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The effects of dietary flavonoids on the regulation of redox inflammatory networks

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1. ABSTRACT

Dietary flavonoids are a large family of polyphenols ubiquitously expressed in plants. Recent evidence show that flavonoids possess several anti-inflammatory activities due to their ability to scavenge reactive oxygen and nitrogen species (ROS and RNS), to inhibit the pro-inflammatory activity of ROS-generating enzymes including cyclooxygenase (COX), lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS) and to modulate different intracellular signaling pathways from NF-kB to mitogen-activated protein kinases (MAPKs) through perturbation of redox-sensible networks in immune cells. This report will review current knowledge on the anti-inflammatory effects of flavonoids on immune cells focusing on their ability to modulate multiple redox-sensible pathways involved in inflammation.

2. INTRODUCTION

The history of reactive oxygen species (ROS) discovery traces back to 1954, when Gerschman *et al.* proposed for the first time a pioneering free radical theory to explain oxygen poisonous properties which was based on the existence of partially reduced forms of oxygen (1). From that moment on, a large number of researchers have explored in depth the biology of ROS and their "close relatives" reactive nitrogen species (RNS), enlightening their physiological roles in triggering immune cells involved in host defense against microorganisms and tumor cells and in activating intracellular second messengers involved in all major cell signaling pathways (2). Furthermore, it has been demonstrated that ROS and RNS are implicated in several pathologic conditions including ageing, cardiovascular disease, ischemia/reperfusion,

diabetes, Alzheimer and Parkinson disease, inflammatory and autoimmune pathologies and cancer (2). Moreover, specific ROS- and RNS-producing enzymes as well as reducing molecules and enzymes (collectively known as antioxidant defenses) have been discovered in a variety of cells, thus depicting a multifaceted role for ROS, RNS and redox-sensitive pathways in cell biology (2,3).

As long as ROS and RNS began to gain attention as noxious agents, it was the aim of researchers to discover new antioxidant molecules which could be used to modulate their production in pathological conditions. Among those, dietary flavonoids represent attractive drugs to counteract ROS and RNS production. Dietary flavonoids, in fact, are a large family of plant polyphenols endowed with potent antioxidant abilities, whose exploitation as free radicals-scavenging agents and regulators of redox-sensitive signaling pathways is of great interest because of their effectiveness, tolerability and dietary availability (4). Accordingly, this review aims to provide an insight on the potential benefits of dietary flavonoids as antioxidant therapeutic agents and to report current knowledge on their ability to modulate ROS and RNS production during inflammation both by directly scavenging reactive oxygen and nitrogen intermediates and by perturbing redox-triggered signaling pathways in immune cells. In addition we also report recent clinical trials involving the use of dietary flavonoids for counteracting inflammation.

3. THE BIOGENESIS OF REACTIVE OXYGEN AND NITROGEN SPECIES

3.1. Reactive oxygen species (ROS)

The radical forms of oxygen represent the most important family of biological free radical molecules, clearly as a function of the unique electronic configuration of molecular oxygen which is a radical on its own, though relatively unreactive, due to the presence of two uncoupled electrons (3,5). From molecular oxygen, then, at least six different radicals are generated, namely superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and ozone (O_3), each with a specific catalytic mechanism and each displaying a different reactivity (3).

Superoxide anion ($O_2^{\cdot-}$) is generated through the addition of an unpaired electron to molecular oxygen, and is the starting point for the biogenesis of other oxygen radicals through metals- and enzyme-catalyzed reactions (6). The primary source of superoxide are cell mitochondria, in which a low level of electron leakage (up to no more than 3% of total transported electrons) occurs during the respiratory process mainly at Complex I and III of the electron transport chain (6,7), or after activation of enzymes such as NADPH oxidase and xanthine oxidase (8). $O2^{\cdot-}$ is also produced during arachidonic acid (AA) metabolism by both cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (9). Prostaglandin (PG) H synthase, in fact, has distinct cyclooxygenase and hydroperoxidase activities which cooperate in the oxygenation of AA to PGG_2 and its subsequent reduction to the corresponding alcohol, PGH_2, which is the starting point for the synthesis of all other PGs (9). During this process, the hydroperoxidase moiety releases oxidizing equivalents mainly in the form of oxygen superoxide (9). Similarly, 5-lipoxygenase (LOX5) converts AA to 5-hydroperoxy eicosatetraenoic acid which is then reduced to leukotriene (LT) A4, the precursor of other LTs isoforms, by a reaction involving NADH or NADPH as reductants and liberating superoxide equivalents (10).

Even though superoxide is a relatively strong reductant, it can behave both as a reductant or an oxidant depending on the redox potential of the molecules with whom it can react, thus providing the starting point for the biosynthesis of more reactive oxygen radicals. Further it acts as a powerful reactant for the iron-sulphur centers of that electron-transporting proteins which serve both as the respiratory chains of bacteria and as key cell enzymes such as NADH dehydrogenase, hydrogenases, coenzyme Q-cytochrome C reductase, succinate-coenzyme Q reductase, aconitase and nitrogenase (11).

Due to its peculiar electronic configuration, spontaneous dismutation of superoxide occurs in protonated media, so that two superoxide molecules can react with two protons to generate molecular oxygen and a more powerful radical molecule, the hydrogen peroxide (H_2O_2) (12). Though spontaneous, this reaction is mainly governed and accelerated by different enzymes inside the cells, which are collectively known as superoxide dismutases (SODs) (12). Being the major sites of oxygen consumption within the cells, peroxisomes are the main H_2O_2 production site too, wherein hydrogen peroxide is used as a reactant for different oxidative reactions. Clearly, as a function of their highly-oxidizing potential, peroxisomes are loaded with effective antioxidant defenses, mainly represented by catalases which reduce the peroxide to water (2). In addition, if not properly scavenged, in the presence of iron H_2O_2 is able to participate to the Fenton reaction with superoxide, yielding the highly-reactive hydroxyl radical ($OH^{\cdot}$) (2). Theoretically, iron atoms are not found free in cells due to their high redox potential (2), but under stress conditions generating high levels of $O_2^{\cdot-}$, superoxide is able to determine iron release from the iron-sulphur centers of the dehydratase-lyase family of enzymes (13). Once released, iron enters a redox cycle, known as the Haber–Weiss reaction, in which superoxide and hydrogen peroxide react with oxidized iron to form hydroxyl radical, hydroxyl anion and molecular oxygen parallel to iron reduction by superoxide (2).

A different fate for H_2O_2 is represented by its conversion to hypochlorous acid (HOCl) by the myeloperoxidase (MPO) enzyme which occurs mainly in polymorphonuclear cells (PMNs) and, to a lesser extent, in monocytes (even though it has also been demonstrated to occur in macrophages under pathological conditions). The production of HOCl represents a key mechanism involved in microorganisms killing processes (14).

Hydroxyl radical ($OH^{\cdot}$), the neutral form of the hydroxyl anion, is the main product of

superoxide/hydrogen peroxide Fenton reactions, and it is far more reactive than the molecules from which it is generated (2). In fact, Pastor *et al.* have demonstrated that $OH^{\cdot}$ half-life is of approximately 10^{-9} seconds, which means that this radical reacts very closely to its generation site (15). Singlet oxygen ($^1O_2$) is an electronically-excited state of molecular oxygen, whose generation can be due both to photochemical absorption of UV or visible radiations by a photosensitizer (PS), which then returns to its ground state by transferring energy to an oxygen molecule, and to a biochemical reaction catalyzed by MPO (16). Singlet oxygen is profoundly different from other ROS for it is the only one being in an excited state, which is responsible for the peculiar features of this molecule in respect to other reactive species. Indeed, singlet oxygen do not interconvert to other radical forms (as it happens for superoxide and hydrogen peroxide), and its reactivity is limited to its time of permanence in the excited state while other ROS retain their proper potential until they react with another molecule (16). The shortness of singlet oxygen half-life, however, limits its diffusion distance and thus recalls the same spatial limitations that compel hydroxyl radical to react closely to its site of production (16). $^1O_2$, moreover, is also a less-representative product of MPO-catalyzed reactions in phagocytes, as the result of H_2O_2 reduction by chlorine monoxide (OCl^-) to water and chloride ion (17). In this context, singlet oxygen still retains a bactericidal activity due to its ability to damage microorganisms' respiratory chain (17).

Ozone (O_3) is another minor ROS which is profoundly linked to the immune system, for it has been variously demonstrated that antibodies, regardless of their antigen specificity, can act as catalytic enzymes for the oxidation of water to ozone by singlet oxygen either generated by photochemical reactions or by MPO (18,19). In this context, ozone appears to have microbicidal features, which empower H_2O_2 antimicrobical effects (17-19).

