects may play a role in the potential toxic effects of EGCG that have been reported in vivo. For example, Galati et al., have reported that treatment of freshly isolated mouse hepatocytes with 200 μM EGCG resulted in time and dose-dependent cytotoxicity that correlated with the production of ROS as measured by oxidation of dichlorofluorescin [Galati G et al.]. Inclusion of GSH, catalase, or ascorbic acid reduced levels of ROS and EGCG-mediated cytoxicity. Following treatment of CF-1 mice with 400 mg/kg, i.p. EGCG, EGCG-2′-cysteine and EGCG-2″-cysteine could be detected in the urine. These metabolites are hypothesized to arise from the reaction of EGCG quinone intermediates with the thiol moiety of cysteine and suggests that at high doses EGCG may have pro-oxidant effects in vivo (Fig. 2.2).

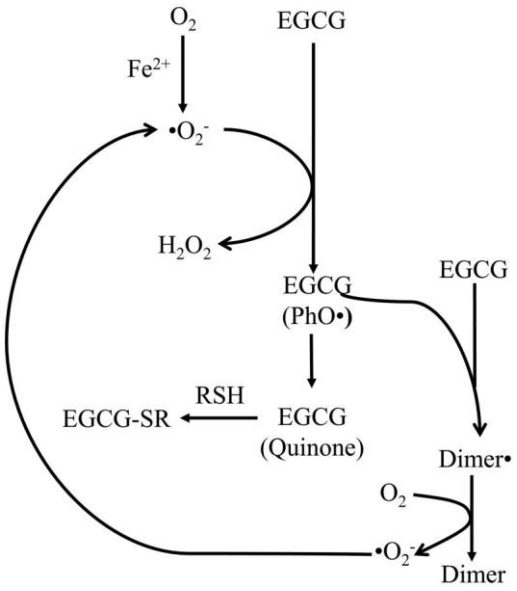

Figure 2.2 Oxidative reaction between EGCG, superoxide, and ferric iron resulting in the production of oxidative stress, EGCG dimers, and EGCG-cysteine conjugates (EGCG-SR). PhO = semiquinone radical. [J. D. Lambert and R. J. Elias, "The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 65–72, 2010]

Lambert JD et al. has reported that treatment of mice with high oral doses of EGCG result in the dosedependent hepatotoxicity [Lambert JD et al.]. These hepatotoxic effects were correlated with increased hepatic lipid peroxidation and expression of hepatic metallothionein I/II and hepatic levels of γH2A.X. These biomarkers all suggest the pro-oxidant effects of EGCG may underlie the hepatotoxicity of EGCG in mice.

Besides, some of the protective effects of EGCG have been ascribed to its capability to reduce excessive UA level [T. Yokozawa et al.]. In particular, flavanols of Camellia sinensis is modulate both xanthine oxidase and urate transport [I. Peluso et al., Green tea and bone marrow transplantation: from antioxidant activity to enzymatic and multidrug-resistance modulation]. Among all tea polyphenols, EGCG, a flavonoid of the flavanols subgroup, has been found to be responsible for much of the antioxidant activity of GT. However, recently data suggest that some effects of EGCG are not imputable to its antioxidant activity). Besides, antioxidant defenses of the body are composed of molecular and enzymatic players; the latter supply protection at the cellular level, together with glutathione (GSH), and include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR) and glutathione S-Transferase (GST), whereas in plasma non-enzymatic antioxidants play the major role. Plasma Non-Enzymatic Antioxidant Capacity (NEAC) is due to endogenous UA, bilirubin and thiols, as well as to dietary-derived molecules such as vitamin E, ascorbic acid, carotenoids, and polyphenols. Some of the protective effects of EGCG have been ascribed to its capability to reduce high UA level.

NEAC assays evaluate both endogenous and exogenous antioxidants, thus if tea affects the levels of endogenous antioxidants it could have both direct and indirect effects on NEAC. In particular, the inhibitory effects of EGCG on XO could decrease UA circulating levels (Figure 2.3).

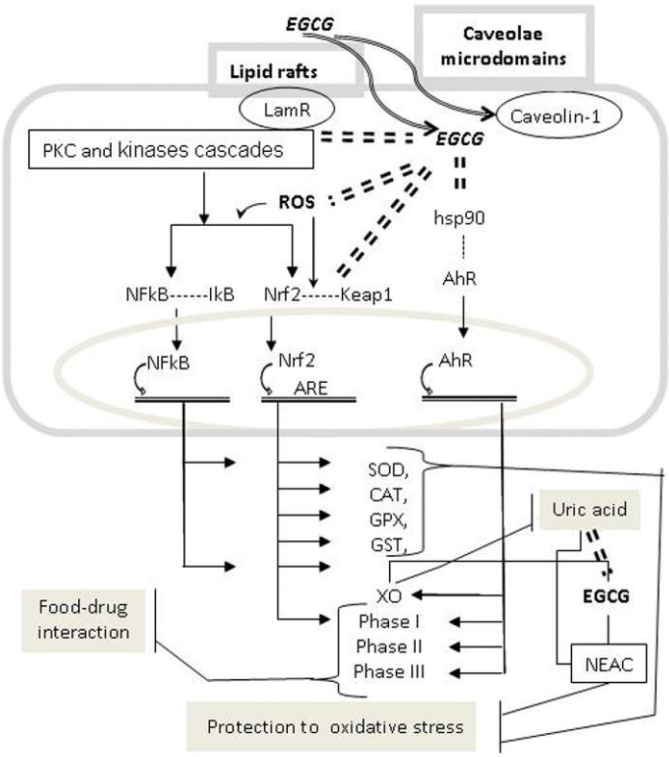

Figure 2.3 Potential molecular mechanisms involved in the modulation by EGCG of redox pathway and metabolism/transport system [I. Peluso, M. Palmery, and A. Vitalone, "Green tea and bone marrow transplantation: from antioxidant activity to enzymatic and multidrug-resistance modulation," *Critical Reviews in Food Science and Nutrition*, vol. 56, no. 14, pp. 2251–2260, 2016]

UA is the major plasma antioxidant and contributes to plasma non-enzymatic antioxidant capacity [Serafini M. et al. 2011]: In terms of the participation of individual components to the network, calculated on the basis of the single individual concentration and respective stoichiometric coefficient, the main contributor to NEAC is UA (40-55 %), followed by thiol groups (10-24 %), ascorbic acid (8-15 %) and vitamin E (less than 10 %).

The peroxidation of leukocytes index ratio (PLIR) measures the resistance of leukocytes to exogenous oxidative stress and their functional capacity of oxidative burst upon activation [I. Peluso et al., The effect of sample storage on the peroxidation of leukocytes index ratio (PLIR) measure]. Therefore, we performed a pilot study in order to evaluate the effect of a single dose of a GTE supplement on the PLIR, in relation to plasma UA and ferric reducing antioxidant potential (FRAP) [I. F. F. Benzie et al.], as well as the sample size to reach statistical significance.
The FRAP assay gives fast, reproducible results with plasma, with single antioxidants in pure solution and with mixtures of antioxidants in aqueous solution and added to plasma.

2.2. MATERIAL AND METHODS

2.2.1. SUBJECTS AND TREATMENT

Participants (6 men and 4 women, 19–35 years old) to the study, who volunteered in response to advertisements, were healthy, nonsmokers and weretakingnosupplements. For two days prior to each feeding study the subjects followed a low antioxidant diet (washout) by avoiding all freshfruit, vegetables, tea, coffee, cocoa, fruitjuices and wine. On the day of the study, after an overnight fast, venous blood samples were collected (in EDTA-tubes) before (T0), 30 minutes (T0.5), and 3 hours (T3) after a single dose of two capsules of a GTE (200mg × 2), commercially available in Italy (cod. 1820, REGISTRO INTEGRATORI https://www.salute.gov.it/imgs/C 17 pagineAree 3668 listaFile itemName 1 file.pdf).

2.2.2. PLASMA URIC ACID AND TAC

The plasma was separated by centrifugation at 1300×g at 4 °C for 15 minutes and stored at -80 °C. Plasma levels of UA were quantified using colorimetric kits (Sentinel CH. SpA, Italy).
Plasma TAC was measured with the FRAP assay [I. F. F. Benzie and J. J. Strain].
We calculated also the uric acid-independent FRAP (FRAP-UA) as previously described [I. Peluso et al. 2013], applying the formula:
FRAP-UA = FRAPμM−2UA μM. (1)

2.2.3. PLIR METHOD

After red blood cells' lysis and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diazas-indacene-3-undecanoic acid (C11-BODIPY, Invitrogen, final concentration 1 μM) staining, leukocytes were treated as previously described [*I. Peluso, H. Manafikhi, **R. Reggi**, Y. Longhitano, C. Zanza, and M. Palmery, 2016"]* with phorbol 12-myristate 13-acetate (PMA, Sigma, final concentration 1 μg/mL), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Sigma, final concentration 10mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, final concentration 10 μM), PMA 1 μg/mL + Trolox 10 μM, or AAPH 10mM + Trolox 10 μM.
After 30 minutes at 37 °C cells were stored in ice, to stop reactions, and rapidly analyzed on an Accuri C6 BD cytometer.
Data acquired on the Accuri C6 was exported in FCS format and analyzed by FCS express software (De Novo Software) to calculate the ratio of oxidation of the probe C11-BODIPY (FL1/FL2).
PLIR was calculated as previously described [*I. Peluso, H. Manafikhi, **R. Reggi**, Y. Longhitano, C. Zanza, and M. Palmery,2016*], applying the formula:
PLIR = (ratio AAPH × ratio PMA Trolox) / (ratio AAPH Trolox × ratio PMA). (2)

2.2.4. STATISTICS

Statistical analysis, carried out with Friedman RM ANOVA on Ranks, revealed a normal distribution for all markers (Normality Test Shapiro-Wilk and Equal variance test passed).
Therefore, statistical analysis was carried out with repeated measures analysis of variance (RM ANOVA), with time or treatment as within-subjects factors. Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure) was used to isolate differences between groups. Spearman correlation was used to evaluate relationships between variables. All statistical evaluations were performed using the SigmaStat and SigmaPlot software (Jandel Scientific, Inc.).

2.3. RESULTS

2.3.1. PLASMA URIC ACID AND TAC

RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure), revealed that GTE consumption did not affect FRAP values, whereas 3 hours (T3) after treatment both a nonsignificant decrease in UA and a significant increase in FRAP-UA were found (Table 2.3). From the difference of means and the standard deviations (power0.8; alpha0.05) we calculated a sample size (to reach statistical significance) of 106 for UA.

	T0	T0.5	T3
UA μM	334.1 ± 7.4	324.9 ± 3.9	324.3 ± 3.7
FRAP μM	1061.5 ± 38.45	1061.48 ± 52.85	1083.33 ± 46.78
FRAP-UA μM	393.23 ± 41.63*	411,57 ± 53.38	434.59 ± 41,75*
PLIR L	2.09 ± 0.17	2.09 ± 0.17	2.37 ± 0.19
PLIR M	1.88 ± 0.13*	1.86 ± 0.12	2.19 ± 0.15*
PLIR G	1.80 ± 0.12*	1.90 ± 0.14	2.17 ± 0.15*

Table 2.3 Effect of GTE consumption on plasma antioxidant markers and PLIR. Plasma antioxidant markers in samples collected from 10 healthy subjects before (T0), 0.5 (T0.5), and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). UA: uric acid, FRAP: ferric reducing antioxidant potential, FRAP-UA: uric acid-independent FRAP, PLIR: peroxidation of leukocytes index ratio, L: lymphocytes, M: monocytes, and G: granulocytes. RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis: T3 versus T0: *p<0.05. [Peluso I, Manafikhi H, Raguzzini A, Longhitano Y, Reggi R, Zanza C, Palmery M. The Peroxidation of Leukocytes Index Ratio Reveals the Prooxidant Effect of Green Tea Extract. Oxid Med Cell Longev. 2016;2016:9139731. doi: 10.1155/2016/9139731.]

2.3.2. PLIR METHOD

Treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3, whereas a nonsignificant increase was observed for PLIR of lymphocytes (Table 2.3).
We calculated a sample size (to reach statistical significance) of 80 for PLIR of lymphocytes.
Pearson Product Moment Correlation revealed an inverse correlation of UA with PLIR L (CC = −0.383, p = 0.0368), PLIR M (CC = −0.474, p = 0.008), and PLIR G (CC = −0.545, p = 0.001) and a direct correlation of FRAP-UA with PLIR L (CC = 0.451, p = 0.012), PLIR M (CC = 0.398, p = 0.029), and PLIR G (CC = 0.434, p = 0.016).

2.3.3. RATIO OF OXIDATION OF THE PROBE C11-BODIPY

Typical overlay dot plots of the four treatments used for PLIR calculation and ratio of fluorescence (FL1/FL2) on single cells, before GTE consumption and 3 hours after, are presented in Figures 2.4 and 2.5, respectively. Trolox inhibited the peroxidation of C11-BODIPY in leukocytes exposed to

AAPH free radicals generating system, but not the PMA-induced oxidation in monocytes and granulocytes, both at T3 and at T0 (Figure 2.4). Considering the major components of PLIR affected by treatment, compared to baseline, the AAPH-induced (exogenous) oxidation appeared greater, whereas the PMA induced oxidative burst appeared lower (Figure 2.4).
Despite the differences of ratio PMA and ratio AAPH between times did not reach significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes (Figure 2.5). We calculated a sample size (to reach statistical significance between times) of 17 and 33 for ratio AAPH and of 21 and 51 for ratio PMA, for granulocytes and monocytes, respectively. Ratio PMA was not related to neither UA nor FRAP-UA, whereas ratio AAPH was inversely correlated with UA on all cells (L: CC = −0.477, $p = 0.007$; M: CC = −0.514, $p = 0.003$; G: CC = −0.511, $p = 0.003$), but not with FRAP-UA.

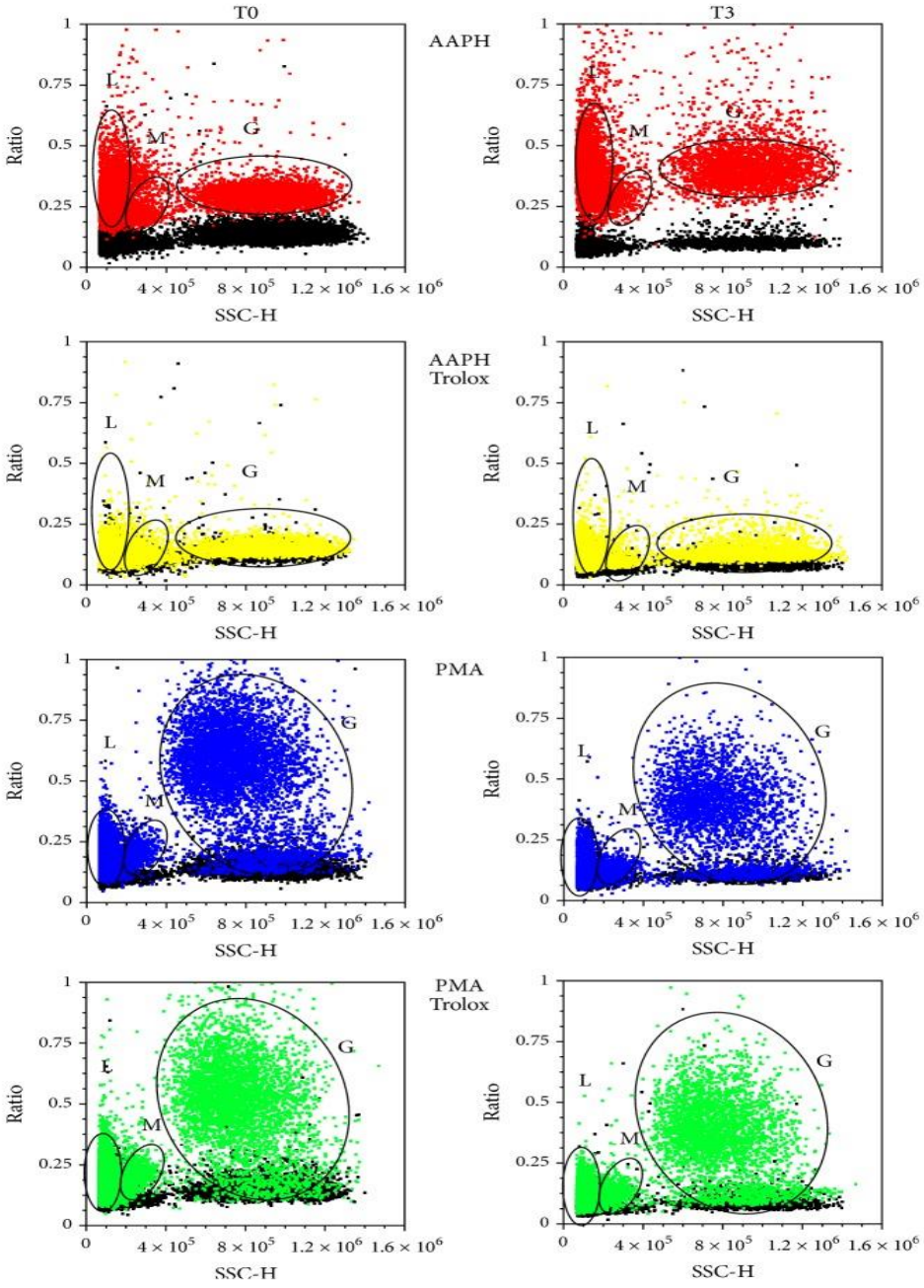

Figure 2.4 Typical overlay dot plots of ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) versus side scatter (SSC): before (T0) and 3 hours (T3) in leukocytes collected after a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). L: lymphocytes, M: monocytes, and G: granulocytes. Unstimulated samples (black) and leukocytes treated with 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 10 mM, red), AAPH (10 mM) + 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μM, yellow), phorbol 12-myristate 13-acetate (PMA, 1 μg/mL, blue), or PMA (1 μg/mL) + Trolox (green). [Peluso I, Manafikhi H, Raguzzini A, Longhitano Y, Reggi R, Zanza C, Palmery M. The Peroxidation of Leukocytes Index Ratio Reveals the Prooxidant Effect of Green Tea Extract. Oxid Med Cell Longev. 2016;2016:9139731. doi: 10.1155/2016/9139731.]

