Anthocyanins As Modulators of Cell Redox-Dependent Pathways in Non-Communicable Diseases

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Abstract: Chronic noncommunicable diseases (NCDs), mostly represented by cardiovascular diseases, diabetes, chronic pulmonary diseases, cancers, and several chronic pathologies, are one of the main causes of morbidity and mortality, and are mainly related to the occurrence of metabolic risk factors. Anthocyanins (ACNs) posses a wide spectrum of biological activities, such as anti-inflammatory, antioxidant, cardioprotective, and chemopreventive properties, which are able to promote human health. Although ACNs present an apparent low bioavailability, their metabolites may play an important role in the *in vivo* protective effects observed.

This article directly addresses the scientific evidences supporting that ACNs could be useful to protect human population against several NCDs not only acting as antioxidant but through their capability to modulate cell redox-dependent signaling. In particular, ACNs interact with the NF-κB and AP-1 signal transduction pathways, which respond to oxidative signals and mediate a proinflammatory effect, and the Nrf2/ARE pathway and its regulated cytoprotective proteins (GST, NQO, HO-1, etc.), involved in both cellular antioxidant defenses and elimination/inactivation of toxic compounds, so countering the alterations caused by conditions of chemical/oxidative stress. In addition, supposed crosstalks could contribute to explain the protective effects of ACNs in different pathological conditions characterized by an altered balance among these pathways. Thus, this review underlines the importance of specific nutritional molecules for human health and focuses on the molecular targets and the underlying mechanisms of ACNs against various diseases.

Keywords: Anthocyanin, chronic diseases, inflammation, antioxidant, NF-κB, AP-1, Nrf2

1. INTRODUCTION

Chronic noncommunicable diseases (NCDs) account for about 70% of all deaths worldwide [1] being one of the main cause of morbidity and mortality. In fact, deaths due to chronic NCDs have increased worldwide by 40% in the last twenty years, and so a largest part of health care expenditure needs to be used for treatment and hospitalizations of patients affected by one or more NCDs [2]. These reasons explain why NCDs represent a major priority for national and pan-national health institutions to establish new interventions finalized to avoid their increase, minimize their incidence, and thus promote human health and well-being [3, 4].

The NCDs are represented mainly by cardiovascular diseases (CVDs), diabetes, chronic pulmonary diseases, and cancers (which account for about 80% of deaths from NCDs) but (as evident by the large definition used to indicate this group of pathologies) include several other chronic pathologies such as arthritis and osteoporosis, neurodegenerative diseases, chronic kidney diseases, eye pathologies, etc.

The NCD incidence and concerning levels are related to the occurrence of metabolic risk factors, present especially when populations adopt Western-style dietary habits and lifestyles, including unhealthful dietary patterns and physical inactivity [5]. Promoting a healthy lifestyle is thus crucial to reach the global Sustainable Development Goals, adopted by the United Nations General Assembly in September 2015, in particular, the target of reducing by one-third worldwide premature mortality from NCDs by 2030 [1] (**figure 1**).

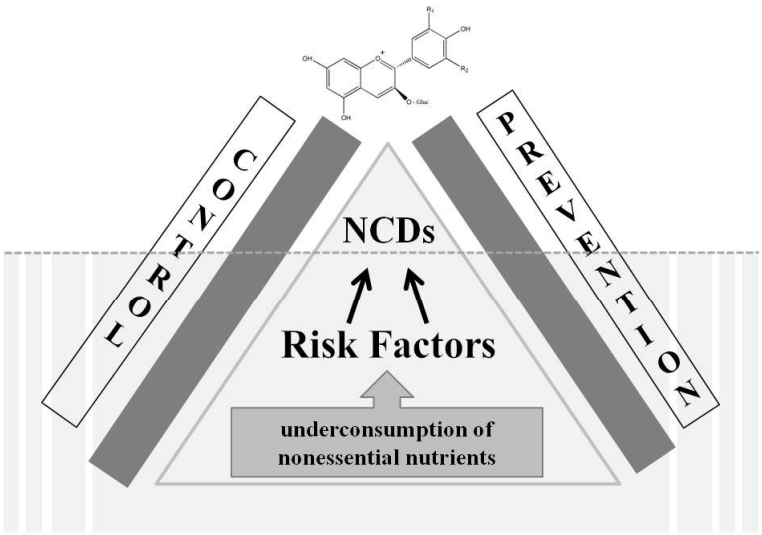


Figure 1. In an iceberg model, chronic NCDs represent the tip of the iceberg, since they may caused, maintained and sustained by several physiological, behavioral and social risk factors (the base of the iceberg) interacting with each other. Suboptimal nutrition and unhealthful dietary intakes have been identified as the most important preventable NCD behavioral risk factor. There is a large consensus that specific non-essential dietary components (such as anthocyanins) may possess both protective and therapeutic functions in the management of chronic NCDs.

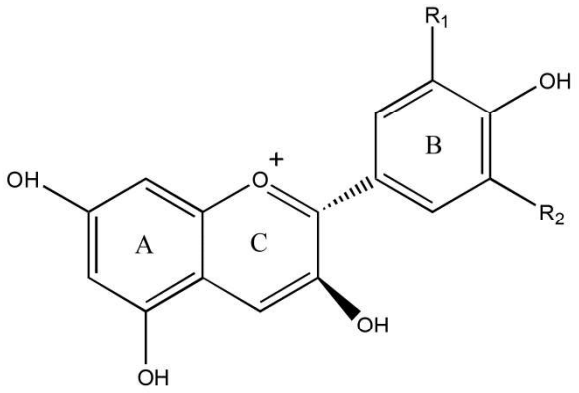
Suboptimal nutrition and unhealthful dietary intakes, together with sedentariness and consequently overweight and obesity, have been identified as the most important preventable NCD risk factor [6, 7]. In fact, it is well known that underconsumption of vitamins A, D, E, and C, folate, calcium, magnesium, fiber, and potassium can be related to adverse health outcomes or prevalent metabolic risk factors [5]. In addition, today there is substantial scientific evidence (coming from health agencies and academic institutions, as well as functional food and dietary supplement manufacturers) to confirm a relationship between enhanced health conditions or reduced risk of a chronic disease and the intake of specific dietary bioactive, nonessential nutrients, defined by the National Institutes of Health (NIH) as "constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, which are responsible for changes in health status" [8].

In this context, it is valuable that dietary fruit consumption, considered the third most important modifiable risk factor for reducing global rates of NCDs, after high blood pressure and smoking [9], contributes to >90% of the intake of ACNs in the habitual diet, one of the main subclasses of bioactive flavonoids [10, 11]. ACNs, as well as other polyphenols with bioactive properties, are mainly found in berries and other fruits and plant foods and are responsible for their reddish-violet color; in recent years they have shown promise in the prevention of NCDs such as arthritis, diabetes, cancer, and CVD. In fact, they possess anti-inflammatory, antioxidant, cardioprotective, and chemopreventive properties, making them an ideal subject for prevention and treatment of chronic diseases [12-17]. Among all the NCDs, most research regarding berry intake (the main dietary source of ACNs) showed its positive impact against undesirable lipid profiles, hypertension, diabetes, and metabolic syndrome [13, 18-23]. Thus, it is evident the existence of a growing interest in better understanding the bioproperties of ACNs, due to their well-proven ability to promote human health and protect against chronic NCDs, when introduced in the organism by a regular intake of colorful fruits and vegetables as well as by functional foods and food supplements [24, 25]. A wide spectrum of biological properties of ACNs was attributed to their capability to act as ''free radical scavengers" and as a result to their antioxidant activity [26-28]. However, there is evidence that *in vivo* beneficial role of ACNs in several human pathological conditions cannot be explained solely on the basis of their antioxidant characteristics. More recently, several papers have reported the involvement of other mechanisms of action beyond such activity [27-30]. ACNs appear able to modulate redox-dependent transcriptional factors and gene expression [12, 31], in particular thanks to the regulation of Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear factor-κB (NF-κB), and Activator protein-1 (AP-1) cell pathways [32].

Herein we review the scientific evidences that ACNs could be useful to protect human population against several NCDs not only acting as antioxidant but through their capability to modulate cell redox-dependent signaling.

2. ANTHOCYANIN CHEMISTRY, SOURCES, AND CONSUMPTION

ACNs are a group of water-soluble pigments, belonging to the chemical class of flavonoids, widely present in various parts of higher-order plants. ACNs are derivatives of 2-phenylbenzopyrylium (flavylium cation) and, due to the resonant structure of the flavylium ion, confer a color, ranging from red-orange to blue, to fruits, vegetables and plant-derived foods, so that they are popular as colorants for the food industry and as alternatives to synthetic colorants. Importantly, the antioxidant/free radical scavenger properties of ACNs are related to their phenolic structure characterized by B ring hydroxyl groups and a conjugated double bond system (**figure 2**).



	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 2. Chemical structures of the most frequently found anthocyanidins in plant-derived food.

ACNs consists of an aglycone (anthocyanidin, which presents the C6-C3-C6 flavonoid backbone structure), sugar(s), and, in many cases, acyl group(s). [33, 34]. ACNs are rarely found as aglycons since ACNs are not-stable and water-insoluble so that they do not usually occur in their free state and they are present in the cell vacuole linked to ≥1 sugar moieties (glucose, but also rhamnose, xylose and galactose) more frequently conjugated to the C3 or C5 hydroxyl group in the C-ring [35]. Furthermore, ACNs can be acylated, by esterification of a cinnamic or aliphatic acid to ≥1 of the sugar substitutions; 50% of ACNs found in nature are acylated. Up to now, more than 700 structurally distinct ACNs derivatives of 27 aglycons have been identified in nature and reported in literature, but only six of these pigments are commonly found in fruits and vegetables: pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin (Pg, Cy, Dp, Pt, Pn, Mv respectively) (**figure 2**) [12, 36], being the glycoside derivatives of the non-methylated anthocyanidins Cy and, at a lower frequency, Dp and Pg, the most common in nature [37].

Important sources of ACNs in the diet of adults include fruits and fruits juice (apples, plums and cherries, grapes, blood oranges), berries (bilberry, blackberry, blueberries, chokeberries, cranberries, elderberries, raspberries, cowberries, strawberries, black currants), red/purple vegetables (red cabbage, eggplants, red onions, radicchio), legumes (beans, cowpeas) and wine [38]. In particular berries with the largest concentrations are elderberries, chokeberries, blackcurrants and blueberries with estimated contents in the range of 160–1300 mg/100 g fresh weight. ACNs-rich fruits have been classified by Fang [39] into three groups, namely the Pg group (strawberries), Cy/Pn group (blackberries, ederberries, cranberries, orange juices), and multiple ACNs group (blueberries, bilberries, black currants, grapes). This classification system could facilitate the understanding of the absorption and metabolic processes of ACNs and health effects of different sources. For example, a recent literature review [40] suggests a trend for berries rich in Cy, Pn, or Pg glycosides (such as cranberries and strawberries) to exhibit more reproducible antiinflammatory effects than blueberries, which contain mostly Dp, Mv, and Pt glycosides.

Although for ACNs dietary reference intakes do not currently exist in North American and European countries and only China has currently defined a specific proposed level of 50 mg/day [41], several Institutions and associations point out the contribution of ACNs as dietary bioactive compounds to protect human health [42, 43].

Values of dietary ACNs intake ranging between 3 and 215 mg/day have been reported [44]. However, ACNs intake, as estimated in different countries, seems to vary within a wide range: 2.9 mg/day in Australia, 3 to 27 mg/day in USA, 28 mg/day in China, 35 mg/day in France, 47 mg/day in Finland [45]. A so wide range mainly depends on the specific dietary habits of the population samples under study but is very likely influenced by other factors, for example geographical, social and cultural diversity of the examined populations [46-48]. For example, the European Prospective Investigation into Cancer and Nutrition (EPIC) study estimated the dietary intake and food sources of ACNs and their derivatives, in a study involving totally 36,037 adult subjects from ten European countries. A clear ‘‘south to north’’ gradient of ACNs intake was demonstrated; it is associated with the consumption of specific food items frequently considered ‘‘healthy food’’ and has been found to be higher in non-obese older females, non-smokers, those having higher education level and those doing moderate or active physical activity. Similarly, the What We Eat In America (WWEIA) survey, National Health and Nutrition Examination Survey (NHANES) 2007–2008, reports that in the USA women had a higher daily ACNs intake compared with men, while white individuals had higher mean daily intake than Hispanic and non-Hispanic black populations [45].

2.1. Anthocyanins bioavailability

A complete knowledge of ACNs pharmacokinetic is fundamental to understand the real impact of the daily intake on health protection, since ACNs blood levels obtained through daily diet or supplement intake have to be higher enough to exert biological effects at the level of target organs or tissues.

Unfortunately, important discrepancies regarding ACNs availability are present in the available literature. This is evidently related to differences in ACNs chemical structures and sources but may be very likely influenced also by interindividual differences at the level of xenobiotic metabolism (e.g., polymorphisms of genes coding for specific drug metabolizing enzymes, or variations in intestinal microflora) [49-52]. Also, the consumption timing (before or after a meal) of ACNs-rich food and the natural micro-oxidative processes that occur during the ageing of ACNs-containing food may impact ACNs bioavailability and bioactivity properties [53, 54].

ACNs as glycosides are transported from the gastrointestinal tract to the liver via the hepatic portal vein and are also subjected to enterohepatic recycling. The ACNs deglycosylated forms are less polar and so can be absorbed from the stomach or intestinal tissue most likely by passive diffusion[55], while the ACNs glycosylated forms may be absorbed through the bilitranslocase transporter; their absorption may be potentially mediated by other transport mechanisms, for example the glucose transporters [56]. The recent study of Mueller and coll. [57], carried out in humans with or without a colon, given a bilberry extract, besides to confirm that higher amounts of metabolites (mainly glucuronides) than ACNs reach the circulation after ACNs consumption, evidenced that colon is a significant site for absorption of these compounds and their metabolites. Finally, ACNs are excreted in urine and in feces, mainly as ACNs conjugates and as phenolic acid metabolites respectively.

ACNs degradation can occur because of their chemical instability or through metabolism by colonic microbiota. Breakdown can lead to ACNs deconjugation or to phenolic acids that do not present the C6-C3-C6 structure typical of ACNs. The bacterial enzymes deglycosylate the compounds, but the microbes can also perform a range of other transformations including hydrolysis, demethylation, reduction, decarboxylation, dehydroxylation, or isomerization of these compounds into simpler components [58]. The first step in ACNs bacterial hydrolysis involves cleavage of the

sugar moiety leading to the formation of ACNs aglycons, and the second phase includes degradation into simple phenolic acids by the activities of two bacterial enzymes, in particular α-L-rhamnosidase and β-D-glucosidase, in the small intestine. The degradation of phenolic acids by enteric bacterial or chemical conversions may produce other metabolites, including protocatechuic acid (PcA), syringic acid, vanillic acid, phloroglucinol aldehyde, phloroglucinol acid, and gallic acid (GA). On the other hand, ACNs and metabolites concurrently formed in the intestine have the ability to promote and inhibit the growth of bacterial groups [59, 60]. ACNs can exert an antimicrobial activity inhibiting growth of several human pathogenic bacteria, both gram-negative (*Citrobacter freundii*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* ser. *typhimurium*) and gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Enterococcus faecalis*) but enhance the growth of some bacterial genera (*Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp.) described as contributing to the health benefits of the host.