3.2. Reactive nitrogen species (RNS)

As for oxygen, nitrogen metabolism is able to produce radical species too, called reactive nitrogen species (RNS). The starting point for RNS synthesis is represented by nitric oxide ($NO^{\cdot}$), whose radical properties depend on the presence of one unpaired electron in the antibonding $2p^*_y$ orbital (2). Unlike superoxide, $NO^{\cdot}$ is not generated by electron leakage or spontaneous redox reactions within the cells. Its synthesis is carefully under the control of different tissue-specific nitric oxide synthases (NOSs) which convert arginine to citrulline and produce nitric oxide via a five-electrons redox process (20). Nitric oxide half-life is rather short, being limited to a few seconds in acqueous solutions, but its stability increases greatly under low oxygen tension conditions (2). Furthermore, since $NO^{\cdot}$ is soluble in both aqueous and lipid solutions, it can readily diffuse through cell membranes and cytosol and react, under different conditions, with molecular oxygen and ROS, thus generating different radical and non-radical compounds (20). Nitric oxide is converted to non-radical molecules such as nitrite (NO_2) and nitrate (NO_3) in the extracellular space through the reaction with oxygen and water. Even though these products have been classically considered as rather inert end products of $NO^{\cdot}$ metabolism, recent findings have reported the existence of a "nitrate-nitrite-nitric oxide pathway" which reduces these molecules back to nitric oxide, which can subsequently re-enter the cell and give rise to new RNS, as it probably occurs in hypoxic conditions (21). The most important fate of nitric oxide, however, resides within the cells, where it can readily react with superoxide anion to form the strongly oxidant peroxynitrite ($ONOO^-$) which is able to cause DNA fragmentation and lipid oxidation and thus accounts for most of $NO^{\cdot}$ toxicity (2). To date, the preferential site of $ONOO^-$ formation inside the cells is still unclear. Recent findings have reported the existence of a calcium-sensitive mitochondrial-specific NOS isoform, called mtNOS, which revealed a new role of nitric oxide functions in the cells (20). $NO^{\cdot}$, in fact, has been reported to bind to mitochondrial cytochrome oxidase, thus competing with O_2 uptake and determining a stress condition (20,22). The level of $NO^{\cdot}$ binding to cytochrome oxidase, however, is fairly below the utilization of $NO^{\cdot}$ by superoxide anion which occurs at the inner mitochondrial membrane (22). Indeed, the reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ is characterized by one of the highest rate constants among known nitric oxide reactions, and thus produces high amounts of peroxynitrite (2). Coherently, it appears that mitochondria, which produce both superoxide and nitric oxide, are the most likely site of $ONOO^-$ production due to the close distance of these two reactants within the same cell compartment (2,20). (Figure 1) shows the reactivity of different ROS and RNS as well as their main biosynthetic pathways.

4. ROS AND RNS AS IMMUNE EFFECTORS IN HOST DEFENSE

ROS and RNS are key players of host defense and inflammatory processes, both under physiological and pathological conditions (2). Upon pro-inflammatory activation, phagocytic cells, such as PMNs, macrophages and monocytes are in fact known to produce large ROS amounts, mainly in the form of superoxide and subsequent radicals, in the "respiratory burst" process (2). According to Baldridge *et al.*, who first defined it in the early 30s, the "respiratory burst" consists in a strong increase in oxygen consumption (23) leading to a dramatic increase in $O_2^{\cdot-}$ production. This process is now widely recognized to be under the control of the NADPH oxidase enzyme complex (24). This abrupt increase in superoxide concentration, together with the concomitant increase in nitric oxide production by the inducible NOS (iNOS) isoform and the subsequent rise of other radical molecules, represents a quick and early response against invading pathogens, bearing pro-inflammatory, microbicidal and virocidal effects (2,25).

In this context, phagocyte oxidase activation within the phagosomes liberates $O_2^{\cdot-}$, which rapidly undergoes dismutation to H_2O_2. Although hydrogen peroxide has a modest antimicrobial activity *per se*, it is essential for the MPO-chloride system to catalyze the two-electrons oxidation of Cl^- to Cl^+ (and, to a lesser extent, that of I^- and Br^-) which leads in turn to the production of

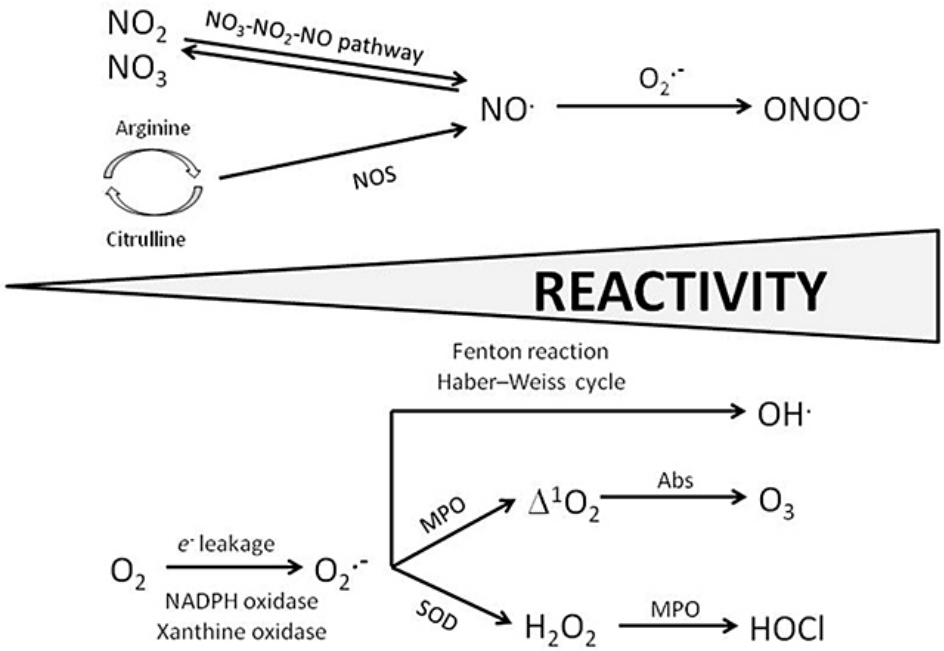

Figure 1. Biosynthesis and reactivity of ROS and RNS. The different radical products of oxygen and nitrogen are depicted according to their reactivity. e^-: electron; MPO: myeloperoxydase; SOD: superoxide dismutase; Abs: antibodies; NOS: nitric oxide synthase; $O_2^{\cdot-}$: superoxide; H_2O_2: hydrogen peroxide; $OH^\cdot$: hydroxyl radical; Δ^1O_2: singlet oxygen; HOCl: hypochlorous acid; O_3 ozone; $NO^\cdot$: nitric oxide; $ONOO^-$: peroxynitrite; NO_2: nitrite; NO_3: nitrate.

HOCl. Hypochlorous acid, then, can cross-link and covalently chlorinate protein targets in microbes as well as oxidize iron centers, sulfhydryl groups, heme-proteins, sulfur-ether groups and lipids, thus affecting pathogens' viability. In addition, HOCl can chlorinate host factors as well to produce weaker but longer-lasting antimicrobial products. Chloramines generated by the action of the MPO-chloride system on cell amines is one of the most important examples (24,26). Similarly, a ROS-dependent host defense system is triggered by the epithelia upon infections, in which H_2O_2 is the lactoperoxidase (LPO) substrate for the oxidization of thiocyanate anions (SCN^-) to hypothiocyanite (HOSCN), a powerful antimicrobial agent which is found in milk, saliva, airway surface liquid and tears (26,27).

The hydroxyl radical has antimicrobial activity too due to its high genotoxicity and its oxidizing effect on microbes' membrane lipids, though its direct cytotoxic effect on pathogens is still discussed (28). It has been reported, in fact, that its extremely high reactivity strongly affects its ability to diffuse along the phagosome, so that it probably acts as a reactant together with chloride and bicarbonate to generate secondary bactericidal compounds such as HOCl (28,29). Similarly, it appears that also oxygen singlet, probably generated by the reaction of hydrogen peroxide with hypoclorous and hypobromous acid within the phagosome, has a limited effect on bacterial killing which is mostly limited to the initiation of membrane peroxidation (28,29). Still, it has been reported to exert genotoxic effects on bacterial and viral plasmids (30).

Nitric oxide and its derivatives have a strong microbicidal effect and provide effective defense against fungal, protozoal and parasitic infections by forming complexes with heme proteins, inactivating iron/sulfur centers and forming nitrosothiols (31). However, there is a clear difference in the microbicidal activity of NO and its derivative $ONOO^-$. In fact, while the former (and its aerobic oxidation products) appears to have none or limited microbicidal properties, peroxynitrite has been variously reported to be far more effective in microbial killing (32) by hydroxylating and nitrating aromatic compounds and thiols (32), and to be produced in a time-dependent manner which overcomes the limitedness of ROS stability (33). Furthermore, it appears that other nitrogen derivatives, such as the radical nitrogen dioxide ($NO_2^\cdot$), empower the microbicidal effect of peroxynitrite, further amplifying the close relationship between ROS and RNS in pathogen killing (33). (Figure 2) summarizes the mechanism of ROS and RNS production in a phagocytic cell and the phagosomal and secretory fate of the different radicals.

