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The Potential of Flavonoids in the Treatment of Non-alcoholic Fatty Liver Disease

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The potential of flavonoids in the treatment of non-alcoholic fatty liver disease

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List of abbreviations:

NAFLD = non-alcoholic fatty liver disease, NASH = non-alcoholic steatohepatitis, FFA = free fatty acids, VLDL = very low density lipoprotein, SREBP-1c = sterol regulatory element binding protein-1c, ChREBP = carbohydrate response element binding protein, gk = glucokinase, FAS = fatty acid synthase, ACC = acetyl-CoA carboxylase, ROS = reactive oxygen species, ER = endoplasmic reticulum, GSH = glutathione, SOD = superoxide dismutase, GPx = glutathione peroxidase, PPAR = peroxisome proliferator activated receptor, LXRα = liver X-receptor α, AMPK = 5' adenosine monophosphate-activated protein kinase, HO-1 = heme-oxygenase 2, AREs = antioxidant response elements, nrf2 = nuclear factor erythroid derived 2, IKK = IκB kinase complex, IκB = NFκB inhibitor protein, PKC = protein kinase C, COX = cyclooxygenase, iNOS = nitric oxide synthase, NO = nitric oxide, TNF-α = tumor necrosis factor α, ALT =

alanine transaminase, AST = aspartate transaminase, γGT = gamma-glutamyl transpeptidase, EGCG = epigallocatechin-gallate, GTE = green tea extract.

Abstract

The contemporary pathophysiological model of non-alcoholic fatty liver disease (NAFLD) consists of multiple parallel pathways with a dynamic cross talk that cumulate in steatosis and inflammation and ultimately fibrosis, cirrhosis, liver failure and hepatocellular carcinoma. So far, no pharmacological treatment has been approved. A major impediment of drugs in general is that they are intended to act on one single target in the pathology of a disease. However, the multitude of pathways involved in the pathogenesis of NAFLD underpins the need for treatments that address these various pathways. Interestingly, flavonoids have been found to have positive effects on lipid metabolism, insulin resistance, inflammation and oxidative stress, the most important pathophysiological pathways in NAFLD. This puts flavonoids in the spotlight for the treatment of NAFLD and prompted us to review the existing evidence for the use of these food derived compounds in the treatment of NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of liver disorders, ranging from steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma. The development of NAFLD is associated with the metabolic syndrome. Around 20 percent of the general population in Western countries has NAFLD (Bedogni et al. 2005) and 2 to 3 percent develops NASH (Neuschwander-Tetri et al. 2003). The pathophysiological model that has evolved for the development of NAFLD includes multiple hits that do not follow a strict sequence (fig.1) (Tilg et al. 2010). In this model, metabolic disorders, oxidative stress and local and systemic inflammation are the major processes involved in the progression of NAFLD. These processes appear to enforce each other and a dynamic cross talk between the different processes exists (fig. 1). Some authors suggest that steatosis and steatohepatitis might be two distinct disease entities (Tilg et al. 2010; Yilmaz 2012). However, progression from steatosis to NASH has been documented in patients (Pais et al. 2011).

To date, no evidence based pharmacological treatment exists for NAFLD. Patients are given life style advice consisting of dietary recommendations as well as encouragement to increase physical exercise to lose weight. That a multitude of pathways has been implicated in the etiology of NAFLD makes the treatment challenging; ideally, the treatment should address all the multiple pathways. The involvement of multiple pathways explains why no effective drug has been found for the treatment of NAFLD. Traditionally, drugs are designed as "silver bullets" that, according to the classical medicinal chemical approach, have a well-defined, specific

biologic target like a receptor (fig. 2). Since such a well-defined, specific biologic target is missing in NAFLD, this traditional approach is deemed to fail.

Natural compounds, such as flavonoids, have frequently been studied in models of NAFLD and seem to display beneficial effects. Flavonoids comprise a diverse group of compounds abundantly found in our diet. The intake of flavonoids has been associated with several health benefits. Initially, their health benefits were attributed to their potent antioxidant activity. However, further research revealed that also other activities contribute, such as anti-inflammatory and metabolic effects. Apparently, flavonoids display a multitude of activities and therefore these compounds were upgraded from antioxidant to bioactive (Bast et al. 2013). A bioactive produces a biological response via an array of subtle effects via different targets (fig. 2). This multifarious mode of action of flavonoids seems to suit seamlessly in the treatment of NAFLD, in which various pathways are involved. This concept will be reviewed in the present paper.

