Dietary Anthocyanins as Nutritional Therapy for Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD), defined by excessive lipid accumulation in the liver, is the hepatic manifestation of insulin resistance and the metabolic syndrome. Due to the epidemics of obesity, NAFLD is rapidly becoming the leading cause of altered liver enzymes in Western countries. NAFLD encompasses a wide spectrum of liver disease ranging from simple uncomplicated steatosis, to steatohepatitis, cirrhosis, and hepatocellular carcinoma. Diet may affect the development of NAFLD either by increasing risk or by providing protective factors. Therefore, it is important to investigate the role of foods and/or food bioactives on the metabolic processes involved in steatohepatitis for preventive strategies. It has been reported that anthocyanins (ACNs) decrease hepatic lipid accumulation and may counteract oxidative stress and hepatic inflammation, but their impact on NAFLD has yet to be fully determined. ACNs are water-soluble bioactive compounds of the polyphenol class present in many vegetable products. Here, we summarize the evidence evaluating the mechanisms of action of ACNs on hepatic lipid metabolism in different experimental setting: in vitro, in vivo, and in human trials. Finally, a working model depicting the possible mechanisms underpinning the beneficial effects of ACNs in NAFLD is proposed, based on the available literature.

1. Introduction

In the last decades, the pandemic of overweight and obesity related to sedentary lifestyle and excess intake of refined foods has led to a dramatic rise in the prevalence of the metabolic syndrome and associated conditions, such as type 2 diabetes and dyslipidemia, leading to accelerated atherosclerosis [1], but also to nonalcoholic fatty liver disease (NAFLD) [2, 3].

Lifestyle and dietary habits represent both major risk and protective factors in the development and progression of degenerative diseases [4].

Diet rich in fruits and vegetables are among the recommended lifestyle modifications to decrease the risk of degenerative diseases, such as cardiovascular disease but also to reduce the complications associated with metabolic disorders and advanced atherosclerosis. Diet is in fact affordable and available and usually does not include the side effects and the metabolic and physiologic burden that medications impose on body systems [5].

In this regard, many different dietary components are under study for their possible pharmacologic activity in several pathophysiological conditions at different levels (e.g., vascular, immune, hepatic, etc.).

Most bioactive compounds have been documented in fruits and vegetables [6] and their mechanisms of action investigated both in in vitro and in in vivo models. In particular, great interest has been devoted to several classes of polyphenols and especially to a specific subset of molecules called anthocyanins (ACNs).
### Table 1: Anthocyanin concentrations in selected food sources.