5. ROS, RNS AND REDOX-SENSITIVE PATHWAYS IN INFLAMMATION

To date it is ascertained that ROS and RNS play major roles in the regulation of the inflammatory network, both in acute and chronic conditions. As a consequence of immune cells recruitment, in fact, ROS and RNS are produced to both clear off the insult and to stimulate the production of inflammatory mediators and to trigger multiple inflammatory factors, thus behaving as second

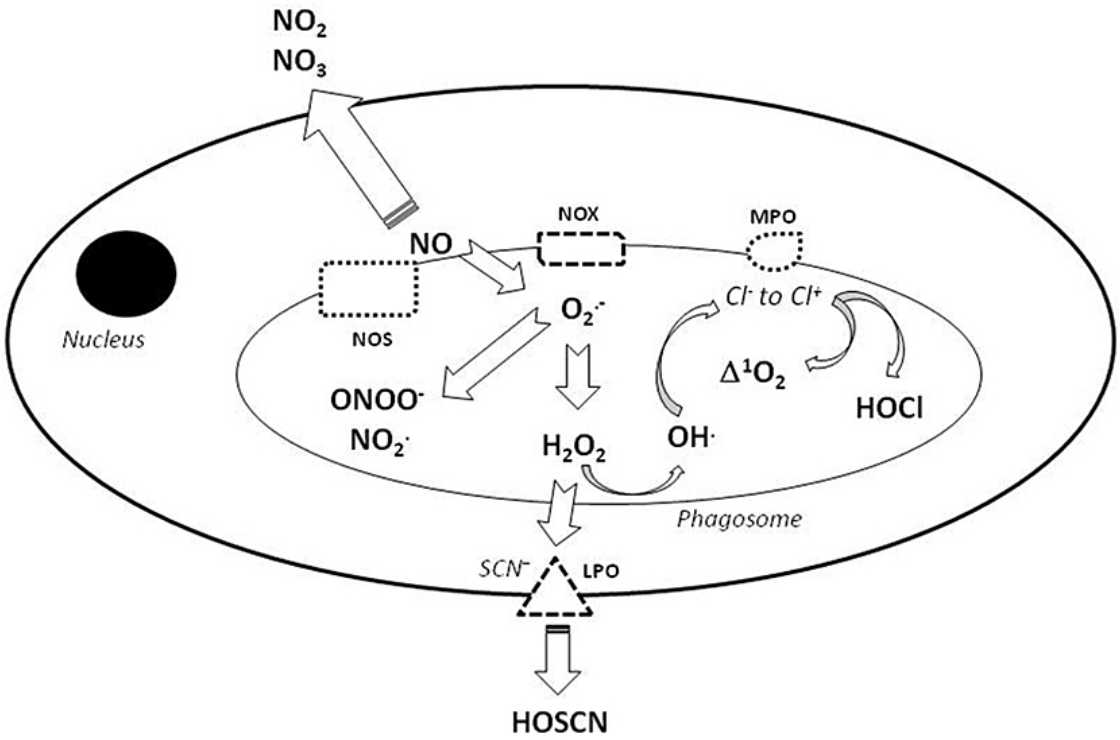

Figure 2. Production of ROS and RNS in phagocytes. ROS and RNS are differentially produced into phagocyte to clear the host off invading pathogenes. MPO: myeloperoxydase; NOS: nitric oxide synthase; NOX: NADPH oxidase; LPO: lactoperoxidase; $O_2^{\cdot-}$: superoxide; H_2O_2: hydrogen peroxide; $OH^{\cdot}$: hydroxyl radical, Δ^1O_2: singlet oxygen; HOCl: hypochlorous acid; O_3 ozone; $NO^{\cdot}$: nitric oxide; $ONOO^{-}$: peroxynitrite; $NO_2^{\cdot}$: nitrogen dioxide; NO_2: nitrite; NO_3: nitrate; SCN^{-}: thiocyanate anions; HOSCN: hypothiocyanite.

messengers to build up and coordinate the inflammatory reaction (2,36-38). Several cytokines and growth factors have been reported to use ROS as second messengers (2,37-39).

For instance, receptor activation by epidermal growth factor (EGF) leads to the production of both H_2O_2 and $O_2^{\cdot-}$, thus determining tyrosine phosphorylation, c-Jun N-terminal kinases (JNK) and mitogen-activated protein kinases (MAPKs) activation, phospholipase A_2 (PLA_2) activation and cell growth (40). Likewise, platelet-derived growth factor receptor activation (PDGF-R) induces superoxide and hydrogen peroxide production which stimulate mitogenesis, MAPK activation, NOS expression and nuclear factor-kB (NF-kB)-mediated signaling (41,42). Other growth factors, including fibroblast growth factor-2 (FGF-2), insulin growth factor-1 (IGF-1) and hepatocyte growth factor (HGF), are known to stimulate ROS production for different purposes such as activation of c-Fos, mitogenesis, differentiation and apoptosis (43,44).

Similarly, many cytokines have been reported to induce ROS production. Indeed, tumor necrosis factor-alpha (TNF-alpha) stimulates the production of H_2O_2 and $O_2^{\cdot-}$, determining either mitogenesis and cell death as well as the production of pro-inflammatory mediators like monocyte chemotactic protein-1 (MCP-1), macrophage colony-stimulating factor (M-CSF), interleukin-6 (IL-6), and activating NF-kB (43,45,46). In this context, it has been also reported that both IL-1 and interferon-gamma (IFN-gamma) have synergistic effects on TNF-alpha signaling through amplification of ROS production, which then induce COX-2 activation and powerful host defense processes (47). IL-1 has been reported to induce ROS production as second messengers too, by determining NADPH oxidase-dependent production of H_2O_2 and $O_2^{\cdot-}$. The result of IL-1 activity is vascular injury and angiogenesis, inflammation, pathogen killing and fever (48-51). NADPH oxidase-dependent ROS-mediated signaling has been also identified for IFN-gamma, where oxidants lead to COX-dependent microbial killing mechanisms and regulate innate defenses of major airways and gastrointestinal tract mucoses, also providing the extracellular LPO substrate to produce antimicrobial hypothiocyanite ions (26). As pleiotropic signaling molecules, ROS can also mediate immunosuppressive and inhibitory effects of cytokines such as the transforming growth factor-beta (TGF-beta). In this context, ROS have been implied in those signaling mechanisms which lead to growth inhibition and apoptosis of several cell types, including osteoblasts, endothelial cells, hepatocytes and pancreatic beta cells (52-55). Moreover, ROS and RNS are also involved in TGF-beta-mediated immunosuppressive effects, as demonstrated by the ability of this cytokine to

Table 1. The role of ROS as second messengers: activation of signaling pathways and effects

Stimulus	ROS	Intracellular signaling	Effects	References
EGFR	H_2O_2 $O_2^{\cdot-}$	Tyrosine phosphorylation, JNK activation, MAPKs activation, PLA_2 activation	Cell growth	(39,40)
PDGF-R	H_2O_2 $O_2^{\cdot-}$	MAPK activation, NOS expression, NF-kB signaling	Mitogenesis	(41,42)
FGF-2, IGF-1, HGF	H_2O_2 $O_2^{\cdot-}$	c-fos activation, MAPK activation	Mitogenesis, differentiation, apoptosis	(43,44)
TNF-alpha	H_2O_2 $O_2^{\cdot-}$	MAPK activation, NF-kB signaling	Mitogenesis, cell death, increase of MCP-1, M-CSF, IL-6	(43,45,46)
TNF-alpha + IL-1/IFN-gamma	H_2O_2 $O_2^{\cdot-}$	COX-2	Host defense	(47)
IL-1	H_2O_2 $O_2^{\cdot-}$	MAPK activation, NF-kB signaling	Vascular injury, angiogenesis, activation of inflammation, pathogen killing, fever	(48-51)
IFN-gamma	H_2O_2 $O_2^{\cdot-}$	COX-2	Pathogen killing, mucosal innate defense, LPO activation	(26)
TGF-beta	H_2O_2 $O_2^{\cdot-}$	MAPK inhibition, NF-kB signaling perturbation	Immunosuppression, cell growth inhibition, apoptosis, respiratory burst depression, Treg and immature myeloid cells development	(52-59)
Serotonin	$O_2^{\cdot-}$	ERK activation	Muscle cell mitogenesis	(60)
Bradykinin	H_2O_2 $O_2^{\cdot-}$	COX-dependent and-independent mechanisms	Vascular functions damage	(61)
Thrombin	H_2O_2 $O_2^{\cdot-}$	MAPKs activation, PLA_2 activation	Cell growth	(62,63)
Endothelin	H_2O_2 $O_2^{\cdot-}$	Ras activity modulation	Myocyte functions regulation	(64)

Abbreviations: EGFR: epidermal growth factor receptor; PDGF-R: platelet-derived growth factor receptor; JNK: c-Jun N-terminal kinases; PLA_2: phospholipase A_2; FGF-2: fibroblast growth factor-2; IGF-1: insulin growth factor-1; HGF: hepatocyte growth factor; TNF-alpha: tumor necrosis factor-alpha; IL-1: interleukin-1; IFN-gamma: interferon-gamma; TGF-beta: transforming growth factor-beta; COX-2: cyclooxygenase-2; MCP-1: monocyte chemotactic protein-1; M-CSF: macrophage colony-stimulating factor; IL-6: interleukin-6; Tregs: regulatory T-cells.

inhibit macrophage oxidative functions aimed at controlling *Mycobacterium tuberculosis* growth, to depress PMNs respiratory burst, to trigger the development of forkhead box P3 (FOXP3)-positive T-regulatory (Treg) cells, and to mediate immature myeloid cells-dependent mechanisms of cancer immune evasion (56-59).

Many soluble mediators of inflammation have also been reported to stimulate ROS production. Serotonin has been demonstrated to stimulate NADPH oxidase-dependent superoxide production, whose effects are mainly directed towards muscle cell mitogenesis through the activation of extracellular signal-regulated kinases (ERK) (60). Similarly, bradykinin stimulates H_2O_2 and $O_2^{\cdot-}$ production by COX-dependent and –independent mechanisms, which may mediate its pathophysiologic effects on vascular functions (61). Thrombin is able to induce ROS release by NADPH oxidase and NADPH oxidase-like enzymes in endothelial and smooth muscle cells, thus regulating cell growth, MAPK activation and phospholipase-dependent signaling pathways (62,63), and endothelin is able to use ROS as signaling molecules to modulate the activity of Rat sarcoma (Ras) kinase on myocytes (64). (Table 1) summarizes the ROS-producing stimuli and ROS-mediated activation of intracellular signaling pathways in inflammation.

Coherently with the large number of identified ROS-inducing signaling pathways, a large number of ROS-targeted signaling molecules have been identified so far (37). The non-receptor protein kinases (PTKs) belonging to the Src and Janus family, have been demonstrated to be activated by ROS (mainly hydrogen peroxide and superoxide) in fibroblasts, T and B cells, macrophages and myeloid cells, thus initiating MAPK-, NF-kB- and phosphoinositide 3-kinase (PI3K)-dependent signaling pathways (2,37,65). Similarly, protein tyrosine phosphatases (PTPs) have been identified as key ROS targets in the redox control of cell signaling (2,37,66).