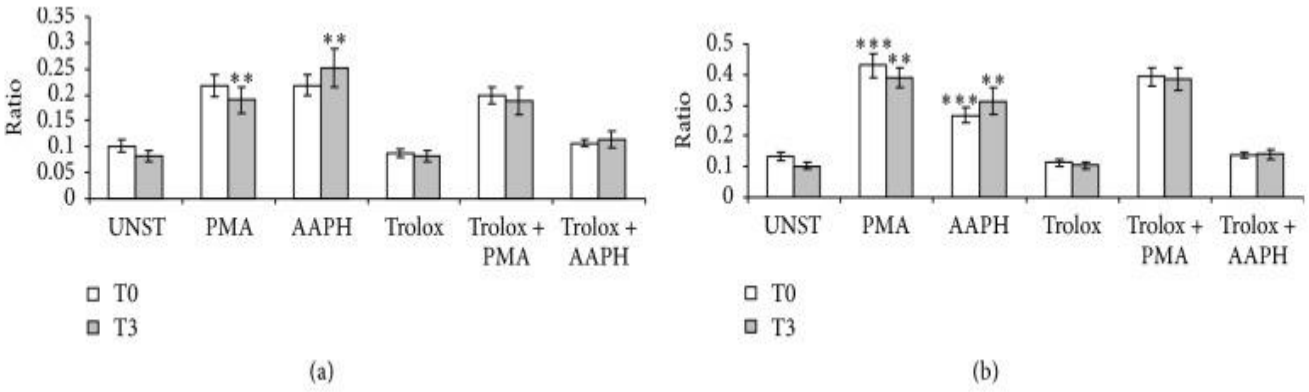

Figure 2.5 Ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) of monocytes (a) and granulocytes (b), in samples collected from 10 healthy subjects before (T0) and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). Cells unstimulated (UNST) or treated with phorbol 12-myristate 13-acetate (PMA, 1 μg/mL), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μM), Trolox + PMA, or Trolox + AAPH. Two-way (2W) RM ANOVA, with time and treatment as within-subjects factors, followed by Student-Newman-Keuls post hoc analysis: AAPH versus PMA within time: $^{**}p<0.01$, $^{***}p<0.001$. [Peluso I, Manafikhi H, Raguzzini A, Longhitano Y, Reggi R, Zanza C, Palmery M. The Peroxidation of Leukocytes Index Ratio Reveals the Prooxidant Effect of Green Tea Extract. Oxid Med Cell Longev. 2016;2016:9139731. doi: 10.1155/2016/9139731.]

2.4. DISCUSSION

2.4.1. EFFECT OF GTE ON PLASMA ANTIOXIDANTS

Previous studies reported decreased, increased, or unchanged UA and NEAC levels after bolus consumption of EGCG or GTE. The purpose of E. J´owko et al. was to evaluate the effect of acute ingestion of green tea polyphenols (GTP) on blood markers of oxidative stress and muscle damage in soccer players exposed to intense exercise. This randomized, double-blinded study was conducted on 16 players during a general preparation period, when all athletes participated in a strength-training program focused on the development of strength endurance. After ingestion of a single dose of GTP (640 mg) or placebo, all athletes performed an intense muscle-endurance test consisting of 3 sets of 2 strength exercises (bench press, back squat) performed to exhaustion, with a load at 60% 1-repetition maximum and 1-min rests between sets. Blood samples were collected preexercise, 5 minutes after the muscle-endurance test, and after 24 hr of recovery. Blood plasma was analyzed for the concentrations of thiobarbituric acid–reacting substances (TBARS), UA, total catechins, total antioxidant status (TAS), and activity of creatine kinase (CK); at the same time, erythrocytes were assayed for the activity of superoxide dismutase (SOD). E. J´owko et al. found that in both groups, plasma TBARS, UA, and TAS increased significantly postexercise and remained elevated after a 24-hr recovery period. SOD activity in erythrocytes did not change significantly in response to the muscle-endurance test, whereas in both groups plasma CK activity increased significantly after 24 hr of recovery. Acute intake of GTP cased a slight but significant increase in total plasma catechins. However, GTP was found not to exert a significant effect on measured parameters. So, acute ingestion of GTP (640 mg) does not attenuate exercise-induced oxidative stress and muscle damage.

A. Rabovsky et al. in their method includes 3 steps:

(1) enzymatic removal of UA

(2) ex vivo free radical oxidation of plasma by the addition of a free-radical generator such as SIN-1 or AAPH

(3) measurement of a marker of lipid peroxidation, 8-isoprostanes.

It has been shown in an in vitro experiment that the addition of various antioxidants to plasma significantly reduced the amount of free radical induced 8-isoprostanes. In a human single dose supplementation clinical study with vitamin C, vitamin E, and grape seed and green tea extracts (on separate days) 8-isoprostane formation was also significantly reduced compared to no supplementation (74.2 to 53.8 ± 5.1pg/ml for vitamin C).

So A. Rabovsky et al. concluded that the reduction in 8-isoprostane formation demonstrates that the total amount of antioxidant protection in the plasma can be increased with supplementation of antioxidants and this new method can effectively measure the plasma antioxidant reserve (PAR) in healthy subjects.

In a thirth study [M. Kimura et al.] the purpose was to investigate the effects of single/double or repeated intake of a normal amount of tea catechin on plasma catechin concentrations and antioxidant activity in young women. First, after an overnight fast, five healthy subjects were given water or single/double dose(s) of tea polyphenol extract (164 mg tea catechins containing 61% epigallocatechin gallate in 190 ml water). Blood samples were taken before and 30, 60 and 180 min after the ingestion. Second, 16 healthy subjects ingested the tea polyphenol extract three times a day at mealtimes for 7 days followed by withdrawal of tea polyphenol extract for 7 days. Blood samples were taken before and after ingestion, and 7 days after the withdrawal of tea catechin. Subjects were prohibited from drinking any beverages containing polyphenols or antioxidant supplements during the study period. Catechin and other antioxidant concentrations in the plasma were measured, and changes in antioxidant activity were evaluated by ferric reducing ability of plasma assay. So, a single/double ingestion of tea polyphenol extract did not cause an increase in the antioxidant

activity. There was no also change in antioxidant activity after the ingestion of tea polyphenol extract for 7 days. Plasma-free epigallocatechin gallate concentration remained at the pre-study level; however, the plasma FRAP value decreased significantly at 7 days after the withdrawal of tea polyphenol extract. Decreases in endogenous antioxidants in the plasma, including vitamin C and bilirubin, were also observed 7 days after withdrawal of tea polyphenol. The results of this study suggest that continuous daily intake of tea catechins affects the concentrations of endogenous antioxidants in the plasma and has the potential to maintain total antioxidant activity.

A human intervention study was performed to evaluate the bioavailability and antioxidant capacity of EGCG administered as a single large dose in the form of either purified EGCG or as green tea extract (Polyphenon E) [S. M. Henning et al.]. Plasma concentrations of tea polyphenols were determined by high-performance liquid chromatography (HPLC) analysis combined with coulometric array electrochemical detection (ECD). They found no differences in plasma EGCG concentrations and trolox equivalents determined by the trolox equivalent antioxidant capacity assay after administration of either form of EGCG. However, they found that the plasma antioxidant activity was significantly affected by changes in the plasma urate concentration, which may have interfered with the effect of tea polyphenols on the antioxidant activity. In addition, lymphocyte 8-hydroxydeoxyguanosine to deoxyguanosine (8-OHdG/106dG) ratios were determined by HPLC with ECD. The 8-OHdG/106dG ratios did not change significantly during the 24 h following both EGCG interventions but correlated significantly within individuals determined during the two interventions separated by 1 week. In summary, changes in plasma UA due to dietary intake were significantly correlated to the plasma antioxidant activity and exerted a stronger influence on the plasma antioxidant activity compared with the EGCG intervention. In future studies of dietary effects on the plasma antioxidant capacity, changes in plasma UA will need to be closely monitored.

However, in some studies, EGCG increased in plasma from 30 minutes to 2.6 hours after GTE consumption, depending on the dose and on the formulation.

A randomized, double-blind, placebo-controlled study [U. Ullmann et al.] assessed the safety, tolerability and plasma kinetic behaviour of single oral doses of 94% pure crystalline bulk EGCG under fasting conditions in 60 healthy male volunteers. In each group of 10 subjects, eight received oral EGCG in single doses of 50 mg, 100 mg, 200 mg, 400 mg, 800 mg or 1600 mg, and two received placebo. Blood samples were taken at intervals until 26 h later. The area under the concentration– time curve from 0 h to infinity, the maximum plasma concentration (Cmax) of EGCG, the time taken to reach the maximum concentration (Tmax), and the terminal elimination half-life (t1/2z) of EGCG were determined. Safety and tolerability were assessed. In each dosage group, the kinetic profile revealed rapid absorption with a one-peak plasma concentration versus time course, followed by a multiphasic decrease consisting of a distribution phase and an elimination phase. The mean AUC of total EGCG varied between 442 and 10 368 ng·h/ml. The according mean Cmax values ranged from 130 to 3392 ng/ml and were observed after 1.3 – 2.2 h. The mean t1/2z values were seen between 1.9 and 4.6 h. Single oral doses of EGCG up to 1600 mg were safe and very well tolerated.

In this report of M. J. Lee et al. [M. J. Lee et al.], the pharmacokinetic parameters of EGCG, (-)-epigallocatechin (EGC), and (-)-epicatechin (EC) were analyzed after administration of a single oral dose of green tea or decaffeinated green tea (20 mg tea solids/ kg) or EGCG (2 mg/kg) to eight subjects. The plasma and urine levels of total EGCG, EGC, and EC (free plus conjugated forms) were quantified by HPLC coupled to an electrochemical detector. The plasma concentration time curves of the catechins were fitted in a one compartment model. The maximum plasma concentrations of EGCG, EGC, and EC in the three repeated experiments with green tea were 77.9 ± 22.2, 223.4 ± 35.2, and 124.03 ± 7.86 ng/ml, respectively, and the corresponding AUC values were 508.2 ± 227, 945.4 ± 438.4, and 529.5 ± 244.4 ng·h·ml^{-}1 , respectively. The time needed to reach the peak concentrations was in the range of 1.3–1.6 h. The elimination half-lives were 3.4 ±

0.3, 1.7 ± 0.4, and 2.0 ± 0.4 h, respectively. Considerable interindividual differences and variations between repeated experiments in the pharmacokinetic parameters were noted. Significant differences in these pharmacokinetic parameters were not observed when EGCG was given in decaffeinated green tea or in pure form. In the plasma, EGCG was mostly present in the free form, whereas EGC and EC were mostly in the conjugated form. Over 90% of the total urinary EGC and EC, almost all in the conjugated forms, were excreted between 0 and 8 h. Substantial amounts of 4-Omethyl EGC, at levels higher than EGC, were detected in the urine and plasma. The plasma level of 4-O-methyl EGC peaked at 1.7 0.5 h with a half life of 4.4 1.1 h. Two ring-fission metabolites, (-)-5-(3,4,5- trihydroxyphenyl)--valerolactone (M4) and (-)-5-(3,4- dihydroxyphenyl)-valerolactone (M6), appeared in significant amounts after 3 h and peaked at 8–15 h in the urine as well as in the plasma.

Chow et al. performed a Phase I pharmacokinetic study to determine the systemic availability of green tea catechins after single oral dose administration of EGCG and Polyphenon E (decaffeinated green tea catechin mixture). Twenty healthy subjects (five subjects/dose level) were randomly assigned to one of the dose levels (200, 400, 600, and 800 mg based on EGCG content). All subjects were randomly crossed-over to receive the two catechin formulations at the same dose level. Blood and urine samples were collected for up to 24 h after oral administration of the study medication. Tea catechin concentrations in plasma and urine samples were determined using high-performance liquid chromatography with the coulometric electrode array detection system. After EGCG versus Polyphenon E administration, the mean area under the plasma concentration-time curves (AUC) of unchanged EGCG were 22.5 versus 21.9, 35.4 versus 52.2, 101.9 versus 79.7, and 167.1 versus 161.4 min·mg/ml at the 200-, 400-, 600-, and 800-mg dose levels, respectively. EGC and EC were not detected in plasma after EGCG administration and were present at low/undetectable levels after Polyphenon E administration. High concentrations of EGC and EC glucuronide/sulfate conjugates were found in plasma and urine samples after Polyphenon E administration. There were no significant differences in the pharmacokinetic characteristics of EGCG between the two study medications. The AUC and Cmax of EGCG after the 800-mg dose of EGCG were found to be significantly higher than those after the 200- and 400-mg dose. The AUC and Cmax of EGCG after the 800-mg dose of Polyphenon E were significantly higher than those after the three lower doses. We conclude that the two catechin formulations resulted in similar plasma EGCG levels. EGC and EC were present in the body after the Polyphenon E administration; however, they were present predominantly in conjugated forms. The systemic availability of EGCG increased at higher doses, possibly due to saturable presystemic elimination of orally administered green tea polyphenols. However, the FRAP value did not increase when free EGCG concentration was at its peak [M. Kimura et al.], probably due to the decrease in UA levels observed after GTE consumption [S. M. Henning et al.].

In agreement with these results, in our study GTE consumption did not affect FRAP values whereas a non-significant decrease in UA and a significant increase in FRAP-UA were found 3 hours after treatment. The increase in FRAP-UA, probably due to the catechins, could counter balance the reduction in FRAP induced by the UA decrease. However, the FRAP assay matches the antioxidant capacity to the reducing ability [I. F. F. Benzie et al.] and the reduced iron is critical in the onset of oxidative stress due to the Fenton reaction that generates the hydroxyl radical initiator of lipid peroxidation [S. Knasm¨uller et al.]. Therefore, an increase in the metal reducing power could be more likely detrimental than beneficial.

2.4.2. EFFECT OF GTE ON AAPH-INDUCED LIPOPEROXIDATION

The increase in FRAP-UA was temporally associated with an increased oxidation of the fluorescent probe C11-BODIPY incorporated into the leukocytes. In this context, the prooxidant effects of tea catechins on cells are supported by the molecular mechanisms involved in their induction of antioxidant enzymes, through the antioxidant responsive elements (ARE) pathway [J. D. Lambert et al.]. In particular, it has been suggested that some derivatives of catechins can oxidize highly reactive cysteine thiol groups of Kelch-like ECH-associated protein1 (Keap1), resulting in disulphide bond formation and nuclear factor-erythroid2-relatedfactor2(Nrf2) release [H.-K. Na et al.]. The redox-sensitive transcription factor Nrf2 plays a key role in regulating induction of phase II detoxifying or antioxidant enzymes. Thus, activation of Nrf2 is considered to be an important molecular target of many chemopreventive and chemoprotective agents. They proposed mechanisms by which EGCG activates Nrf2, leading to up-regulation of ARE-driven antioxidant gene expression (Fig. 2.6). It is plausible that reactive forms of EGCG can conjugate with GSH, thereby lowering the cellular GSH level which may lead to transient disruption of redox-status with concomitant activation of MAPK cascades triggering Nrf2 phosphorylation. Alternatively, some electrophilic forms of EGCG may directly interact with cysteine residue present in Keap1, thereby inducing Nrf2 dissociation. Likewise, ROS derived as a consequence of auto-oxidation of EGCG can oxidize the cysteine thiols of Keap1, which will lead to diminished affinity of Nrf2 for Keap1, facilitating the release of Nrf2 for nuclear translocation.