| Food description          | Cyanidin mg/100g | Delphinidin mg/100g | Malvidin mg/100g | Pelargonidin mg/100g | Peonidin mg/100g | Petunidin mg/100g |
|---------------------------|------------------|---------------------|------------------|----------------------|------------------|------------------|
| **Berries**               |                  |                     |                  |                      |                  |                  |
| Arctic bramble berries    | 88.3             | 0.7                 |                  |                      |                  |                  |
| (Rubus arcticus)          |                  |                     |                  |                      |                  |                  |
| Bilberry (Vaccinium myrtillus) | 85.3           | 97.6                | 39.2             | 20.4                 | 42.7             |                  |
| Blackberries (Rubus spp.) | 99.9             | 0                   | 0                | 0.4                  | 0.2              | 0                |
| Blueberries (Vaccinium spp.) |            |                     |                  |                      |                  |                  |
|   Cultivated              | 8.5              | 35.4                | 67.6             | 0                    | 20.3             | 31.5             |
|   Wild                    | 19.4             | 37.6                | 57.2             | 2.6                  | 10               | 23.5             |
| Chokeberry                | 344.1            | 0.6                 | 1.2              | 1                    | 0.1              | 2.8              |
| Cranberries (Vaccinium macrocarpon) | 46.4          | 7.7                 | 0.4              | 0                    | 49.2             | 0                |
|   Currants                |                  |                     |                  |                      |                  |                  |
|   Black (Ribes nigrum)    | 61.3             | 87.9                | 1.2              | 0.6                  | 3.9              |                  |
|   Red                     | 65.5             | 9.3                 |                  | 0.2                  |                  |                  |
|   Golden (Ribes aureum)   | 108.8            | 0.7                 |                  | 0.1                  |                  |                  |
| Elderberries (Sambucus spp.) | 485.3         | 0                   | 0                | 0                    | 0                |                  |
| **Raspberries**           |                  |                     |                  |                      |                  |                  |
| Black                     | 669              | 16.7                | 1.1              |                      |                  |                  |
| Raspberries (Rubus spp.)  | 45.8             | 1.3                 | 0.1              | 1                    | 0.1              | 0.3              |
| Saskatoon berries         | 110.6            | 50.4                | 10.6             | 0                    | 3                | 6.3              |
| (Amelanchier canadensis)  |                  |                     |                  |                      |                  |                  |
| Strawberries (Fragaria X ananassa) | 1.7            | 0.3                 | 0                | 24.8                 | 0                | 0.1              |
| **Other fruits**          |                  |                     |                  |                      |                  |                  |
| Cherries, sweet           | 30.2             | 0                   | 0                | 1.4                  | 1.5              | 0                |
| **Grape**                 |                  |                     |                  |                      |                  |                  |
| Red                       | 1.2              | 2.3                 | 39               | 0                    | 3.6              | 2                |
| Concord (Vitis vinifera)  | 23.8             | 70.6                | 6                |                      | 4.8              | 14.9             |
| Pistachio nuts, raw       | 7.3              | 0                   | 0                | 0                    | 0                | 0                |
| (Pistacia vera)           |                  |                     |                  |                      |                  |                  |
| **Plums**                 |                  |                     |                  |                      |                  |                  |
| Black diamond (with peel) | 56               | 0                   | 0                | 0                    | 0                | 0                |
| Purple                    | 179              | 5.2                 |                  |                      |                  |                  |
| Plums (Prunus spp.)       | 5.63             | 0                   | 0                | 0.3                  | 0                | 0                |
| **Vegetables**            |                  |                     |                  |                      |                  |                  |
| Black beans (P. vulgaris) | 18.5             | 10.6                |                  |                      | 15.4             |                  |
| Cabbage red picked        | 11.8             |                     |                  |                      |                  |                  |
| Eggplant raw (Solanum melongena) | 3.2          | 85.7                |                  |                      |                  |                  |
| Onions red                | 3.2              | 4.3                 | 0                | 2.1                  |                  |                  |
| Radicchio (Cichorium intybus) | 127            | 7.7                 |                  |                      |                  |                  |
| Radishes (Raphanus sativus) | 0              | 0                   | 63.1             | 0                    | 0                | 0                |
| Sweet potato purple (cooked) | 10.6           | 0.9                 |                  |                      |                  |                  |

### 2. Anthocyanins

ACNs are water-soluble bioactive compounds, which belong to the widespread group named flavonoids within the polyphenol class. Chemically, ACNs consist of two aromatic rings linked by three carbons in an oxygenated heterocycle. The chromophore of ACNs is the 7-hydroxyflavylium ion. In particular, ACNs consist of an aglycon base or flavylium ring (anthocyanidins), sugars, and possibly acylating groups (Figure 1) [7]. ACNs are responsible for the red, purple and blue colors of many flowers, cereal grains, fruit, and vegetable. They are generally found in the skins, and their content is usually proportional to color intensity. ACN content varies greatly depending on the different food sources considered (Table 1) [8]. More than 600 different ACNs have been identified in vegetables, derived from twenty-three different aglycones (anthocyanidins) classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus. The six anthocyanidins commonly found in fruit and vegetables are pelargonidin, cyanidin, delphinidin,
peonidin, petunidin, and malvidin which are combined with sugars (mostly glucose, galactose, and arabinose) (Figure 1) [8].