Thus, although ACNs present an apparent lack of bioavailability, estimated in ranges of 2% to less than 1%, with only trace quantities detected in the expected target organs or in the bloodstream, ACNs metabolites formed in the proximal and distal gastrointestinal tract through phase I and phase II metabolism, appear in the circulatory system and may play an important role in the bioavailability of ACNs and very likely also in their protective effects on human health [61-63]. For example, Warner and coll. [64] demonstrated that physiologically relevant ACNs metabolite signatures, derived from a previous pharmacokinetics study of cyanidin-3-glucoside (C3G) in healthy subjects, reduce Vascular Cell Adhesion Molecule-1 (VCAM-1) and Interleukin-6 (IL-6) production in *in vitro* cultured human endothelial cells. Recent researches suggest the hypothesis that ACNs bioactivity is likely mediated by the high concentrations and longer half-lives of its microbial-derived phenolic metabolites [65]. Findings by Esposito and coll. [66] showed that administration of ACNs-rich black currant powdered extract to obese mice were clearly protective against obesity and associated insulin resistance in animals without antibiotic pretreatment (when gut microbiota was intact) but failed to be active in animals with disrupted microbioma. Furthermore nutritionally relevant amounts of ACNs colonic metabolites exert greater vascular and anti-inflammatory activity than do the metabolites that are formed and absorbed in the small intestine [67, 68], thereby providing additional evidence that the bioactivity of ACNs is highly likely attributed to their microbial-derived metabolites.

3. REDOX-DEPENDENT CELL SIGNALLING PATHWAYS: THE TRANSCRIPTIONAL FACTORS NRF2, NF-KB, AND AP-1

3.1 Mechanisms of redox signalling

Reactive oxygen species (ROS), originally thought to cause exclusively harmful effects, are today considered involved in the regulation of many cell signalling pathways. The classical opinion was that ROS generation was uncontrolled, and that their targets were casual. ROS, in fact, are highly reactive and can damage cellular macromolecules, such as proteins, lipids, and nucleic acids. Resulting oxidized/damaged macromolecules, and their accumulation, were considered factors triggering various pathologies, from inflammatory to neurodegenerative diseases, from atherosclerosis to cancer, and including ageing [69].

At present, however, it is generally accepted that ROS can play the part of second messengers in various extracellular signals, and thus display even desirable effects [70].

In physiological conditions, intracellular ROS levels are balanced by cellular processes producing and eliminating them. The intracellular sources of ROS are various, and could be divided into: (a) biological processes releasing ROS as byproducts, such as the mitochondrial oxidative metabolism, considered the most important producer of intracellular

ROS, and, (b) processes producing ROS on purpose, either as part of cell defence mechanisms, or as part of signal transduction pathways [71, 72].

The mechanisms of ROS-dependent signalling implicate the reversible oxidation/reduction of specific amino acids, especially cysteine (Cys) residues. From its reduced form (SH) the sulfur atom of Cys can be subjected to several oxidative reversible or irreversible modifications [73], all of which can alter the function or activity of proteins. The reversibility of many of these reactions renders Cys an effective molecular switch for regulating protein function. The main ROS-dependent cell signalling pathways include the NF-κB, mitogen-activated protein kinases (MAPKs)/AP-1, Kelch-like ECH-associated protein 1 (Keap1)-Nrf2-antioxidant response elements (ARE) (Keap1-Nrf2-ARE), and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways.

3.2. Structure of Nrf2 pathway and mechanisms underlying its regulation

A relevant example of transcriptional regulation using Cys as a redox switch sensing ROS levels is the mammalian Keap1/Nrf2 pathway. In this case, however, the target of oxidants is not the Nrf2 transcription factor but its interacting partner Keap1. Keap1/Nrf2 pathway has a key role in protecting cells by controlling gene expression of many antioxidant and detoxification enzymes through ARE (also known as the Electrophile Response Element, EpRE) [74-76]. Nrf2 is found in most tissues and organs and in total it controls the expression of nearly 250 genes encoding proteins with many cellular functions, such as redox balancing factors, detoxifying enzymes, stress response proteins and metabolic enzymes [77].

Nrf2 belongs to the bZIP factors of the Cap'n'collar (CNC) family of proteins. The members of this family heterodimerise with small musculoaponeurotic fibrosarcoma (sMaf) proteins [78] and have high homology in their DNA binding and bZIP domains. The Nrf2 protein comprises seven highly conserved Nrf2–ECH homology (Neh) domains, Neh1–Neh7, either involved in regulating Nrf2 stability or transcriptional activity.

Neh2, the N-terminal, modulates Nrf2 binding with its negative regulator Keap1 [79] thanks to the presence of two conserved motifs known as DLG and ETGE [80]. The DLG motif is essential for Nrf2 ubiquitination and degradation [81], while the ETGE for the interaction with Keap1 [82]. At the carboxy-terminal of Nrf2 lies instead the Neh3 domain, acting as a transactivation domain to promote the transcription of ARE-dependent genes [83]. Keap1, the main intracellular Nrf2 regulator, is a dimeric protein of 624 amino acids presenting 27 cysteine residues working as ROS sensors in cellular homeostasis regulation [84-88].

Also called inhibitor of Nrf2 (INrf2) [89], Keap1 is normally associated with Nrf2 (the majority of which is located in the cytoplasm), and interacts with Cullin-3-based ubiquitin E3 ligase complex (Cul3) [90, 91], thus stimulating its ubiquitination and consequent proteasomal degradation by the 26S proteasome [87]. Under basal conditions, in fact, the function of Nrf2 is mainly regulated by the ubiquitin-proteasome system.

Under oxidative stress conditions, or through the activity of Nrf2 inducers, this transcription factor is activated and released from Keap1 and Cul3, allowing *de novo* synthesized Nrf2 to translocate and accumulate into the nucleus [92]. This Nrf2 activation mechanism is known as “canonical activation”, and is the primary mechanism of Nrf2 activation.

Alteration of the reducing status of certain critical cysteine residues of Keap1 can result in conformational changes [93] leading to Nrf2 escape. [87, 88].

Another proposed mechanism for Nrf2-Keap1 complex response to different stimuli is known as the “hinge and latch” model. In this model Nrf2 binds to two Keap1 molecules with the DLG and ETGE motifs of the Neh2 domain [85]. However, the binding affinity of the two motifs is different: ETGE, having a higher affinity, serves as a hinge, whereas DLG, for its weaker affinity for Keap1, acts as a latch that can be easily disturbed by Keap1 conformational changes

[93]. Since DLG binding is essential for Cul3-dependent stimulation of Nrf2 proteasomal degradation, disturbing DLG-Keap1 binding can lead to Nrf2 rescuing from degradation, and to its following accumulation into the nucleus.

Nrf2 can also be regulated through phosphorylation by various protein kinases, such as protein kinase C (PKC), PKR-like endoplasmic reticulum-resident kinase (PERK), as well as PI3K, c-jun N-terminal kinase (JNKs), and extracellular signal regulated kinase (ERKs) [85, 94-98]. This post-translational modification, in fact, can induce Nrf2 release from the complex with Keap1, and thus the activation of the pathway.

A part from the above described canonical activation of Nrf2, there is another activation pathway, called "non-canonical" and mediated by various proteins presenting motifs similar to the ETGE motif in Nrf2. Proteins such as dipeptidylpeptidase 3 (DPP3), Wilms tumor gene on the X chromosome protein (WTX), and partner and localizer of Breast Related Cancer Antigens (BRCA)2 (breast cancer susceptibility gene 2) protein (PALB2), can compete with Nrf2 for Keap1 binding thanks to the presence of the ETGE motif in their structure, thus stabilizing and activating Nrf2 [99]. Probably the better known positive regulator of Nrf2 is p62 (or p62/SQSTM1/sequestosome 1), a protein essential to autophagosomal formation [100, 101].

Activation of Nrf2, by whatever mechanisms involved, leads to its translocation and accumulation into the nucleus where it transactivates genes containing the ARE/EpRE. The ARE sequence is the cis-regulatory DNA enhancer sequence recognized by Nrf2 and present in many genes encoding detoxification and cytoprotective proteins [102, 103]. Most of the genes containing the ARE promoter can be assigned to one of the following groups based on the gene product function: a) antioxidant proteins [*e.g.* glutamate-cysteine ligase catalytic subunit (GCLC), glutathione peroxidase 2 (GPX2), heme oxygenase 1 (HO-1)]; b) phase I drug-metabolizing enzymes [*e.g.* NAD(P)H quinone dehydrogenase 1 (NQO1)]; c) phase II detoxifying enzymes [*e.g.* microsomal glutathione S-transferase 1 (MGST1)] drug transporters (efflux pumps) [e.g. ATP binding cassette subfamily B member 6 (ABCB6), ATP binding cassette subfamily C member 2 (ABCC2)]; e) NADPH generating enzymes [*e.g.* glucose-6-phosphate dehydrogenase (G6PD)] (see the review by Hayes and coll. [104] for a more exhaustive list of the ARE-driven genes).

3.3. Structure of NF-κB pathway and mechanisms underlying its regulation

NF-κB is a family of critical transcription factors involved in a broad range of biological processes, such as inflammatory and immune responses, differentiation, proliferation, and survival of various cell types, as well as stress responses [105]. NF-κB pathway activation is tightly regulated in order to protect the organism, and its misregulation seems to be often implicated in many diseases including inflammation, immune disorders, and cancer [106].

The NF-κB signaling network comprises five protein monomers, namely, p65/RelA, RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), assembling to form active homo- and heterodimers, able to bind to κB sites and then exert positive or negative effects on target genes transcription [107, 108]. All these five proteins share a conserved N-terminal Rel Homology Region (RHR), responsible for dimerization, DNA binding, nuclear localization, and IκB inhibitor proteins binding [109].

Signals from tumor necrosis factor receptor (TNFR), toll-like receptor (TLR) superfamilies, IL-1 receptor (IL-1R) and stress factors are regulated by the IκB/NF-κB signaling network resulting in signal-, context-, and cell type-specific transcriptional responses [110, 111].In its resting state, in fact, NF-κB is retained in the cytoplasm by IκBs (IκBα, IκBβ, and IκBε), which bind to the RHR domain of NF-κB dimers, and mask the nuclear localization signal [112], thus preventing DNA binding and transcriptional activation. Stimuli inducing activation of IκB kinase (IKK), consisting of two subunits (IKK1 and IKK2), and a regulatory subunit, NEMO (NF-κB essential modifier), lead to the degradation of the IκBs through phosphorylation on conserved serine residues, and thus to the release of NF-κB [113].The signaling of

NF-κB is mediated through either the NEMO-dependent canonical pathway, or the NEMO-independent non-canonical pathway. In the canonical pathway, NF-κB activation follows stimulation by proinflammatory signals such as cytokines (such as tumor necrosis factor-alpha, TNF-α), pathogen-associated molecular patterns (PAMPs), and some danger-associated molecular patterns (DAMPs), leading to the activation of IKK. This pathway has a major role in the control of innate immunity and inflammation [114].

The non-canonical pathway, instead, depends on the activation of a NEMO-independent kinase complex involving IKK1 and the NF-κB-inducing kinase (NIK) [115, 116]. This pathway is stimulated by developmental signals that activate TNF receptors (such as BAFF receptor, CD40L receptor, Lymphotoxin-beta receptor, TNFR 11A, TNFR1B, and TNFR12A), some of which also activate the canonical NF-κB pathway [117].

Following removal of IκB, NF-κB dimers are able to translocate into the nucleus and bind to DNA in κB sites in the promoters/enhancers of its target genes [118]. Among the many target genes of NF-κB there are inflammatory cytokines, chemokines, immunoreceptors, cell adhesion molecules, stress-response genes, regulators of apoptosis, growth factors, and transcription factors [119].

3.4. Crosstalk between Nrf2 and NF-κB pathways

Efficient regulation of NF-κB and Nrf2 pathways activity is essential for maintaining redox homeostasis in healthy cells. Some pathological conditions, such as neurodegeneration, autoimmune disorders, and cancer, are characterized by a perturbation of the balance between these two pathways [120]. Many experimental studies support the presence of a putative crosstalk between Nrf2 and NF-κB pathways that can take place at different levels and regulate transcription or function of target proteins. It has been demonstrated, for example, that many phytochemicals are able to suppress NF-κB and activate Nrf2 pathway [27, 121, 122], supporting the hypothesis of the crosstalk. Different levels of oxidative stress can also regulate these two redox-sensitive pathways. A low oxidative stress induces Nrf2, an intermediate level triggers an inflammatory response through the activation of NF-κB, whereas a high oxidative stress can result in apoptosis or necrosis [123].

Moreover, there are genes presenting both ARE as well as κB sites, and thus regulated by both Nrf2 and NF-κB. For example, HO-1 has been shown to present also a functional NF-κB site [124].

Even though there is evidence of functional interactions between the Nrf2 and NF-κB pathways, many aspects of this crosstalk remain unknown, and therefore further investigation is needed.

3.5. AP-1 pathway

The mammalian AP-1 is a family of bZIP transcription factors consisting of Fos, Jun, Maf, and activating transcription factor (ATF) sub-families [125]. These usually form homo- or hetero-dimers that bind to DNA at the level of the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE), and thus activating the transcription of target genes responsible for the regulation of several cellular processes, such as cell differentiation, death, proliferation, transformation, survival, and apoptosis [126-128].

AP-1 can be activated by a variety of factors, such as cytokines, chemokines, growth factors, and oxidative stress [129, 130], both through an increase in the production of its components, and through their phosphorylation. The major signalling cascades activating AP-1 are JNK, ERK, and p38 MAPK [131].

Fujioka and coll. [132] reported the existence of a cross-talk between NF-κB and AP-1 pathways. Although these two pathways are regulated by distinct mechanisms, they seem to be activated at the same time by the same stimuli, and

many genes require the concomitant activation of both of them, suggesting they work cooperatively [133-136]. Moreover, response to AP-1 is markedly improved by the presence of NF-κB subunits and vice versa [137].

4. ANTHOCYANINS IN NCDS

4.1. Anthocyanins as anti-inflammatory agents

Epidemiological and experimental animal studies have shown that inflammation is strongly correlated to a group of diseases, particularly in metabolic syndromes such as obesity, type 2 diabetes and cardiovascular disease [138, 139]. In particular, chronic inflammation is reported as a causative factor [140]. Interestingly, systemic metabolic functions can be negatively affected by persistent exposure to low-dose endotoxin, which creates a chronic subacute inflammatory situation in the body [141].

The pathogenesis of inflammation involves activation of inflammatory immune cells producing mediators and cytokines. Monocytes are well known to induce inflammatory response by producing proinflammatory cytokines, including TNF-α, IL-1β, IL-8, and IL-6 [142]. During inflammatory disease, circulating monocytes are mobilized and rapidly recruited to the site of inflamed tissue where, following conditioning by the local growth factors and pro-inflammatory cytokines, they differentiate into macrophages. Furthermore, activated macrophages, the primary cells of chronic inflammation, release a broad spectrum of mediators such as nitric oxide (NO) and proinflammatory cytokines such as TNF-α, IL-6, and Monocyte chemotactic protein-1 (MCP-1) [142]. In particular, TNF-α plays a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases [143]. MCP-1 is a potent chemoattractant capable of promoting monocyte recruitment while IL-6, an important mediator of fever and of the acute phase response, acts on monocytes to induce their differentiation to macrophages to potentiate inflammation [143].