Protein kinase B and C (PKB and PKC) have also been demonstrated to undergo ROS-dependent regulation, as it occurs in VEGF-mediated cell growth via the PI3K/PKB pathway, in the ROS-dependent release of calcium ions with the subsequent activation of PKC and in the redox regulation of both the catalytic and regulatory domains of PKC, which then triggers MAPKs, transcription factors and proto-oncogenes (2,67,68).

MAPKs are undoubtedly major targets of redox-dependent regulation of cell functions (69). In this context, superoxide and hydrogen peroxide have been shown to activate the MAP kinase kinase (MKK) and ERK1/2 (2) in a stimulus-specific manner. Indeed, hydrogen peroxide produced during the respiratory burst appears to activate ERK but not p38 MAPK (70). Conversely, macrophage stimulation with exogenous H_2O_2 appears to activate p38 MAPK instead of ERK (2). Moreover, it has been reported that macrophage responses to prostaglandins and inflammatory cytokines such as IL-12 and IL-6 depend on the redox status of the macrophages themselves. In addition, differences in macrophages' redox balance have

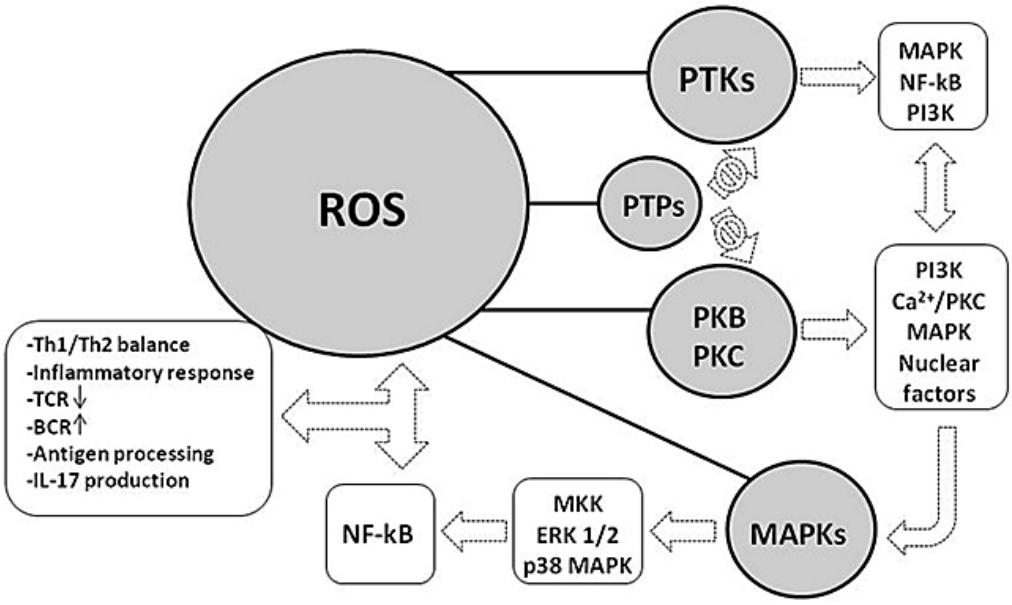

Figure 3. ROS-activated signaling pathways in inflammation. Downstream of ROS production, several signaling pathways are activated to regulate the inflammatory process. PTKs: non-receptor protein kinases; PTPs: protein phosphatases; PKB: protein kinase B; PKC: protein kinase C; MAPKS: mitogen-activated protein kinases; NF-kB: nuclear factor kB; PI3K: phosphoinositide 3-kinase; PKC: protein kinase C; MKK; MAP kinase kinase; ERK: extracellular signal-regulated kinases; TCR: T-cell receptor; BCR: B-cell receptor; IL-17: interleukin-17.

been implicated in the regulation of Th1/Th2 balance (8). Nuclear factors activation is the endpoint of MAPK signaling pathways and, in the context of redox-sensitive regulation, different transcriptions factors have been identified so far (2,37). NF-kB is a major target in ROS-regulated inflammatory pathways, as demonstrated by the ability of pro-inflammatory cytokines like TNF-alpha and IL-1 to trigger its activity by stimulating ROS production. In turn NF-kB induces different genes involved in chronic and acute inflammatory responses (71).

Likewise, hydrogen peroxide production in T cells has been reported to negatively regulate T cell receptor (TCR) assembly in membrane rafts and downstream signaling to MAPK and NF-kB. Conversely, B cell receptor (BCR) activity appears to be increased by ROS through a synergic effect with calcium signaling (72). Finally, recent findings demonstrate that antigen processing in dendritic cells is under the control of ROS, which modulate phagosomal pH, and that components of the NADPH machinery are involved in the control of IL-17 production by gamma/delta-T cells during fungal infection (24). (Figure 3) represents signaling pathways downstream of ROS production and their effects on nuclear transcription and the control of the inflammatory reaction.

6. DIETARY FLAVONOIDS

Flavonoids are a large group of polyphenolic compounds ubiquitously expressed in plants as secondary metabolites of phenylalanine (73,74). They are present in edible fruits, vegetables, herbs, spices, legumes, nuts, and in plant-derived beverages such as tea and wine, and retain various biological activities involved in host defense against pathogens and signal transduction (73,74). All flavonoids consist of 15 carbon atoms arranged into three aromatic rings, termed A, B and C respectively, with the B-ring being linked to the A-ring by a three-carbon bridge that binds with one oxygen and two carbons of the A-ring thus forming the C-ring. Flavonoid classification depends on the different functional groups and oxidation level of the C-ring and on different connections between the B- and the C-ring. Differences between compounds within a class are, instead, due to the differences in the substituents of the A- and B-rings (4,75).

The most important classes of dietary flavonoids are flavonols, flavones, flavan-3-ols, anthocyanins, flavanones and isoflavones (76). Conversely dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones and aurones are infrequently introduced with the diet (76).

Flavonols are the most common class of flavonoids found in plant foods, mainly as O-glycosides of glucose or rhamnose, even though galactose, arabinose, xylose and glucuronic acid are also found (77). The main members of this class are quercetin, kaempferol and myricetin, with quercetin being predominant in respect to kaempferol and myricetin in plants (78). These flavonols differ for the substituents at the 5-,5'-,7-,4'- and 3'- carbons of the A- and B-ring (76), and are mostly intaken by consuming fruits, plants, wine and tea (77,79).

Flavones are present in plants mainly as 7-O-glycosides bearing hydroxyl, methyl, O- and C-alkyl and

glycosylic groups as common substituents (76,80). Their major dietary representatives in foods are apigenin and luteolin, the former being abundant in parsley, celery, onion, garlic, pepper and in plant-derived beverages such as chamomile (77,78,81), the latter being found in bird chili, onion leaves and celery (78,81). Other flavones, less abundant in edible plants, are tangeretin and nobiletin, found in citrus fruits, baicalein and wogonin from *Scutellaria,* and chrysin, contained in *Passiflora* (76-78,80,82).

Flavan-3-ols contain one hydroxyl group in the 3-position of the C-ring and exhibit the highest range of chemical complexity among flavonoids. This class, in fact, includes several different compounds that can be divided into monomers and polymers. Simplest monomers are (+)-Catechin and its isomer (-)-Epicatechin, whose hydroxylation generates (+)-Gallocatechin and (-)-Epigallocatechin, respectively. The additional esterification with gallic acid in the 3-position of the C-ring transforms these monomers in (-)-Epicatechin-3-O-gallate and (-)-Epigallocatechin-3-O-gallate. Furthermore, monomers can bind each other through C-C or, less frequently, C-O-C bonds to form polymers named proanthocyanidins (76,83), which are subdivided into type A, B and C. Type A proanthocyanidins are double-bound dimers, exhibiting both a C-C bridge between a monomer's C4 and the C6 or C8 from the other one and a C-O-C bridge between the C2 of a unit and the oxygen in C7 or C5 position of the other unit. Type B dimers, conversely, only show the C4-C6/C8 bond in between the monomers. Type C proanthocyanidins are trimers, formed by three flavan-3-ols joined by two C4-C8 bonds (76,83,84). The most common proanthocyanidins found in plants are procyanidins B1, B2, B3 and B4, with Flavan-3-ols being mainly found in fruits, berries, cereals, nuts and also in chocolate, red wine and tea (76,77).

Anthocyanins, whose most abundant representatives in plants are cyanidin, pelargonidin, delphinidin, peodinin, petunidin and malvidin (76,85), are water-soluble and widespread compounds, which can be found in the aglycone (anthocyanidin) and the heteroside (anthocyanin) form. Aglycones represent the basic chemical structure, while heterosides, which are mainly present in nature, are formed when a sugar (glucose, galactose, arabinose, rhamnose and xylose) is linked to an aglycone through the C3 hydroxyl group of the C-ring (85). More than 550 anthocyanins have been identified in nature so far differing for the number of hydroxyl groups and methylation degree in the aglycone moiety, the number and position of sugars linked to the aglycone molecule and the number and nature of aliphatic or aromatics acids linked to these sugars (76,85). The main dietary sources of anthocyanins are fruits of the berries family, red pigmented varieties of oranges, vegetables (cabbage, beans, onions, radishes), grains (corn and rice) and potato (85).