ACN intake has been estimated to range between 180 mg/day and 215 mg/day, but these values can be 10 times lower in industrialized countries [9–11]. ACN bioavailability is reported to be lower than that of other polyphenols, and less than 1% of consumed ACNs is generally absorbed, reaching plasma concentrations in the nanomolar order [12]. In addition, ACNs are rapidly metabolized and their presence in the circulation is limited to a few hours. Despite their low absorption and rapid metabolism, the regular intake of ACNs may result in beneficial effects on human health by reducing the risks of cardiovascular disease and cancer [13–15]. Indeed, they possess high antioxidant capacity and can play a key role in the prevention of oxidative stress by scavenging reactive oxygen species and free radicals and by modulating endogenous defense system, as demonstrated in several in vitro and in vivo studies [16–18]. ACNs have also been documented to ameliorate hyperglycemia, to modulate endogenous defense system, as demonstrated in several in vitro and in vivo studies [16–18]. ACNs have also been documented to ameliorate hyperglycemia, to modulate endothelial function, and to decrease inflammation [19–24]. Moreover recently ACNs have been studied for their role in the modulation of lipid metabolism and fat deposition [25–27] in different tissues, including the liver.

### 3. Nonalcoholic Fatty Liver Disease

NAFLD is characterized by liver fat deposition, that is, steatosis, related to systemic insulin resistance (IR) [28]. In susceptible individuals, steatosis may be associated with oxidative hepatocellular damage, inflammation, and activation of fibrogenesis, defining nonalcoholic steatohepatitis (NASH) [29, 30]. NASH, but not simple steatosis, is a potentially progressive liver disease leading to cirrhosis and hepatocellular carcinoma [31]. Following the epidemics of obesity and the metabolic syndrome, NAFLD is rapidly becoming the leading cause of altered liver enzymes in Western countries [2, 32, 33], and NASH will become the major cause of end-stage liver disease and hepatocellular carcinoma within the next 10–20 years.

Fatty liver, that is, hepatic fat accumulation exceeding 5% of total liver mass, results from an unbalance between triglyceride deposition and synthesis on one hand and oxidation and secretion by lipoproteins on the other hand [34] and initially represents a protective mechanism against the toxicity resulting from an increased flux of free fatty acids (FFAs) to the liver [35]. Most of excess hepatic lipid content derives from increased peripheral lipolysis [36], which is caused by adipose tissue insulin resistance [37], and is a typical feature of obesity. Other contributing factors are increased lipogenesis induced by hyperinsulinemia or directly by diet. Indeed, the major risk factor for NAFLD is systemic IR due to central obesity and the metabolic syndrome [28, 38]. Impaired ability to secrete lipoproteins [39] and changes in fattyacid oxidation also contribute to hepatic fat accumulation [40].

Development of NASH has classically been explained by the occurrence of a so-called second-hit, leading to the activation of inflammation, in the context of hepatic steatosis (the “first hit”) [41]. This second insult likely represents a combination of insults related to (a) direct hepatic lipotoxicity, (b) hepatocellular oxidative stress secondary to free radicals produced during β- and ω-oxidation of FFAs, (c) inflammation triggered by endotoxins engaging Toll-like receptor-4 (TLR-4) in Kupffer cells (the hepatic macrophages) and hepatocytes due to increased intestinal permeability, bacterial overgrowth, and altered intestinal flora [42–44], (d) cytokine release, and (e) endoplasmic reticulum stress. These combine to produce inflammation, cellular damage, and activation of fibrogenesis. Genetic factors, and in particular the I148M variant of Patatin-like phospholipase domain containing-3 (PNPLA3), play a major role in determining individual susceptibility to develop steatosis or NASH and progressive liver disease, interacting with dietary factors [45, 46].