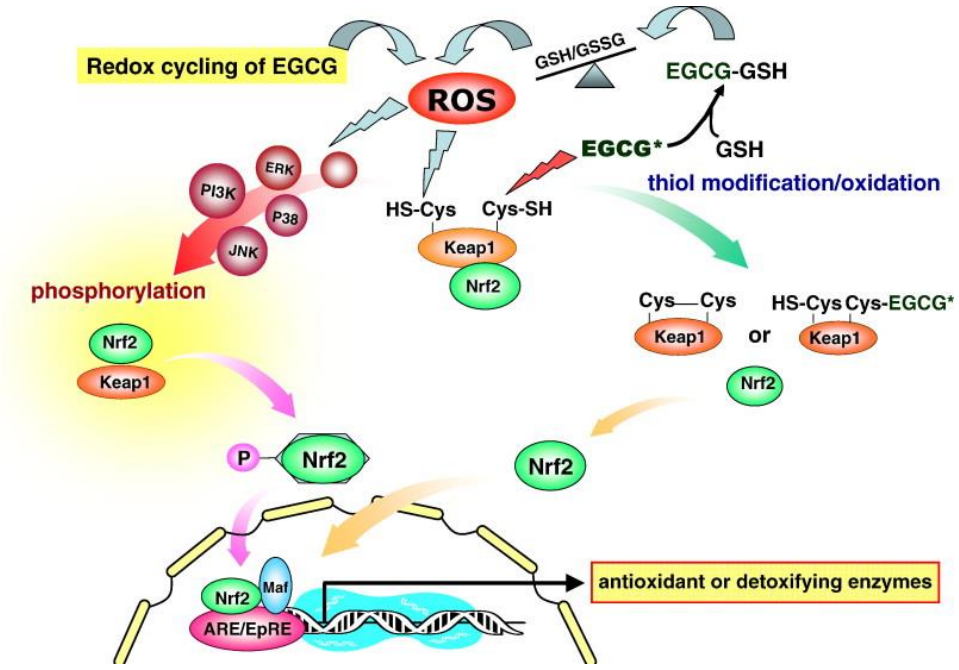

Figure 2.6 EGCG-induced upregulation of antioxidant or detoxifying enzymes via Nrf2-ARE signaling. Nrf2 is a transcription factor that regulates expression of many detoxification or antioxidant enzymes. The Kelch-like-ECH-associated protein 1 (Keap1) is a cytoplasmic repressor of Nrf2 that inhibits translocation of Nrf2 to the nucleus. It is plausible that oxidized or other reactive forms of EGCG (EGCG*) can conjugate with GSH thereby lowering the cellular GSH level which may lead to disruption of redox-status with subsequent activation of upstream kinases including phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and MAPKs, such as c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), triggering Nrf2 phosphorylation. Alternatively, some reactive forms of EGCG (EGCG*) may directly interact with cysteine residues present in Keap1, thereby stimulating Nrf2 dissociation. Likewise, ROS produced by auto-oxidation of EGCG may not only stimulate phosphorylation of Nrf2 through activation of upstream kinases or oxidize the cysteine thiols of Keap1. Both events can facilitate the

nuclear translocation of Nrf2. In the nucleus, Nrf2 associates with small Maf (the term derived from MusculoAponeurotic-Fibrosarcoma virus), forming a heterodimer that binds to antioxidant-response element (ARE) or electrophile-responsive element (EpRE) to stimulate phase II detoxification or antioxidant enzymes. [H.-K. Na and Y.-J. Surh, "Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG," *Food and Chemical Toxicology*, vol. 46, no. 4, pp. 1271–1278, 20]

However, the ratio AAPH was inversely correlated with UA on all cells, but not with FRAP-UA. In agreement with our results the consumption for 112 days of a lutein (12mg/d) plus GTE (200mg/d) supplement did not reduce the oxidation of the C11-BODIY incorporated into the lipid compartment of plasma [L. Li, C.-Y. O. Chen et al.].
Epigallocatechin gallate, a major component of green tea polyphenols, protects against the oxidation of fat-soluble antioxidants including lutein. The study of L. Li et al. [L. Li, C.-Y. O. Chen et al.] determined the effect of a relatively high but a dietary achievable dose of lutein or lutein plus green tea extract on antioxidant status. Healthy subjects (50– 70 years) were randomly assigned to one of two groups (n=20 in each group): (1) a lutein (12 mg/day) supplemented group or (2) a lutein (12 mg/day) plus green tea extract (200 mg/day) supplemented group. After 2 weeks of run-in period consuming less than two servings of lightly colored fruits and vegetables in their diet, each group was treated for 112 days while on their customary regular diets. Plasma carotenoids including lutein, tocopherols, flavanols and ascorbic acid were analyzed by HPLC-UVD and HPLC-electrochemical detector systems; total antioxidant capacity by fluorometry; lipid peroxidation by malondialdehyde using a HPLC system with a fluorescent detector and by total hydroxyoctadecadienoic acids using a GC/MS. Plasma lutein, total carotenoids and ascorbic acid concentrations of subjects in either the lutein group or the lutein plus green tea extract group were significantly increased (Pb.05) at 4 weeks and throughout the 16-week study period. However, no significant changes from baseline in any biomarker of overall antioxidant activity or lipid peroxidation of the subjects were seen in either group. Their results indicate that an increase of antioxidant concentrations within a range that could readily be achieved in a healthful diet does not affect in vivo antioxidant status in normal healthy subjects when sufficient amounts of antioxidants already exist.

2.4.3. EFFECT OF GTE ON PMA-INDUCED OXIDATIVE BURST

We have found a non-significant decrease of PMA-induced lipoperoxidation after GTE consumption, contrarily with the increase of the oxidative burst of granulocyte, observed in cyclists when quercetin was administered with the tea flavanol epigallocatechin 3-gallate, by using dihydrorhodamine 123 (DHR123) as fluorescence probe [D. C. Nieman et al. 2009].
D. C. Nieman et al. wanted to test the influence of 1000 mg of quercetin (Q) with or without 120 mg of epigallocatechin 3-gallate (EGCG), 400 mg of isoquercetin, and 400 mg of eicosapentaenoic acid and docosahexaenoic acid (Q–EGCG) on exercise performance, muscle mitochondrial biogenesis, and changes in measures of immunity and inflammation before and after a 3-d period of heavy exertion. So, trained cyclists (N = 39) were randomized to placebo (P), Q, or Q–EGCG and ingested supplements in a double-blinded fashion for 2 weeks before, during, and 1 week after a 3-d period in which subjects cycled for 3 $h \cdot d^{-1}$ at ~57% Wmax. Blood, saliva, and muscle biopsy samples were collected before and after 2 weeks of supplementation and immediately after the exercise bout on the third day. Blood and saliva samples were also collected 14 h after exercise. D. C. Nieman et al. Founded that two-week supplementation resulted in a significant increase in plasma quercetin for Q and Q–EGCG and granulocyte oxidative burst activity (GOBA) in Q–EGCG. Immediately after the third exercise bout, significant decreases for C-reactive protein (CRP), and plasma interleukin 6 (IL-6) and interleukin 10 (IL-10) were measured in Q–EGCG

compared with P. Granulocyte colony-stimulating factor and CRP were reduced in Q–EGCG 14 h after exercise. No group differences were measured in muscle messenger RNA expression for peroxisome proliferator-activated receptor F coactivator >, citrate synthase, or cytochrome c. In conclusion, two-week supplementation with Q–EGCG was effective in augmenting GOBA and in countering inflammation after 3 d of heavy exertion in trained cyclists.
However, the post-exercise induced decrease in oxidative burst was unaffected after bolus consumption of the same supplement [M. Konrad et al.], when hydroethidine was used as probe.
Therefore, our results confirm that the plasma membrane C-11BODIPY is a suitable probe in the evaluation of the effects on the oxidative burst of flavonoids, which increase DHR123 accumulation [*I. Peluso, H. Manafikhi, **R. Reggi**, and M. Palmery, 2014]*. Though the effect of GTE consumption on oxidative burst requires more subjects to reach statistical significance, our results are in agreement with the reduction of the p22phox subunit of the NADPH oxidase observed in hemodialysis patients after 6 months of treatment with GT [U. Vertolli et al.].
As green tea is increasingly well recognized for its antioxidant properties, U. Vertolli et al. probed the effect of consumption of 1 capsule daily of green tea as a commercially available, decaffeinated green tea capsule (1 g, catechin content 68 mg) for 6 months on fibrinogen and inflammation in dialysis patients. Chronic hemodialysis patients (N = 25) were recruited and fibrinogen, FDP-D-dimer, high sensitivity (hs) CRP and the mononuclear cell protein expression of p22phox, were assessed before, i.e. baseline and after 6 months of ingestion of 1 green tea capsule per day. After 6 months of daily green tea capsule ingestion, dialysis patients showed reduced protein expression of p22phox ($p < 0.0001$), reduced hsCPR ($p = 0.032$) and fibrinogen ($p = 0.022$) levels and increased FDP-D-dimer ($p = 0.0019$) compared to their values at baseline. These results document lower oxidative stress and inflammation with green tea capsule ingestion and suggest a likely positive impact of green tea treatment on the atherosclerotic process of ESRD patients under dialysis.

2.4.4. EFFECT OF GTE ON PLIR

In a postprandial study [*I. Peluso, H. Manafikhi, **R. Reggi**, Y. Longhitano, C. Zanza, and M. Palmery 2016*] we observed that a functional food covered by dark chocolate and containing glucomannan, inulin, fructooligosaccharides, and Bacillus coagulans strain GanedenBC30 significantly improved postprandial metabolic stress (insulin, glucose, and triglycerides), reduced the postprandial increase of UA, and improved PLIR of lymphocytes, but not of monocytes and granulocytes. We suggested that, although PLIR is a functional index that is independent of baseline levels of oxidation, measuring the ratio between the resistance to exogenous and the resistance to endogenous ROS injury, this ratio calculation could mask the effect of foods that inhibit both the exogenous ROS injury and the oxidative burst.

On the contrary, in the present study, treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3 after ingestion, whereas a non-significant increase was observed for PLIR of lymphocytes. An inverse correlation of UA with PLIR and a direct correlation of FRAP-UA with PLIR of all leukocytes were found. Therefore, though some of the protective effects of catechins have been ascribed to their capability to reduce excessive UA level [T. Yokozawa et al.], in our study the inverse correlation of PLIR with UA levels, in particular with the ratio AAPH component of PLIR, confirms that UA is a major circulating antioxidant as suggested by Fabbrini et al..
These authors evaluated whether alterations in levels of circulating UA, a systemic antioxidant, affects the following:
1) systemic (plasma and saliva) non-enzymatic antioxidant capacity (NEAC);

2) markers of systemic (urinary 8-iso-prostaglandin-F2a) and muscle (carbonylated protein content) oxidative stress;
3) whole-body insulin sensitivity (percentage increase in glucose uptake during a hyperinsulinemic-euglycemic clamp procedure).
Thirty-one obese subjects (BMI 37.1 6 0.7 kg/m2) with either high serum UA (HUA; 7.1 6 0.4 mg/dL; n = 15) or normal serum UA (NUA; 4.5 6 0.2 mg/dL; n = 16) levels were studied; 13 subjects with HUA levels were studied again after reduction of serum UA levels to 0 by infusing a recombinant urate oxidase. HUA subjects had 20–90% greater NEAC, but lower insulin sensitivity (40%) and levels of markers of oxidative stress (30%) than subjects in the NUA group (all $P < 0.05$). Acute UA reduction caused a 45–95% decrease in NEAC and a 25–40% increase in levels of systemic and muscle markers of oxidative stress (all $P < 0.05$) but did not affect insulin sensitivity (from 168 6 25% to 156 6 17%, P = NS).
E. Fabbrini et al. reported that rasburicase treatment, in subjects who had high serum UA concentrations, caused a marked decrease in plasma FRAP and a significant increase in urinary isoprostanes/creatinine ratio and in skeletal muscle protein carbonylation. On the other hand, though the effects of GTE consumption on oxidative burst and AAPH-induced lipoperoxidation require more subjects to reach statistical significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes. Therefore, the ratio-based calculation of the PLIR is able to appreciate differences also with a low number of subjects in monocytes and granulocytes.
Our results suggest that PLIR reveals the prooxidant effect of a green tea extract, a result in agreement with the Food and Drug Administration and the European Food Safety Administration (EFSA) [Dwyer JT et al.] that denied the proposed health claims and decreased risk of non-communicable diseases for green tea (https://www.fda.gov/food/ingredientspackaginglabeling/labelingnutrition/ucm073207.htm, https://www.fda.gov/food/ingredientspackaginglabeling/ labelingnutrition/ucm301644.htm, http://onlinelibrary.wiley. com/doi/10.2903/j.efsa.2011.2055/epdf).

Furthermore, the Supplement Information Expert Committee suggests that consumption of green tea extract can induce liver damage [Sarma DN et al.] and EFSA recently launched a public call for data to acquire documented information for the risk assessment of green teacatechins (http://www.efsa.europa.eu/it/data/call/170410).

This was followed by publication of adverse event case reports involving green tea products. In response, the US Pharmacopeia (USP) Dietary Supplement Information Expert Committee (DSI EC) systematically reviewed the safety information for green tea products in order to re-evaluate the current safety class to which these products are assigned. DSI EC searched PubMed (January 1966–June 2007) and EMBASE (January 1988–June 2007) for clinical case reports and animal pharmacological or toxicological information. Reports were also obtained from a diverse range of other sources, including published reviews, the US FDA MedWatch programme, USP's MEDMARX® adverse event reporting system, the Australian Therapeutic Goods Administration, the UK Medicines and Healthcare Products Regulatory Agency, and Health Canada's Canadian Adverse Drug Reaction Monitoring Program.

In addition, the Committee analysed information concerning historical use, regulatory status, and current extent of use of green tea products. A total of 216 case reports on green tea products were analysed, including 34 reports concerning liver damage. Twenty-seven reports pertaining to liver damage were categorized as possible causality and seven as probable causality. Clinical pharmacokinetic and animal toxicological information indicated that consumption of green tea

concentrated extracts on an empty stomach is more likely to lead to adverse effects than consumption in the fed state. Based on this safety review, the DSI EC determined that when dietary supplement products containing green tea extracts are used and formulated appropriately the Committee is unaware of significant safety issues that would prohibit monograph development, provided a caution statement is included in the labelling section. Following this decision, USP's DSI ECs may develop monographs for green tea extracts, and USP may offer its verification programmes related to that dietary ingredient [Sarma DN et al.].

Green tea catechins have the ability to lower UA levels [Peluso I, Serafini M (2017)], the major plasma antioxidant.

3. AIM 2:
RELATIONSHIP BETWEEN THE PEROXIDATION OF LEUKOCYTES INDEX RATIO AND A FUNCTIONAL MATHEMATICAL INDEX INCLUDING URIC ACID LEVELS AND HEALTH-RELATED HABITS: A PILOT STUDY

3.1. INTRODUCTION

An inverse correlation of UA with PLIR has been found in healthy subjects [*Peluso I, Manafikhi H, Raguzzini A, Longhitano Y, **Reggi R**, Zanza C, Palmery M (2016)*] and we previously suggested that UA could affect PLIR in two different ways: acting as antioxidant (at physiological concentration) on all leukocytes and inducing oxidative burst in reactive oxygen species (ROS)-producing cells (at values higher than the saturation concentrations) *[Peluso I, Manafikhi H, **Reggi R**, Longhitano Y, Zanza C, Palmery M (2016)*].

3.2. URIC ACID AND OXIDATIVE STRESS

UA, the end product of purine metabolism in humans produced by hypoxanthine and xanthine under the catalysis of xanthine oxidase (XO), is a potent endogenous antioxidant in the extracellular environment [Peluso I, Raguzzini A. 2016]. UA is the final product of endogenous and dietary purine metabolism. UA is a weak acid with pKa of 5.75 in the blood and 5.25 in the urine [Desideri G et al.]. UA was initially considered an inert waste product of purine metabolism able to crystallize at high concentrations, causing gouty arthritis and renal stones. Recent scientific evidences demonstrated that UA exerts different biological effects, depending on its chemical microenvironment, including a protective antioxidant activity as well as a dangerous pro-oxidant action. It has been proposed that higher serum levels of urate may be of selective advantage in the evolution of hominids because of its antioxidant effects. On the other hand, hyperuricemia is associated with multiple diseasesin humans and points to the deleterious effects of high concentrations of urate (Figure 3.1) [So A et al.].
The relative hyperuricemia in humans has raised questions about its evolutionary advantages, and its association with diseases requires understanding how it can become deleterious at high concentrations. Initially, UA was considered an inert waste product that crystallizes at high concentrations to form renal stones and provoke gouty arthritis. Subsequently, UA was recognized to be a powerful antioxidant that scavenges singlet oxygen, oxygen radicals, and peroxynitrite and chelates transition metals, to reduce, for instance, iron ion–mediated ascorbic acid oxidation.