MAPKs and NF-κB signaling represent two key pathways in the expression of inflammatory mediators including inducible nitric oxide synthase (iNOS) and cytokines [144, 145] (**figure 3**).

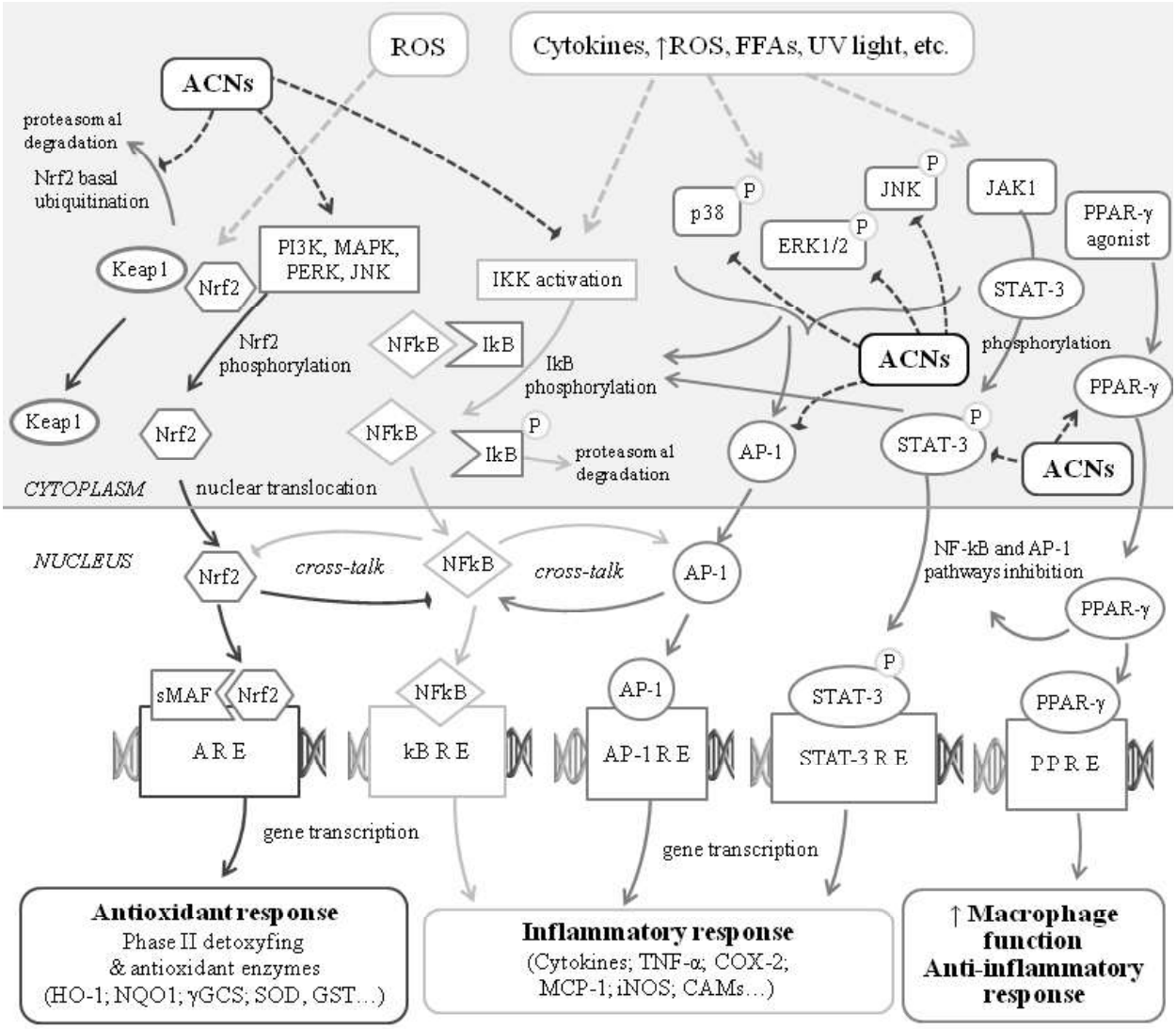


Figure 3. ACNs can inhibit cell inflammatory and oxidative response by interacting (directly or indirectly) with pathways controlled by redox-sensitive transcription factors. These mechanisms involve inhibition of kinases (such as IKK, p38, ERK1/2, JNK and JAK) affecting NF-κB, AP-1 and STAT-3 activation. NF-κB, AP-1 and STAT3 are key transcription factors that orchestrate expression of many genes involved in inflammation. Their activities are induced by a plethora of physiological and environmental stimuli. Other mechanisms involve acute activation of antioxidant and detoxifying enzymes modulated by Nrf2 transcription factor, and PPARg activation.

ACNs: Anthocyanins; AP-1 RE: AP-1 responsive element; AP-1: Activator protein-1; ARE: Antioxidant Responsive Element; CAMs: cell adhesion molecules; COX-2: cyclooxygenase-2; ERK: extracellular signal–regulated kinases; FFAs: Free fatty acids; GST: Glutathione S-transferase; HO-1: heme oxygenase-1; IkB: inhibitor of kappa B; IKK: IκB kinase; iNOS: inducible nitric oxide synthase; JAK1:Janus kinase 1; JNK: Jun N-terminal kinases; kB RE: kB Responive elements; Keap1: Kelch-like ECH-associated protein 1; MAPK: mitogen-activated protein kinase; NF-kB: Nuclear factor-kappa B; NQO1: NAD(P)H quinine dehydrogenase 1; Nrf2: nuclear related factor 2; P: phosphorylation; PERK: PKR-like endoplasmic reticulum-resident kinase; PI3K: phosphoinositide 3-kinase; PPARγ: peroxisome proliferator-activated receptor gamma; PPRE: PPAR responsive element; ROS: Reactive oxygen species; sMAF: small musculoaponeurotic fibrosarcoma protein; SOD: superoxide dismutase; STAT-3: Signal transducer and activator of transcription-3; TNF-α: tumor necrosis factor-alpha; UV: ultraviolet; g-GCS: gamma-glutamylcysteine synthetase.

MAPKs signal pathway includes ERK, p38 MAPK and JNKs [145] and can be activated after phosphorylation by diverse extracellular and intracellular stimuli including peptide, growth factors, cytokines and bacterial lipopolysaccharide (LPS). In macrophages, MAPKs activation induces inflammatory mediators such as iNOS and cytokines. NF-κB activation by LPS, cytokines, and virus involves IKKα/β triggering that then phosphorylate IκB leading to IκB degradation. Finally, NF-κB released from the complex is able to translocate into the nucleus and then to transactivate a wide range of target proinflammatory genes such as iNOS and cytokines [144] (**figure 3**).

In recent years the association between ACNs and inflammation has been evaluated in some cellular and animal models. Lee and coll. demonstrated that blueberry (BBA), blackberry (BKA), and blackcurrant (BCA) (being major ACNs in BBA, BKA and BCA, malvidin-3-glucoside (M3G), C3G and delphinidin-3-rutinoside, respectively) exert anti-inflammatory effects in LPS-activated RAW 264.7 murine macrophages, at least in part, by inhibiting nuclear translocation of NF-κB independent of the Nrf2-mediated pathways [30]. In bone marrow-derived macrophages (BMMs) from Nrf2 wild-type ($Nrf2^{+/+}$) mice, BBA, BKA and BCA significantly decreased cellular ROS levels with a concomitant decrease in IL-1β mRNA levels upon LPS stimulation, while in BMMs from $Nrf2^{-/-}$ mice, the ACN fractions were able to considerably decrease IL-1β mRNA while ROS levels were not significantly affected. NF-κB pathway inhibition was also confirmed by using blueberry-blackberry fermented berry beverages in LPS-activated RAW 264.7 macrophages [146]. Furthermore, the polar fraction of black rice whole grain extracts, enriched with ACNs, significantly inhibited LPS-induced proinflammatory mediators in RAW 264.7 cells, via the inhibition of the MAPK signaling pathway leading to decrease of NF-κB and AP-1 translocation [147]. Interestingly, Appel and coll. reported that a commercial chokeberry (CBA) concentrate (being cyanidin-3-O-galactoside and caffeoylquinic acids the major compounds) was able to reduce LPS-induced NF-κB activation and release of pro-inflammatory mediators in RAW 264.7 macrophages; this product synergizes with sodium selenite (an essential micronutrient found in the body in the form of selenoproteins such as GPx,) to inhibit NF-κB activation, cytokine release and PGE_2 synthesis [148], so suggesting that the use of traditional herbals may be improved by combining them with micronutrients. Partially disagree with these data, there are the findings from Roth and coll. which studied the molecular mechanisms through which ACNs-containing bilberry extract (BE) can exert anti-inflammatory activity in human monocytic THP-1 cells stimulated with TNF-α or Interferon- γ (IFN-γ). In fact they demonstrated that BE effects seem to be dependent on the respective stimulus [149]. In particular, BE (10 μg/ml) is able to ameliorate IFN-γ-induced proinflammatory cytokines gene expression and secretion but was unable to ameliorate TNF-α-induced cellular response strengthening NF-κB phosphorylation, and IL-6 and IL-8gene expression [149].

Delphinidin 3-sambubioside (D3S), isolated from the dried calices of *Hibiscus sabdariffa* L, prodelphinidin B-4 3'-O-gallate (PDG) and Dp inhibited NF-κB pathway and ERK1/2 signaling induced by LPS in RAW 264.7 cells [150, 151]. These effects were ascribed to the inhibition of two main kinases involved in phosphorylation and degradation of IkBα, and subsequent nuclear translocation of p65 (**figure 3**). Similarly, in RAW 264.7 macrophages stimulated by LPS, Mv (the most abundant polyphenol in red wine) attenuated LPS-induced NF-κB, poly ADP-ribose polymerase (PARP) and ROS production. Interestingly the authors observed that Mv attenuated LPS induced activation of JNK, p38 and ERK, three MAPKs involved in activation of NF-κB and AP-1, via MAPK phosphatase-1, the major enzyme responsible for the dephosphorylation [152]. However, Hamalainen and coll. reported that in murine J774 macrophages exposed to LPS, Pg reduced iNOS protein and mRNA expression and also NO production inhibiting speciafically the NF-κB pathway but not that of Signal transducer and activator of transcription-1 (STAT-1) [153].

Studies explored the effect of C3G on LPS-stimulated human THP-1 derived macrophages (differentiated through exposure to phorbol myristate acetate) [154, 155]. C3G, at a concentration ranging from 0.005 and 10 μM, significantly

inhibited LPS-stimulated TNF-α and IL-6 mRNA levels and protein release in THP-1 cells; phosphorylation of IkBα and NF-κB nuclear translocation was blocked by C3G already at 0.5 μM [154]. However, not only C3G (major constituent of black rice) but also its metabolites Cy and PcA suppressed, via NF-κB pathway, the production of proinflammatory mediators and the gene expression of iNOS and cyclooxygenase-2 (COX-2) at concentrations ranging between 2 and 5μM [156]. Furthermore, these compounds significantly inhibited the leukocyte number and the levels of IL-1ß, TNF-α and PGE_2 in the exudates of the air pouch in carrageenan-treated BALB/c mice, as well as COX-2 expression and NF-κB activation [156]. Among the tested agents, PcA strongly inhibited these inflammatory mediators *in vivo* and *in vitro*. In addition, submicromolar concentrations of C3G, Cy and cyanidin-3-rutinoside (C3R) from black raspberry (*Rubus coreanus* Miquel) reduced LPS-induced protein level of iNOS and suppressed release of pro-inflammatory mediators through inhibition of MAPKs and STAT-3 phosphorylation in RAW 264.7 cells [157]; interestingly, a mixture of the key components C3G and C3R was more effective than Cy alone, explaining the health beneficial activity of black raspberry. Furthermore, the study from Varì and coll. provided evidence that 25μM of PcA improved the cellular endogenous antioxidant potential through the overexpression of glutathione peroxidase (GPx) and GSH reductase (GRx) in J774 A.1 macrophages by inducing JNK2-mediated phosphorylation of Nrf2 [158].

A strong confirmation of the NF-κB involvement in ACNs anti-inflammatory properties is reported by Karlsen and coll. which demonstrated that in cultured U937–3×κB-LUC cells (monocytes stably transfected with a luciferase reporter that contained 3 NF-κB binding sites) ACNs isolated from bilberries (*Vaccinium myrtillus*) and black currants (*Ribes nigrum*) (Medox®; a mixture of 3-*O*-rutinosides of Cy and Dp, and 3-*O*-*β*-galactosides, 3-*O*-*β*-glucosides, and 3-*O*-*β*-arabinosides of Cy, Pn, Dp, Pt, and Mv) efficiently suppressed LPS-induced activation of NF-κB [159]. The same Authors demonstrated, in a parallel-designed, placebo-controlled clinical trial on a total of 120 healthy subjects, that supplementation with Medox® (300 mg/d for 3 weeks) produced a decrease in circulating levels of pro-inflammatory chemokines IL-8 and Interferon-α (IFN-α), IL-4, and IL-13 (3 mediators inducing NF-*κ*B pathway), at an extent higher than that observed in placebo receiving group [159].

4.2. Anthocyanins protective effects against endothelial dysfunction

ACNs have been reported to induce the expression of enzymes involved in both cellular antioxidant defenses and attenuating inflammation-associated pathogenesis (**figure 3**); induction of such enzymes largely accounts for their atherosclerosis chemo-protective activities [160]. Natural Nrf2 and HO-1 inducers, such as C3G, might be a potential therapeutic strategy to protect vascular system against various stressors preventing several pathological conditions [121] through modulation of Nrf2 pathway and consequently of genes regulated by the ARE/EpRE motif. Table 1 summarizes the *in vivo* and *in vitro* studies of the beneficial effects of ACNs on endothelial and metabolic dysfunction.