Flavanones are non-planar flavonoids mainly found in citrus fruits, where they exist prevalently as mono- and diglycosides or, less frequently, in aglycone forms. Naringenin and hesperetin are the most important flavanones in aglycone forms, whose correspondent glycated forms (in which sugars are attached to the C7 oxygen) are neohesperidosides, such as naringin (naringenin-7-O-neohesperidoside) and neohesperidin (hesperetin-7-O-neohesperidoside), and rutinosides, such as narirutin (naringenin-7-O-rutinoside) and hesperidin (hesperetin-7-O-rutinoside) (76,86). Hesperetin is abundant in oranges, while naringenin is abundant in grapefruit and tomatoes (77,86). The main food sources of hesperidin are sweet orange, lemon and mandarin, while narirutin is found mainly in grapefruit and mandarin (86). Neohesperidin and naringin can be found mainly in grapefruit and orange (86).

Isoflavones differ from other flavonoids because of the B-C rings bond in the C3 position instead of the C2 position (76). This peculiar chemical structure resembles that of the human hormone 17-beta-estradiol (87), conferring them a pseudohormonal activity (76) which could be useful in the treatment of osteoporosis and menopausal symptoms (88), and allowing to classify this compounds as phyto-estrogens (87). The most common isoflavones are daidzein, genistein and glycitein, which are found mainly in leguminous plants among which soy bean and its products bear the highest isoflavones levels (89). Along with the common aglycones genistein and daidzein, soy products may also carry corresponding glycosides such as genistin and daidzin, depending on the soy preparation (89). (Table 2) summarizes the main classes, forms and sources of dietary flavonoids.

Several studies showed that the dietary intake of flavonoids is highly variable around the world and such a variability also characterizes the different classes of flavonoids (4). Recent epidemiological studies show that the bioavailability of dietary flavonoids (the quantity of compound that is absorbed and metabolized within the body after dietary intake, usually measured as maximum plasma concentration reached after the intake) is an important topic in the prevention of diseases, and that their effects also depend on the proportion of active substances that are absorbed from the gastrointestinal tract (90). In foods, flavonoids occur mainly in native non-glycosylated forms, termed aglycones, and in glycosylated forms whose glycosyl moiety affects intestinal absorption and bioavailability. While aglycones can in fact be directly absorbed by passive diffusion from the small intestine, flavonoid glycosides must be hydrolyzed to aglycones by intestinal enzymes or microflora prior to absorption. Once uptaken, flavonoids are metabolized in both the small intestine and the liver by methylation, sulfation or glucuronidation and enter the blood flow, thus reaching target tissues (77,91). However, several mechanisms limit the bioavailability of flavonoids, such as their metabolism in the gastrointestinal tract and liver, their binding on the surface of blood cells as well as on the surface of the microbial flora of the oral cavity and the gut, and the regulatory mechanisms of the body triggered to prevent the toxicity of high flavonoid levels. For all these reasons, only nano or micromolar quantities of flavonoids are found in the blood, as recently reviewed by Manach *et al.* who reported that the plasma concentrations of total flavonoid metabolites reached after a dietary intake of 50 mg of a single molecule ranged from 0 to 4 micromol/L (92).

Table 2. Main classes, forms and sources of dietary flavonoids

Flavonoid class	Main form(s)	Glucide(s)	Main members	Dietary sources	References
Flavonols	Glycosides	Glucose, rhamnose, galactose, arabinose, xylose, glucuronic acid	Quercetin, kaempferol, myricetin	Fruits, plants, wine, tea	(76-79)
Flavones	Glycosides	Glucose, rhamnose, neohesperidose, glucuronic acid	Apigenin, luteolin, tangeretin, nobiletin, baicalein, wogonin, chrysin	Parsley, celery, onion, garlic, pepper, chamomile, bird chili	(76-78,80-82)
Flavan-3-ols	Aglycones	None	(+)-Catechin, (-)-Epicatechin, (+)-Gallocatechin, (-)-Epigallocatechin, (-)-Epicatechin-3-O-gallate, (-)- Epigallocatechin-3-O-gallate, Proanthocyanidins	Fruits, berries, cereals, nuts, chocolate, red wine, tea	(76,77,83,84)
Anthocyanins	Aglycones, heterosides	Glucose, galactose, arabinose, rhamnose, xylose	Cyanidin, pelargonidin, delphinidin, peodinin, petunidin, malvidin	Fruits, vegetables, red wine	(76-78,85)
Flavanones	Aglycones, Monoglycosides, diglycosides	Neohesperidose, rhamnose, rutinose	Naringenin, hesperetin, naringin, neohesperidin, narirutin, hesperidin	Oranges, grapefruit, tomatoes, lemons, mandarins	(76-78,86)
Isoflavones	Aglycones, glycosides	Glucose	Daidzein, genistein, glycitein, genistin, daidzin	Leguminous plants	(76,87-89)

Moreover, it appears that even a long-term consumption of flavonoid-rich foods is unable to overcome this problem, and the low availability of bioactive flavonoid metabolites explains why also the most abundant flavonoids comprised in our diet are often devoided of a beneficial effect (4,90).

Nevertheless, flavonoids are characterized by effective antioxidant and anti-inflammatory activities (93-95), as confirmed by epidemiological studies evidencing an inverse correlation between the consumption of flavonoid-rich fruits and vegetables and the incidence of chronic diseases and cancer, even though the mechanisms underlying these beneficial effects are still poorly clarified (96,97).

7. DIETARY FLAVONOIDS AS ROS/RNS SCAVENGERS AND INHIBITORS

Several mechanisms have been described so far to explain the anti-inflammatory activity of flavonoids. In the oral cavity and in the intestine, these compounds are likely to form stable antioxidant complexes interacting with local microbial flora, whereas in the stomach they act as direct scavengers of hydroperoxides and aldehydes deriving from fatty acids metabolism. The latter finding suggests that tissue damages associated with oxidative stresses induced by the ingestion of fatty foods might be prevented by simultaneous consumption of flavonoid-rich beverages (98). Moreover, flavonoids are able to bind erythrocytes and plasma proteins, which might then act as carriers, and synergize with plasmatic low molecular weight antioxidants, blood cells and albumin to enhance their scavenging activity (4). Flavonoids also exert direct antioxidant activities on immune cells, as demonstrated by their ability to scavenge ROS generated by activated neutrophils and macrophages and to impair their production by inhibiting NADPH oxidase, xanthine oxidase and myeloperoxidase (99).

In this context, however, the most important effect of flavonoids is represented by their ability to significantly modulate the activity of those AA-metabolizing enzymes, such as COX and LOX, which are ROS-generating enzymes too (100,101). Some flavonoids such as luteolin, galangin and morin have been reported to have an inhibitory effect on COX enzymes (102), with the flavone wogonin displaying an inhibitory effect on both COX-2 activity and its mRNA expression in LPS-stimulated macrophages (103) and fibroblasts (104). Several studies have confirmed the ability of different flavonoids to suppress COX-2 at the transcriptional level, as observed with genistein and kaempferol in LPS-stimulated macrophages, with apigenin and quercetin in LPS-J774A.1 cells, with quercetin, kaempferol, naringenin and nobiletin in mouse macrophages and human synovial fibroblasts, with luteolin in RAW 264.7 cells, and with genistein and the catechin EGCG in human chondrocytes and synovial fibroblasts, respectively (105,106). The COX-2–inhibiting effect of flavonoids has been also confirmed *in vivo* in murine models of acute and chronic inflammation (107) and in SNF1 mice with established lupus-like disease (108).

Similarly, different classes of flavonoids have been reported to have inhibitory effects on 5-LOX too (109). Apigenin and luteolin have been demonstrated to inhibit 5-LOX in mouse mast cells (110), while genistein was shown to inhibit the synthesis of leukotriene C4 in eosinophils by blocking 5-LOX activation (111). Finally, dual inhibition of COX-LOX enzymes has been reported *in vitro* for apigenin and luteolin and in *in vivo* inflammatory models for baicalin and catechin (110,112).

Different flavonoids have been shown to inhibit NO production from activated macrophages or macrophage-like cells, as demonstrated by *in vitro* studies showing that quercetin and apigenin can effectively inhibit iNOS expression in the RAW 264.7 cell line (105,106), and

that flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin can inhibit iNOS protein and its mRNA expression as well as NO production in a dose-dependent manner in activated macrophages (113). To date, it appears that the anti-RNS effects of flavonoids depend on their ability to decrease both iNOS expression and activity, though reduction of iNOS expression is more frequently observed than the decrease in enzyme activity (106,113,114). Only a few studies, in fact, reported a direct effect of certain flavonoids, such as soy isoflavones, prenilated flavonoids and biflavonoid, on NO release from lipopolysaccharide (LPS)-stimulated macrophages (115).

Conversely, different studies demonstrated that the iNOS inhibitory effects of flavonoids occur at the transcriptional level (116). In this context, quercetin has been reported to significantly decrease iNOS mRNA level in IL-1beta-activated hepatocytes (117), whereas quercetin and kaempferol have been demonstrated to have the same effects in Chang liver cells (118). Moreover, Lee *et al.* recently reported that the flavones chrysin, apigenin and luteolin and the flavonols kaempferol and quercetin share the ability to dose-dependently decrease NO production in activated BV-2 microglia cells through a marked reduction of iNOS (119). Coherently, quercetin-dependent iNOS downregulation has been confirmed *in vivo* in rats (120). (Table 3) summarizes the effects of dietary flavonoids as direct ROS/RNS scavengers and inhibitors.

8. EFFECTS OF DIETARY FLAVONOIDS ON CYTOKINES AND INFLAMMATION

A key role for dietary flavonoid in the control of inflammatory processes is undoubtedly represented by their ability to inhibit the production of pro-inflammatory cytokines, such as IL-1beta, IL-2, IL-6, IFN-gamma, TNF-alpha, and chemokines in different cell types (4). These cytokines are able to trigger ROS production, which serve as second messengers, in a different variety of inflammatory cells (2).