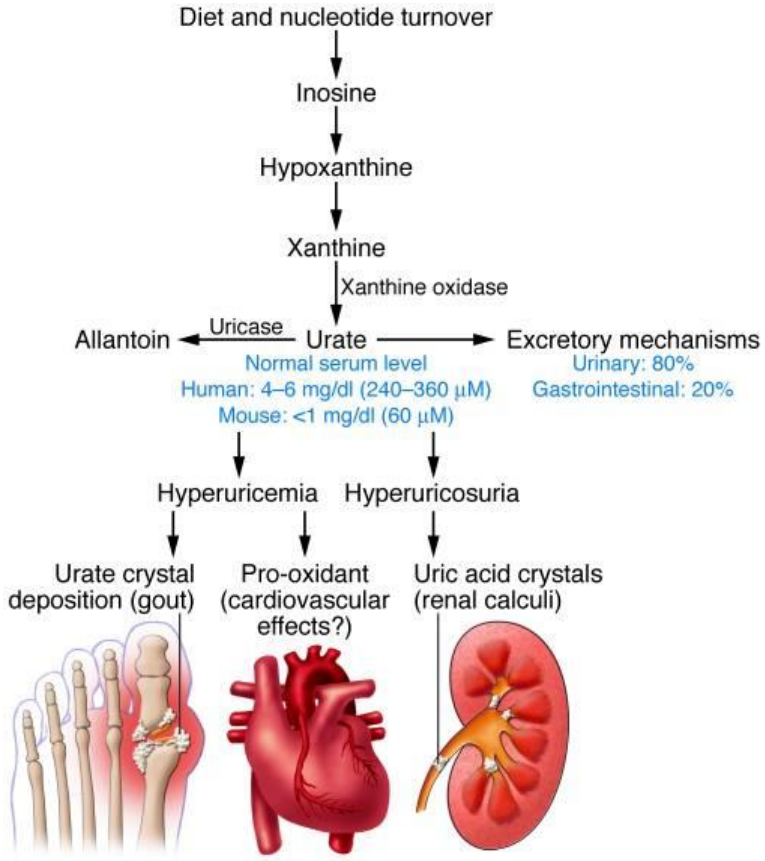

Figure 3.1 Pathways of urate homeostasis. Summary scheme of the pathways to produce uric acid, to convert it into allantoin by the liver enzyme uricase, and to excrete it. The balance between these pathways regulates blood urate concentrations, which are higher in humans and apes due to inactivation of the uricase genes. Hyperuricemia can lead to gout and possibly to cardiovascular effects, whereas hyperuricosuria may lead to uric acid crystal–induced pathologies. [So A, Thorens B. Uric acid transport and disease. J Clin Invest. 2010 Jun;120(6):1791-9.]

Urate thus accounts for approximately half of the antioxidant capacity of human plasma, and its antioxidant properties are as powerful as those of ascorbic acid [Ames BN et al.]. As illustrated in Figure 3.2A, UA can prevent peroxynitrite-induced protein nitrosation [Whiteman M et al.], lipid and protein peroxidation [Muraoka S et al.], and inactivation of tetrahydrobiopterin [Kuzkaya N et al.], a cofactor necessary for NOS. UA also protects LDL from Cu^{2+}-mediated oxidation (Figure 3.2B). Together, these antioxidant actions underlie the protective effects of UA action in cardiovascular diseases, aging, and cancer [Ames BN et al.].

In vitro and cellular studies have nevertheless demonstrated that depending on its chemical microenvironment, UA may also be pro-oxidant. For instance, although UA can protect native LDL particles against Cu^{2+}-induced oxidation, it also increases the oxidation of already oxidized LDLs, which contain lipid peroxidation products [Bagnati M et al.] and this dual role appears to depend on the presence of transition metals. As illustrated in Figure 3.2A, when UA is oxidized by peroxynitrites, urate radicals are produced that could propagate the pro-oxidant state [Santos CX et al.], but in the plasma they are rapidly inactivated by reaction with ascorbic acid. NO, described initially as an endothelial cell–derived relaxing factor, is an important regulatory molecule in the cardiovascular system, and reduced NO levels are associated with hypertension and insulin resistance. Urate can react directly with NO under aerobic conditions to generate an unstable nitrosated UA product that can transfer NO to other molecules such as glutathione (Figure 3.2). Under anaerobic conditions, urate is converted in the presence of NO into stable 6-aminouracil

[Gersch C et al.]. The possibility that increased urate plasma levels can reduce NO bioavailability has been tested in rats treated with the uricase inhibitor oxonic acid. The consequent increase in plasma UA was indeed associated with a decrease in plasma nitrites/nitrates (NOx). Similarly, direct exposure of endothelial cells to UA slightly reduces basal or VEGF-stimulated NO production [Khosla UM, et al.]. Thus, UA can dose-dependently reduce NO bioavailability. Although a direct chemical reaction of urate with NO could explain the decrease in plasma NOx, there is evidence that in vivo urate can decrease NO production by interfering with its biosynthesis. For instance, in pulmonary endothelial cells, UA reduces NO production by a mechanism that depends on UA increasing the activity of arginase, which diverts l-arginine to urea production instead of to NO production by eNOS (Figure 3.2C). Another pro-oxidant action of urate has been described during adipogenic differentiation of 3T3-L1 cells (Figure 3.2). When these cells are induced to differentiate into adipocytes, addition of UA at physiological concentrations further increases ROS production by a mechanism that involves activation of NADPH oxidase. This effect in adipocytes may participate in the induction of inflammation and insulin resistance of adipose tissue observed in obesity [Hotamisligil GS.]. Together, the available information indicates that UA has complex chemical and biological effects and that its pro-oxidant or NO-reducing properties may explain the association among hyperuricemia, hypertension, the metabolic syndrome, and cardiovascular disease. In addition, when hyperuricemia leads to the formation of microcrystals, it leads to joint and renal inflammation. Chronic inflammation leads to bone and cartilage destruction, and chronic hyperuricemia and hyperuricosuria in gouty patients are also frequently associated with tubulointerstitial fibrosis and glomerulosclerosis, signs of local renal inflammation [Kang DH et al.].

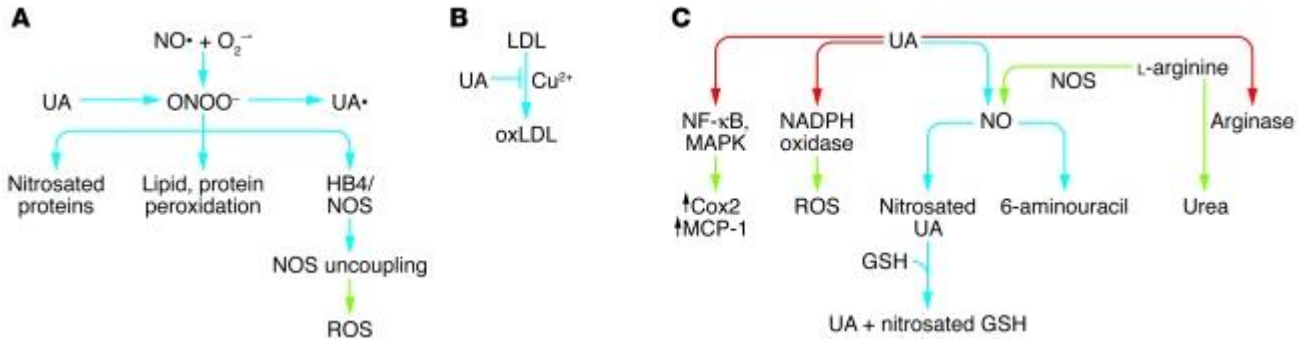

Figure 3.2 Antioxidant and pro-oxidant effect of uric acid. Antioxidant activities. (A) Peroxynitrites ($ONOO^-$) are produced from the reaction of nitric oxide (NO•) with superoxide ($O_2^{-\bullet}$). Peroxynitrites can induce protein nitrosation and lipid and protein peroxidation and block tetrahydrobiopterin (HB4), a cofactor necessary for NOS activity. In the absence of HB4, NOS produces ROS. Uric acid (UA) can directly inactivate peroxynitrite by a reaction that generates uric acid radicals (UA•); these can be rapidly eliminated by plasma ascorbic acid. (B) Uric acid can also prevent Cu2+-induced oxidation of LDL, a reaction that may protect against atherosclerosis development. (C) By enhancing arginase activity, uric acid diverts l-arginine from NO production to urea production. Uric acid can also directly react with NO to generate nitrosated uric acid, and the nitroso group can then be transferred to glutathione (GSH) for transport to another recipient molecule. In the presence of oxygen, uric acid reacts with NO to produce the stable species 6- aminouracil. Uric acid uptake in adipocytes activates NADPH oxidase and increase production of ROS, which can initiate an inflammatory reaction. In vascular smooth muscle cells, uric acid can activate the NF-κB and MAPK pathway and increase cyclooxygenase and MCP-1 production. Blue arrows, chemical reactions; green arrows, products from enzymatic or signaling pathways; red arrows, activation of enzymatic activities. [So A, Thorens B. Uric acid transport and disease. J Clin Invest. 2010 Jun;120(6):1791-9.]

Oxidative stress is associated with the metabolic syndrome, a cluster of cardiovascular risk factors including dyslipidemia, abnormal glucose tolerance, hypertension, and obesity [F. Bonomini et al.]. Despite the antioxidant effect of UA, hyperuricaemia is associated with obesity and insulin

resistance [W. Y. Tae et al.] and has been proposed as a component of the metabolic syndrome [R. Kawamoto et al.]. A great number of physiological functions are controlled by redox-responsive signalling pathways. These, for example involve:
(i) redox regulated production of NO
(ii) ROS production by phagocytic NAD(P)H oxidase (oxidative burst)
(iii) ROS production by NAD(P)H oxidases in nonphagocytic cells
(iv) regulation of vascular tone and other regulatory functions of NO•
(v) ROS production as a sensor for changes of oxygen concentration
(vi) redox regulation of cell adhesion
(vii) redox regulation of immune responses
(viii) ROS-induced apoptosis and other mechanisms.
Systemic oxidative stress is common in patients with cognitive impairment and markers of lipid peroxidation are elevated and total antioxidant capacity is decreased in Alzheimer's disease (AD) and mild cognitive impairment (MCI) [Schrag M et al.].
The effect of UA on oxidative stress depends on its concentration [*Palmery M.,* ***Reggi R.,*** *Peluso I. Uric acid and cognition: what is the connection?*]. UA at concentrations below the saturation level for urate precipitation (6.0 mg/dl) inhibits significantly oxidations caused by the Fenton reaction [Waugh WH.] and by the peroxyl radicals [Muraoka S et al.]. In their study, they observed that UA efficiently scavenged carbon-centered and peroxyl radicals derived from the hydrophilic free radical generator 2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH). All damage to biological molecules, including protein, DNA and lipids induced by AAPH, was strongly prevented by UA. In contrast, alpha-tocopherol had little effect on damage to biological molecules. Lipid peroxidation by the lipophilic free radical generator 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) was little inhibited by UA, but not by alpha-tocopherol. Copper-induced lipid peroxidation was inhibited by UA and alpha-tocopherol. NADPH- and ADP-$Fe^{(3+)}$-dependent microsomal lipid peroxidation was efficiently inhibited by alpha-tocopherol, but not by UA. On the other hand, UA induces the nuclear translocation of the nuclear factor (erythroid- derived 2)-like 2 (Nrf2) protein in primary astrocytic cultures [Bakshi R et al.], leading to the transcription of antioxidant genes through the antioxidant responsive elements (ARE) (Figure 3.3).

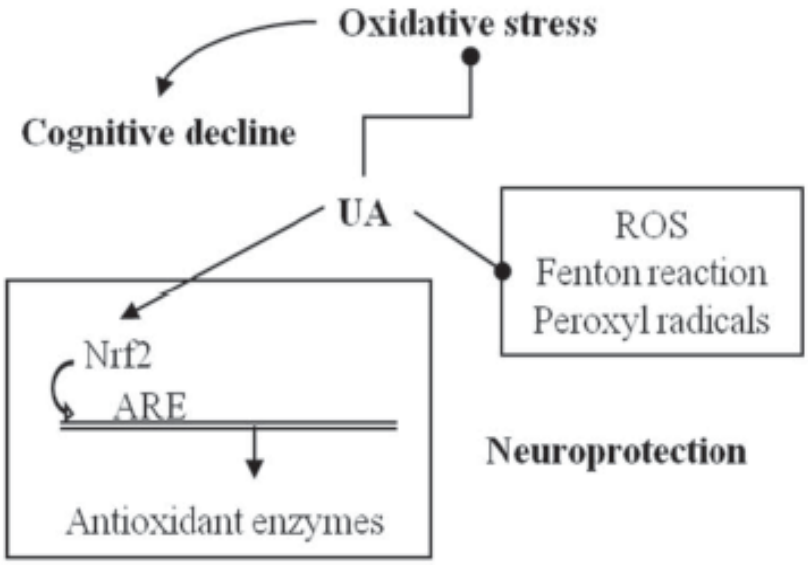

Figure 3.3 Uric acid and neuroprotection against oxidative stress. [Palmery M., Reggi R., Peluso I. Uric acid and cognition: what is the connection? Biomedical Reviews 2016; 27: 51 – 57]

Although, low levels of UA are detrimental to the neurons, UA has been identified as an endogenous danger signal for immune system [Fang P et al.] (Figure 3.4).

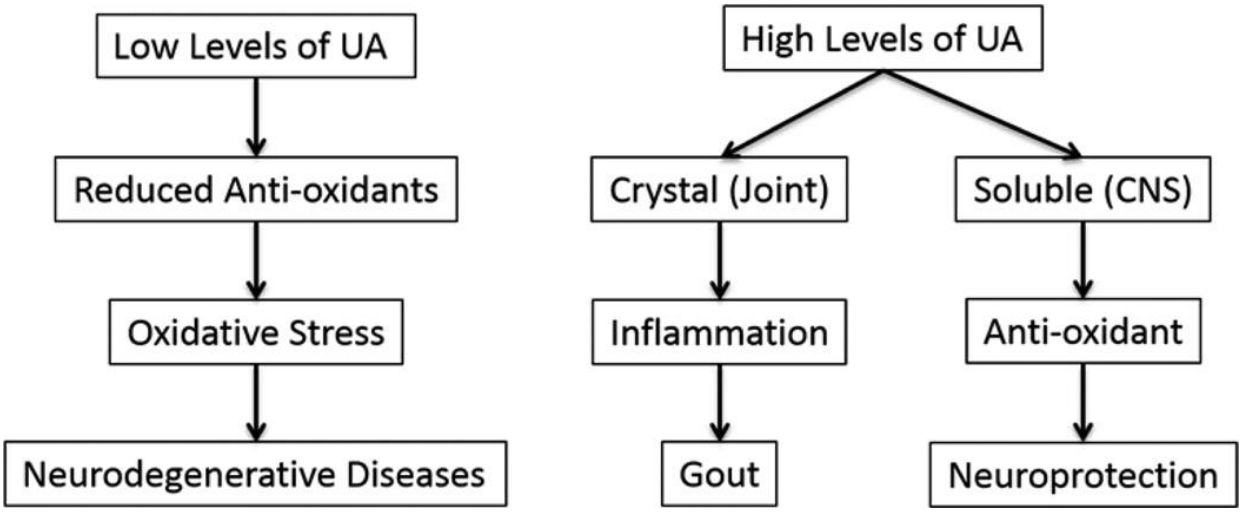

Figure 3.4 UA's effect is organ-dependent, plasma level dependent and soluble/crystal status dependent. UA-uric acid; CNS-central nervous system [Fang P, Li X, Luo JJ, Wang H, Yang XF. A Double-edged Sword: Uric Acid and Neurological Disorders. Brain Disord Ther. 2013 Nov 1;2(2):109.]

Although our previous studies (postprandial and GTE) suggest that UA has a key role in PLIR also life styles, including dietary habit and physical activity (PA) have a well-known role in the development of oxidative stress and immune impairment. In addition to a low PA and a low adherence to Mediterranean diet, eating out of home (EOH) is significantly increasing worldwide and has been associated with risk of becoming overweight or obese [Nago ES et al.]. In this pilot study, we aimed to investigate the relationship between the PLIR and various potential determinants, such as UA levels, PA, and dietary habits and the possibility to apply, in humans, the functional mathematical index (FMI) previously used as "global quality" index for foods [Finotti E et al.]. With the FMI approach, based on the concept of Euclidean distance [Finotti E et al.], it is possible to obtain an adimensional index including various health-related aspects having different units of measurement, such as serum clinical markers and life style factors.