1 **Table 1**: *In vitro* and *in vivo* studies of the biological effects of ACNs.
2

ANTHOCYANINS	EXPERIMENTAL MODEL	AGENT/INDUCER	REFERENCES
Endothelial dysfunction			
Dp and Cy	EA.hy926 endothelial cells	OxLDL	Yi et al., 2012[161] Chen et al., 2011[162]
Purple sweet potato leaf extract and Cy	Human aortic endothelial cells (HAECs)	TNF-α	Chao et al., 2013[163]
C3G	Human umbilical vein endothelial cells (HUVECs)	TNF-α	Speciale et al., 2010[164] Speciale et al., 2013[121]
C3G	Rat aortic smooth muscle cells (RASMCs)	TNF-α	Yan et al., 2016[165]
ACNs isolated from black soybean seed coat	Bovine aortic endothelial cells (BAECs)	TNF-α	Kim et al., 2006[166]
C3G	Human vascular smooth muscle cells (HVSMCs)	Angiotensin II	Pantan et al., 2016[167]
Chokeberry fruit extract	Endothelial progenitor cells (EPCs)	Angiotensin	Parzonko et al., 2015[168]
Pg	Human umbilical vessel endothelial cells (HUVECs)	Polyp	Lee and Bae, 2017[169]
Mv, M3G and M3Ga	Human umbilical vessel endothelial cells (HUVECs)	TNF-α	Huang et al., 2014[170] Huang et al., 2014[171]
M3G	Bovine aortic endothelial cells (BAECs)	Peroxynitrite	Paixao et al., 2012[172]
Metabolic syndrome			
C3G	Human umbilical vessel endothelial cells (HUVECs)	PA	Fratantonio et al., 2015[173] Fratantonio et al., 2017[174]
Purple sweet potato flavonoids	Mice kidney dysfunction	HFD	Shan et al., 2014[175]
Abacopterin A from *Abacopteris penangiana* (Hook.) Ching	Liver inflammation in C57BL mice	HFD	Lei et al., 2011[176]
Purple corn ACNs	Human mesangial cells	HG	Li et al., 2012[177]
C3G from mulberry fruits	MIN6N pancreatic β-cells	HG	Lee et al., 2015[178]
Chinese bayberry fruit extract	INS-1 pancreatic β cells	H_2O_2	Zhang et al., 2013[179]
Chinese bayberry fruit extract	Beta-cell transplantation in ICR mice	Streptozotocin	Zhang et al., 2013[179]
Osteoarticular diseases			
ACNs from black soybean seed coats	Affected joints in DBA/1J mice	Collagen-induced arthritis	Min et al., 2015[180]
Dp from *Punica granatum*	MH7A human rheumatoid arthritis synovial cells	TNF-α	Seong et al., 2011[181]
Dp	Human chondrocytes	IL-1β	Haseeb et al., 2013[182]
Dp	RAW264.7 monocyte/macrophage mouse cells	RANKL-mediated osteoclast formation	Moriwaki et al., 2014[183]

Intestinal inflammatory disorders			
C3G	Differentiated Caco-2 human colonic cells	TNF-α	Ferrari et al., 2016[184]
C3G	HT-29 human intestinal cells	Different cytokines	Serra et al., 2013[185] Serra et al., 2016[186]
ACN-rich fraction from a wild blueberry powder	Caco-2 intestinal epithelial cells	IL-1β	Taverniti et al., 2014[187]
Grape seed and grape marc extract	Pigs duodenal mucosa	-	Gessner et al., 2013[188]
ACN-rich bilberry extract	Colonic tissue in patients with ulcerative colitis	-	Roth et al., 2016[189]
C3G	Coculture of differentiated Caco-2 intestinal epithelial cells and human umbilical vessel endothelial cells (HUVECs)	TNF-α	Ferrari et al., 2017[190]
ACN-rich grape extract and M3G	Cocultures of Caco-2/HT29-B6 cells and human umbilical vessel endothelial cells (HUVECs)	TNF-α	Kuntz et al., 2015[191]
Liver injury			
Cy	HepG2 human hepatoblastoma cells	H_2O_2	Shih et al., 2012[192]
ACN fraction from purple sweet potato	HepG2 human hepatoblastoma cells	T-BHP	Hwang et al., 2011[193]
ACN fraction from purple sweet potato	Liver injury in rats	DMN	Hwang et al., 2011[194]
Bilberry extract	Liver injury in mice	*Propionibacterium acnes* plus LPS	Luo et al., 2014[195]
ACNs from purple sweet potato and purple potato	Liver injury in mice	Alcohol	Jiang et al., 2016[196]
Central Nervous System disorders			
ACNs from korean black bean	Postnatal day 7 rat brain	Glutamate	Shah et al., 2016[197]
ACNs from korean black bean	SH-SY5Y human neuroblastoma cells	Glutamate	Shah et al., 2016[197]
ACNs from black soybean seed coats	BV2 murine microglial cells	LPS	Jeong et al., 2013[198] Wang et al., 2015[199]
ACNs from black soybean	LPS-injected adult mice	Cerebral cortex	Khan et al., 2016[200]
C3G	SH-SY5Y human neuroblastoma cells	Aβ25–35 oligomers and aβ1–42 oligomers	Tarozzi et al., 2010[201]
Mix of different ACNs	SK-N-SH human neuroblastoma cells	Acrolein	Belkacemi and Ramassamy, 2016[202]
Retinal diseases			
Black rice anthocyanidins	Retinal damage in rats	Fluorescent light	Jia et al., 2013[203]
C3G	Retinal damage in pigmented rabbits	Visible light	Wang et al., 2016[204]
Bilberry extract and	661 W cultured murine	Blue light	Ogawa et al., 2014[205]

lingonberry extract	photoreceptor cells		
Bilberry extract	C57Bl/6 uveitis in mice	Endotoxin	Miyake et al., 2012[206]
Blueberry ACNs	Diabetic rats	Retinal cell damage	Song et al., 2016[207]
Skin damage			
ACNs from black soybean seed coats	HaCaT human keratinocyte cells	UVB	Tsoyi et al., 2008[208]
Extract from red orange	HaCaT human keratinocyte cells	UVB	Cimino et al., 2007[209]
C3G	HaCaT human keratinocyte cells	UVB	Cimino et al., 2006[210]
ACNs-rich extract from bog blueberry	Human dermal fibroblasts	UVB	Bae, Lim et al., 2009[211]
Extract from black raspberries	JB6 Clone 41 mouse epidermal cells	UVB and UVC	Huang et al., 2007[212]
Pomegranate fruit extract	Normal human epidermal keratinocyte	UVB	Afaq et al., 2005[213]
Cy	WI-38 human diploid fibroblast (HDF) cells	H_2O_2	Choi et al., 2010[214]
C3G	SKH-1 hairless mice	UVB	Pratheeshkumar et al., 2014[215]
ACNs from black soybean seed coats	Hairless mice topical	UVB	Tsoyi et al., 2008[208]

ACNs: Anthocyanins; Cy: cyanidin; C3G: cyanidin-3-glucoside; DMN: dimethylnitrosamine; Dp: delphinidin; HFD: high fat diet; HG: high-glucose; H_2O_2:hydrogen peroxide; IL:interleukin ; LPS: lipopolysaccharide; Mv:malvidin; M3Ga:malvidin-3-galactoside; M3G: malvidin-3-glucoside; oxLDL: oxidized low-density lipoprotein; PA: palmitic acid; Pg: pelargonidin; PolyP: PolyPhosphate; RANKL: Receptor Activator of NF-κB ligand; t-BHP: tert-butyl hydroperoxide; TNF: tumor necrosis factor ; UV ultraviolet light.

Pretreatment with Dp and Cy (40 μM) protects endothelial EA.hy926 cells against oxidized low-density lipoprotein (oxLDL)-induced decrease of cell viability, ROS generation and p38 MAPK activation [161]. Furthermore, these two ACNs reduced NF-κB nuclear translocation and its transcriptional activity as well as the mRNA expression of genes including intercellular adhesion molecule 1 (ICAM-1), VCAM-1, E-selectin, matrix metalloproteinase (MMP)-1, MMP-2 and MMP-9 through blunting ROS-triggered signaling pathway induced by oxLDL. Similarly, Dp attenuates oxLDL induced adhesion of monocytes to EA.hy926_cells by inhibiting ROS/p38 MAPK/NF-κB pathway at concentrations ≥ 50μM [162]. As to the structure-activity relationship, the number of hydroxyl groups in total, 3',4'-ortho-dihydroxyl groups in B-ring and 3-hydroxyl group in C-ring are important structure characteristics for the inhibitory effects [161].

Consistent results were obtained when human aortic endothelial cells (HAECs) were treated with purple sweet potato leaf extract (PSPLE, 100μg/ml) or Cy (10μM) [163], which can significantly inhibit TNF-α-induced monocyte-endothelial cell adhesion and attenuate VCAM-1, IL-8 and CD40 expression. In this research, significant reductions in NF-κB nuclear translocation and DNA binding activity by Cy were also observed in addition to decreased expression of ERK1, ERK2 and p38 MAPK.

Several researches have been carried out to demonstrate the antiatherogenic effect of C3G. In fact, C3G (20–40 μM) is able to protect human umbilical vessel endothelial cells (HUVECs) against alterations induced by TNF-α, including the activation of NF-κB, increased gene expression of adhesion molecules and leukocyte adhesion [164]; furthermore, C3G

improved antioxidant systems, and activated Nrf2 pathway, at baseline and after TNF-α treatment, through the involvement of specific MAPKs (ERK1/2) [121] (**figure 3**).

The anti-inflammatory capability of C3G was shown also in rat aortic smooth muscle cells (RASMCs) challenged by TNF-α [165]. C3G pretreatment (≥ 25 μM) induced apoptosis and decreased migration of TNF-α-stimulated RASMCs. In particular, TNF-α-induced nuclear translocation of NF-κB subunit p65 and phosphorylation of IκBα in RASMCs were decreased by C3G. Finally, in TNF-α-treated bovine aortic endothelial cells (BAOECs) ACNs isolated from black soybean seed coat [rich in C3G, delphinidin-3-glucoside (D3G) and petunidin-3-glucoside (Pt3G)] inhibited, at concentrations ≥ 10μg/ml, TNF-α-induced VCAM -1, ICAM-1, and COX-2 levels, through inhibition of NF-κB nuclear translocation [166]. Further, doses ≥ 25mg/kg of this ACNs mixture protected myocardiac injury in rats subjected to 30-min occlusion of left descending coronary artery followed by 24-h reperfusion [166].

Statins have often been used in atherosclerosis treatment because of their pleiotropic effects on inflammation. Pantan and coll. [167] suggest that a combination of low dose statins and C3G might act as a potential modulator of the atherosclerosis process. In fact, in human vascular smooth muscle cells (HVSMCs) exposed to angiotensin II (Ang II) atorvastatin and C3G ≥ 2μM produces synergism against inflammation and oxidative stress, by suppressing NF-κB nuclear translocation, and attenuating the expression of iNOS, ICAM-1, and VCAM-1. Moreover, C3G improves the antioxidative properties of atorvastatin promoting the activity of the Nrf2/ARE signaling pathway and downstream proteins including HO-1 and NQO-1 [167]. Endothelial progenitor cells (EPCs), thanks to their innate ability to substitute damaged or dysfunctional endothelial cells in plaque microvessels, can afford protection against plaque rupture and atherosclerosis. Recent evidences supported that Ang II is able to increase oxidative stress and to accelerate the process of ageing of EPCs so impairing the angiogenic functions in the atherosclerotic plaque [216]. Chokeberry (*Aronia melanocarpa*) fruit extract, containing mainly C3G, protects EPCs (isolated from peripheral blood of young healthy volunteers and cultured *in vitro*) against angiotensin-induced oxidative stress at concentrations ≥ 1 μg/ml [168]. This effect was related to the activation of the Nrf2 transcription factor and the increase of HO-1 expression.

Pelargonidin can modulate inflammatory responses mediated by human endothelial cells-derived PolyPhosphate (PolyP, one of the pro-inflammatory mediators) both *in vitro* on HUVECs and *in vivo* in mice [169]. Pg concentrations ≥ 10μM inhibit PolyP-mediated endothelial barrier disruption, adhesion molecules gene expression, and adhesion/migration of leukocyte to HUVECs. PolyP-induced NF-κB activation and the productions of TNF-α and IL-6 were also inhibited by Pg [169]. These anti-inflammatory effects of Pg were confirmed also in PolyP injected mice.

Huang and coll. investigated the inhibitory effect of Mv, M3G and malvidin-3-galactoside (M3Ga) (two main blueberry ACNs) on inflammatory response in TNF-α exposed HUVECs [170, 171]. These ACNs, at concentrations ≥ 1 μM, inhibit TNF-α induced increase of mRNA and protein levels of MCP-1, ICAM-1, and VCAM-1 in a concentration-dependent manner. In addition, the anti-inflammatory activity was attributed to the modulation of NF-κB pathway since they reduced IκBα degradation and p65 nuclear translocation induced by TNF-α. In general, M3G had better anti-inflammatory effect than M3Ga. Furthermore, 25μM of M3G up-regulated eNOS mRNA in BAOECs, leading to the enhancement of eNOS activity and NO production, an effect even greater when cells were further stimulated with peroxynitrite [172]. On the other hand, in peroxynitrite-activated endothelial cells, M3G suppressed pro-inflammatory mediators, namely iNOS expression/NO biosynthesis, COX-2 expression and IL-6 production, through inhibition of NF-κB activation [172]. These findings suggest a potential role of M3G in NO balance and in inhibition of pro-inflammatory signaling pathways. Conversely, unlike of a wild blueberry extract rich in ACNs, M3G increases the adhesion of human THP-1 monocytes to TNF-α–treated HUVECs, starting from 20 nM, while other ACNs and their metabolites (including PcA) had no effect at the same concentrations [217].

Unfortunately, these results all evidence that the effects of the single ACNs and their metabolites are controversial and merit further exploration.

4.3. The health effects of anthocyanins in metabolic syndrome

Several studies have shown that chronic overfeeding leads to immune cell infiltration in different tissues, resulting in the production of cytokines, chemokines, and other mediators, which are implicated in oxidative stress and inflammatory response associated with obesity and may be major cause of tissue and organ damage (table 1).

Free fatty acids (FFAs), generally elevated in diabetes and obesity, have been shown to affect endothelial functions and trigger oxidative stress, inflammation, and insulin resistance (**figures 3** and **4**).

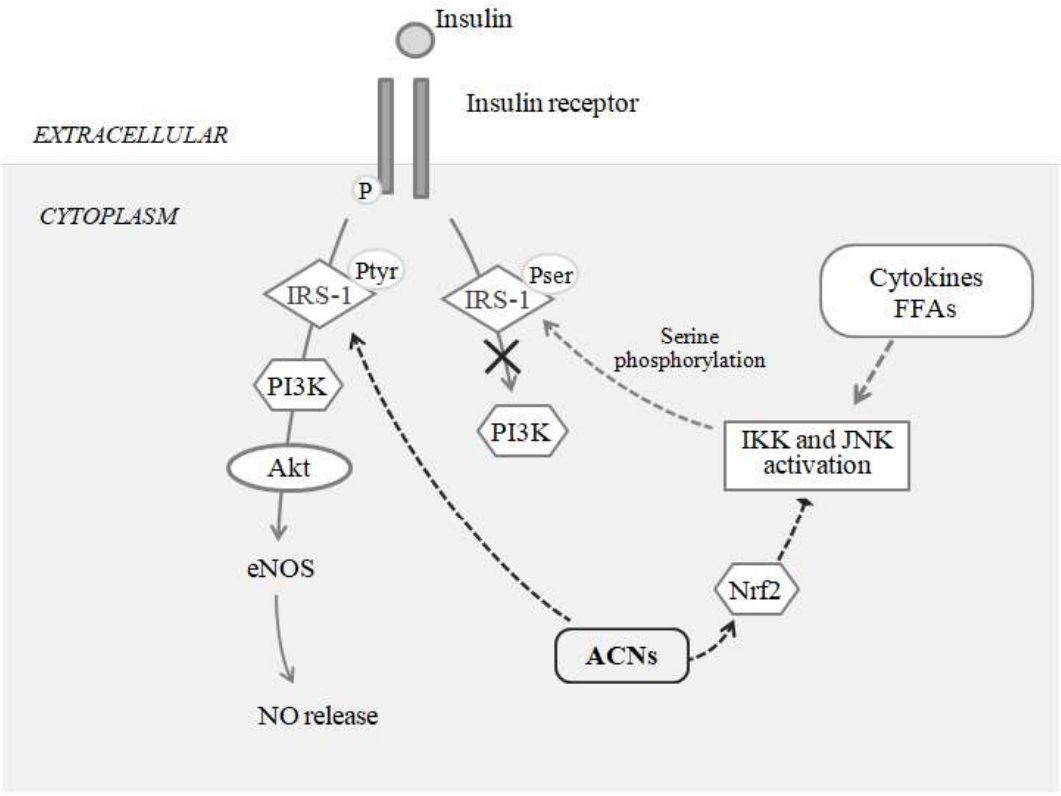


Figure 4. ACNs modulate insulin IRS1/Akt signalling in insulin resistance. Under inflammatory conditions, ACNs inhibit insulin serine phosphorylation of IRS-1 via suppressed IKK and JNK phosphorylation, restoring transduction of the insulin IRS-1/Akt pathway ACNs: Anthocyanins; Akt: protein kinase B; eNOS: Endothelial nitric oxide synthase; FFAs: Free fatty acids; IKK: IκB kinase; IRS-1: Insulin receptor substrate 1; JNK: Jun N-terminal kinases; NO:nitric oxide; Nrf2: nuclear related factor 2; P: phosphorylation; PI3K: phosphoinositide 3-kinase; Pser:serine phosphorylation; Ptyr: Tyrosine phosphorylation.