Firstly, an inventory of the various pathways identified in the etiology of NAFLD will be made. Secondly, the several biological activities of flavonoids will be presented. These activities will be linked to the pathways involved in NAFLD explaining the rational for the use of flavonoids in the treatment of NAFLD. Finally, the clinical studies on the efficacy of flavonoids in NAFLD will be evaluated, with the focus on the effect of flavonoids on the different pathways.

To this end, a literature search was conducted on PubMed in November 2013 using the search terms: flavonoids, nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. To increase the number of studies found, a search using the name of specific flavonoids was also included.

Pathogenesis of nonalcoholic fatty liver disease

The pathogenesis of NAFLD is complex and multifactorial, comprising multiple hits that lead to steatosis and NASH (Tilg et al. 2010; Polyzos et al. 2012). Although various pathways have been identified, the list is not complete as the etiology still is enigmatic and our knowledge of the disease is progressing.

A hallmark in the development of NAFLD is the accumulation of fat in the liver. Factors that are known to contribute to this accumulation include: (1) high free fatty acids (FFA) supply due to increased lipolysis from visceral and subcutaneous adipose tissue and dietary intake, (2) low FFA oxidation in relation to the supply of FFAs, (3) high hepatic lipogenesis and (4) low hepatic excretion of very low density lipoprotein (VLDL) (fig. 3) (Tilg et al. 2010). Another hallmark is chronic inflammation causing fibrosis. Underlying processes include oxidative stress and lipid peroxidation, mitochondrial dysfunction, adipocytokine/cytokine imbalance, gut-derived bacterial endotoxins, hepatic stellate cell activation and genetic factors (Polyzos et al. 2012). Also activation of Kupffer cells by cholesterol crystals is suggested to be a trigger for hepatic

inflammation (Bieghs et al. 2013). Insulin resistance plays an important role in the development of both steatosis and inflammation (Polyzos et al. 2012). Due to insulin resistance, lipolysis is not inhibited by insulin. The FFA's released cause inflammation, promote ectopic fat deposition and further enhance insulin resistance, creating a self-propelling feed forward process (fig. 3) (Polyzos et al. 2012). Furthermore, insulin resistance stimulates gluconeogenesis in hepatocytes and reduces glycogen formation. Increased glucose and insulin levels stimulate de novo lipogenesis via hepatic transcription factors such as sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP). This causes stimulation of lipogenic enzymes such as glucokinase (gk), fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC) (fig. 3) (Polyzos et al. 2012).

In the multifactorial etiology of NASH, oxidative stress represents a crucial process (Koek et al. 2011; Rolo et al. 2012; Ucar et al. 2013): the production of reactive oxygen species (ROS) not balanced by the protection against ROS by antioxidants. Various potential sources of oxidative stress have been reported in NAFLD. ROS are produced during the mitochondrial and peroxisomal beta oxidation of FFAs and during the metabolism of FFAs by cytochrome P450 2E1 and 4A. ROS cause endoplasmic reticulum (ER) stress, which further promotes the accumulation of ROS within the cell (Ucar et al. 2013). Also reduction in antioxidant defenses will contribute to oxidative stress. Reduced glutathione (GSH) levels and decreased superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and glutathione transferase activities are found in NASH and appear to be correlated to disease severity (Rolo et al. 2012). ROS react with biological compounds including fatty acids, proteins and DNA, causing lipid peroxidation, mitochondrial dysfunction, stellate cell activation, inflammation (via NF-κB activation) and

apoptosis (fig. 3) (Koek et al. 2011; Ucar et al. 2013). Mitochondrial dysfunction and inflammation will lead to the formation of more ROS, further fueling the self-propelling feed forward process.

Iron has also been implemented in the pathogenesis of NAFLD. In patients with NAFLD elevated hepatic iron levels have been found (Valenti et al. 2010; Nelson et al. 2011). The precise role of iron in the pathogenesis of NAFLD has not yet been established, but it is well documented that iron increases oxidative stress, e.g. by its ability to generate hydroxyl radicals via the Fenton reaction (Fenton 1894). Citrate, an intermediate product of lipid metabolism found to be elevated in NAFLD patients, has the ability to further increase this iron-induced oxidative stress by stimulation of the Fenton reaction (van de Wier et al. 2013).

Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitously found in nature. They are secondary metabolites in plants and are frequently bound to sugars (glycosides) (Ross et al. 2002). Flavonoids also occur as aglycones (without a sugar group) (Ross et al. 2002). Most flavonoids (fig. 4) consist of three rings: two aromatic rings (A and B) and one heterocyclic ring (C). Flavonoids are categorized into subclasses based on variations in the C ring. The major subclasses are flavones, isoflavones, flavanols, flavanones, anthocyanidins and chalcones (fig. 4) (Ross et al. 2002).