3.2.1. METHODS

We selected 19 subjects (12 men and 7 women), who volunteered in response to advertisements at "La Sapienza" University of Rome, according to the following criteria: being healthy, being aged between 25 and 50 years, and taking no drugs, supplements, probiotics, or functional foods. Exclusion criteria include smoking habits and adherence to special diets (vegetarian, vegan). Body Mass Index was calculated as weight (in kilograms, Kg) divided by height (in meters' square, m^2). Adherence level to Mediterranean Diet (Ad-MD) and PA were calculated by the MedDietScore Software [Panagiotakos DB et al.] and the "Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire" (IPAQ) (https://

www.academia.edu/5346814/Guidelines_for_Data_Processing_and_Analysis_of_the_International_Physical_ Activity_Questionnaire_IPAQ_Short_and_Long_Forms_ Contents).
The IPAQ assesses physical activity undertaken across a comprehensive set of domains including:
a. leisure time physical activity
b. domestic and gardening (yard) activities
c. work-related physical activity
d. transport-related physical activity;
We used the the IPAQ Scoring Protocol (Short Forms) in order to evaluate the Continuous Score expressed as Metabolic Equivalent of Task-min per week: MET level x minutes of activity/day x days per week
Calculation:
Walking MET-minutes/week = 3.3 * walking minutes * walking days
Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days
Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days
Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores.
There are three levels of physical activity proposed to classify populations:
1. Low: Total physical activity of < 600 MET-minutes/week.
2. Moderate: Total physical activity of at least 600 MET-minutes/week (OR 3 or more days of vigorous activity of at least 20 minutes per day OR 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day)
3. High:
A separate category labelled 'high' can be computed to describe higher levels of PA.
The two criteria for classification as 'high' are:
a) vigorous-intensity activity on at least 3 days achieving a minimum Total physical activity of at least 1500 MET-minutes/week
OR
b) 7 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 3000 MET-minutes/week.

The Mediterranean dietary pattern has become customary to be represented in the form of a pyramid, the base of which refers to foods which are suggested to be consumed most frequently and the top of the pyramid to those foods consumed rarely. An index (diet score) that estimates the adherence level to Mediterranean diet was developed and associated with cardiovascular disease risk and biomarkers. Panagiotakos DB et al. presented a computer program that can easily calculate this diet score, as well as its association with cardiovascular disease risk.
The base of pyramid refers to foods which are suggested to be consumed most frequently and the top of the pyramid to those foods consumed rarely. The remaining food patterns occupy intermediate positions (Figure 3.5). In particular, this dietary pattern consists of:
(a) daily consumption of non-refined cereals and products (8 servings/day), vegetables (2–3 servings/day), fruits (4–6 servings/day), olive oil (in daily cooking as the main added lipid) and nonfat or low fat dairy products (1–2 servings/day);
(b) weekly consumption: of potatoes (4–5 servings/week), fish (4–5 servings/week), olives, beans, pulses and nuts (>4 servings/week) and more rare poultry (1–3 servings/week), eggs and sweets (1–3 servings/week)
(c) monthly consumption: of red meat and meat products (4–5 servings/month).
This pattern is also characterized by moderate consumption of wine (1–2 wineglasses/day), which usually accompanies meals. In addition, although intake of milk is moderate, the consumption of cheese and yogurt is relatively high. White cheese is regularly added to salads and accompanies vegetable stews. It has to be noticed that the described dietary pattern is low in saturated fat (≤7–8%

of energy), with total fat ranging from <25% to >35% of energy, and ratio of monounsaturated-to-saturated fats >2.

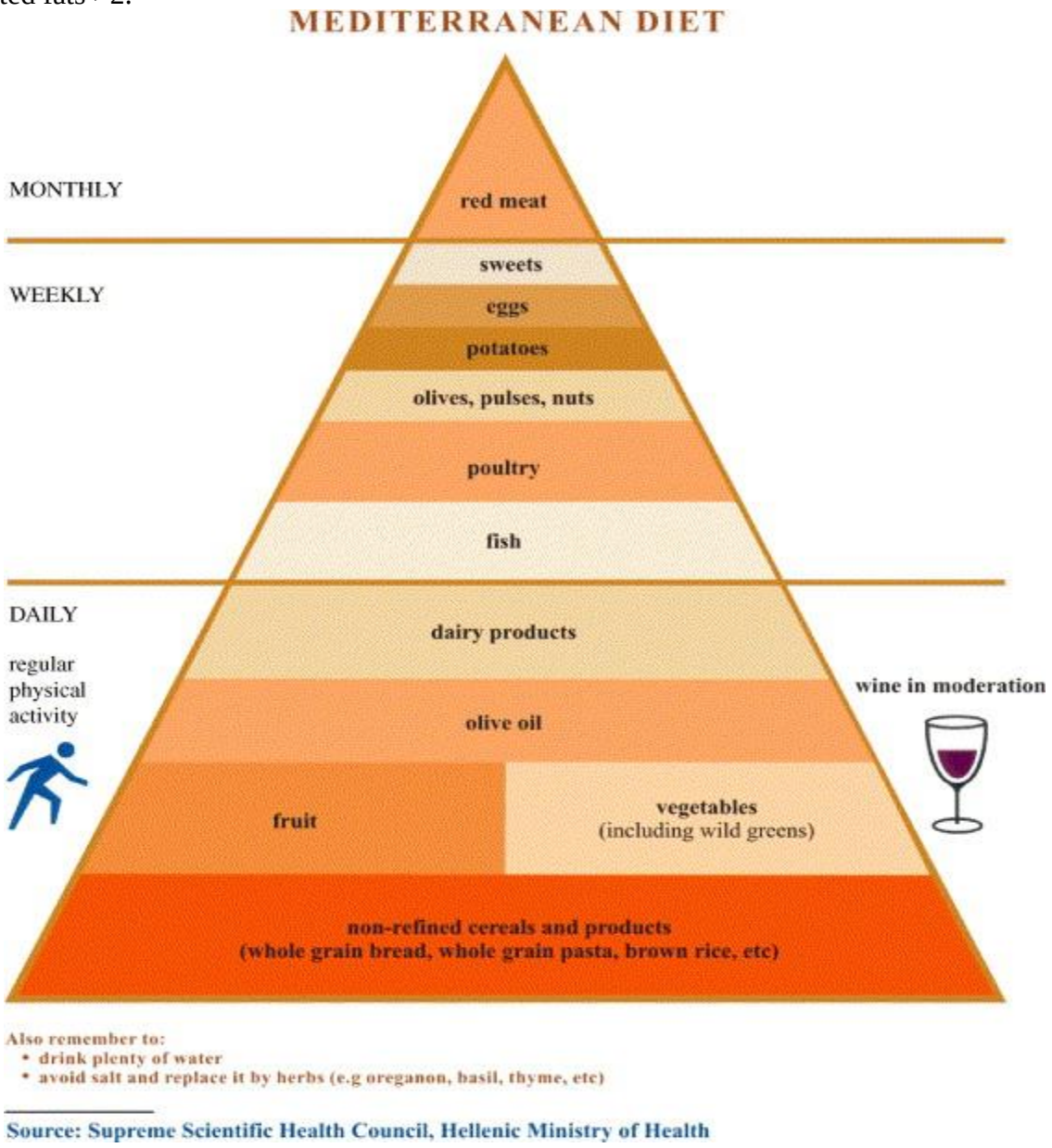

Figure 3.5 The traditional Mediterranean diet pyramid. [Panagiotakos DB, Milias GA, Pitsavos C, Stefanadis C (2008) MedDietScore: a computer program that evaluates the adherence to the Mediterranean dietary pattern and its relation to cardiovascular disease risk. Comput Methods Progr Biomed 83(1):73–77.]

A software program in Microsoft Visual Basic 6.0 (Service Pack 6) was developed for calculation of the proposed Mediterranean diet score (Table 3.1). In the main form (Figure 3.6) the user is asked to state the frequency of consumption of every listed food, by clicking with the mouse at the respective option button. Every time, that an option button is selected, the respective red circle turns to green color indicating that selection has been made. This feature makes it easier to the user to find out which food items remain unanswered. When frequency of consumption for all listed foods has been selected, the program automatically calculates the diet score (a value from 0 to 55), based on the theoretical background described above. Moreover, if we assume that a score equal to 55

represents 100% adherence to the Mediterranean dietary pattern, then a score equal to *k* represents (*k*/55) × 100% agreement to this pattern.

How often do you consume:	Frequency of consumption (servings/month):					
	Never	1-4	5-8	9-12	13-18	>18
Non-refined cereals (whole grain bread, pasta, rice, etc.)	0	1	2	3	4	5
Potatoes	0	1	2	3	4	5
Fruits	0	1	2	3	4	5
Vegetables	0	1	2	3	4	5
Legumes	0	1	2	3	4	5
Fish	0	1	2	3	4	5
Red meat and products	5	4	3	2	1	0
Poultry	5	4	3	2	1	0
Full fat dairy products (cheese, yoghurt, milk)	5	4	3	2	1	0
Use of olive oil in cooking (times/week)	Never	Rare	<1	1-3	3-5	Daily
	0	1	2	3	4	5
Alcoholic beverages (ml/day, 100 ml = 12 g ethanol)	<300	300	400	500	600	>700 or 0
	5	4	3	2	1	0

Table 3.1 The Mediterranean diet score. [Panagiotakos DB, Milias GA, Pitsavos C, Stefanadis C (2008) MedDietScore: a computer program that evaluates the adherence to the Mediterranean dietary pattern and its relation to cardiovascular disease risk. Comput Methods Progr Biomed 83(1):73–77.]

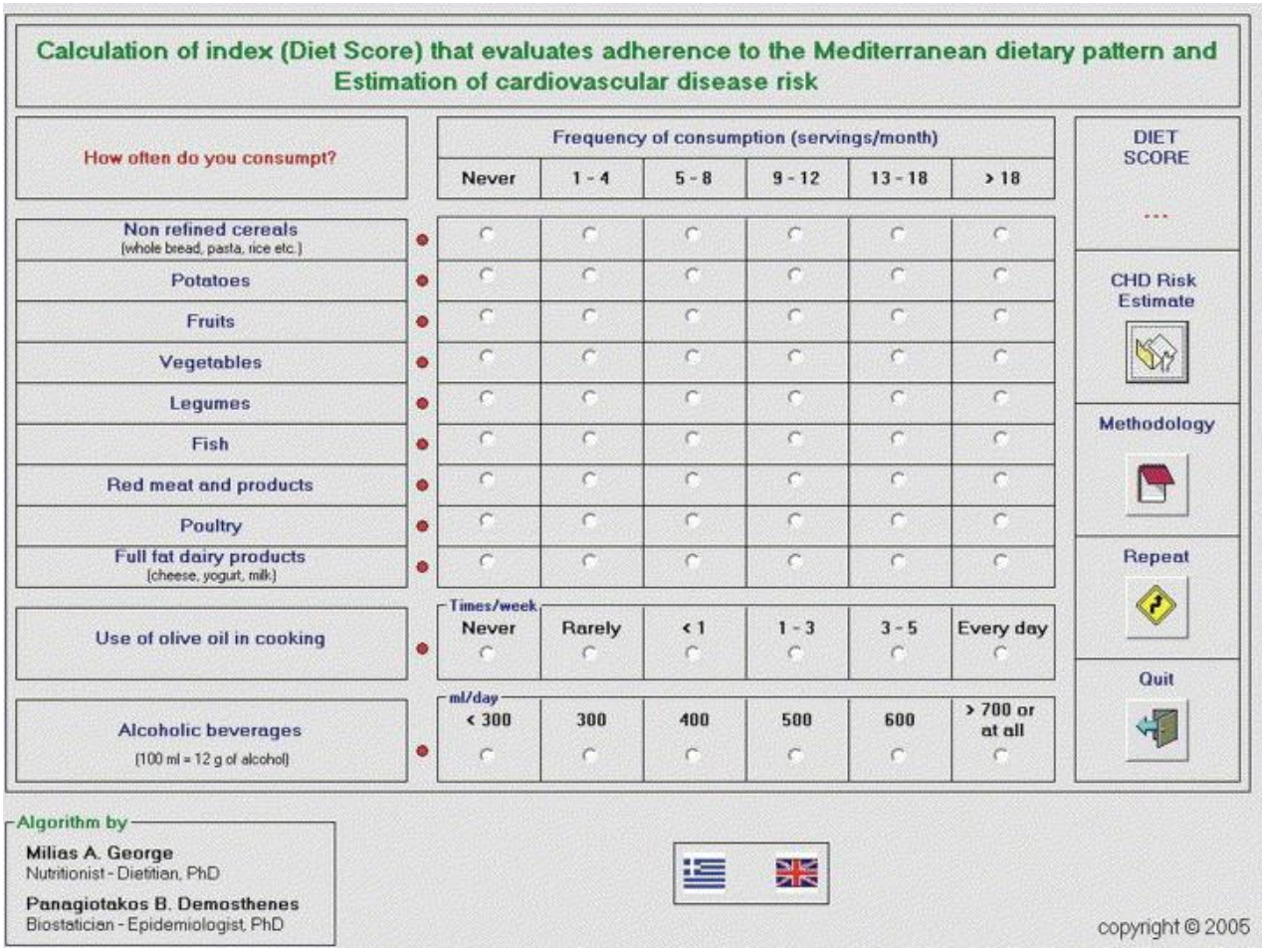

Figure 3.6 Main form of the program. [Panagiotakos DB, Milias GA, Pitsavos C, Stefanadis C (2008) MedDietScore: a computer program that evaluates the adherence to the Mediterranean dietary pattern and its relation to cardiovascular disease risk. Comput Methods Progr Biomed 83(1):73–77.]

Besides the calculation of the diet score, the program calculates also an estimation of the cardiovascular disease risk based on the values of the score (Figure 3.7).

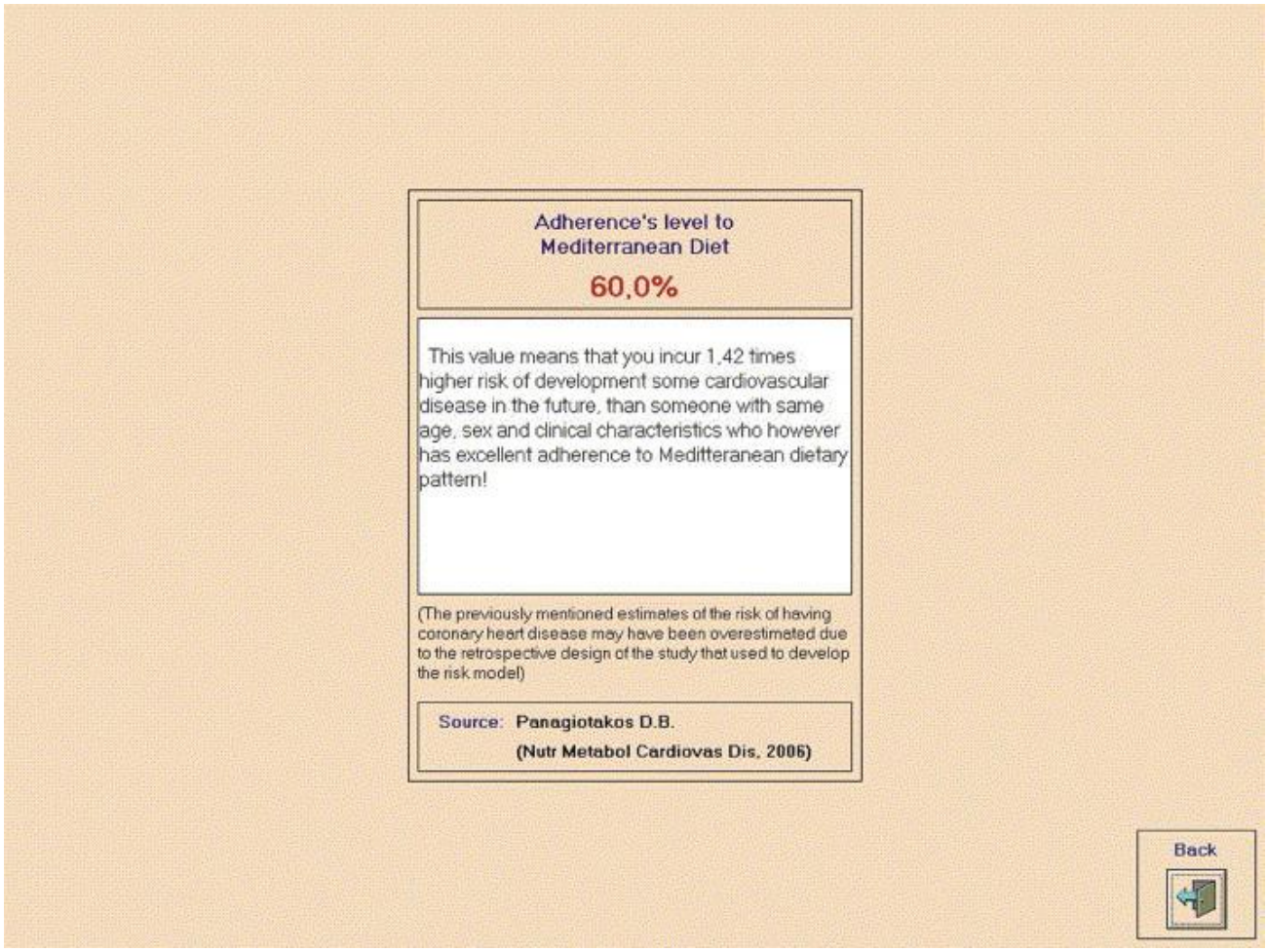

Figure 3.7 Sample of advice given after diet score has been calculated. [Panagiotakos DB, Milias GA, Pitsavos C, Stefanadis C (2008) MedDietScore: a computer program that evaluates the adherence to the Mediterranean dietary pattern and its relation to cardiovascular disease risk. Comput Methods Progr Biomed 83(1):73–77. doi:10.1016/j.cmpb.2006.05.003]

Participants were also asked to indicate their usual weekly frequency (every day, 2–3 times, once, or never) of EOH at fast food (EOH-F) or other EOH (EOH-R: restaurant and EOH-B: bar).
UA and PLIR measurements were performed as previously described, at "La Sapienza" university of Rome. In particular, PLIR was calculated applying the previously described:

PLIR = (RATIO AAPH × RATIO PMA Trolox) /

(RATIO AAPH Trolox × RATIO PMA).