A significant augmentation of free radicals and oxidative stress was demonstrated in HUVECs exposed to palmitic acid (PA, the most prevalent saturated FFA in circulation), while C3G (20 and 40 μM) pretreatment improved intracellular redox status altered by FFA [173], and significantly inhibited NF-κB pathway and adhesion molecules gene expression induced by PA. Interestingly, C3G induced Nrf2 nuclear localization and activation of cellular antioxidant and cytoprotective genes at baseline and after PA exposure in endothelial cells. Furthermore, FFAs produce insulin resistance in endothelium inducing a decreased synthesis of insulin-mediated vasodilator NO (**figure 4**), and an increase of the vasoconstrictor protein, endothelin-1. Fratantonio and coll. demonstrated that C3G 20 μM reverses not only endothelial dysfunction but also insulin resistance induced by PA in HUVECs [174], restoring PI3K/Akt/eNOS axis and NO release. Interestingly, C3G induced phosphorylation of specific tyrosine residue of Insulin receptor substrate 1 (IRS-1), reduced by PA, through the modulation of JNK and IKK activity (**figure 4**). Importantly, silencing of Nrf2 transcripts demonstrated that also these effects may be attributed to the activation of Nrf2/EpRE pathway exerted by C3G

Shan and coll. showed that the beneficial effects of purple sweet potato color (PSPC; flavonoids isolated from purple sweet potato) on kidney dysfunction and damage induced by high fat diet (HFD) in mice [175]. In particular these authors demonstrated that PSPC (700 mg/kg) reduced, in mice fed an HFD, the expression level of kidney Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome and restored its downstream cell signaling (such as NF-κB pathway) and upstream modulators [such as oxidative stress-associated Receptors for Advanced Glycation Endproducts (RAGEs) and thioredoxin interacting protein (TXNIP)], which have also been demonstrated to be involved in obesity process, resulting in kidney injury and insulin resistance.

Similarly, Lei and coll. demonstrated the hypolipidemic and anti-inflammatory properties of abacopterin A (APA. 40 or 20 mg/kg) [an ACN isolated from *Abacopteris penangiana* (Hook.) Ching] in male C57BL/6J mice fed with HFD for 8 weeks [176], reducing NF-κB expression, TNF-α and IL-6 serum levels, and triacylglycerol and total cholesterol levels induced by HFD.

Hyperglycemia causes toxicity in vulnerable cell types and induces intracellular ROS generation resulting in cell damage and death. Purple corn anthocyanins (PCA, 1 to 20 μg/ml) mainly comprising C3G and cyanidin-3-(6″-malonylglucoside) can attenuate high-glucose (HG)-induced human mesangial cell (HMC) hyperplasia and matrix accumulation, typical condition of chronic renal failure [177]. PCA ameliorated HG-induced mesangial inflammation by reducing ICAM-1 and MCP-1 through the suppression of transforming growth factor β (TGF-β), a fibrogenic cytokine having a key role in the development of renal hypertrophy in diabetes. These PCA effects were attributed to dampened NF-κB translocation in HG-exposed HMC since a crosstalk between TGF-β and NF-κB pathways has been demonstrated [177].

The study by Lee and coll. investigated the cytoprotective effects of C3G, isolated from mulberry fruits, on the HG-induced apoptosis in mouse insulin-producing pancreatic β-cells (MIN6N cells) [178]. C3G (concentrations of 103 and 144 μM) protected the pancreatic β-cells by reducing apoptosis through the modulation of the intrinsic apoptotic pathway-mediated proteins. In addition, C3G dose-dependently reduced nuclear translocation of NF-κB in HG treated cells. Finally, insulin secretion, significantly inhibited in the HG-exposed MIN6N cells, was partially restored by pretreatment with 144 μM of C3G [178].

However, ACNs are able to protect, in other several stress conditions, pancreatic β cells against damage. These cells are particularly susceptible to oxidative stress, due to a low expression of antioxidant enzymes, representing one of the major factors causing cell damage [218]. An extract obtained from Chinese bayberry fruits rich in ACNs (especially C3G) was shown to protect β cells (INS-1 cell line) from H_2O_2-induced apoptosis and decreased cells grafts apoptosis in a β cell transplantation model in ICR mice rendered diabetic using streptozotocin [179]. In particular, ACNs pretreatment (1–2 μM concentrations calculated as C3G content) attenuated H_2O_2-mediated autophagic cell death in INS-1 cells through the activation of the transcription factor Nrf2 and its regulated gene HO-1, with a resulting negative modulation of the autophagy process [179].

4.4. Protective effects of anthocyanins on osteoarticular diseases

Rheumatoid arthritis (RA) is the most common form of the autoimmune rheumatic diseases, and affects about 1% of the world's population. The main problems connected with RA are systemic inflammation and progressive joint destruction that cause functional impairment in RA patients [219]. Min and coll. investigated the therapeutic effects of ACN extracted from black soybean seed coats (AEBS, containing C3G, D3G and Pt3G) in a murine model of collagen-induced arthritis (CIA); [180]. To induce CIA DBA/1J mice were intradermally immunized with bovine type II collagen suspended in complete Freund's adjuvant. AEBS reduced the occurrence of arthritis, decreased proinflammatory

cytokines levels in affected joints of CIA mice and suppressed NF-κB signaling. Additionally, AEBS decreased Th17 cell numbers in spleen of CIA mice and inhibited *in vitro* differentiation of Th17 cells and expression of Th17-associated genes in splenocytes of naïve DBA/1J mice.

Histone acetyltransferase inhibitors (HATi) can suppress inflammatory signaling, which contributes to RA. Seong and coll. identified Dp, present in *Punica granatum* L., as a novel HATi [181]. Dp, at concentrations ≥ 10 μM, inhibited TNF-α-induced p65 acetylation in MH7A cells (a human RA synovial cell line), together with cytosolic accumulation of p65 and nuclear localization of IκBα; these effects are mediated by inhibition of p300 and CBP acetyltransferase activities, two coactivators of NF-κB [181].

In osteoarthritis (OA), there is enhanced expression of pro-inflammatory cytokines such as IL-1β in the affected joint. Haseeb and coll. demonstrated that Dp 29 μM inhibited IL-1β-induced expression of COX-2 and production of cartilage-degrading molecule PGE_2 in human chondrocytes [182]. Dp, tested at 148 μM concentration, also inhibited IL-1β-mediated phosphorylation of IL-1R-associated kinase-1(Ser376) and the downstream NF-κB pathway.

Dp showed also anti-osteoporotic bone resorption through the suppression of osteoclast formation so preventing bone loss in postmenopausal osteoporosis [183]. In fact, Dp, at concentration starting from 0.7 μM, markedly inhibited the *in vitro* differentiation of RAW 264.7 cells [induced by treatment with receptor activator of NF-κB ligand, Receptor Activator of NF-κB ligand (RANKL)] into osteoclasts. Furthermore, oral administration of Dp (1–10 mg/kg) significantly prevented bone loss in mice with soluble RANKL-induced osteoporosis and in ovariectomized mice, a standard model of osteoporosis. Additionally, Dp suppressed the activity of NF-κB, c-fos, and nuclear factor of activated T cells 1 (NFATc1), which are the main transcriptional factors in osteoclastogenesis.

4.5. The role of anthocyanins in the prevention and treatment of intestinal inflammatory disorders

Chronic intestinal inflammatory disorders, such as inflammatory bowel diseases (IBDs), are characterized by excessive release of proinflammatory mediators, intestinal barrier dysfunction and excessive activation of NF-κB cascade.

Since dietary ACNs can potentially reach the gastrointestinal tract at high concentrations, C3G may be considered as a promising dietary compound giving complementary benefits in the IBD context, especially due to the ability to activate cellular adaptive responses regulated by Nrf2 (**figure 3**).

In *in vitro* model of acute intestinal inflammation using differentiated human intestinal colon carcinoma cell line (Caco-2 cells) exposed to TNF-α [184], cell pretreatment with 20 and 40μM of C3G prevented TNF-α-induced changes (NF-κB pathway activation and increased IL6 and COX-2 expression), improving intracellular redox status. These effects were attributed to the activation of Nrf2 pathway since C3G, also without any kind of stimulus, increased the translocation of Nrf2 into the nucleus so triggering antioxidant and detoxifying genes [184].

C3G was shown to protect human intestinal HT-29 cells against inflammatory response triggered by different cytokines (IL-1α, TNF-α and IFN-γ) [185], in terms of NO, PGE_2 and IL-8 release and of iNOS and COX-2 protein expressions, at a much lower concentration than 5-aminosalicylic acid (a well-known anti-inflammatory drug used in IBD). Interestingly, C3G was not able to prevent IkB-α degradation or the activation of NF-κB, but significantly reduced cytokine-induced nuclear levels of activated STAT-1. Furthermore, in cytokine-challenged HT-29 cells, C3G 25 μM induced Nrf2 activation [(without increasing nuclear levels of peroxisome proliferator-activated receptor (PPAR) -γ)] and its downstream genes HO-1 and glutamate cysteine ligase, enhanced glutathione reduced (GSH)/ glutathione oxidized (GSSG) ratio and inhibited reactive species production [186].

In addition, the effects of ACNs were evident also when contained in a plant-derived complex matrix (table 1); for example, the ACNs-rich fraction from a wild blueberry powder (containing mainly glycosides of Mv, Dp and Cy,

together with lower amounts of Pt and Pn glycosides) reduced the activation of NF-κB, induced by IL-1β in intestinal epithelial Caco-2 cells, at concentrations ≥ 50 μg/ml [187].

The benefic effect of ACNs against chronic gut diseases was confirmed also by *in vivo* studies (table 1). Roth and coll. studied the molecular mechanisms of ACNs-rich bilberry extract (ARBE) by analyzing colonic tissue and serum samples of ulcerative colitis (UC) patients treated with an oral ARBE during an open label clinical trial [189]. Reduced levels of the pro-inflammatory cytokines IFN-γ and TNF-α and of phosphorylated p65/NF-κB were detected in colon tissue of UC patients in remission after ARBE treatment. Further, patients with successful ARBE treatment featured enhanced IL-17A , IL-22 and IL-10 cytokines levels, fundamental for host defence, together with reduced serum levels of TNF-α and MCP-1, in comparison with who did not benefit from ARBE treatment [189].

The study from Gessner and coll. 2013 evidences that ACNs intake may be useful dietary approach also in animals, for example to inhibit inflammation in the gut that regularly occurs in pigs**.** In fact, pigs administered 1% grape seed and grape marc extract (GSGME) rich in M3G and containing 8.5% of polyphenols, showed a higher ratio of villus height/crypt depth and of the gain/feed, together with a lower transactivation of NF-κB and its target genes in the duodenal mucosa, when compared to controls [188]. Surprisingly, GSGME administration did not increase but even reduced transactivation of Nrf2 and expression of Nrf2 target genes in duodenum, very likely due to the decrease in levels of ROS or pro-inflammatory cytokines that are known to activate Nrf2 [188].

Intestinal epithelium is considered a defensive physical barrier and it contributes to the mucosal immune system. Polarized basolateral intestinal release of pro-inflammatory molecules, followed by activation of NF-κB pathway in endothelial cells, powerfully induces extravasation of neutrophils, so contributing to the progression and maintenance of intestinal inflammation. By using a coculture *in vitro* system (Caco-2 cells and HUVECs), Ferrari and coll. evaluated if C3G can prevent the effect of TNF-α-stimulated intestinal cells on endothelial cells activation [190]. In this model, C3G (20 and 40 μM) reduced TNF-α stimulated nuclear translocation of NF-κB and expression of TNF-α and IL-8 gene in Caco-2 cells cultured alone. Furthermore, Caco-2 cells, exposed to TNF-α, stimulated endothelial cells activation, as observed by higher E-selectin and VCAM-1 mRNA levels, leukocyte adhesion, and nuclear translocation of NF-κB in HUVECs; these effects were reduced by C3G treatment. Thus, C3G selective modulation of the NF-κB pathway in colonic epithelial cells represents the principal mechanism of the observed protective effects.

A similar coculture model was employed to demonstrate that ACNs, in physiological concentrations, can reach the serosal compartment and reduce inflammatory markers involved in the first steps of atherosclerosis [191]. Kuntz and coll. established a transwell epithelial-endothelial co-culture system with Caco-2 alone or with HT29-B6 cells (in order to simulate different metabolization sides of the gut), mimicking the intestinal barrier, and HUVECs, mimicking the vasculature. ACNs-rich grape extract as well as M3G and PCA were applied to the luminal compartment of the transwell in order to mimic intestinal absorption. HUVECs were then stimulated for 3 hours with TNF-α (1 ng/mL for a low-grade or 10 ng/mL for a high-grade inflammation). ACNs-rich extract as well as M3G and PCA significantly inhibited leukocyte adhesion when being transported across the intestinal layer. This effect was only observed when Caco-2 cells as single cell type served as an absorptive cell model; furthermore no effect was observed with short-term exposure and high-grade inflammation. Furthermore, ACNs extract and M3G significantly attenuated TNF-α-induced E-selectin, VCAM-1 and ICAM-1 gene expression and cytokine expression and secretion (IL-8 and IL-6) as well as NF-κB mRNA expression. Using the same model, the Authors demonstrated that ACN metabolites generated by *E. faecalis* and *H. alvei* significantly attenuate low-grade stimulated leukocyte adhesion, adhesion molecules E-selectin, VCAM-1 and ICAM-1 gene expression and cytokine secretion, as well as NF-κB mRNA expression [220].