Different flavonoids, especially flavone derivatives, have been demonstrated to inhibit TNF-alpha release by activated RAW 264.7 cells (121). Moreover, fisetin, luteolin and apigenin have been reported to effectively inhibit the production of Th-2-type cytokines by activated human basophils (122), whereas epigallocatechin-3-gallate (EGCG) was able to inhibit IL-8 production by human epithelial cells (123), the secretion of TNF-alpha and IL-6 from human mast cells (124), and the production of IL-1beta, TNF-alpha and IL-6 from synovial fibroblasts and chondrocytes (125,126). Similarly, it has been recently reported that the flavonols quercetin and kaempferol inhibited both expression and secretion of TNF-alpha, IL-6 and IFN-gamma in mast cells (127).

Anthocyanins have been reported to inhibit the production of IL-13 and of IL-13 receptor 2a (IL-13R2a) and to decrease mRNA expression of pro-inflammatory cytokines such as IL-6 and TNF-alpha in a mouse model of ovalbumin (OVA)-induced asthma (128). In the same experimental model, naringenin exhibited the ability to attenuate OVA-induced airway inflammation by significantly reducing the levels of IL-4 and IL-13 (129). Moreover, quercetin has been demonstrated to affect the production of interferon-inducible protein 10 (IP-10) and of macrophage inflammatory protein 2 (MIP-2) in murine intestinal epithelial cells (130).

Flavonoids can inhibit chemokines expression as well. In this context, it has been demonstrated that apigenin was able to inhibit the production of monocyte chemoattractant protein (MCP-1) at the transcriptional level in J774.2 macrophages (131), an effect which was also reported for EGCG in vascular endothelial cells (132) and for isoflavones in 3T3-L1 mature adypocytes (133). Similarly, apigenin has been also reported to inhibit the expression of macrophages-derived chemokine (MDC) and of interferon-inducible protein 10 (IP10/CXCL10) in THP-1 monocytes (134), and naringenin has been shown to significantly reduce the production of chemokine ligands CCL5 and CCL11 in OVA-stimulated mice (129).

Since the activation of NF-kB is responsible for the transcription of many inflammatory factors, including TNF-alpha, IL-6, IL-8, chemokines, adhesion molecules, iNOS and COX-2 (135), it is not surprising to find that the transcriptional machinery of this nuclear factor is one of the most studied target of dietary flavonoids effects (4). Several flavonoids, in fact, have been demonstrated to modulate NF-kB activity in macrophage cell lines through different mechanisms (136). Apigenin was found to inhibit IkB kinase (IKK) activity, thus depressing NF-kB activation, to block LPS-induced phosphorylation of the p65 subunit of NF-kB and to inhibit LPS-induced production of TNF-alpha *in vivo* (137). Similarly, quercetin and kaempferol have been demonstrated to inhibit gene expression of both iNOS and COX-2 by reducing IkB degradation and the consequent activation of NF-kB in Chang liver cells (118), an effect also displayed by quercetin on RAW 264.7 cells (138). Moreover, it has also been reported that the flavone chrysin inhibits NF-kB activity in human intestinal Caco-2 cells and in mast cells (139,140), and that EGCG inhibits NF-kB activity in osteoclasts (141). Furthermore, EGCG has also been shown to inhibit IKK activity, IkB phophorylation and NF-kB activation, as well as to decrease the DNA-binding activity of NF- kB, in a wide range of cells, including intestinal epithelial cells (142), respiratory epithelial cells (143), endothelial cells (144), mast cells (124) and human articular chondrocytes (126). A similar anti-inflammatory role has also been reported for blueberry anthocyanins, which were reported to inhibit NF-kB translocation to the nucleus in LPS-activated BV-2 cells (145).

Perturbation of NF-kB signaling is also responsible for the effects of dietary flavonoids on the expression of chemokines and adhesion molecules. It has been recently demonstrated that the soy isoflavone genistein decreases the production of pro-inflammatory and adhesion molecules by inhibiting NF-kB translocation in hemolysate-stimulated brain microvascular endothelial cells (146). In a similar way, daidzein has been reported to

Table 3. Dietary flavonoids as ROS/RNS scavengers and inhibitors

Flavonoids	Target cells and body district	Action(s)	References
Red wine polyphenols	Oral cavity, intestine	Stable antioxidant complexes with microbial flora	(98)
Red wine polyphenols	Stomach	Scavenging of hydroperoxides and aldehydes	(98)
Resveratrol, quercetin, gallic acid, polyphenols	Blood	Synergy with plasmatic low molecular weight antioxidants, blood cells and albumin	(4)
Taxifolin, eriodictyol, hesperetin, luteolin	Activated neutrophils, macrophages	Inhibition of NADPHox, xanthine oxidase and myeloperoxidase	(99)
Quercetin, apigenin, daidzein, genistein, isorhamnetin, kaempferol, naringenin, pelargonidin, chrysin, luteolin	Activated macrophages, RAW 264.7 cells	iNOS mRNA downregulation	(105,106,113,116)
Soy isoflavones, prenilated flavonoids, biflavonoids	LPS-stimulated macrophages	iNOS activity inhibition	(115)
Apigenin, luteolin, genistein, baicalin, catechin	Mast cells, eosinophils	5-LOX inhibition	(109-112)
Wogonin, genistein, kaempferol, apigenin, quercetin, naringenin, nobiletin, luteolin, EGCG	LPS-stimulated macrophages, fibroblasts, J774A.1, synovial fibroblasts, RAW 264.7 cells, chondrocytes	COX-2 mRNA downregulation	(105-108)
Wogonin, luteolin, galangin, morin	LPS-stimulated macrophages, fibroblasts	COX-2 activity inhibition	(100-104,110-112)

Abbreviations: iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; 5-LOX: lipooxygenase-5; EGCG: epigallocatechin-3-gallate.

have an *in vivo* protective effect against ischemia/reperfusion-induced myocardial damage through its ability to modulate NF-kB nuclear translocation, which in turn suppresses the expression of inflammatory cytokines and chemokines (147). Such observation closely matches with the ability of quercetin and kaempferol to down-regulate VCAM-1, ICAM-1 and E-selectin expression by blocking NF-kB binding in activated HUVEC cells (148).

Another key inflammatory checkpoint which is closely interlaced with NF-kB and can be regulated by flavonoids is represented by the MAPK family, whose members have been variously reported to be inhibited by these dietary compounds. Indeed, quercetin has been demonstrated to block iNOS expression in stimulated RAW cells through inhibition of p38 MAPK activation (149); almost parallel, quercetin has also been demonstrated to inhibit the production of pro-inflammatory cytokines and NF-kB activation through ERK and p38 MAPK blockade in LPS-activated macrophages (150). Moreover, it has been recently reported that the ability of quercetin to inhibit ICAM-1 expression by IL-1beta-stimulated human A549 cells relies, at least partly, on p38 MAPK inhibition (151). In a similar way, kaempferol has been reported to suppress chemokine expression from human THP-1 cells through the suppression of MAPK pathways activation (152). Green tea proanthocyanidins inhibit COX-2 expression in LPS-stimulated mouse macrophages by blocking the MAPK-mediated activation of NF-kB (153), thus almost exactly overlying with the mechanisms by whom luteolin suppresses LPS-stimulated pathways in RAW 264.7 cells (154). A widespread MAPK inhibition has also been reported for apigenin, which has been proven both to inhibit p38 MAPK and JNK activity induced by LPS in BV-2 microglia and head and neck carcinomas cells (119,155) and, more recently, to suppress the Th1- and Th2-related chemokine production by human THP-1 monocytes through the inhibition of JNK, ERK and p38 MAPK phosphorylation (134). Finally, different reports underlying the interest for EGCG as a biological chemopreventive agent for arthritis and other inflammatory diseases have pointed out MAPK inhibition. Indeed, EGCG has been reported to interfere with the regulation of various inflammatory genes by MAPKs via inhibition of p38, JNK and ERK phosphorylation in human dermal fibroblasts (156), as it has also been demonstrated to reduce the synthesis of IL-6 in osteoblast-like MC3T3-E1 cells and in primary cultures of mouse osteoblasts through the inhibition of p44/p42 MAPK-dependent pathway activation (158). (Table 4) summarizes main flavonoid inhibitory effects on cytokines, chemokines and signaling pathways.

Despite the great number of studies demonstrating the antinflammatory effects of dietary flavonoids *in vitro* and *in vivo*, human studies are still controversial and insufficient and most of them have been carried out using a diet based on flavonoid-rich foods and not on a single flavonoid (121).