### 4. Anthocyanins in NAFLD

Recent studies documented that ACNs can reduce hepatic lipid accumulation, but their impact on NAFLD has yet to be determined.

We have classified the available evidence according to the experimental setting: in vitro, in vivo, and in human trials. For the revision of the literature, the PubMed database was searched up to June 2013 (keywords: steatosis or nonalcoholic fatty liver disease or steatohepatitis plus anthocyanins or single anthocyanin names). No publication data restrictions

| Anthocyanidin  | R₁  | R₂  |
|----------------|-----|-----|
| Pelargonidin    | H   | H   |
| Cyanidin        | OH  | H   |
| Delphinidin     | OH  | OH  |
| Peonidin        | OCH₃| H   |
| Petunidin       | OCH₃| OH  |
| Malvidin        | OCH₃| OCH₃|

![Figure 1: General chemical structures of anthocyanins in the diet.](image)
were applied. Papers were selected for inclusion in this review on the basis of their relevance, and additional papers were obtained from their reference lists.

4.1. In Vitro. Studies evaluating the effect of ACNs in vitro on lipid metabolism and oxidative stress in hepatocytes, typical of NAFLD and NASH, are presented in Table 2. Most studies were conducted in human hepatoma HepG2 cells [47–55], an established model of hepatic lipid metabolism. Both ACN-rich extracts of foods (berries and potatoes) and synthetic ACNs (cyanidin hydrochloride and cyanidin-3-O-β-glucoside) were employed. Unfortunately, interpretation of the overall evidence is hindered by differences in cellular models, experimental protocols, and the molecular pathways evaluated. However, most studies are concordant on the fact that ACNs reduce hepatocellular lipid accumulation [48–50, 53–55] by inhibiting lipogenesis [49] and possibly by promoting lipolysis [53–55], although the different aspects of lipid metabolism were not evaluated in all studies. Furthermore, ACNs also reduce cellular oxidative stress by promoting the antioxidant response [47, 51, 52]. Interestingly, three independent studies reported that activation of the adenosine monophosphate protein kinase (AMPK) pathway was implicated in mediating the effect of ACNs on hepatic lipid metabolism and antioxidant response [49, 51, 53, 54]. However, another study suggested that ACNs may act as direct agonist of PPAR receptors in hepatocytes [55].

4.2. In Vivo. Studies evaluating the effect of ACNs in vivo on hepatic lipid metabolism, steatosis, oxidative stress, and steatohepatitis are presented in Table 3. Also in this case, the interpretation of the overall evidence is difficult, due to the very different experimental models of NAFLD and metabolic syndrome employed and to the different outcomes for the evaluation of lipid metabolism, oxidative stress, and liver damage. In addition, in some studies, animals were exposed to synthetic ACNs (i.e., cyanidin-3-O-β-glucoside) [50, 52, 56, 57], whereas in others they were exposed to extracts of ACN-rich foods (e.g., sweet potato, berries, and oranges) [27, 49, 58–62]. Mirroring the results obtained in vitro, there is ample convergence supporting an effect of ACNs in reducing hepatic lipid accumulation, that is, steatosis [49, 50, 52, 56–58, 60–63]. In addition, the majority of studies also reported an improvement in hepatic and systemic IR and serum lipids, often related to reduced weight gain [57, 58, 60–62]. Again, increased activation of PPARα inducing lipolysis and reduced lipogenesis were postulated to be responsible for decreased hepatic fat content [27, 59–61]. Increased activity of

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Table 2: Studies evaluating the effect of anthocyanins on hepatic lipid metabolism and hepatocellular lipotoxicity in vitro.