Interestingly, there is evidence that maternal diet supplementation with ACN-rich products could contribute to reduce colonic low-grade inflammation in offspring, and thus could contribute to reducing chronic disease development in later life. Morais and coll. [221, 222] investigated the effect of jussara (Euterpe edulis Mart., rich in C3G and C3R) supplementation in the maternal diet on the proinflammatory state of the colon in 21-day-old offspring exposed to trans-fatty acids (TFAs) during pregnancy and lactation. In fact, maternal intake of TFAs in the perinatal period triggers a proinflammatory state in offspring. Jussara supplementation in maternal diet reduced serum IL-6 and TNF-α and gene expression of IL-6 and TNF-α receptors, and increased *Lactobacillus* spp. in the colon of offspring [221]. Furthermore, jussara supplementation restored the fecal content of *Bifidobacterium* spp., increased colonic ZO-1 mRNA expression, and reduced NF-κB pathway activation by decreasing MyD88, phosphorylated NF-κB p65 subunit, and TNF-α receptor I in the liver [222].

Several researches demonstrate that gut dysbiosis (caused, for example by a diet high in saturated/trans fat and simple sugars and low in fibers) may contribute to development of chronic inflammation. In fact microorganisms, particularly Bifidobacterium strains, are able to regulate expression of tight junction proteins [223], which are peripheral membrane proteins involved in the regulation of paracellular permeability. Furthermore, gut microbiota (*e.g. Akkermansia muciniphala* and *Bifidobacterium* spp.) can stimulate mucus secretion and contribute to thickening of the mucus layer [58]. Thus, gut dysbiosis can lead to a status of metabolic endotoxemia (characterized by higher plasma LPS levels) that promotes low-grade inflammation-induced metabolic disorders (such as insulin resistance, diabetes, and obesity). Prebiotics can lead to compositional changes in gut microbiota, and so can decrease plasma LPS levels and normalize low-grade inflammation. As recently reviewed by Jamar and coll. [58], ACNs and metabolites formed in the intestine can change the composition of the gut microbiota, so restoring distribution and localization of tight-junction proteins and increasing mucus layer thickness, and consequently decreasing gut permeability and improving metabolic endotoxemia and low-grade inflammation.

4.6 Hepatoprotective effects of anthocyanins

Chronic nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis (NASH), is an indicator of metabolic syndrome. Oxidative stress can induce NASH, which might further lead to hepatic fibrogenesis, and both PPAR and Nrf2 play roles in hepatic dysfunction by modulating cellular redox status (**figure 3**).

The protective effects of ACN against hepatotoxicity may be due to its ability to modulate oxidative stress by scavenging ROS, to decrease expression of inflammatory factors and to induce a cellular adaptive response modulated by Nrf2. In human hepatoblastoma cells (HepG2) exposed to H_2O_2 [192], Cy (50 μM) promoted antioxidant enzyme expression through the ERK and JNK pathways and Nrf2 activation; in fact cell treatment with Nrf2 siRNA significantly inhibited C3G-induced PPAR-γ expression. Hwang and coll. reported the protective effects of ACN fraction (AF; at doses > 10 μg/ml) from purple sweet potato (PSP) on tert-butyl hydroperoxide (t-BHP)-induced hepatotoxicity in HepG2 cells [193], notably decreasing t-BHP-induced oxidative damage and inducing antioxidant enzymes such as HO-1, NQO1, and Glutathione S-transferase (GST). Furthermore, AF was able to induce Nrf2 nuclear translocation, Akt and ERK1/2.

The results have been confirmed also *in vivo* against dimethylnitrosamine (DMN)-induced liver injury [193]. In fact, AF treatment (50, 100, or 200 mg/kg) attenuated the DMN-induced increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, reduced hepatic malondialdehyde (MDA) and GSH depletion while preserved normal GST activity in the rat livers. Also in this case, AF induced Nrf2 pathway, which was reduced by DMN administration, also preventing the nuclear translocation of NF-κB induced by DMN.

Similar results were obtained testing a BE (*Vaccinium myrtillus L.*), containing 42.04% ACNs on liver injury in mice exposed to *Propionibacterium acnes* plus LPS [195]. In this model BE (50, 100, or 200 mg/kg) significantly reduced liver inflammation, by suppressing liver iNOS, TNF-α, IL-1β and IL-6 genes expression like also the protein levels of iNOS, TNF-α and NF-κB.

Finally, ACNs can have inhibitory effects also on inflammatory response in alcoholic liver disease, as demonstrated by Jiang and coll. [196] in a murine model. In fact, serum alanine aminotransferase (AST) and (alanine aminotransferase) ALT levels in animals treated with ACNs from purple potato (PP) and PSP were lower than those of alcohol-treated mice. PPAs and PSPAs could decrease mRNA levels of NF-κB, STAT, and TLR and inhibit mRNA expressions of inflammatory factors TNF-α, VCAM-1, IFN-γ and CXCL-1, in comparison with alcohol treated mice.

4.7. Management of central nervous system disorders by anthocyanins

Glutamate-induced neural death, oxidative stress, and neuroinflammation are supposed to have an important role in many central nervous system (CNS) disorders, including neurodegenerative diseases [224]. ACNs can minimize the severity of glutamate-induced neurotoxicity, as demonstrated by Shah and coll. in the brain of developing rats [197] (table 1). In fact, korean black bean-derived ACNs (100 mg/kg) significantly reduced glutamate-induced AMP-activated protein kinase (AMPK) induction and ROS production in the postnatal day 7 (PND7) rat brain; furthermore, ACNs increased cellular GSH content and GSH/GSSG ratio and induced Nrf2 pathway and the endogenous antioxidant system HO-1. Similar results were obtained also in SH-SY5Y cells by silencing AMPK, very likely the key mediator in glutamate-induced neurotoxicity [197].

ACNs can also protect the inflammatory effect induced by LPS in cerebral tissue, as demonstrated through *in vitro* and *in vivo* models. For example, ACNs isolated from black soybean seed coats as well as PcA (a major metabolite of ACNs) were able to protect against LPS-stimulated murine BV2 microglial cells [198, 199], suggesting that ACNs may represent a therapeutic approach against microglial activation accompanying inflammatory and neurodegenerative diseases. In BV2 microglial cells, ACNs extract (50 and 100 μg/ml) inhibited LPS-induced pro-inflammatory mediators (iNOS, COX-2, TNF-α, and IL-1β) and nuclear translocation of NF-κB by reducing IκBα degradation as well as phosphorylating ERK, c-Jun N-terminal kinase, p38 MAPK, and Akt [198]. Similarly, in the same experimental models, PA (5, 10 and 20 μM) inhibited LPS-induced TNF-α, IL-6, IL-1β, and PGE_2 production, suppressed LPS-induced toll like receptor 4 (TLR4) expression, NF-κB and MAPKs activation. *In vivo* administration of ACNs extracted from black soybean (24 mg/kg intraperitoneally for 14 days) was able to produce a protective effect against LPS-induced-ROS-mediated neuroinflammation and neurodegeneration in cerebral cortex of LPS-injected adult mice [200] by inhibiting inflammatory mediators, such as IL-1β, TNF-α and NF-κB**,** and preventing apoptotic markers overexpression. In the same experimental model, ACNs extract was able to reduced activated astrocytes and microglia [200].

Alzheimer disease (AD) is a neurodegenerative progressive disorder affecting an estimated 40 million individuals worldwide and is characterized by accumulation of amyloid-β (Aβ) plaques and high levels of oxidative stress damage. Oligomers of short and full-length Aβ peptides, such as Aβ(25-35) and Aβ(1-40) and Aβ(1-42) respectively, seem to be accountable for synaptic dysfunction and/or neuronal loss in AD, by inducing redox imbalance, mitochondrial dysfunction and caspase activation [201]. It has been demonstrated that induction of Nrf2 pathway, both *in vitro* and *in vivo* AD model, is able to decrease Aβ-induced neurodegeneration and oxidative stress [225]. For example, ACNs (concentrations ranging from 50 to 200 μg/ml) increased SH-SY5Y cell viability against Aβ1-42 by preventing the activation of the p38-MAPK/JNK pathways and the down-regulation of endogenous Nrf2 and HO-1 and attenuating

neuroinflammatory markers such as NF-κB, TNF-α and iNOS [225] (**figure 3**). Furthermore, the effects of ACNs were improved by loading equimolar concentration into biodegradable nanoparticles based on poly (lactide-co-glycolide) and polyethylene glycol-2000 [225]. In fact, since ACNs are not stable because their phenolic hydroxyl groups are easily oxidized into quinones, nanotechnological approach can improve their bioavailability and increase the biological activity.

Lipid peroxidation by-products, such as the highly reactive aldehyde acrolein, was shown to accumulate in vulnerable regions of the brain from mild cognitive impairment (MCI) and preclinical AD patients. Acrolein is able to quickly deplete the intracellular GSH level and up-regulates GST activity in human neuroblastoma SK-N-SH cells since it reacts rapidly with GSH to form the irreversible acrolein-GSH adduct [202]. Belkacemi and Ramassamy showed that a equimolar mix of different ACNs (C3G, M3G, pelargonidin-3-glucoside and Pn; MAF14001), from 5μM, protected SK-N-SH cells against acrolein-induced toxicity, reducing glutathione depletion and activation of both NF-κB and the mitochondrial adaptor protein p66HSC, and inducing Nrf2 pathway and expression of γ-glutamylcystein synthetase [202].

4.8. Protective effects of anthocyanins in retinal diseases

The retina is the most metabolically active tissue in the body. Due to high oxygen tension, high levels of light exposure, and high concentration of polyunsaturated fatty acids, photoreceptors are particularly vulnerable to light damage. In particular age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the elderly worldwide [226].

ACNs are helpful for vision because they stimulate the reproduction of rhodopsin, and improve the circulation and the impaired night vision. Several *in vivo* and *in vitro* studies have demonstrated that ACNs can possess protective effects against retinal cell damage and dysfunction following oxidative stress and inflammatory conditions [226] (table 1).

High-energy visible light (from 380 to 530 nm) typically present in sunlight, fluorescent light, and light-emitting diode (LED) light may induce retinal damage [205]. This damage hits via thermal, mechanical, and especially photochemical mechanisms. Photochemical injury causes a reduced visual function and retinal degeneration, leading also to blindness. Apoptosis of photoreceptor cells, probably regulated by reduction of NF-κB activation and increased AP-1 and caspase-1, seems to be the main cause of retinal photochemical damage (RPD).

Jia and coll. demonstrated that black rice ACNs (1 g in 100 g diet for 15 days) improve the RPD induced in rats by exposure to fluorescent light (3,000 ± 200 lux for 3-24 h) by inhibiting the AP-1/NF-κB/Caspase-1 apoptotic pathway in photoreceptor cells [203]. The protective effect of ACNs was confirmed also by Wang and coll. who demonstrated, by means of *in vivo* experiments on pigmented rabbits orally administered with C3G or its metabolites PcA or ferulic acid (FA) (0.11 mmol/kg/day for 3 weeks) [204]. C3G and FA reduced visible light (18 000 lx for 2 h)-induced retinal oxidative stress by activating the Nrf2/HO-1 antioxidant pathway, while FA decreased the light-induced retinal inflammation by inhibiting NF-κB activation.

Blue light (from 450 to 495 nm) can induce damage in retinal pigment endothelial cells by the accumulation of lipofuscin [205]; this lead to ROS generation in mitochondria and to apoptotic cell death via activation of caspase-3. The study by Ogawa and coll. determined the protective effects of BE (*Vaccinium myrtillus L.*) and lingonberry (*Vaccinium vitis-idaea*) extract (LE) and their active components (Cy, Dp, Mv, trans-resveratrol, and procyanidin B2) against blue LED light-induced damage on cultured murine photoreceptor (661 W) cells [205]. BE and LE (10μg/ml), and their active components (10μM) improved the viability of 661W cells and inhibited the generation of intracellular ROS and the activation of p38 MAPK and NF-κB induced by light exposure [205].

Diabetic retinopathy, caused by long-term elevated blood glucose levels that induce dysfunction of the retinal microvasculature, is one of the most common forms of diabetic eye disease, and if untreated, can cause blindness. Vascular-Endothelial Growth Factor (VEGF) as well as other inflammatory mediators, such as ICAM-1, IL-1β, TNF-α, and IL-6, play an important role in diabetic retinopathy development. Blueberry anthocyanins (BA) (20, 40 and 80 mg/kg) protect retinal cells from diabetes-induced oxidative stress and inflammation, as demonstrated in rats intraperitoneally injected with streptozotocin [207]. In fact, BA increased GSH and GPx activity, and decreased MDA and ROS levels in the retina. Furthermore, BA increased the mRNA levels and nuclear location of Nrf2 and HO-1 protein and gene expression.

Uveitis is an intraocular inflammatory disease that can affect any part of the eye and cause serious complications such as blindness (10–15% of the number of cases of total blindness in developed countries). Endotoxin-induced uveitis (EIU) in rodents is an *in vivo* model mimicking the pathological conditions of human acute uveitis [206]; it is characterized by inflammatory reactions in the anterior uvea and in the posterior segments of the eye, leading to altered visual function, abnormal leukocyte adhesion and, at molecular level, activation of NF-κB pathway. In EIU, an ACNs-rich bilberry extract (500 mg/kg body weight for 4 days) prevented the impairment of photoreceptor cell function in C57BL/6 mice [206]. At molecular level the extract avoided both STAT-3 activation, able to induce rhodopsin decrease, and IL-6 gene expression, which in turn triggers STAT-3; in addition, the ACNs-rich bilberry extract reduced the intracellular elevation of ROS and NF-κB activation [206].

4.9. Anthocyanins in skin protection

Excessive exposure to solar ultraviolet (UV) radiation, particularly its UV-B component, to humans causes many adverse effects that include erythema, hyperplasia, hyperpigmentation, immunosuppression, photoaging and skin cancer. Oxidative stress and inflammatory condition are known to play a central role in initiating and driving the signaling events that lead to cellular response following UV irradiation [227].

Furthermore, impairment of the skin extracellular matrix (ECM) integrity is a critical event involving skin wrinkle and blister formation, hallmarks of photoaging [227]. In premature skin aging and in aged skin, UV-induced ECM damage is considered as the result of the production of various matrix MMP by the cells.

Recently, there is an increase in the use of skin care products containing botanical agents and ACNs are known to possess great antioxidant power able to protect plants from UV damage. For this reason, they are supposed to be useful also in protecting human skin. Table 1 presents the studies regarding the beneficial effects of ACNs on skin damage in cells and animals.

ACNs from black soybean [*Glycine max* (L.) Merr] seed coats (10, 50 and 100 μg/ml) were able to protect human keratinocyte HaCaT cells against UV-B radiation-induced inflammatory responses, by inhibiting COX-2 and PGE_2 production through a NF-κB-dependent pathway and regulating the PI3K/Akt pathway [208] (**figure 3**). Accordingly, to these findings, in hairless mice topical application of 50 and 100 mg/kg ACNs prior to UVB irradiation also inhibited induction of COX-2 and PGE_2 [208]. At the same extent, *in vivo* investigations using female ICR mice exposed to UV-B revealed that the upregulation of COX-2 expression, MAPK Kinase (MAPKK) 4 activity and PI3K activity induced by UV-B in the skin was abolished with Dp treatment [228]. Furthermore, a standardized extract from red oranges, containing ACNs as main active principles, reduced UVB-induced response in HaCaT cells, and in particular those events associated to inflammation and apoptosis, such as NF-κB and AP-1 pathways and procaspase-3 cleavage [209]. Under the same experimental conditions, UVB-induced increase of the translocation of NF-κB and AP-1, overexpression of the pro-inflammatory cytokine IL-8, cleavage of procaspase-3 and DNA fragmentation were clearly

inhibited by pretreating HaCaT cells with C3G 80 μM [210] (**figure 3**). However, Ernst and coll. demonstrated that Cy (ranging from 1 to 100 μM) is unable to induce the activation of Nrf2 and its target genes in HaCaT cells and does not enhance the Nrf2 activation mediated by the Nrf2 agonist sulforaphane and the expression of its target gene [229]. Finally, in normal human epidermal keratinocytes (NHEK) pomegranate fruit extract (PFE) (10-40 μg/ml for 24h) protected against the adverse effects of UVB by inhibiting UVB-induced modulations of NF-κB and MAPKs pathways [213]; in fact, the treatment of NHEK with PFE inhibited UVB-mediated phosphorylation of ERKl/2, JNK1/2 and p38 MAPK protein, nuclear translocation and phosphorylation of p65/NF-κB at Ser(536), IkBα degradation and phosphorylation and IKKα activation.