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Over 5,000 different flavonoids have been identified. Because the group of flavonoids is a very heterogenic group of compounds that act on multiple biological targets, various, sometimes even paradoxical, effects have been described for different classes of flavonoids. Also, the wide variation in models used to study the effects of flavonoids contributes to the variation in the effects found. This review addresses the potential of flavonoids and focusses on the reported positive effects of these bioactives in the treatment of NAFLD. These effects are differentiated in metabolic, antioxidant and anti-inflammatory effects.

Metabolic effects

PPARs

The peroxisome proliferator activated receptors (PPARs) are promising targets in the treatment of NAFLD. PPARs are nuclear receptors that play a role in the regulation of lipid and glucose metabolism as well as inflammation (Tailleux et al. 2012). PPARα is highly expressed in the liver and regulates FFA transport and stimulates enzymes involved in β-oxidation (Kallwitz et al. 2008). Furthermore, it limits inflammation by inhibition of NF-κB and reduction of C-reactive protein expression (Tailleux et al. 2012). Studies have found evidences for a role of PPARα in NAFLD and its treatment (Macdonald et al. 2004; Kallwitz et al. 2008). PPARα$^{-/-}$ mice have increased susceptibility for development of NAFLD when fed a high fat diet compared to wildtype mice (Kallwitz et al. 2008). In addition, PPARα agonists, such as fibrates, reduce

steatosis, inflammation and fibrosis in models of NASH (Shiri-Sverdlov et al. 2006; Tailleux et al. 2012). Stimulation of PPARα is expected to decrease steatosis by stimulation of β-oxidation and to mitigate inflammation by inhibition of NF-κB. Several studies have demonstrated that flavonoids stimulate PPARα (Medjakovic et al. 2010; Chang et al. 2011; Cho et al. 2011; Goto et al. 2012; Lee et al. 2012; Jia et al. 2013; Malek et al. 2013). For this stimulation various mechanisms of action have been proposed. Some studies claim that flavonoids are ligands and (partial) agonists of PPARα (Medjakovic et al. 2010; Jia et al. 2013; Malek et al. 2013). Other studies conclude that flavonoids upregulate PPARα gene and/or protein expression (Chang et al. 2011; Cho et al. 2011; Goto et al. 2012; Lee et al. 2012), possibly involving adiponectin, a stimulator of PPARα (Goto et al. 2012). As stated above, the variation in reported mechanisms might originate from the variation in chemical structure between flavonoids and the multitude of targets the flavonoids act on.

PPARγ is mainly expressed in adipose tissue and its activation results in adipocyte differentiation and insulin sensitization (Medjakovic et al. 2010). Secretion of insulin resistance promoting factors by adipose tissue is reduced and secretion of insulin sensitivity promoting factors is increased (fig. 5). By upregulation of adiponectin, PPARγ also activates PPARα. Furthermore, by activation of the involved genes (FABP, PEPCK, Acyl-CoA synthase, DGAT, FATP and LPL) adipogenesis and lipid storage in subcutaneous adipose tissue is stimulated (fig 5). Consequently, fat from harmful visceral adipose tissue is redistributed to subcutaneous fat depots (Medjakovic et al. 2010). Moreover, FFA delivery to the liver is reduced (Medjakovic et

al. 2010; Tailleux et al. 2012). PPARγ has also been reported to increase energy expenditure by induction of uncoupling protein-2 (UCP-2) (Kallwitz et al. 2008). Mutations in the PPARγ gene increase the risk of developing metabolic syndrome and NAFLD (Savage et al. 2003). Additionally, glitazones, PPARγ agonists, improve insulin resistance and decrease aminotransferase levels and liver fat in NASH patients, whereas positive effects on histological markers of NASH are not always noted (Tailleux et al. 2012). These findings substantiate the use of PPARγ agonists in the treatment of NASH. Several studies report that flavonoids stimulate PPARγ (Xia et al. 2005; Chen et al. 2009; Medjakovic et al. 2010; Puhl et al. 2012; Lee et al. 2013; Sharma et al. 2013). Similar as for PPARα stimulation, various mechanisms of action are described. Flavonoids have been found to upregulate PPARγ gene expression (Xia et al. 2005; Chen et al. 2009; Sharma et al. 2011; Lee et al. 2013). Also, some flavonoids were observed to be agonists of PPARγ (Medjakovic et al. 2010; Puhl et al. 2012). An advantage of flavonoids over other drugs, like the glitazones, could be that the bioactives only partially activate PPARγ. This reduces the risk of serious side effects seen with the use of full agonists. For example, weight gain, an important side effect of glitazones, is not associated with intake of isoflavones that also activate PPARγ. In several studies, intake of isoflavones even leads to a slight weight reduction (Medjakovic et al. 2010).