In this context, a milestone is represented by the work of Hanninen *et al.*, who reported that a vegan diet consisting of uncooked berries, fruits, vegetables and roots, nuts, germinated seeds and sprouts determined a decrease of joint stiffness and pain in fibromyalgic subjects and in rheumatoid arthritis patients (158). In addition, Jenkins *et*

Table 4. Cytokines, chemokines and signaling pathways inhibition by flavonoids

Flavonoid	Cell type or animal model	Inhibited cytokines and chemokines	Inhibited signaling pathways	References
Flavone derivatives	RAW 264.7	TNF-alpha	AP-1,Nrf2	(121)
Fisetin	Basophils	Th-2 cytokines	AP-1	(122)
Luteolin	Basophils, RAW 264.7 cells	Th-2 cytokines	MAPK/NF-kB	(122,154)
Apigenin	Basophils, THP-1 cells, J774.2 macrophages, BV-2 microglia, head and neck carcinomas	Th-2 cytokines, MCP-1, MDC, CXCL10	IKK, p65, p38 MAPK, JNK, ERK	(119,122,131,134,137,155)
EGCG	Fibroblasts, epithelial cells, mast cells, chondrocytes, endothelial cells, MC3T3-E1 cells, primary osteoblasts, RAW 264.7 cells	IL-8, TNF-alpha, IL-6, IL-1beta, MCP-1	NF-kB-p65, IKK, IkB, NF-kB/DNA-binding, JNK, ERK, p38 MAPK	(123,123,125,131,140-143,154,155)
Quercetin	Mast cells, epithelial cells, HUVEC cells, Chang liver cells, RAW 264.7 cells, A549 cells, LPS-activated macrophages	TNF-alpha, IL-6, IFN-gamma, IP-10, MIP-2, VCAM-1, ICAM-1, E-selectin	IkB, NF-kB/DNA-binding, p38 MAPK, ERK	(118,127,130,138,148-151)
Kaempferol	Mast cells, HUVEC cells, Chang liver cells, THP-1 cells	TNF-alpha, IL-6, IFN-gamma, VCAM-1, ICAM-1, E-selectin	IkB, NF-kB/DNA-binding inhibition, MAPK	(118,127,148,152)
Anthocyanins	Murine asthma model, BV-2 cells	IL-13, IL-6, IL-13R2a, TNF-alpha	NF-kB	(128,145)
Isoflavones Genistein Daidzein	3T3-L1 adypocytes, brain endothelial cells, *In vivo* ischemia/reperfusion model	MCP-1	NF-kB	(133,146,147)
Naringenin	Murine asthma model	CCL5, CCL11	NF-kB	(129)
Proanthocyanidins	LPS-stimulated macrophages	COX-2	MAPK/NF-kB	(153)
Chrysin	Caco-2 cells	TNF-alpha, IL -1beta, IL-4, IL-6	NF-kB	(139,140)

Abbreviations: AP-1: activating protein-1; nuclear factor-erythroid 2-related factor 2; MDC: macrophages-derived chemokine; CXCL10: CXC ligand 10; IP10: interferon-inducible protein 10; MIP-2: macrophage inducible protein-2; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: Inter-Cellular Adhesion Molecule 1; CCL5: CC ligand 5; CCL11: CC ligand 11; IkB: inhibitor of kB; IKK: IkB kinase.

al., found that soy isoflavones consumption increased serum concentrations of IL-6 in women, although it had no effect on acute-phase proteins or other proinflammatory cytokines. Nevertheless, the estrogenic effect of isoflavones was proposed to be a mechanism of immune surveillance potentiation, which could possibly explain the lower incidence of certain cancer types in soy-eating parts of the world (159). However, uncertainty exists about flavonoid effects on serum inflammatory markers, since Phillips *et al.* reported that a supplement containing mixed tocopherols, flavonoids and docosahexaenoate significantly decreased IL-6 and C-reactive protein (CRP) serum levels after eccentric exercise in untrained males (160). Sex-dependent differences in bioflavonoid effects had been detected by Jenkins and coworkers (159), and Song *et al.* reported that flavonoid-rich foods reduced the risk of type-2 diabetes not affecting serum levels of both IL-6 an CRP (161). Similarly, Bogani *et al.* recently reported that extra vergine olive oil consumption significantly reduced thromboxane B2 (TXB_2) and leukotriene B4 (LTB_4) levels in the postprandial phase and Fanti *et al.* reported that soy flavonoids significantly decrease CRP levels in haemodialysis patients (162,163). Conversely, Ryan-Borchers *et al.* reported that soy isoflavones did not significantly affect plasma concentrations of IFN-gamma, IL-2, TNF-alpha and C-reactive protein, or of 8-isoprostane in urine, and Greany *et al.* found that soy isoflavones did not alter plasma levels of CRP, E-selectin, VCAM-1 and ICAM-1 (164,165). A larger randomized study on soy dietary intake also reported that no significant differences could be observed in the levels of leptin, adiponectin, monocyte attractant protein 1 (MCP-1), macrophage inflammatory protein-1 beta (MIP-1beta), IL-6 and CRP after supplementation (166). However, these observations have been recently challenged by the works of Lyall *et al.* and Nieman *et al.*, who reported a decrease in TNF-alpha and oxidant production after blackcurrant supplementation and significant decreases in granulocyte colony-stimulating factor, CRP, IL-6 and IL-10 plasma levels, respectively (167,168). Recently Monagas *et al.* reported that the expression of VLA-4, CD40, and CD36 in monocytes, as well as serum concentrations of the soluble endothelium-derived adhesion molecules P-selectin and intercellular adhesion molecule, were significantly lowered in atherosclerosis high-risk patients who continuously assumed cocoa powder (169). On the other hand, another recent study from Heinz *et al.* showed that supplementation with quercetin did not affect the activity of natural killer cells nor the granulocyte oxidative burst activity or phagocytosis in human females (170).

Nevertheless, different studies suggest that a high consumption of vegetables, fruits and legumes in healthy volunteers inversely correlates with blood inflammation markers (171,172). Moreover, it has been demonstrated that red wine consumption decreases the expression of major adhesion molecules on monocytes and T-lymphocytes (173), and a significant reduction in blood levels of ICAM-1 and VCAM-1 has been observed in a group of 48 healthy volunteers who consumed a polyphenol-rich food

concentrate (174). In addition, it has been reported that dietary supplementation with a grape polyphenol extract containing anthocyanins, quercetin, myricetin, kaempferol and resveratrol led to a significant decrease in plasma TNF-alpha and IL-6 levels (175). A study carried out on 285 teenagers corroborated these findings (176). Furthermore, a survey performed in 120 volunteers reported that the intake of a blueberries-derived anthocyanin-rich extract significantly decreased plasma levels of pro-inflammatory cytokines and chemokines regulated by NF-kB signaling (177).

Although preliminary, a few studies have been carried out to evaluate the beneficial effects of dietary flavonoids in chronic inflammatory diseases and cancer. In two independent studies the oral administration of a bioflavonoid-rich purple passion fruit peel extract has been shown to diminish the clinical symptoms of asthma and osteoarthritis (178,179), a phenomenon which is consistent with the reported ability of apple polyphenols to decrease the clinical symptoms of allergic rhinitis and of muscadine grape seeds to significantly increase resting brachial diameter in subjects with increased cardiovascular risk (180,181). Recent results from the Polyp Prevention Trial indicate that decreased cytokine concentrations during high flavonol consumption may prevent colorectal neoplasms (182).

9. ONGOING CLINICAL TRIALS

Apart from the published human studies reported above, other performed and/or ongoing clinical trials are registered on the clinicaltrials.gov website, a registry of federally and privately supported clinical trials conducted in the United States and around the world. These trials aim to ascertain the potential role of dietary flavonoids alone or in combination with classical treatments in different physiological and inflammatory conditions. A non-negligible part of these studies aims at verifying flavonoid efficacy as modulators of oxidative stress and inflammatory markers in human pathologies. However, the outcome of these trials has not been published yet.

A first authoritative trial is the FLAVO trial, held by the University of East Anglia (NCT00677599). In this study, 152 postmenopausal women with type 2 diabetes at a high risk of cardiovascular disease receive flavonoid compounds from cocoa, including epicatechin, and soy through a vehicle of 27 grams chocolate bar for 365 days versus placebo (a chocolate bar not enriched with flavonoids), in order to determine whether these flavonoids could be more effective in reducing the risk of cardiovascular disease than standard therapy with statin.

Similar to the FLAVO trial is the one held by the Texas Tech University Health Sciences Center (NCT01307917), actually enrolling participants by invitation, in which 80 adolescents, divided equally in healthy and suffering from type 1 or type 2 diabetes mellitus, will receive a capsule containing 500 mg of flavonoids or a placebo for 14 days, twice a day. This trials aims at measuring flavonoid effects on renal nitric oxide synthesis, IL-1beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-gamma, TNF-alpha, MIP-1alpha and beta, and RANTES, in order to ascertain whether flavonoids with anti-inflammatory and antioxidant activities could be used to protect endothelial function and prevent the development and progression of nephropathy. Another ongoing phase II trial (actually enrolling patients) is the one held by the Shiraz University of Medical Sciences (NCT01003236), which aims at verifying the renoprotective effect of the flavonoid complex silymarin, an extract from the milk thistle, and its major pharmacological active component silibinin, on the urinary levels of TNF-alpha and TGF-beta as well as on blood glucose and lipid profile.

The Shiraz University of Medical Sciences has also recently completed a phase II trial (NCT01001845) in which vitamin E (200 mg twice a day for 3 weeks) plus milk thistle extract (140 mg of silymarin, 3 times daily for 3 weeks) have been evaluated in 80 patients suffering from end-stage renal disease for their ability to modulate oxidative stress and inflammation.

A phase IV trial held by the Carolinas Healthcare System (NCT00331227) enrolled 25 healthy volunteers for 10 weeks to ingest a supplement made by NutraMetrix (called OPC-3), consisting of oligomeric proanthocyanidins derived from grape seed, pine bark, bilberry, citrus and red wine extracts. The aim of the trial was to determine the effects of the supplement on endothelial function, lipoproteins and inflammation during the fasting state and after a single standardized high-fat meal, in order to verify its efficacy on serum levels of CRP and PLA_2. Although completed in December 2006, the results of the trial have not been published yet.

With a different endpoint is the trial held by Tufts University (NCT00740077), which aims at studying the pharmacokinetics of phenolic acids and flavonoids, including anthocyanins, flavanols, flavonols, and proanthocyanidins, as well as of their *in vivo* metabolites, in blood, urine, and feces during the 24 h following a single-dose consumption of a cranberry juice cocktail (54% juice). Unfortunately, results about these important kinetic measurements, which will improve our knowledge about dietary flavonoids bioavailability and metabolism, have not been published yet.