There are results suggesting that berries differ in their ability to influence signaling pathways leading to activation of NF-κB induced by UV light. Huang and coll. demonstrated that, unlike extracts from strawberries and blueberries, 50 μg/ml of a methanolic extract from black raspberries (*Rubus occidentalis*) may reduce IKKβ phosphorylation and consequently inhibits the NF-*κ*B pathway in mouse epidermal JB6 Clone 41 cells exposed to UV-B and UV-C irradiation [212]. However the black raspberry extract was unable to inhibit UVB-induced ERKs and AP-1 activation. In fact, some dark colored polyacylated ACNs can screen UV-B and then reduce UV-B damage powerfully due to aromatic acyl residues [212], while some other ACNs, such as C3G (mainly present in black raspberries and not in strawberries or high-bush blueberries), protect UV-B irradiation-induced cell damages by modulating NF-*κ*B pathway. However ACNs have been shown able to modulate also the AP-1 activation. In fact, C3G (≥ 40 μM) demonstrated a protective effect against UVB-exposed human keratinocyte (HaCaT) cells, reducing translocation of NF-κB and AP-1, overexpression of the proinflammatory cytokine IL-8, cleavage of procaspase-3 (a key step in apoptotic pathway), and DNA fragmentation [210]. All these effects elicited by UV-B exposure were also inhibited by pretreating HaCaT cells with 15-30 μg/ml of an extract from red orange [209].

The effects of C3G on UV-B irradiation-induced chronic inflammatory responses were confirmed also *in vivo* in SKH-1 hairless mice [215]. Topical application of C3G (250 – 500 μM) protected against the negative effects of UV-B radiation by modulating UVB-induced MAPKs and NF-κB signaling pathways. In fact, UVB-induced inflammatory responses were diminished by C3G as observed by a remarkable reduction in the levels of phosphorylated ERK1/2, p38, JNK1/2 and MKK4 [215]. Furthermore, C3G also decreased UVB-induced COX-2, PGE_2 and iNOS levels through NF-κB pathway inhibition, as evidenced by reduced p65 nuclear levels and degradation of IκBα.

The study by Bae and coll. examined the capacity of 1, 5 and 10 μg/ml of an ACNs-rich extract from bog blueberry (ATH-BBe), containing C3G, Pt3G, M3G, and D3G, to inhibit photoaging in UVB-irradiated human dermal fibroblasts [211], by attenuating UVB-induced ROS production and the resultant DNA damage accountable for p53 and Bad activation, collagen degradation via MMPs reduction, nuclear translocation of NF-*κ*B and phosphorylation of c-Jun, p53, and STAT-1 (which are linked to MAPKs signaling cascades involved in MMP-promoted collagen degradation), and release of inflammatory IL-6 and IL-8 [211].

Human diploid fibroblasts (HDFs) may be used as an *in vitro* experimental model of cellular aging [214], since they lose the ability to proliferate and become senescent, showing cellular modifications related to the aging process, if exposed to sub-lethal stressors. So WI-38 HDF cells exposed to H_2O_2 were used to investigate the anti-aging effects of Cy [214]. Treatment with Cy (1.7 to 34.8 μM) ameliorated cellular oxidative stress and prolonged the life spans of young-, middle-, and old-aged WI-38 cells. Furthermore, Cy significantly decreased expressions of NF-*κ*B, COX-2 and iNOS in H_2O_2-treated WI-38 cells [214].

ACNs may be a beneficial food for diseases involving scratching behaviors, such as chronic dermatitis and psoriasis. C3G isolated from black-colored rice (which exhibits an anti-allergic effect) and its metabolites (5 to 25 mg/kg)

obtained by its anaerobical incubation with fecal microflora showed inhibitory effects against histamine-induced scratching behaviors in mice [230]. C3G strongly prevented scratching behaviors following oral administration than following intraperitoneal administration. C3G metabolites also inhibited the expression of allergic cytokines, IL-4 and TNF-α, and the activation of NF-κB in RBL-2H3 mast cells (a histamine-releasing cell line commonly used in inflammation, allergy and immunological research) stimulated with IgE-antigen.

4.10. Anticancer activities of anthocyanins

Cancer is the second leading cause of death globally. Lung, colon, prostate, and breast cancers are the most common causes of cancer death, accounting for almost half of the total cancer deaths among men and women. Furthermore, tumor metastasis is a leading cause of cancer death and many treatment strategies are targeted on metastasis prevention. One has to stress that the NF-κB-regulated protein matrix MMP-9 plays an important role in the invasion and metastasis of cancer cells.

Dietary agents from fruits and vegetables have received extensive consideration for the prevention and treatment of cancers [231]. The potential antitumor effects of ACNs are based on a wide variety of biological activities, including antioxidant, anti-inflammatory, anti-mutagenic, antiproliferative and anti-metastasis, stimulation of apoptosis or autophagy of cancer cells, and increase of cancer cells sensitivity to chemotherapy [232]. Current molecular bases involved in cancer chemoprevention by anthocyanidins include: inhibition of cell transformation through targeting MAPK pathway and AP-1 factor; suppression of inflammation and carcinogenesis through targeting NF-κB pathway; apoptotic induction of cancer cells through ROS/c-JNK-mediated caspase activation [233] (**figure 5**). Table 2 presents the anticancer effects of ACNs in cells and animals.

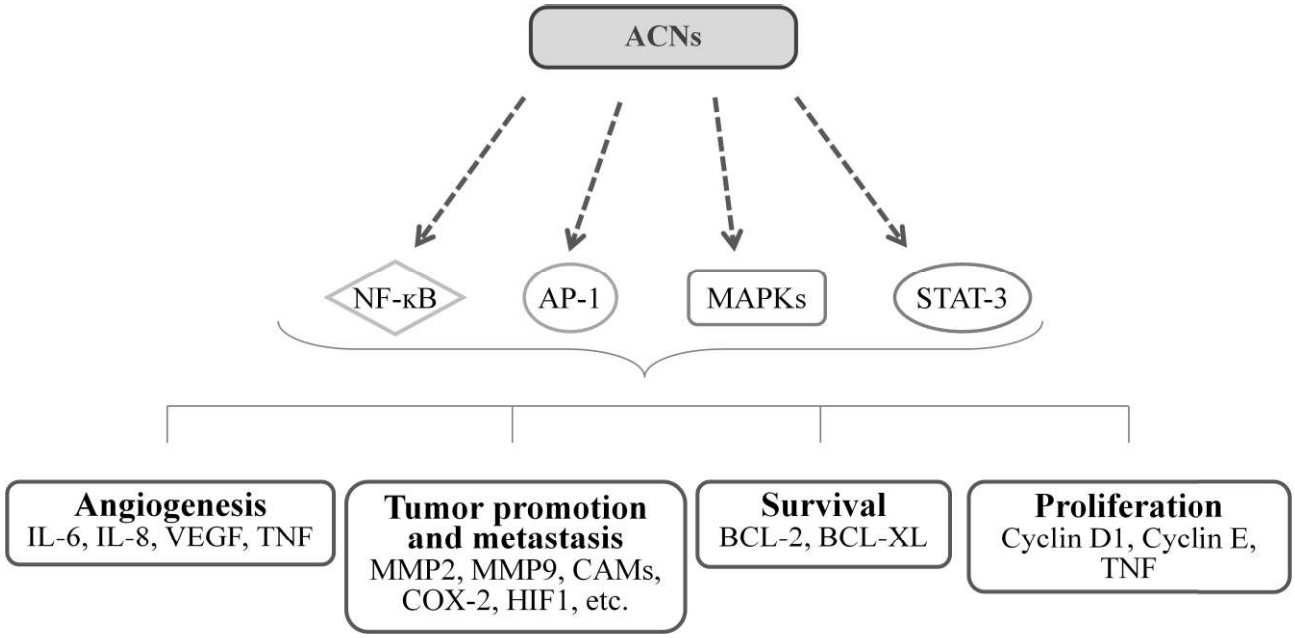


Figure 5. ACNs possess a wide spectrum of biological activities, including the capability to modulate signalling pathways involved in the development and progression of cancer. The significant anticancer properties of ACNs may be due to frank apoptosis by inhibiting prosurvival proteins such as BCL-2 and BCLXL. Furthermore, perturbations in cell cycle progression may account for the anticarcinogenic effects of ACNs by modulating a series of regulated steps allowing traverse of the cell cycle, such as those dependent on cyclin-dependent kinases that are recognized as key regulators of cell cycle progression. Finally, ACNs can affect cancer progression by modulating MMPs, COX-2, adhesion molecules and angiogenic proteins.

AP1: Activator protein 1; BCL-2: B-cell lymphoma-2; Bcl-xL: B-cell lymphoma-extra large; CAMs: cell adhesion molecules; COX-2: cyclooxygenase-2; HIF-1: Hypoxia-inducible factor-1; IL: interleukin; MAPKs mitogen-activated protein kinases; MMP: Matrix metalloproteinase; NF-kB: Nuclear factor-kappa B; STAT-3: Signal transducer and activator of transcription-3; TNF: tumor necrosis factor; VEGF: Vascular-Endothelial Growth Factor.

Table 2: *In vitro* and *in vivo* studies of the anticancer effects of ACNs.

ANTHOCYANINS	EXPERIMENTAL MODEL	CANCEROGENIC AGENT	REFERENCES
Skin cancer			
Lingonberry extracts	JB6 P+ mouse epidermal cells	TPA, UVB	Wang et al., 2005[234]
Dp	JB6 P+ mouse epidermal cells	UVB	Kwon et al., 2009[228]
Pomegranate fruit extract	CD-1 mice	TPA	Afaq et al., 2005[213]
Digestive system cancer			
Dp	HCT116 human colon cancer cells	-	Yun et al., 2009[235].
Liofenol™	HCT116 human colon cancer cells	-	Signorelli et al., 2015[236]
ACNs from *Vitis coignetiae* Pulliat fruits	HT-29 human colon cancer cells	-	Yun et al., 2010[237]
ACNs from black rice	CAL 27 human oral cells	-	Fan et al., 2015[238]
ACNs from plasma of healthy volunteers after ingestion of an ACNs-rich juice	PANC-1 human pancreatic cancer cells and AsPC-1 human pancreatic cancer cells	-	Kuntz et al., 2017[239]
Black raspberries	Rat esophagus	NMBA	Chen et al., 2006[240]
Liver cancer			
ACNs from *Vitis coignetiae* Pulliat fruits	Hep3B human hepatoma cells	-	Shin et al., 2009[241]
ACNs fraction from black rice	SKHep-1 human hepatocellular carcinoma cells	-	Chen et al., 2006[242]
P3G and C3G from black rice	SKHep-1 human hepatocellular carcinoma cells	-	Chen et al., 2006[242]
ACNs -rich black currant skin extract	Rat liver cancer	DENA	Thoppil et al., 2012[243]
Lung cancer			
C3R and C3G from *Morus alba* L.	A549 human lung carcinoma cells	-	Chen et al., 2006[244]
Cy, Mv, Pn, Pt and Dp, as a mixture, as present in blueberry, bilberry and Indian blackberry	NSCLC H1299 (p53null/EGFRWT) non-small-cell lung cancer and A549 (p53WT/EGFRWT) adenocarcinomic human alveolar basal epithelial cells	-	Kausar et al., 2012[245]
Anthocyanidins from bilberry and Dp	H1299 xenografts in nude mice	-	Kausar et al., 2012[245]
Reproductive cancer			
Dp	MCF-7 human breast carcinoma cells	-	Im et al., 2014[246]
Dp	MCF-10A human mammary epithelial cells	HGF	Syed et al., 2008[247]
Dp	22Rnu1 human prostate cancer cells	-	Hafeez et al., 2008[248]
Dp	PC3 human prostate cancer cells	-	Hafeez et al., 2008[248]
Cy	LNCaP human prostate adenocarcinoma cells	-	Munoz-Espada and Watkins, 2006[249]
C3S from *Acanthopanax sessiliflorus* fruits	MDA-MB-231 human breast cancer cells	-	Lee et al., 2013[250]
ACNs from *Vitis coignetiae* fruits	HeLa human uterine cervical cancer cells	-	Lu et al., 2013[251]

ACNs: Anthocyanins; Cy: cyanidin; C3G: cyanidin-3-glucoside; C3R: cyanidin-3-rutinoside; C3S: cyanidin-3-O-sambubioside; Dp: delphinidin; DENA: diethylnitrosamine; HGF; hepatocyte growth factor; Mv: malvidin; NMBA: N-nitrosomethylbenzylamine; Pg: pelargonidin; Pn: peonidin; Pt: petunidin; P3G: peonidin 3-glucoside; TPA: tetradecanoylphorbol-13-acetate; UVB: ultraviolet B light.

However, the anticancer efficacy of ACNs-rich products widely differs as dependent on their sources and composition. For example, the inclusion of berries in the diet might be surely useful for preventing the development of cancer but berry juices have outstanding differences in their potential chemopreventive activity. Boivin and coll. showed that the growth of various cancer cell lines, including those of stomach, prostate, intestine and breast, was strongly inhibited by raspberry, black currant, white currant, gooseberry, velvet leaf blueberry, low-bush blueberry, sea buckthorn and cranberry juice, but not (or only slightly) by strawberry, high-bush blueberry, serviceberry, red currant, or blackberry juice [252]. Interestingly, of the 13 berries tested, juice of 6 significantly inhibited the TNF-induced activation of COX-2 expression and NF-κB pathway [252].

4.10.1 Skin cancer

Skin cancer is one of the most commonly diagnosed cancers in several countries. UV-B is the most carcinogenic component of solar irradiation and exposure to this radiation induces inflammation and photocarcinogenesis in mammalian skin [209].

Pretreatment of promotion-sensitive mouse epidermal JB6 P+ cells with lingonberry extracts dose-dependently reduced the activation of AP-1 and NF-κB induced by either 12-O-tetradecanoylphorbol-13-acetate (TPA) or UV-B [234], blocking phosphorylation of MAPKs signaling members such as ERK1/2, p38, and MEK1/2. In agreement with these data, topical application of pomegranate fruit (*Punica granatum*) extract (20μg/ml) to CD-1 mice inhibited TPA-induced markers of skin tumor promotion, such as phosphorylation of ERK1/2, p38 and JNK1/2, activation of NF-κB, IKKα and phosphorylation and degradation of IkBα [213].