SREBP-1c

Another target identified in the treatment of NAFLD is SREBP-1c. SREBP-1c is a transcription factor that controls de novo lipogenesis via induction of the lipogenic enzymes (FAS, ACC, gk)

(Ferre et al. 2010), which stimulates steatosis. In liver biopsies of NAFLD patients, the expression of SREBP-1c and liver X receptor α (LXRα), which controls SREBP-1c transcription, as well as the expression of ACC and FAS are found to be significantly higher than in control biopsies (Higuchi et al. 2008). Several flavonoids inhibit SREBP-1c (Shin et al. 2007; Hwang et al. 2011; Liu et al. 2011; Sharma et al. 2011; Wu et al. 2011; Ahn et al. 2013). Multiple mechanisms of SREBP-1c inhibition have been implicated. Various studies have found that flavonoids downregulate SREBP-1c protein and gene expression (Hwang et al. 2011; Liu et al. 2011; Sharma et al. 2011; Wu et al. 2011; Ahn et al. 2013). The isoflavone genistein reduces the expression of site-1 proteases, which are necessary for SREBP-1c to act as a transcription factor (Shin et al. 2007). Furthermore, SREBP-1c is inhibited by activation of 5' adenosine monophosphate-activated protein kinase (AMPK) (Hwang et al. 2011; Liu et al. 2011; Wu et al. 2011). Activation of AMPK inhibits LXRα, which controls SREBP-1c transcription (Yap et al. 2011). Flavonoids stimulate activation of AMPK (Hwang et al. 2011; Liu et al. 2011; Wu et al. 2011; Lee et al. 2012). Additionally, flavonoids inhibit LXRα (Goldwasser et al. 2010; Sharma et al. 2011; Ahn et al. 2013). Since SREBP-1c transcription is stimulated by hyperinsulinemia, flavonoids might also reduce SREBP-1c expression by improving insulin sensitivity and normalizing insulin levels. Recently, activation of SREBP-1c was suggested to be one of the consequences of ER stress in the steatotic liver (Ferre et al. 2010). Inhibition of ER stress is another way in which flavonoids might inhibit SREBP-1c.

Antioxidant effects

As a first line of defense, flavonoids reduce the production of radicals and other reactive species. Flavonoids inhibit pro-oxidant enzymes, such as xanthine oxidase (Garcia-Lafuente et al. 2009). Also inhibition of lipoxygenases and cyclooxygenases (see anti-inflammatory effects), enzymes that are capable to co-oxidize molecules other than their usual substrates, reduces the production of reactive species (Garcia-Lafuente et al. 2009).

Flavonoids have been found to be very effective scavengers. This is a pivotal biochemical mode of action of bioactives, although the importance of radical scavenging is exaggerated as well as undervalued (Bast et al. 2013). Amongst antioxidants, flavonoids are at the top of the pecking order, meaning that they are the first in line to scavenge radicals. They scavenge a wide array of reactive species including superoxide, hydroxyl, peroxyl and peroxynitrite radicals (Bors et al. 1997; Haenen et al. 1997; Duthie et al. 2000). During this scavenging, flavonoids are oxidized by the radical, resulting in a more stable, less reactive radical (Garcia-Lafuente et al. 2009). Among the various subclasses of flavonoids, the flavonols that comprises quercetin and related flavonoids display superior scavenging activity. This is due to a large conjugated π–system that delocalizes electrons over the entire molecule. Structure-activity relationship studies reveal that two pharmacophores are present in the flavonols, (1) a catechol moiety in ring B and (2) a hydroxyl (OH) group at the 3 position at a 2,3-double bound, which is activated by the hydroxyl groups at the 5 and 7 position (Heijnen et al. 2002). In NAFLD, radicals produced during peroxisomal and mitochondrial β-oxidation and the metabolism of FFA by Cytochrome P450 2E1 and 4A can be scavenged by flavonoids, which will result in a reduction of oxidative stress.