Moreover, the Universidad de Antioquia is actually recruiting participants suffering from stage I or II essential arterial hypertension for a trial (NCT01276951) in which different doses of cocoa (ranging from 6,5 to 50 grams per day) will be consumed for 18 weeks in order to determine whether ingested flavonoids are able to modulate markers of oxidative stress and inflammation like the oxidation of low density lipoproteins, platelet aggregation ability and the levels of IL-1beta, IL-2 and TNF-alpha produced by the mononuclear cells of these patients. Similar to the one mentioned above are the trials held by LifeBridge Health (NCT00559663), which investigates the effects of a one-week flavonoid supplementation through green tea and 70% dark chocolate on platelet activity, HDL, LDL and CRP levels in 35 healthy volunteers, and by

Table 5. Effects of flavonoids on inflammation: human studies and clinical trials

Reference or ClinicalTrial.gov identifier	Flavonoid(s) and dietary sources	Effects and Goals	Subjects
Hanninen et al (158)	Vegan diet	Decrease of joint stiffness and pain	Fibromyalgic and rheumatoid arthritis patients
Jenkins et al. (159)	Soy isoflavones	Increase of IL-6 serum concentrations	Hypercholesterolemic men and postmenopausal women
Phillips et al. (160)	Mixed tocopherols, flavonoids and docosahexaenoate	Decrease of IL-6 and CRP serum levels	Post eccentric exercise-fatigued untrained man
Song et al. (161)	Flavonoid-rich foods	No effects on IL-6 and CRP serum levels	Type-2 diabetes patients
Bogani et al. (162)	Extra vergine olive oil	Decrease of TXB_2 and LTB_4 levels	Normolipemic healthy subjects
Fanti et al. (163)	Soy isoflavones	Decrease of CRP levels	Haemodialysis patients
Ryan-Borchers et al. (164)	Soy isoflavones	No effects on IFN-gamma, IL-2, TNF-alpha and CRP plasma concentrations and urinary 8-isoprostane	Postmenopausal women
Greany et al. (165)	Soy isoflavones	No effects on CRP, E-selectin, VCAM-1 and ICAM-1 plasma levels	Postmenopausal women
Maskarinec et al. (166)	Soya foods	No effects on leptin, adiponectin, MCP-1, MIP-1alpha, IL-6 and CRP levels	Overweight men
Lyall et al. (167)	Blackcurrant supplementation	TNF-alpha and oxidant production decrease	Exercise-fatigued men
Nieman et al. (168)	Quercetin +/- EGCG +/- isoquercetin +/- eicosapentaenoic acid +/- Q-EGCG	Decrease of GM-CSF, CRP, IL-6 and IL-10 plasma levels	Trained cyclists
Monagas et al. (169)	Cocoa powder	Decrease of VLA-4, CD40 and CD36 in monocytes, decrease of P-selectin serum levels	Atherosclerosis high-risk patients
Heinz et al. (170)	Quercetin	No effects on natural killer cells activity, granulocyte oxidative burst and phagocytosis	Healthy women
Estruch et al. (173)	Red wine	Decreases of major adhesion molecules on monocytes and T-lymphocytes	Healthy men
Schoen et al. (174)	Polyphenol-rich food concentrate	Reduction of ICAM-1 and VCAM-1 blood levels	Healthy men
Zern et al. (175)	Grape polyphenol extract	Decrease of TNF-alpha and IL-6 plasma levels	Pre- and post-menopausal women
Holt et al. (176)	Fruit and vegetables, antioxidants, folate and total flavonoids	Decrease of CRP, TNF-alpha and IL-6 plasma levels and urinary F(2)-isoprostane	Healthy adolescent boys and girls
Karlsen et al. (177)	Blueberries-derived anthocyanin-rich extract	IL-4, IL-8, IL-13, RANTES and IFN-gamma	Healthy adults
NCT00677599	Flavonoid compounds from cocoa and soy	Reduction of cardiovascular disease risk	Type 2 diabetes
NCT01307917	500 mg of flavonoids capsule	Prevention of renal endothelial function	Type 1 or type 2 diabetes mellitus
NCT01003236	Silymarin	Renoprotection	Nephropathy
NCT01001845	Vitamin E + silymarin	Modulation of oxidative stress and inflammation	End-stage renal disease
NCT00331227	OPC-3	Endothelial functions, lipoproteins levels and inflammation	Healthy volunteers
NCT00740077	Phenolic acids and flavonoids	Flavonoid pharmacokinetics	Healthy volunteers
NCT01276951	Cocoa	Oxidative stress and inflammation	Essential arterial hypertension
NCT00559663	Green tea and 70% dark chocolate	Platelet activity, HDL, LDL and CRP levels	Healthy volunteers
NCT00302809	Concord grape juice	Blood pressure and vascular functions	Pre-hypertension and stage 1 hypertension
NCT00512967	Quercetin	*Ex vivo* LPS-induced cytokine production	Interstitial lung disease
NCT00402623	1000 mg quercetin	Modulation of oxidative stress and inflammation	Pulmonary sarcoidosis
NCT01038362	3 oz of almonds	Lipid profiles and blood markers of inflammation and oxidative stress	Coronary artery disease
NCT00554242	450 mg of grape seed extract + 1500 mg of vitamin C	Hemodynamic parameters and serum markers of inflammation	Coronary artery disease
NCT00538941	80 gr of commercially available dark chocolate	Platelet function, oxidative stress, CRP, 8-Isoprostanes and CD40 ligand levels	Heart failure
NCT01162174	100 or 200 mg Oligonol	Endothelial functions, platelet reactivity and circulating flavonoids	Healthy volunteers
NCT00914576	VITAMAC©	Oxidative stress, endothelial dysfunction and vascular reactivity	100% oxygen and *E.Coli* LPS-mediated hypoxia and inflammation

Boston University (NCT00302809), which evaluated the effects of approximately 16 oz of concord grape juice on blood pressure and vascular functions in subjects with pre-hypertension and stage 1 hypertension.

Two studies which are more strictly related to inflammation are the ones performed by Maastricht University. The former (NCT00512967), started in September 2005 and concluded in June 2006, aimed at determining the antioxidant and inflammatory status in interstitial lung disease (ILD), i.e. sarcoidosis and idiopathic pulmonary fibrosis, and to evaluate the possible anti-inflammatory effects of quercetin on *ex vivo* LPS-induced cytokine production in ILD in a cohort of 51 patients. The latter (NCT00402623), started and completed in January 2006, focused more specifically on quercetin, at a dose of 1000 mg, as modulator of the oxidative and inflammatory state on 18 subjects affected by pulmonary sarcoidosis.

More recently, the Boston University held two trials on subjects suffering from coronary artery disease. In the first one (NCT01038362), 52 patients were treated with National Cholesterol Education Program (NCEP) Step 1 diet plus or minus 3 oz of almonds for 6 weeks, in order to verify the potential of almond flavonoids to aid NECP step

1 diet in regulating lipid profiles and blood markers of inflammation and oxidative stress. In the second one (NCT00554242), 40 participants took a food supplement containing 450 mg of grape seed extract and 1500 mg of vitamin C or matching placebo for four weeks and then crossed over to the alternative treatment (active supplement or placebo) for four weeks, to evaluate whether flavonoid supplementation could modulate hemodynamic parameters and serum markers of inflammation.

Flavonoids as regulators of inflammation and oxidative stress are also the topic of the NCT00538941 trial held by the University of Zurich. In this study, 22 patients suffering from heart failure (NYHA ≥II, LVEF<50%) have been enrolled to verify whether a daily 80 gr intake of commercially-available dark chocolate could modulate platelet function, oxidative stress, CRP, 8-isoprostanes and CD-40 ligand levels.

A pilot study (Phase I) on the benefits of flavonoid consumption has been also recently completed by the University of California (NCT01162174), which evaluated the effect of Oligonol, a patented lychee fruit extract produced by Amino Up Chemical Co. and particularly rich in low molecular weight flavanols, on the improvement of endothelial functions and platelet reactivity and on the levels of circulating flavonoids after a single intake of 100 or 200 mg Oligonol.

Soon to be completed is another trial (NCT00914576), held by the Medical University of Vienna, which proposes to investigate the effects of VITAMAC®, a combination of vitamins and minerals, in a systemic in-vivo inflammation model in which 40 volunteers will be exposed to 100% oxygen and *E.Coli* LPS to evaluate oxidative stress, endothelial dysfunction and the vascular reactivity of retinal vessels. (Table 5) summarizes published human studies and ongoing clinical trials.

10. PERSPECTIVE

Accumulating knowledge shows that ROS and RNS are not only involved in virtually all inflammatory pathologies and immune processes, but that they also provide a key bridge between extracellular microenvironment and nuclear transcription by acting as second messengers in the control of intracellular pathways triggered by cytokines, growth factors and other inflammatory mediators in the complete spectrum of immune and stromal cells.

In this context, flavonoids have to date emerged as strong *in vitro* and *in vivo* anti-inflammatory compounds due to their pleiotropic ability to scavenge ROS and RNS, to reduce the activities of arachidonic acid-metabolizing enzymes (phospholipase A_2, COX, LOX), to depress the expression and the activity of nitric oxide synthases and to modulate the production of proinflammatory cytokines and the expression of proinflammatory genes.

In light of these findings, flavonoids have a promising therapeutic role as novel anti-inflammatory drugs which is now widely studied in several clinical trials worldwide. Their potential beneficial effects should encourage researchers to envisage novel therapeutic protocols employing different flavonoids to target multiple inflammatory networks.

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