Dp, at concentrations ≥ 5μM, suppressed UVB-induced COX-2 expression and PGE_2 production in JB6 P+ mouse epidermal cells through downregulation of AP-1 and NF-κB transcription factors [228]. In addition, UVB-induced phosphorylation of JNK, p38 kinase and Akt, essential to the development and progression of tumors, was inhibited by Dp. The authors demonstrated that all these effects were mediated by mitogen-activated protein kinase kinase 4 (MAPKK4) and PI3K inhibition induced by Dp through a direct interaction with these two kinases in a manner that was competitive with adenosine triphosphate [228].

4.10.2. Digestive system cancer

Dp in human colon cancer HCT116 cells produced decrease in viability, induction of apoptosis, inhibition of IKKα, phosphorylation and degradation of IkBα, phosphorylation and nuclear translocation of p65/NF-κB, increase in Bax with a concomitant decrease in Bcl-2 protein, and G2/M phase cell cycle arrest at concentrations ≥ 60μM [235]. Furthermore, experiments on mouse epidermal JB6 Cl41 cells stably transfected with either a NF-κB- or an AP-1-luciferase reporter, and treated with racemic anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), demonstrated that Cy glycosides (≥ 1.25 μg/ml) from black raspberries are good inhibitors of BPDE-induced NF-κB activity [253].

ACNs, isolated from fruits of *Vitis coignetiae* Pulliat, possess anti-invasive effects on HT-29 human colon cancer cells at concentrations ≥ 50 μg/ml [237] by inhibition of constitutive NF-κB activation through suppression of IkBα phosphorylation. Liofenol™, a red wine lyophilized extract containing polyphenols, flavonoids and ACNs, at concentrations ranging between 300 and 600 μg/ml, reduced HCT116 clorectal cell proliferation, in association with an increase of p53 and p21 cell cycle gate keepers, a strong induction of antioxidant response, the activation of the transcriptional factor Nrf2, without altering tumor sensitivity to chemotherapy [236]. In the research by Fan and coll., ACNs from a species of black rice (*O. sativa* L. cultivar) appeared to have an antimetastatic effect inhibiting the

invasion/migration of human oral CAL 27 cells [238], by inhibiting MMP-2, MMP-9, NF-κB and PI3K/Akt pathways at concentrations ≥ 400μg/ml.

Since gut microflora metabolize ACNs to phenolic acids and aldehydes, Forester and coll. investigated the effects of GA, 3-O-methylgallic acid (3MGA), and 2,4,6-trihydroxybenzaldehyde (THBA), at concentrations of 10-100 μM, on the cell cycle modulation and apoptotic cell death induction on dividing Caco-2 cells [254]. These metabolites caused cell cycle arrest at G0/G1 and inhibited transcription factors NF-κB, AP-1 and STAT-1 [254].

Pancreatic cancer is an aggressive cancer type, whose most important characteristics are migration and metastasis. Kuntz and coll. evaluated whether ACNs and/or their metabolites from plasma, isolated from blood of healthy volunteers after ingestion of an ACNs-rich juice, were effective in modulating cancer PANC-1 and AsPC-1 cells migration *in vitro* [239]. After application of metabolites to PANC-1, a reduced cell migration, associated with reduced levels of endogenously generated ROS, and reduced NF-κB as well as MMP-2 and MMP-9 mRNA expression levels, was observed. However, in AsPC-1 cells, however, migration was not affected by metabolites. So, ACNs and their metabolites are very likely able to inhibit pancreatic cancer cell migration but only in dependency of the phenotype [239].

4.10.3. Liver cancer

Hepatocellular carcinoma (HCC) represents one of the most fatal cancers and more than 1 million deaths are reported annually worldwide [255]. Shin and coll. investigated anti-invasive effects of the ACNs from fruits of *Vitis coignetiae* Pulliat (100–400 μg/ml) on human hepatoma Hep3B cells [241]. The results of this study showed that these ACNs possess anti-invasive effects and inhibit MMP-2 and MMP-9 gene expression at least in part through the involvement of NF-κB pathway inhibition.

Anti-metastatic effects of peonidin-3-glucoside (Pn3G - 100μM), C3G (50 – 100μM) and ACNs fraction (100 – 200 μg/ml) from black rice (*Oryza sativa* L. indica) have been demonstrated and molecular evidences showed a marked inhibition on the invasion and motility of human hepatocellular carcinoma SKHep-1 cells [242] with an inhibitory effect on the DNA binding activity and the nuclear translocation of AP-1. Furthermore, the tumor growth suppression effect of ACNs fraction was confirmed in a nude mice xenograft model by a subcutaneous inoculation of SKHep-1 cells [242].

Thoppil and coll. demonstrated the chemopreventive effect of ACNs-rich black currant skin extract (BCSE, 100 and 500 mg/kg) against diethylnitrosamine (DENA)-initiated hepatocarcinogenesis in rats by reducing oxidative stress through activation of Nrf2 signaling pathway [243] upregulation of gene expression codifying for antioxidant and detoxifying enzymes, such as GST,, in DENA-initiated animals [243].

4.10.4. Lung cancer

C3R and C3G (25 – 100μM) extracted from *Morus alba* L. exerted a dose-dependent inhibitory effect on the migration and invasion, of highly metastatic A549 human lung carcinoma cells [244] by decreasing the expressions of MMP-2 and u-PA reduction (the last one at lower concentrations 2.5–10 μM) and the activation of c-Jun and NF-κB.

The combinatorial effects of a mixture of berry anthocyanidins (Cy, Mv, Pn, Pt and Dp), similar to that present in blueberry, bilberry and Indian blackberry ('Jamun'), was studied by Kausar and coll. [245]. The combination of equimolar concentrations of anthocyanidins (25 – 100 μM) synergistically reduced the growth of two non-small-cell lung cancer (NSCLC) cell lines [the tumorigenic NSCLC H1299 (p53null/EGFRWT) and A549 (p53WT/EGFRWT) cells], without affecting non-tumorigenic cell viability. The greater effects of the combinatorial treatment were due to

interactions on the oncogenic neurogenic locus notch homolog (Notch) and WNT pathways and their downstream targets, increased activation of the proapoptotic Bcl2 and PARP proteins and inhibition of TNFα-induced NF-κB activation. In addition, *in vivo* studies reported an inhibition of the growth of H1299 xenografts in nude mice by 60% both for the native mix of anthocyanidins from bilberry (0.5 mg/animal) and Dp (1.5 mg/animal) [245]. Interestingly, the effective dose of Dp in the anthocyanidin mixture was 8-fold lower than that of Dp alone, so demonstrating a synergism between all the compounds.

4.10.5. Reproductive cancer

Reproductive cancers can affect men and women. In women, uterine, cervical, ovarian, vaginal, and vulval cancers, as well as breast cancer, are the most common. In men, the most common are prostate and testicular cancer [256].

Lee and coll. suggest that cyanidin-3-sambubioside (1–30 μg/ml) from *Acanthopanax sessiliflorus* fruits inhibits metastasis processes in breast cancer cells MDA-MB-231 through regulation of MMP-9 activity and an inhibitory effect on Akt phosphorylation [250].

Also Dp is considered a potential antimetastatic agent; in fact, it suppresses PMA-induced MMP-9 expression by blocking the NF-κB activation through MAPKs signaling pathways in MCF-7 human breast carcinoma cells at concentrations ≥ 30μM [246].

The hepatocyte growth factor (HGF)-Met signaling pathway is altered in many cancers and is associated with poor prognosis in breast cancer. Pretreatment of MCF-10A breast cell line (expressing Met receptor) with Dp (5–40 μM) caused an increased expression of Met receptor, inhibition of HGF-activated Ras-ERK MAPKs and PI3K/Akt/mTOR/p70S6K pathways, and decreased HGF-activated phosphorylation of IKKα/β and IkBα and nuclear translocation of NF-κB/p65 [247].

Lu and coll. suggested that ACNs from fruits of *Vitis coignetiae* (AIMs) should exert anticancer effects in cervical cancer by suppressing NF-κB-regulated genes and epithelial-mesenchymal transition (EMT, which is linked to cancer metastasis). In fact, in human uterine cervical cancer HeLa cells. , AIMs inhibited the invasion and EMT of HeLa cells and MMP-9 expression at concentration ≥ 25 μg/ml, and suppressed NF-κB activation induced by TNF. [251].

In the United States, the primary cancer in elderly men is prostate cancer (33% of newly diagnosed malignancies), but the prevalence is 75% lower in some Mediterranean countries; this significant difference could be associated to the bioactive compounds present in the Mediterranean diet.

Two reports demonstrated that Dp (30–180 μM) induces apoptosis of both highly metastatic androgen independent and androgen refractory human prostate cancer PCa cells (PC3 and PCa 22Rnu1) via activation of caspases and this effect was probably due to inhibition of NF-κB signaling pathway [248, 257]. Dp treatment to cells resulted in a dose-dependent decrease in IKKγ and IkBα phosphorylation like also phosphorylation of NF-κB/p65 and NF-κB/p50 and nuclear translocation and DNA binding activity of NF-κB/p65. Dp administration (2 mg/animal) to athymic nude mice implanted with PC3 cells resulted in a significant inhibition of tumor growth [248], with a significant decrease in cell expression of NF-κB/p65, Bcl2, Ki67, and PCNA.

Conversely, Cy (0.5–1 μM) reduced the level of PGE_2 in LNCaP cell cultures, a nonaggressive androgen-dependent cell model that expresses moderate levels of COX-2 and reduced the levels of COX-2 protein [249] but not NF-κB mRNA levels.

5. OPEN QUESTIONS AND FUTURE PERSPECTIVES

As demonstrated also by the large number of papers reported in this review, ACNs possess a wide range of health-promoting properties and may have important implications in the prevention of chronic diseases through their capability to modulate cell redox-dependent signaling pathways. Nevertheless, much remains to be elucidated before a rational employment of these drugs for human health benefits.

First, more studies on the effects of the ACNs metabolites are desired. In fact, ACNs have a relatively low bioavailability, but there is evidence that they generate bioactive metabolites which may totally or partially contribute to the beneficial effects described for the parent drugs. However, just a few number of researches have evaluated the effect (and eventually characterized the action mechanisms) of ACNs metabolites, such as PcA, in particular those derived from intestinal microbial metabolism.

Furthermore, the largest part of available studies on this topic has been carried out *in vitro* on suitable models of cultured cells exposed to a wide range of ACNs concentrations. However, up to date the exact molecular interactions through which ACNs exert their actions are not exhaustively understood. This may be of particular importance if we suppose an employment of ACNs as adjuvant anticancer therapy together with conventional drugs. ROS are considered oncogenic acting through numerous signaling pathways [258, 259]. While the constitutive high production of ROS in some cancer cells appears to promote their proliferation, additional amounts of ROS above a certain threshold cause cell cycle arrest and/or apoptosis (the so-called 'persistent oxidative stress in cancer' hypothesis). Thus, wide interest is focused also on the evaluation of the interaction of putative antioxidants with chemotherapy drugs or radiation therapy. In fact, depending on the type of cancer, the mechanisms of action of the drug(s) and of the antioxidants used and the doses employed [260], in some cases administration of antioxidants could ameliorate the toxicity of antineoplastic drugs on cancer cells and/or counteract their toxicity on non tumoral cells, but in others could also interfere with the cytotoxic effect of ROS-inducing chemotherapy drugs. So, it is crucial to confirm that the anticancer activities of ACNs are related to modulation of cell signaling pathways and not merely to a decrease of cell ROS accumulation.

In addition, the limits intrinsic in *in vitro* experimentation (including also the short-term exposure of cells to the investigated drugs, the instability of ACNs in cell culture medium, and their possible conversion in other degradation products perhaps responsible for the observed effects) cannot allow us to clearly identify the dosage of these drugs, which could be really effective in producing the described beneficial effect in humans affected by, or with one or more risk factors for, chronic NCDs. It is also evident that more *in vivo* studies (on animals and in humans) are needed to clarify not only the efficacy but also the safety of ACNs in management of human NCDs.

Another important aspect is that, in this context, Cy and Dp as well as their glycosides are the pure ACNs more frequently subjected to extensive mechanistic studies; on the other hand, the largest part of available information concerns the effects of products derived from berries (such as bilberry and blackberry). However, purified ACNs might exert different biological activities due to their specific chemical structures, and also the effect of ACNs-rich products are different or better than that of single pure ACNs, due to interactions amongst the several components of the complex vegetable matrix. Thus, future studies should focus on accurate characterization of different ACNs in order to better elucidate the molecular mechanism underlining their effects and to individuate new ACN-rich plants/plant derivatives useful to be introduced in human daily diet or to be used as source of ACNs dietary supplements or of pure ACNs.

6. CONCLUSIONS

The evidences reviewed in the present paper strongly demonstrate that ACNs possess both protective and therapeutic functions useful in the management of chronic NCDs, including not only those more strictly related to suboptimal nutrition and unhealthful dietary intakes, such as metabolic syndrome and atherosclerosis, but also skin disorders, eye pathologies, osteoarticular diseases, intestinal inflammatory disorders, neurodegenerative diseases and cancer, all pathogical conditions involving oxidative stress, inflammatory damage and immune dysregulation. The incidence and concerning levels of these pathologies is one of the main reasons that justify the increased strong interest in dietary ACNs during the last years, being their health properties supported by wide scientific evidences. Several cellular signaling pathways, as well as crucial cellular processes, such as cell cycle, apoptosis, autophagy, and biochemical metabolism, are involved in ACNs beneficial effects. In particular, as described in detail in this review, in many cases the ACNs protective effect against chronic NCDs is clearly related not to a mere radical-scavenging effect counteracting oxidant species-induced damage but to their capability to modulate the main redox-dependent cellular signaling pathways, due to their antioxidant and hormetic properties (**Figure 3** and **4**). These findings explain why, despite their poor bioavailability, ACNs can induce beneficial effects acting at concentrations below those required for a direct radical-scavenging action. As a consequence, it is also evident that the opportune use of ACNs-based dietary supplements can allow to reach serum and tissue concentrations of these compounds sufficient to induce their beneficial effects. In fact, besides having a chemical structure compatible with a putative antiradical capacity, ACNs can actually perform activities and roles independent of such capacity. There is evidence that, very likely as consequence of their capability to directly quench intracellular ROS, ACNs can down-regulate the NF-κB and AP-1 signal transduction pathways, which are functionally dependent on cellular redox status and respond to oxidative signals. On the other hand, ACNs can induce expression of Nrf2/ARE-regulated cytoprotective proteins (GST, NQO, HO-1, etc.) involved in both cellular antioxidant defenses, and elimination/inactivation of toxic compounds, so countering the alterations caused by conditions of chemical/oxidative stress. One has also to mention that there is evidence of functional interactions between Nrf2 and NF-κB pathways and between NF-κB and AP-1 pathways which can take place at different levels and regulate transcription or function of target proteins; such putative crosstalks could contribute to explain the protective effects of ACNs in different pathological conditions characterized by a perturbation of the balance between these two pathways. Thus single ACNs or ACNs-rich products able to target, through different mechanisms and/or at different steps, the redox-related Nrf2, NF-κB and AP-1 pathways could have remarkable therapeutic potential for the prevention and treatment of NCDs in future.

Conflicts of interest

There are no conflicts to declare.

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