PLIR components are described in Table 3.2. The RATIO is the ratio of green (as a result of oxidation) to red fluorescence (the fluorescence of the non-oxidized probe) of the lipophilic 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY).
The RATIO was evaluated in leukocytes treated as previously described with phorbomyristate13-acetate (PMA), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), and/or the standard antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

	Mean	SD
PLIR-L components		
RATIO PMA	0.16	0.05
RATIO AAPH	0.48	0.21
RATIO Trolox PMA	0.16	0.05
RATIO Trolox AAPH	0.16	0.08
PLIR-M components		
RATIO PMA	0.21	0.06
RATIO AAPH	0.42	0.20
RATIO Trolox PMA	0.20	0.06
RATIO Trolox AAPH	0.15	0.06
PLIR-G components		
RATIO PMA	0.49	0.21
RATIO AAPH	0.45	0.19
RATIO Trolox PMA	0.46	0.20
RATIO Trolox AAPH	0.19	0.08
FMI components		
BMI (centred: 18.5–24.9 Kg/m^2)	23.76	4.11
Ad-MD (more: 62–100%)	52.53	6.15
EOH-F (less: 0–2 times/week)	0.74	0.73
EOH-R (less: 0–3 times/week)	1.74	1.33
EOH-B (less: 0–2 times/week)	4.47	3.10
PA (centred: 600–1499 MET/min/week)	1608.95	914.12
UA (sex centred: 3.5–7.2 mg/dl M 2.6–6 mg/dl W)	M 5.43	0.88
	W 4.96	0.71

Table 3.2 PLIR and FMI components. AAPH 2,2′-azobis(2-methylpropionamidine) dihydrochloride; Ad-MD adherence's level to mediterranean diet; BMI body mass index; EOH eating out of home at fast food (EOH-F), at restaurant (EOH-R) and at bar (EOH-B), FMI functional mathematical index, G granulocytes; L lymphocytes; M monocytes; PA physical activity; PLIR peroxidation of leukocytes index ratio; PMA phorbo-myristate13-acetate; RATIO the ratio of fluorescence of the lipophilic 4,4-difluoro-5-(4-phenyl-1 ,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY); SD standard deviation; Trolox 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UA uric acid. [Peluso I, Reggi R, Yarla NS, Longhitano Y, Palmery M. Relationship between the peroxidation of leukocytes index ratio and a functional mathematical index including uric acid levels and health-related habits: a pilot study. Eat Weight Disord. 2017 Sep 15. doi: 10.1007/s40519-017-0441-6.]

FMI is the square root of a finite sum of quantities, and each one is a function of a specific parameter of interest [Finotti E et al.].
In this study, Finotti E. et al. extended the concept of a FMI for the assessment and prediction of food quality and safety of jujube fruit, a medicinal food widely consumed in Asian countries. In this study the index has been applied to one field-grown jujube fruit harvested at eight stages of maturity and three commercial Korean jujube cultivars. The index allows quantitative evaluation of nutritional, health-promoting, and safety aspects based on reported essential amino acid and phenolic content and antioxidative and cancer-cell-inhibiting activities of the test substances.
For example, the FMI values for the antioxidative capacities ranged from 0.36 to 0.87 and for the inhibition normal and cancer cells from 0.35 to 0.86, suggesting that consumers have a choice of

selecting growth (maturity) stages of jujube fruit with optimum beneficial properties. The use of specific performance FMI values seems to be a better tool for predicting relative beneficial and adverse effects than prediction on the basis of concentrations of the nutritional and bioactive compounds. The FMI approach, that numerically scores compositional, nutritional, and health-related aspects of food, complements but does not replace standard statistical analysis of the original compositional analytical data from which this value is derived.

FMI values are higher according to the number of parameters differing from the "better condition". Each parameter contributes to the FMI on the basis of its distance from the "optimal" value (zero distance is the better condition). All parameters have two extreme acceptable values maximum and minimum. The optimum value for each parameter could be the average of the two extreme values ("centred" parameter), as well as the minimum ("less" parameter) or maximum ("more" parameter) value. In the present study, we have chosen UA, BMI, and PA as "centred" parameters, Ad-MD as "more" parameter, and EOH-F, EOH-R and EOH-B as "less" parameters (Table 3.2).

The FMI concept developed is not based on additive effects of experimental concentrations or antioxidative- or bioactivity-related parameters. The FMI concept complements and extends but does not replace statistical and other methods used to define quality parameters of food. The FMI calculations can be used for any food when all parameters are of the same type (all *more*, all *less*, or all *centered*). The mathematical FMI approach buffers (smooths) the interrelated experimentally determined concentration values, thus providing a more useful approach to determine and predict effects on chemical composition and related nutritional, health, and other quality aspects [Finotti E et al.]. Normal values of UA were fixed according to the gender's ranges and BMI according to the normal weigh definition.

We fixed PA in the range of the moderate PA levels, because it is well known that regular exercise and overtraining have opposite effects on oxidative stress and that only moderate PA has favourable effects on health [Pingitore A et al.]: although exercise leads to increate oxidative stress, the same exercise stimulus appears necessary to allow an up-regulation in endogenous antioxidant defenses according to the hormesis theory (Fig. 3.8).

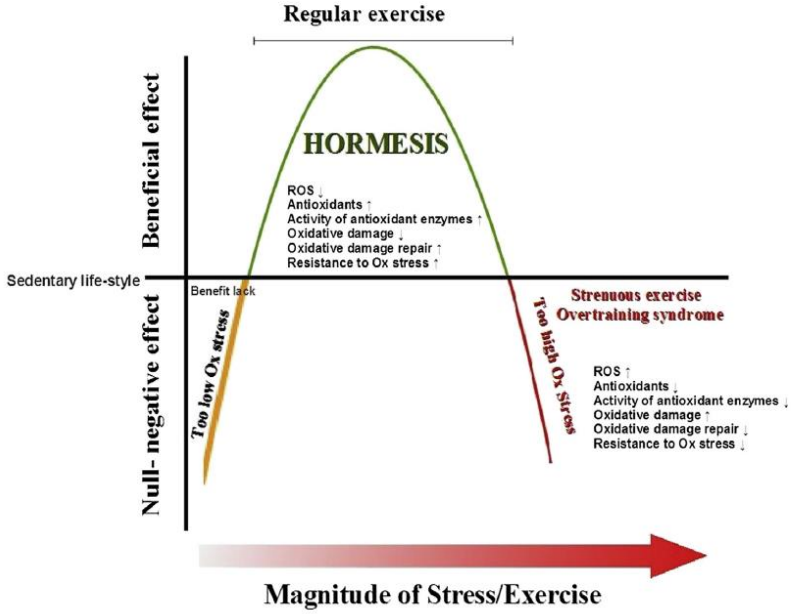

Figure 3.8 Pingitore A, Lima GP, Mastorci F, Quinones A, Iervasi G, Vassalle C (2015) Exercise and oxidative stress: potential effects of antioxidant dietary strategies in sports. Nutrition 31(7–8):916–922.

Free radicals are produced during aerobic cellular metabolism and have key roles as regulatory mediators in signaling processes. Oxidative stress reflects an imbalance between production of reactive oxygen species and an adequate antioxidant defense. This adverse condition may lead to cellular and tissue damage of components, and is involved in different physiopathological states, including aging, exercise, inflammatory, cardiovascular and neurodegenerative diseases, and cancer. In particular, the relationship between exercise and oxidative stress is extremely complex, depending on the mode, intensity, and duration of exercise. Regular moderate training appears beneficial for oxidative stress and health. Conversely, acute exercise leads to increased oxidative stress, although this same stimulus is necessary to allow an up-regulation in endogenous antioxidant defenses (hormesis). Supporting endogenous defenses with additional oral antioxidant supplementation may represent a suitable noninvasive tool for preventing or reducing oxidative stress during training. However, excess of exogenous antioxidants may have detrimental effects on health and performance. Whole foods, rather than capsules, contain antioxidants in natural ratios and proportions, which may act in synergy to optimize the antioxidant effect [Pingitore A et al.].
Ad-MD values were fixed according to the advices suggested by the MedDietScore Software. The ranges of EOH were defined on the basis of the systematic review by Nago et al. [Nago ES et al.]. It has been reported that eating at fast-food outlets (twice a week) is associated with a greater increase in body weight and waist circumference over time than eating at restaurants. However, an increased risk of obesity has been reported also for EOH breakfast [Nago ES et al.].
FMI calculation was performed using the FMI Workbench v1.1.0 software (http://sourceforge.net/projects/fmiworkbench). Spearman correlation was used to evaluate relationships between variables (Sigma Stat and Sigma plot software, JandelScientific,Inc.).

3.3. RESULTS

In the subjects participating to our study, Ad-MD, EOHB, and PA were within the parameters included in the FMI, whose mean values were more distant from the “optimum” values (Table 3.2 pag. 51).
In Table 3.3, age and values of FMI, PLIR of lymphocytes (PLIR-L), monocytes (PLIR-M), and granulocytes (PLIR-G) of subjects are reported.

	Mean	SD
Age	32.05	8.61
FMI	3.26	2.27
PLIR-L	3.16	1.20
PLIR-M	2.68	0.97
PLIR-G	2.27	0.60

FMI functional mathematical index, *G* granulocytes, *L* lymphocytes; *M* monocytes, *PLIR* peroxidation of leukocytes index ratio, *SD* standard deviation

Table 3.3

Correlation analysis showed that FMI is associated with PLIR-L and age (Table 3.4). The latter was positively associated with PA and EOH-B, suggesting that the consciousness of unhealthy dietary habit induces a compensatory PA increase to maintain weight.
With regard to Ad-MD, we did not observe significant relationship with PLIR or major PLIR components and only a weak correlation between Ad-MD and the PMA-induced oxidative burst of monocytes (RATIO PMA-M) was found (Table 3.8).

	FMI	EOH-B	PA	Ad-MD	UA
Age	0.575 (0.010)	0.664 (0.002)	0.473 (0.041)		
BMI					0.560 (0.013)
PLIR-L	0.768 (<0.001)		0.571 (0.011)		–0.468 (0.042)
RATIO AAPH-M					–0.463 (0.045)
RATIO AAPH-G					–0.465 (0.044)
RATIO PMA-M				–0.418 (0.074)	

Data are expressed as coefficient of correlation (*p*)

AAPH 2,2'-azobis(2-methylpropionamidine) dihydrochloride; *Ad-MD* adherence's level to mediterranean diet; *BMI* body mass index; *EOH-B* eating out of home at bar; *FMI* functional mathematical index; *G* granulocytes; *L* lymphocytes; *M* monocytes; *PA* physical activity; *PLIR* peroxidation of leukocytes index ratio; *PMA* phorbo-myristate13-acetate; *RATIO* the ratio of fluorescence of the lipophilic 4,4-difluoro-5-(4-phenyl-1 ,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY); *SD* standard deviation; *UA* uric acid

Table 3.4 Spearman rank-order correlation.

3.4. DISCUSSION

In this preliminary report, UA levels were positively associated with BMI, but they were inversely correlated with the PLIR of PLIR-L and to the AAPH-induced (exogenous) oxidation of monocytes and granulocytes, confirming the major role of UA as antioxidant when it is in the normal ranges, as previously suggested [***see Aim 1*** *and Peluso I, Manafikhi H,* ***Reggi*** *R, Longhitano Y, Zanza C, Palmery M (2016)*]. We observed that the weekly frequency of EOH-F was low, probably because fast food is generally perceived as unhealthy and consists of energy dense meals that are rich in saturated fats (hamburgers, French fries, and sauces). However, pizza and sandwiches, easily accessible from bar and closely linked to fast food and street food (SF) [Buscemi S et al.], could affect health. In their cross-sectional study, frequent consumers of SF were found to also frequently consume pasta/rice, bread, cured meats, eggs, cheese, seeds oil and fried foods, and rarely consume vegetables. This dietary pattern is generally considered unhealthy and is almost the opposite of a traditional, healthy Mediterranean diet [Kris-Etherton P et al.]. This finding supports the hypothesis that SF may be a proxy indicator of other components of an unhealthy lifestyle [Malik VS et al.]. They also found that with increased levels of habitual SF consumption, the HDL-cholesterol blood concentrations decreased and those of UA rose, two conditions that are associated with atherosclerosis and increased cardiovascular risk [Grundy SM et al.]. With regard to the weak correlation between Ad-MD and the PMA-induced oxidative burst of monocytes (RATIO PMA-M), it must be taken into account that catechins could affect PLIR [***see Aim 1***] and that the consumptions of catechin-rich food and beverage, such as chocolate (0–7 times/week), coffee (0–3 times/day), and tea (0–3 times/day), were variable between subjects.However, in the absence of recommended dietary allowance (RDA) concerning the consumption of polyphenols, the intake of these phytochemicals cannot be included in the FMI. On the other hand, a direct relationship was found between PA and PLIR-L. This result is in line with the oxidative stress induced by high PA when not accompanied by healthy dietary habits [Pingitore A et al.]. Most studies concerning the area of recovery from exercise in athletes focus on the use of nutritional supplements rather than on foods [Vassalle C et al.]. The study of the effects of natural food is difficult because food products are difficult to group according to the type and content of antioxidants. A diet rich in antioxidants may really be a nonpharmacologic and natural opportunity to maintain a physiological antioxidant status (Fig. 3.9) [Pingitore A et al.].

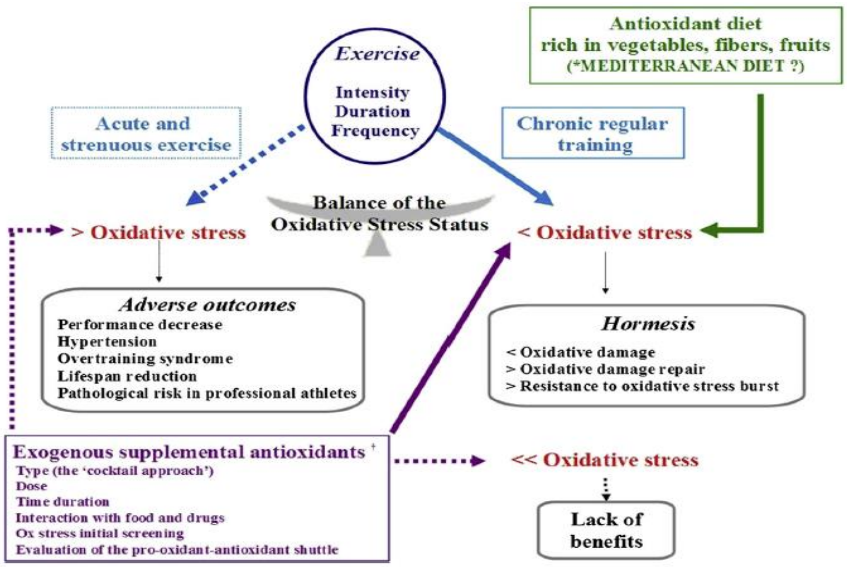

Figure 3.9 [Pingitore A, Lima GP, Mastorci F, Quinones A, Iervasi G, Vassalle C (2015) Exercise and oxidative stress: potential effects of antioxidant dietary strategies in sports. Nutrition 31(7–8):916–922.].

The benefit of antioxidant supplementation is controversial. Antioxidant treatment could eliminate the adaptive response, which appears to be systemic and reportedly reduces the incidence of a wide range of diseases. Radak Z et al. suggested that if the antioxidant treatment occurs before the physiological function-ROS dose-response curve reaches peak level, the antioxidants can attenuate function. On the other hand, if the antioxidant treatment takes place after the summit of the bell-shaped dose response curve (Fig. 3.10), antioxidant treatment would have beneficial effects on function.