| Paper | Anthocyanin | Food | Model | Effects | Mechanism |
|-------|-------------|------|-------|---------|-----------|
| 46    | ACN-rich extract | Bilberry | Primary rat hepatocytes | ↓ tBH induced damage ↓ MTT, LDH, TBARS | Antioxidant |
| 47    | ACN-rich fraction | Blueberry | HepG2 cells | ↓ OA induced TG accumulation at high doses | ? |
| 48    | Anthocyanin factor | Sweet potato | HepG2 cells | ↑ pAMPK ↓ Srebp1c, FAS | ↑ pAMPK |
| 49    | Cyanidin-3-O-β-glucoside | — | HepG2 cells | ↓ lipogenesis | |
| 50    | Cyanidin chloride | Blackberry | HepG2 cells | ↑ antioxidants (SOD, catalase) | ↑ pMAPK, ↑ Nrf2 and PPARα |
| 51    | Cyanidin-3-O-β-glucoside | — | HepG2 cells | ↓ ROS induced by glucose ↑ antioxidants (GSH) | ↑ PKA and CREB |
| 52    | Cyanidin-3-O-β-glucoside | — | HepG2 cells | ↑ pAMPK and pACC, ↑ CPT1 and FFAs oxidation | AMPK activation mediated by calmodulin kinase kinase |
| 53    | ACN-rich extract | Mulberry | HepG2 cells | ↑ pAMPK and pACC, ↑ PPARα, CPT1 and FFAs oxidation ↓ Srebp1c and lipogenesis | AMPK activation |
| 54    | Cyanidin | — | HepG2 cells | ↓ lipogenesis ↓ lipolysis | PPARα/β/γ agonist |

AMPK: adenosine monophosphate protein kinase; Srebp1c: sterol regulated element binding protein 1c; ACC: acetyl-coenzyme A carboxylase; p: phospho; glycerol 3 phosphate acyl transferase; PKC: protein kinase C; OMM: outer mitochondrial membrane; SOD: superoxide dismutase; MAPK: mitogen associated protein kinase; Nrf2: nuclear factor erythroid 2-related factor 2; PPARα: β/δ peroxisomes proliferator activated receptor α; ROS: reactive oxygen species; GSH: reduced glutathione; PKA: protein kinase A; CREB: cAMP-response element binding protein; CPT-1: carnitine-palmytoil-transferase-1; ACN: anthocyanins; OA: oleic acid; tBH: tert-butylhydroperoxide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; LDH: lactate dehydrogenase; TBARS: thiobarbituric acid reacting substances.
**Table 3: Studies evaluating the effect of anthocyanins on hepatic steatosis and steatohepatitis in vivo.**

| Paper  | Anthocyanin | Food | Model | Metabolic effects | Molecular effects |
|--------|-------------|------|-------|-------------------|-------------------|
| 48     | Anthocyanin factor | Sweet potato | Mice fed HFD | ▼ weight gain ▼ steatosis | ▲ pAMPK and pACC ▲ Srebp1c, FAS, ACC |
| 49     | Cyaniding-3-O-β-glucoside | — | KKAy mice | ▼ weight gain | ▼ steatosis |
| 51     | Cyaniding-3-O-β-glucoside | — | db/db mice | ▼ weight gain ▼ steatosis | ▲ antioxidants (GSH) ▲ weight gain ▲ steatosis, ROS, and inflammation |
| 55     | Cyaniding-3-O-β-glucoside | Blackcurrant | Rats | ▼ weight gain ▼ steatosis | ▲ antioxidants (GSH) ▲ weight gain ▼ steatosis, ROS, and inflammation |
| 56     | Cyaniding-3-O-β-glucoside | — | C57Bl/6 on HFD and db/db | ▼ cytokines and adipose tissue inflammation | ▲ antioxidants (GSH) ▲ weight gain ▼ steatosis |
| 57     | Several  | Tart cherry | Dahl Salt-Sensitive rat | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 58     | — | Vitis coignetiae Pulliat leaves (yama-budo) | Rats on HFD choline deficient diet | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 59     | Several  | Moro orange juice | C57Bl/6 mice on HFD | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 60     | — | Blueberry | Zucker rats on HFD | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 61     | — | Mulberry | Hamsters on HFD | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 62     | Several  | Elderberry | Hamsters fed high fat fish oil | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |
| 63     | — | Mulberry | Rats on HFD | ▼ weight gain ▼ steatosis | ▲ cytokines and adipose tissue inflammation |

HFD: high fat diet; IR: insulin resistance; TGs: triglycerides; LXR: liver X receptor; FAS: fatty acid synthase; GAPT1: glycerol 3 phosphate acyl transferase; PPARα: peroxisomes proliferator activated receptor α; chol: cholesterol; FFAs: free fatty acids; CPT-1: carnitine-palmitoyl-transferase-1; HMG-CoA red: 3-hydroxymethyl-3-glutaryl-coenzyme A reductase; p: phospho; AMPK: adenosine monophosphate activated protein kinase; Srebp1c: sterol regulated element binding protein 1c; ACC: acetyl-coenzyme A carboxylase; ROS: reactive oxygen species; JNK: c-Jun N-terminal kinase; FOXO1: forkhead box O1.

the AMPK pathway was confirmed in vivo in one study [49], and increased hepatic antioxidant activity after exposure to ACN was also widely confirmed in experimental models of NAFLD [52, 56, 59, 63, 64]. However, whether improved redox status was secondary to or independent of reduced hepatic lipids and improved metabolic status was not tested. In some studies, these effects of ACN exposure translated in an improvement in inflammation, that is, in reduced severity of steatohapatitis [53, 58, 60]. The involvement of AMPK activation in mediating the beneficial effect of ACN on insulin sensitivity is also supported by evidence that bilberry extract ameliorates insulin resistance and hepatic lipid metabolism via this pathway [65].

4.3. Clinical Studies. There is only one study evaluating the effect of ACN on NAFLD patients, which is summarized in Table 4 [66]. Suda and coworkers recruited 48 adult men with increased liver enzymes negative for viral hepatitis, thereby likely affected by NAFLD. During a eight-week intervention, about 200 mg of acylated ACNs or placebo was administered twice daily. Acylated ACN intake was associated with reduced levels of liver enzymes, in particular gamma-glutamyltransferases. However, liver damage was not directly assessed, fatty liver was not confirmed by direct imaging, and the effect of acylated ACNs was not compared to that of a control food or to the lack of intervention.
5. Conclusions

It is widely accepted that exploring the role of foods and more specifically the effect of bioactive compounds such as ACNs on the metabolic processes involved in chronic diseases is critical for preventive strategies. For instance, similar therapeutic activities have been shown for docosahexaenoic acid on steatosis severity in children with NAFLD [67]. The availability of data demonstrating cause-effect relationships and the specific mode of action of such compounds are of paramount importance in order to support any dietary recommendation or supplementation.

A working model depicting the possible mechanisms underpinning the beneficial effects of anthocyanins in NAFLD and NASH: a Srebp1c working model based on available studies. Anthocyanins may prevent the progression of liver damage related to NAFLD by three independent mechanisms: inhibition of lipogenesis by reducing Srebp1c, promotion of lipolysis by induction of PPARα activity, and reduction of oxidative stress by induction of anti-oxidant enzymes. The effects of anthocyanins on lipid metabolism seem to be dependent on the activation of the AMPK pathway in hepatocytes.

On the basis of these data, it seems that ACN-rich foods can be promising for the prevention of NAFLD and its complications. Additional studies are required to clarify the molecular mechanisms and to test the specific effect of single compounds and food extracts in vitro and in vivo. Randomized controlled studies are warranted to test foods on histological damage or noninvasive biomarkers of liver damage progression in patients with NASH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Luca Valenti and Patrizia Riso have contributed equally to this paper. They designed the study, independently reviewed the literature, and wrote the first paper draft. Anna Mazzocchi performed the literature search. Silvia Fargion, Marisa Por-rini, and Carlo Agostoni critically reviewed the paper and supervised the study.

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