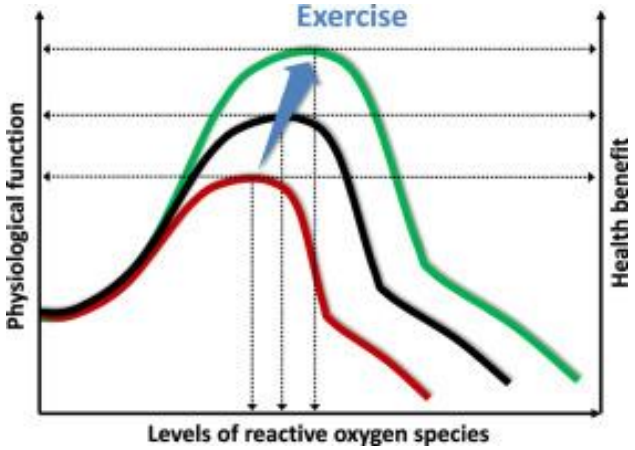

Figure 3.10 Radak Z, Ishihara K, Tekus E, Varga C, Posa A, Balogh L, Boldogh I, Koltai E (2017) Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. Redox Biol 12:285–290.

The effects of antioxidant treatment are dependent on the intensity of exercise, since the adaptive response is strongly influenced by exercise intensity. It is further suggested that levels of ROS concentration are associated with peak physiological function and can be extended by physical fitness level and this could be the basis for exercise pre-conditioning. Physical inactivity, aging or pathological disorders increase the sensitivity to oxidative stress by altering the bell-shaped dose response curve. The middle of the graph Fig. 3.11 [Radak Z et al.] represents the optimal zone of the dynamic homeostasis, while the outer line indicates the biological limitations, which cannot be reached without risk of death. The line, called functional limitation, shows the capacity of each individual and it is a mobile value. The functional/actual limit can be readily altered by exercise training. Aging decreases the rate of adaptive response, and the capacity to maintain homeostasis is decreasing, as demonstrated by the white arrows.

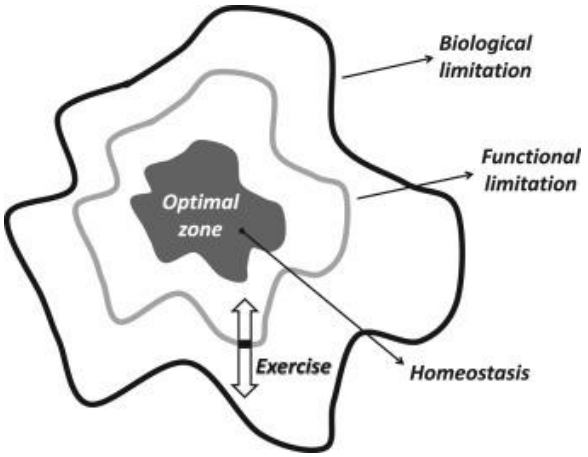

Figure 3.11 The hypothetical adaptive range Radak Z, Ishihara K, Tekus E, Varga C, Posa A, Balogh L, Boldogh I, Koltai E (2017) Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. Redox Biol 12:285–290.

Functional/actual endpoints demark the limits of individual tolerance, which are naturally below biological endpoints and are dynamic, variable values. The distance between the optimal zone and biological end points represents the zone which can be targeted to induce adaptations to extend functional/actual endpoints. In the case of a high degree of adaptation, the distance between the biological endpoints and the functional endpoints can be narrowed. In other words, the distance between the optimal zone and functional/actual endpoints can be increased (Fig. 3.12).

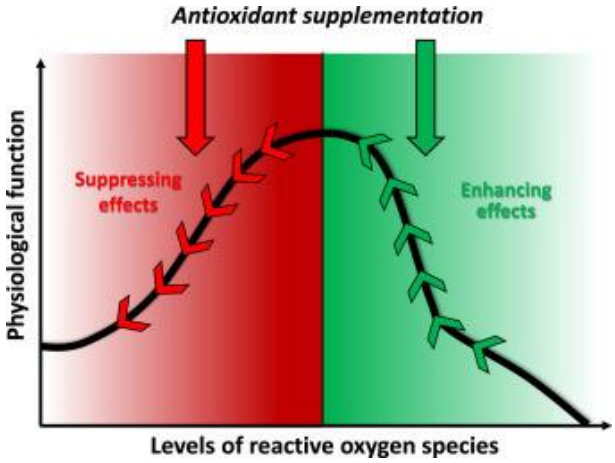

Figure 3.12 Supplementation of antioxidants before (-) the ROS levels reach the value associated with peak physiological function that can attenuate the beneficial effects of exercise. On the other hand, antioxidant treatment, after (+) the period of maximum ROS-associated function can result in decreased appearance of fatigue and/or improved function [Radak Z, Ishihara K, Tekus E, Varga C, Posa A, Balogh L, Boldogh I, Koltai E (2017) Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. Redox Biol 12:285–290]

He F. et al. concluded that, collectively, mixed results from antioxidant intervention studies may be interpreted by the variances in participants' baseline redox status, the dose and length of the antioxidant supplementation, and the choice of oxidative stress markers. Instead of antioxidant

supplements, a balanced diet consisting natural antioxidants from fruits and vegetables is sufficient to meet the dietary requirement for physically active individuals [He F et al.].
In their review Mason SA et al. [Mason SA et al.] have discussed some potentially important redox signalling pathways in skeletal muscle that are involved in acute and chronic adaptive responses to contraction and exercise. Furthermore, we have reviewed evidence investigating the impact of major exogenous antioxidants on these acute and chronic responses to exercise. The potential impact of these antioxidants on exercise responses is summarized in Table 3.5. A bulk of evidence suggests that NAC could be ergogenic through its effects on the maintenance of muscle force production during sustained fatiguing events. However, potential safety risks with higher intakes and a current lack of supportive evidence from studies using performance tests representative of typical athletic events currently warrants a conservative approach by athletes. Evidence also shows that high dose vitamins C (1 g) and E (≥260 IU) supplementation can impair some of the skeletal muscle adaptations to both endurance and resistance exercise training. Thus, while NAC might be beneficial acutely in relation to maintenance of redox state and improved muscle contraction force during a strenuous performance event, the prolonged supplementation of high doses of vitamins C and E during exercise training might promote a less oxidative redox state in muscle, thus facilitating hampered adaptive responses. Additional research is required to better establish effects of antioxidants such as α-lipoic acid, β-carotene and resveratrol on acute and chronic skeletal muscle responses to contraction and exercise. Future research should also focus on establishing a better understanding of mechanisms of action of specific antioxidants *in vivo*. Mason SA et al. feel that this is critical to establishing the utility of antioxidant supplementation in athletes, since evidence suggests that different antioxidant compounds have different dose-related effects and different redox-modulating biological properties that may affect the optimum timing of their use by athletes looking to maximize their training and competition performance.

Summary of effects of discussed antioxidants in skeletal muscle during exercise.

Antioxidant	Key findings in relation to exercise
NAC	• Human studies using both oral and intravenous NAC show improvements in time to fatigue and improved maintenance of muscle contractile force/power during exercise, particularly during sustained submaximal bouts. • Findings of studies using performance tests more representative of athletic competition are conflicting and require additional research. • The most optimal NAC dosing prior to a competitive athletic bout is currently unclear. • Currently no evidence indicates that NAC infusion impairs muscle glucose uptake, although studies using oral supplementation and higher exercise intensities are warranted. • There are potential adverse effects such as gastrointestinal distress with NAC supplementation. However, in the context of fatiguing exercise, acute oral intakes of 70 mg/kg body mass or less appear to minimize adverse effects [172].
Vitamins C and E	• Evidence is equivocal for use of vitamin C in relation to muscle contractile force and its recovery after fatiguing exercise. Studies of vitamin E supplementation currently show no evidence of benefit in relation to muscle contractile force in humans. • Use of high dose vitamin C (1 g/day) alone or in combination with at least 260 IU/day vitamin E impairs some molecular markers of mitochondrial biogenesis and antioxidant enzyme induction. However, doses of 500 mg/day vitamin C do not appear to hamper these measures. • Limited evidence in humans and rodents suggest a potential impairment in muscle hypertrophy and associated markers of redox signalling during overload, although additional research is warranted. • Despite potential cellular impairments, exercise performance outcomes with supplementation are equivocal.
Coenzyme Q10	• Findings are currently not convincing in terms of improvement in performance and maintenance of power during sustained intense and intermittent exercise with oral supplementation in humans. • Well-controlled dose-titrated studies in athletes are required to better evaluate its ergogenic potential. • Effects in skeletal muscle are unclear given the apparent poor bioavailability of oral supplementation in human skeletal muscle.
Resveratrol	• Limited evidence suggests that supplementation might impair expression of key genes involved in mitochondrial biogenesis and antioxidant enzyme induction. However, it is less likely that these changes translate into impairments in whole muscle function and whole body performance in humans. Additional studies are required in humans to more thoroughly investigate effects of resveratrol on muscle function with exercise. • While systemic bioavailability of oral supplements appears poor, studies using higher but relatively safe doses (i.e. 1–2 g/day) are scant in relation to effects on human muscle performance during exercise. • Studies are required to investigate the bioavailability and antioxidant actions of resveratrol and its metabolites in human skeletal muscle.

Table 3.5 Mason SA, Morrison D, McConell GK, Wadley GD (2016) Muscle redox signalling pathways in exercise. Role of antioxidants. Free Radic Biol Med 98:29–45.

Draeger CL et al. included 12 studies published in the last years that addressed the supplementation of antioxidant vitamins in trained volunteers (n = 05; Table 3.6) and in volunteers submitted to

endurance exercise (n = 07; Table 3.7). There are controversial results about antioxidant supplementation during high-intensity exercise

Results of the studies with endurance trained volunteers supplemented with vitamins A, C, and E

Study	Experimental design	Sample	Duration	Suplementation protocol			Result	
				Vitamin A	Vitamin C	Vitamin E	Ergogenic	Ergolytic
Tauler et al. [6]	Randomized, double-blind	15 athletes	90 d*	30 mg (β-caroten)	1000 mg	500 mg	↔	↔
Gauche et al. [9]	Randomized, double-blind	22 athletes	21 d (pre-exercise) + 2 dias (post-exercise)	6 mg (β-caroten)	200 mg	32 mg	↑	N/R
Nielsen et al. [10]	Randomized, double-blind, cross-over	15 athletes	28 d	-	400 mg	180 mg	↔	↔
Patil et al. [11]	Randomized, double-blind	37 athletes	21 d	-	-	200 mg	↔	↔
Louis et al. [12]	Randomized, double-blind	16 athletes	21 d	17.1 mg (β-caroten)	319.2 mg	48 mg	↑	N/R

* Vitamin C supplementation occurred only in the last 15 days of the study; ↑ Improved exercise performance; ↔ No results on exercise performance; N/R – not reported.

Table 3.6 Draeger CL, Naves A, Marques N, Baptistella AB, Carnauba RA, Paschoal V, Nicastro H (2014) Controversies of antioxidant vitamins supplementation in exercise: ergogenic or ergolytic effects in humans?. J Int Soc Sports Nutr 11(1):4. doi:10.1186/1550-2783-11-4

Results of the studies with untrained volunteers submitted to endurance exercise and supplemented with vitamins C e E

Study	Experimental design	Sample	Duration	Supplementation protocol		Result	
				Vitamin C	Vitamin E	Ergogenic	Ergolytic
Bloomer et al. [13]	Randomized, double-blind	15 trained and e 15 untrained subjects	14 d (pre-exercise) + 2 d (post-exercise)	2000 mg	835 mg	↔	↔
Gomez-Cabrera et al. [7]	Randomized, double-blind	14 untrained subjects e 36 rats	8 weeks	1 g (humans) and 0.24 $mg \cdot cm^{-2}$ (rodents)	-	N/R	↓
Ristow et al. [3]	Randomized, double-blind	20 trained and e 20 untrained subjects	4 weeks	1000 mg	440 mg	N/R	↓
Yfanti et al. [14]	Randomized, double-blind	21 untrained subjects	16 weeks	500 mg	400 IU	↔	↔
Yfanti et al. [5]	Randomized, double-blind	21 untrained subjects	16 weeks	500 mg	400 IU	↔	↔
Nalbant et al. [8]	Randomized	57 elderly	6 months		900 IU	↔	↔
Nakhostin et al. [15]	Randomized	16 untrained subjects	N/R	1000 mg	-	↑↓	N/R

↑ Improved exercise performance; ↓ Impaired exercise performance; ↑↓ Partial result; ↔ No results on exercise performance; IU – International Units; N/R – not reported.

Table 3.7 Draeger CL, Naves A, Marques N, Baptistella AB, Carnauba RA, Paschoal V, Nicastro H (2014) Controversies of antioxidant vitamins supplementation in exercise: ergogenic or ergolytic effects in humans?. J Int Soc Sports Nutr 11(1):4. doi:10.1186/1550-2783-11-4

The role of appropriate nutrition to counteract the oxidative stress and immune impairment in athletes is well known. Nieman DC et al. described [Nieman DC et al. 2017] effective and ineffective immunonutrition support strategies for the athlete, with a focus on the benefits of carbohydrates and polyphenols as determined from metabolomics-based procedures. Athletes experience regular cycles of physiological stress accompanied by transient inflammation, oxidative stress, and immune perturbations, and there are increasing data indicating that these are sensitive to nutritional influences. The most effective nutritional countermeasures, especially when considered from a metabolomics perspective, include acute and chronic increases in dietary carbohydrate and polyphenols. Carbohydrate supplementation reduces post-exercise stress hormone levels, inflammation, and fatty acid mobilization and oxidation. A series of studies dating back to the mid-1990s showed that ingestion of carbohydrate supplements (30–60 grams carbohydrate per hour) during prolonged, intensive exercise attenuated increases in blood neutrophil and monocyte counts, grsanulocyte phagocytosis, stress hormones, and anti-inflammatory cytokines such as IL-6, IL-10, and IL-1ra (Figure 3.13) [Nehlsen-Cannarella, S.L et al.]. At the same time, however, null effects of carbohydrate ingestion were measured for exercise-induced decrements in natural killer cell lytic activity, salivary IgA output, and T lymphocyte proliferative capacity. Thus, carbohydrate ingestion emerged as an effective but partial countermeasure to immune dysfunction during recovery from heavy exertion [Bermon S et al.].

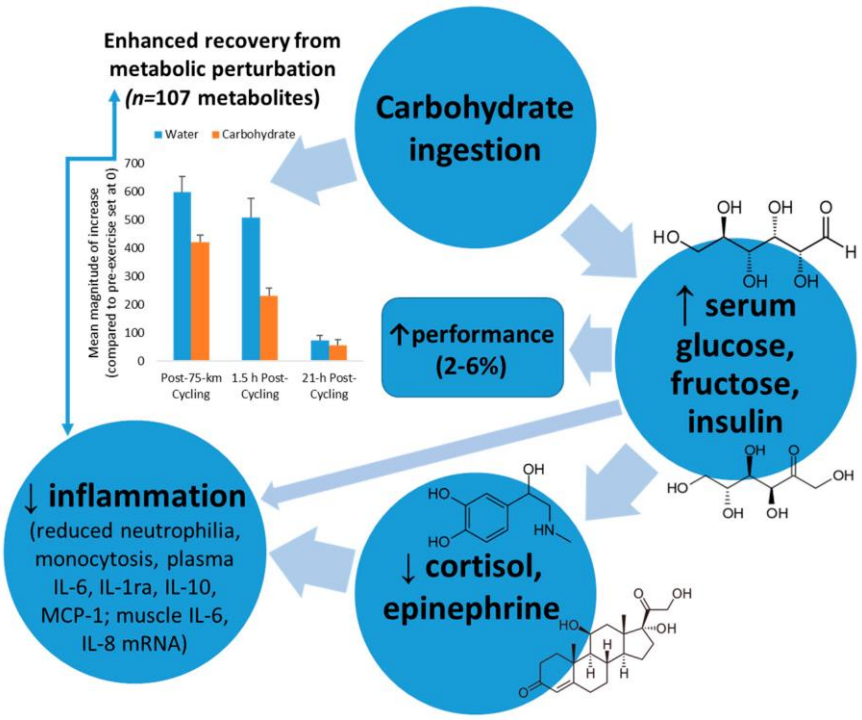

Figure 3.13 Model linking carbohydrate ingestion with attenuated inflammation and enhanced recovery from metabolic perturbation. [Nieman DC, Mitmesser SH (2017) Potential impact of nutrition on immune system recovery from heavy exertion: a metabolomics perspective. Nutrients 9(5):E513. doi:10.3390/nu9050513].

Mechanisms through which carbohydrate may exert these impressive countermeasure effects include increasing blood glucose and tissue glucose uptake leading to diminished central nervous system activation and stress hormone output, inhibiting cytokine mRNA expression, lowering beta-oxidation of lipid fuels, reducing pro-inflammatory signals, and attenuating IL-6 release from the working muscle tissue [Nieman, D.C. 2008]. Exercising with higher blood glucose levels decreases hypothalamic-pituitary-adrenal activation, leading to moderated release of adrenocorticotrophic hormone and cortisol, growth hormone, and epinephrine (Figure 3.13). Stress hormones have an intimate link with genes that control cytokine production, and the function of multiple cell types of the immune system. Exercise-carbohydrate interactions, especially during exercise and the early post-exercise recovery period, may modulate signal transduction cascades that influence protein regulatory systems [Bartlett J.D et al.].

Ingestion of fruits high in carbohydrates, polyphenols, and metabolites effectively supports performance, with added benefits including enhancement of oxidative and anti-viral capacity through fruit metabolites, and increased plasma levels of gut-derived phenolics. Metabolomics and lipidomics data indicate that intensive and prolonged exercise is associated with extensive lipid mobilization and oxidation, including many components of the linoleic acid conversion pathway and related oxidized derivatives called oxylipins. Many of the oxylipins are elevated with increased adiposity, and although low in resting athletes, rise to high levels during recovery [Nieman DC, Mitmesser SH (2017)]. Certain dietary supplements may boost immune function and reduce infection risk in individuals who are subjected to stress [Gleeson M (2016) Immunological aspects of sport nutrition]. Although there are many nutritional supplements that are claimed to boost immunity (Table 3.8), such claims are often based on very limited and often selective evidence of efficacy in animals, *in vitro* experiments, children, the elderly or clinical patients in severe catabolic states and direct evidence for their efficacy for boosting immunity or limiting exercise-induced immune depression in athletes is usually lacking.

In conclusion, it is difficult to make firm judgments about which nutritional supplements are really effective in boosting immunity or reducing infection risk in athletes. It is safe to say with reasonable confidence that individual amino acids, *Echinacea*, vitamin E and zinc are unlikely to be of significant clinical benefit [Gleeson M 2016].

Current recommendations for immuno-nutrition support in athletes include:

- Overall daily energy intake should match energy needs with >50% coming from carbohydrate
- Ingest 30–60 g of carbohydrate per hour during strenuous training sessions
- Ingest of adequate amounts of protein (1.2–1.6 g kg^{-1} per day), which should include ingestion of 0.3 g kg^{-1} in meals following training sessions
- Ingest adequate amounts of micronutrients (this can be ensured by taking a daily multi-vitamin/mineral tablet that meets the RDAs
- Take a daily oral vitamin D3 supplement of 1000 IU at the start of autumn until early spring
- Take a daily probiotic supplement containing at least 10^{10} live bacteria
- Include a variety of fruit and vegetables as part of the normal diet (at least on 5 days per week); this can be supplemented with plant polyphenol supplements or beverages (for example, green tea and non-alcoholic beer) or concentrated fruit/vegetable extracts
- Consider taking a daily 10–20 g bovine colostrum powder supplement
- Consider taking zinc and Kaloba supplements in the days leading up to an important competition in case cold symptoms should begin at that important time

This approach is likely to be of most benefit to those individuals who are particularly prone to illness. It is important to remember that nutrition is only one factor with regard to infection risk and there are several other strategies listed below that can minimise the risk of developing immune function depression or reduce the degree of exposure to pathogens and thus limit infection risk.

Minimise the chances of developing immunodepression:

- Avoid very prolonged training sessions (>2 h), overtraining and chronic fatigue
- Keep other life stresses to a minimum
- Get adequate sleep quantity (at least 7 h) and quality
- Avoid rapid weight loss
- Avoid binge drinking of alcohol
- Vaccinate against influenza if competing in the winter

Minimise the potential for transmission of infectious agents:

- Avoid sick people and large crowds in enclosed spaces if possible
- Good personal skin and oral hygiene (wash hands and use antimicrobial gels on hands; brush teeth regularly and use an antibacterial mouth rinse)
- Never share drink bottles, cutlery, towels and so on
- Avoid putting hands to eyes and nose (a major route of viral self-inoculation)

Nutrition supplement and evidence rating	Proposed mechanism of action	Evidence of efficacy
β-glucans ●●○○○	Polysaccharides derived from the cell walls of yeast, fungi and oats that stimulate innate immunity	Effective in mice inoculated with influenza virus but mixed results from human studies for immune modulation and URS incidence
Bovine colostrums ●●●○○	First milk of the cow that contains antibodies, growth factors and cytokines. Claimed to boost mucosal immunity and increase resistance to infection	Several studies in athletes that indicate some immune boosting effects and reduced URS incidence and duration
Carbohydrate ●●●○○	Maintains blood glucose during exercise, lowers stress hormone and anti-inflammatory cytokine responses, and thus counters immune dysfunction	Ingestion of carbohydrate (30–60 g h^{-1}) attenuates stress hormone and some (but not all) immune pertubations during exercise but only very limited evidence that this modifies infection risk in human athletes
Echinacea ●○○○○	Herbal extract that is a popular supplement among athletes. Claimed to boost immunity via stimulatory effects on macrophages and there is some *in vitro* evidence for this	Early human studies indicated possible beneficial effects but more recent, larger scale and better controlled studies indicate no effect of *Echinacea* on infection incidence or cold symptom severity
Glutamine ●○○○○	Nonessential amino acid that is a precursor in the synthesis of nucleic acids and important for rapidly dividing cells. Also an important fuel for immune cells. Plasma concentration of glutamine falls during prolonged exercise	Supplementation before and after exercise does not alter immune perturbations despite maintenance of plasma glutamine
Kaloba ●○○○○	Herbal medicine that has been shown to boost some aspects of immunity *in vitro* via stimulatory effects on macrophages	Evidence from human studies for reduction in severity and duration of symptoms of sinusitis and common cold but used as a treatment rather than as a preventative
N-3 polyunsaturated fatty acids ○○○○○	Exert anti-inflammatory effects post exercise	No evidence in exercising humans
Probiotics ●●●○○	Probiotics are live microorganisms, which when administered orally for several weeks, can increase the numbers of beneficial bacteria in the gut. This has been associated with a range of potential benefits to gut health, as well as modulation of immune function	Human studies show improvements in some aspects of acquired immunity and reduced incidence of URS and gastrointestinal problems
Quercetin ●●●○○	A plant flavonoid; *in vitro* studies show strong anti-inflammatory, anti-oxidative and anti-pathogenic effects. Animal data indicate increase in mitochondrial biogenesis and endurance performance	Human studies show some reduction in URS incidence during short periods of intensified training and mild stimulation of mitochondrial biogenesis and endurance performance in untrained subjects
Quercetin with epigallocatechin gallate ●●●○○	Flavonoid mixture promotes anti-inflammatory and anti-oxidative effects, and immune function improvement, above that of quercetin alone	Human study showed strong anti-inflammatory effect, with modest anti-oxidative effect and improvement in innate immunity but no data on URS incidence
Vitamin C ●●○○○	An essential water-soluble antioxidant vitamin that quenches reactive oxygen species and augments immunity. Reduces interleukin-6 and cortisol responses to exercise in humans	Relatively small effects on cortisol compared with carbohydrate; immune measures no different from placebo. Some evidence of efficacy in reducing URS incidence after ultramarathon events
Vitamin D ●●●●○	Fat-soluble vitamin that is mostly produced via the action of sunlight in the skin. Induces production of antimicrobial proteins, enhances natural killer cell cytolytic activity, increases the generation of reactive oxygen species in phagocytic cells, augments macrophage interleukin-1β secretion and upregulates the expression of CD14, the lipopolysaccharide receptor	Low vitamin D status is associated with low saliva immunoglobulin A secretion, low pro-inflammatory cytokine production by antigen-stimulated mononuclear cells and increased respiratory infection risk with longer lasting illness symptoms. Oral vitamin D3 supplements of around 4000 IU per day can reduce URS incidence
Vitamin E ●○○○○	An essential fat-soluble antioxidant vitamin that quenches exercise-induced reactive oxygen species and augments immunity. Good evidence for some immune boosting effects in the frail elderly	No evidence of similar benefit for younger healthy humans or athletes
Zinc ●○○○○	Zinc deficiency results in impaired immunity and zinc deficiency is not uncommon in athletes. An essential mineral that is claimed to reduce incidence and duration of colds	No evidence for reduced infection incidence with zinc supplementation in adult humans. Some (but not all) human studies suggest a reduction in duration of cold symptoms if zinc gluconate lozenges are administered within 24 h of cold symptom onset. Unlikely to be of any real benefit to athletes unless they are zinc deficient

The scientific evidence is indicated with ●●●●● meaning very strong evidence and ○○○○○ meaning limited to no evidence.

Table 3.8 Nutrition supplements (listed in alphabetical order) that are claimed to boost immunity and reduce URS incidence in athletes: proposed mechanisms of action and summary of evidence for efficacy [Gleeson M (2016) Immunological aspects of sport nutrition. Immunol Cell Biol. 94(2):117–123.]

CONCLUSION

Our previous postprandial study aimed at evaluating the effect of a functional food, the latter reduced the postprandial insulin, glucose, triglycerides, and UA levels and improved PLIR of lymphocytes [*Peluso I, Manafikhi H,* ***Reggi R****, Longhitano Y, Zanza C, Palmery M (2016)*]. The study also suggested that the reducing effect on UA of cocoa catechins can affect PLIR. From that in this Phd thesis, we keep on the evaluation of the possible use of the PLIR as redox marker in humans. In the first step we evaluate the relationships between PLIR and FRAP, its major endogenous determinant UA and FRAP-UA, by using a GTE due to its reported UA-lowering and potential pro-oxidant effects. In our study GTE consumption did not affect FRAP values whereas a nonsignificant decrease in UA and a significant increase in FRAP-UA. The latter, probably due to the catechins, could counterbalance the reduction in FRAP induced by the UA decrease. However, the FRAP assay matches the antioxidant capacity to the reducing ability and the reduced iron is critical in the onset of oxidative stress due to the Fenton reaction that generates the hydroxyl radical initiator of lipid peroxidation. The direct correlation of FRAP-UA with PLIR suggests that the iron reducing power of GTE could be more likely detrimental than beneficial. Our results suggest that PLIR, in particular PLIR of granulocytes where the differences between ratio AAPH and ratio PMA are more evident, reveals the prooxidant effect of GTE. This result is in agreement with the FDA, the EFSA, and the DSI EC conclusions. In this study a single dose of two capsules of a GTE (200mg × 2) commercially available in Italy (cod. 1820, REGISTRO INTEGRATORI https://www.salute.gov.it/imgs/C 17 pagineAree 3668 listaFile itemName 1 file.pdf) was used according to the suggested dose on the product label. Due to the pro-oxidant effect observed, no higher doses were used for ethical problem. In agreement it has been recently proposed EGCG UL of 300 mg/day based on human intervention data [Yates et al.].

Furthermore, the inverse correlation of UA with PLIR confirms the primary role of UA in the antioxidant defences. Dietary habit and physical activity are well known factors involved in redox balance. From that, in the second step we studied the relationships between PLIR and a mathematical index that considers health-related habits and UA plasma levels.

Indexes of oxidative stress, such as the OXY-SCORE and the oxidative-INDEX, use markers that do not have at the moment normal values and consider high antioxidant capacity always beneficial. However, the antioxidant capacity is strongly influenced by UA. The latter is a well known pathogenic factor when at high concentrations.

The strength of the present pilot study is that we extend the concept of the FMI, introduced in the assessment of foods' quality [Finotti E et al.] to human investigations. Each parameter contributes to the FMI on the basis of its distance from the "optimal" value (zero distance is the better condition). All parameters have two extreme acceptable values maximum and minimum. The optimum value for each parameter could be the average of the two extreme values ("centred" parameter), as well as the minimum ("less" parameter) or maximum ("more" parameter) value. In the present study, we have chosen UA, BMI, and PA as "centred" parameters, Ad-MD as "more" parameter, and EOH-F, EOH-R and EOH-B as "less" parameters. Normal values of UA were fixed according to the gender's ranges, in order to consider sex differences in the potential dangerous effects of hyperuricaemia. Although further studies are needed to evaluate PLIR as redox marker, a direct relationship was found between PA and PLIR-L. This result is in line with the oxidative stress induced by high PA when not accompanied by healthy dietary habits. On the other hand, the FMI calculation can be used for other factors associated with health and PLIR. In a postprandial study aimed at evaluating the effect of a functional food, the latter reduced the postprandial insulin,

glucose, triglycerides, and UA levels and improved PLIR of lymphocytes [*Peluso I, Manafikhi H, **Reggi** R, Longhitano Y, Zanza C, Palmery M (2016)*], but we suggested that further studies on subjects who are at risk of cardiovascular diseases are needed to investigate the relationship between postprandial dysmetabolism and PLIR of monocytes and granulocytes. Not only clinical markers (lipids, glucose, leukocytes count…), but also behavioural, psychological, genetic, and epigenetic factors affecting health could be included in the FMI in the future and could be considered as possible determinants of PLIR.

In conclusion, although further studies are needed to evaluate PLIR as redox marker, we suggest that FMI could be used as global index that integrate clinical parameters, as well as life style, genetic, and epigenetic factors to evaluate the overall effects of the many determinants of health.

LIST OF ABBREVIATION

AAPH: 2,2’-azobis(2-methylpropionamidine) dihydrochloride

ABC: ATP binding cassette

AD: Alzheimer's disease

Ad-MD: Adherence level to Mediterranean Diet

AhR: aryl hydrocarbon receptor

AP-1: activation protein-1

ARE: antioxidant responsive elements

AUC: plasma concentration-time curves

BCRP: Breast Cancer Resistance Protein

BODIPY: boron dipyrromethene difluoride

CAT: catalase

CBG: cytosolic b-glucosidase

CK: creatine kinase

CLP: cacao liquor polyphenol

Cmax: plasma concentration

COMT: catechol-O-methyltransferases

CSA: cyclosporin A

CVD: cardiovascular diseases

CYP450: cytochrome P450

DAF-2 DA: 4,5-diaminofluorescein diacetate

DAF-FMDA: 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate

DCF: 2′,7′-dichlorofluorescein

DCFH-DA: dihydrochlorofluorescein diacetate

DHEAS: [3H]-dehydroepiandrosterone sulfate

DHR123: dihydrorhodamine 123

DRW: dealcoholized red wine

DSI EC: Dietary Supplement Information Expert Committee

E+: ethidium cation

EC: (-)-epicatechin

ECD: electrochemical detection

EFSA: European Food Safety Administration

EGC: (-)-epigallocatechin

EGCG: epigallocatechin-3-gallate

EOH: eating out of home

EtOH: Ethanol

FCM: flow cytometry

FDA: Food and Drug Administration

FMI: functional mathematical index

FRAP: ferric reducing antioxidant potential

GPX: glutathione peroxidase

GSH: glutathione

GSR: glutathione reductase

GST: glutathione S-Transferase

GT: green tea

GTE: green tea extract

GTP: green tea polyphenols

H_2O_2: hydrogen peroxide

HE: hydroethidine

HIV: human immunodeficiency virus

HIV-1: human immunodeficiency virus type 1

HOCl: hypochlorous acid

HPLC: high-performance liquid chromatography

IgG: immunoglobulins G

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase

Keap1: Kelch-like ECH-associated protein1

LDL: low density lipoprotein

LPH: lactase phloridzin hydrolase

MAPK: mitogen activate protein kinase

MCI: mild cognitive impairment

MCT-1: monocarboxylate transporter 1

MDR: multidrug resistance transport proteins

MPO: myeloperoxidase enzyme

MRP: Multidrug Resistance associated Proteins

NASH: non-alcoholic steatohepatitis

NEAC: Non Enzymatic Antioxidant Capacity

NF-kB: nuclear factor kB

NOX: NADPH oxidase

NOx: nitrites/nitrates

Nrf2: NF-E2 related factor 2

$O_2^{-\bullet}$: superoxide anion

OAT: Organic Anion-Transporters

OB: Oxidative burst

$OH^{\bullet}$: hydroxyl radical

oxLDL: oxidized low density lipoproteins

P: placebo

PA: physical activity

PAMP: pathogen associated molecular patterns

Pgp: P-glycoprotein

PKC: protein kinase C

PLIR: peroxidation of leukocytes index ratio

PMA: phorbol 12-myristate 13-acetate

PMN: polymorphonuclear neutrophil

PMNs: polymorphonuclear neutrophils

PP: PolyPhenols

PRR: pattern recognition receptors

PUFA: polyunsaturated fatty acids

PXR: pregnane X receptor

Q: quercetin

Rho123: rhodamine 123

RNS: reactive nitrogen species

ROO•: peroxyl radicals

ROS: reactive oxygen species

RW: Red wine

SGLT1: sodium-dependent glucose transporter 1

SOD: superoxide dismutase

SULT: sulfotransferases

TAS: total antioxidant status

TBARS: acid–reacting substances

TG: Triglycerides

TLR: Toll-like receptors

Tmax: time taken to reach the maximum concentration

TNF-α: tumor necrosis factor-α

Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UA: uric acid

UGT: uridine-5'-diphosphate glucuronosyltransferases

XO: xanthine oxidase

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