In addition to the direct radical scavenging effect, several flavonoids have the ability to chelate iron and other transition metals that contribute to the formation of radicals (Pietta 2000; Ross et al. 2002). The quercetin derived semi-synthetic flavonoid monoHER can scavenge OH-radicals at an extremely high apparent rate - ks = 980 X 10^8 $M^{-1}s^{-1}$ - which is even quicker than the diffusion rate (~ 100 X 10^8 $M^{-1}s^{-1}$) (Lemmens et al.). This can be explained by 'site-specific scavenging'. Essential for this activity is that monoHER can chelate iron. The result of this chelation is that monoHER is present at the site of the radical formation, i.e. the iron ion. This enables monoHER to immediately scavenge the newly formed radical. By this site-specific scavenging, monoHER is able to prevent damage to critical biomolecules such as lipids, proteins or DNA, despite the high reactivity of the radical (Lemmens et al. ; Haenen et al. 1993). Since iron mediated hydroxyl radical formation has been implied in NAFLD (O'Brien et al. 2011; van de Wier et al. 2013), this action of flavonoids is expected to be meaningful for the treatment of NAFLD.

A third mode of action is that flavonoids can protect or enhance the endogenous antioxidant defense (Ross et al. 2002; Stevenson et al. 2007). Several flavonoids induce glutathione S-transferase, heme-oxygenase 1 (HO-1) and other antioxidants (Ross et al. 2002; Yang et al. 2011; Sun et al. 2012; Zhang et al. 2012; Huang et al. 2013). An important pathway in this response is stimulation of nuclear factor erythroid derived 2 (nrf2), a transcription factor that binds to antioxidant response elements (AREs) in the promoter region of genes encoding various

antioxidants and phase II detoxifying enzymes. This leads to the transcription of those enzymes, e.g. HO-1 and NAD(P)H-quinone oxidoreductase (Mann et al. 2009). Stimulation of nrf2 by flavonoids is reported in several studies (Yang et al. 2011; Sun et al. 2012; Zhang et al. 2012; Huang et al. 2013). Flavonoids increase nrf2 nuclear translocation to the nucleus and the binding of nrf2 to AREs (Yang et al. 2011; Huang et al. 2013). Since depletion of antioxidant defenses is seen in NASH patients, upregulation of antioxidants could be beneficial in the treatment of NASH (Rolo et al. 2012).

Anti-inflammatory effects

Anti-inflammatory effects of flavonoids have been mainly subscribed to inhibition of the NF-κB pathway (Gonzalez et al. 2011). The NF-κB pathway consists of a canonical and non-canonical pathway. The canonical pathway is involved in inflammatory responses, while the non-canonical pathway regulates immunce cell differentiation and maturation and lymphoid organogenesis (Shih et al. 2011). Since the canonical pathway is most important for initiation of inflammation and effects of flavonoids focus on this part of the NF-κB pathway, our review is restricted to the canonical pathway. The canonical pathway is activated by pro-inflammatory signals, such as cytokines and oxidative stress (van den Berg et al. 2001). This causes the IKK complex to phosphorylate and designate the IκBs (α, β or ε) for degradation. Degradation of the IκBs releases NF-κB into the nucleus. Consequently, transcriptional activity occurs resulting in an inflammatory response (Shih et al. 2011).

NF-κB activation occurs in various inflammatory diseases. Activation of the NF-κB pathway is observed in animal models of NASH as well as in NASH patients (Marra 2008), illustrating a role for NF-κB activation in NASH. Flavonoids interfere with the NF-κB pathway in several ways (fig. 6) (Gonzalez et al. 2011). Flavonoids inhibit the IKK complex and IκB-phosphorylation, thereby preventing NF-κB translocation to the nucleus and the transcription of genes involved in the inflammatory response. Flavonoids also inhibit protein kinases that control the activity of NF-κB (Kim et al. 2005). In addition, flavonoids inhibit protein kinase C (PKC), mitogen activated protein kinases (MAPKs), extracellular signal related kinase (ERK) and Jun N-terminal kinase (JNK) (Kim et al. 2005). A mechanism proposed for inhibition of protein kinases is competitive binding to nucleotide binding sites (Manthey 2000). Since oxidative stress also results in NF-κB activation (van den Berg et al. 2001), the antioxidant potency of flavonoids is also implicated in inhibition of the NF-κB pathway (Boots et al. 2008; Gloire et al. 2009; Salamone et al. 2012).

Besides inhibitory effects on the NF-κB pathway, flavonoids also inhibit the activity of regulatory enzymes involved in the induction of the inflammatory response, such as protein tyrosine kinases, PKC, phosphodiesterase, phospholipase A_2, lipoxygenases and cyclooxygenase (COX) (Manthey 2000; Kim et al. 2005). These enzymes are responsible for the activation of specialized cells involved in inflammation, e.g. by prostanoid biosynthesis via arachidonic acid metabolism (Manthey 2000). Also, the production of various cytokines is inhibited by

flavonoids, possibly involving inhibition of phosphodiesterase (Manthey 2000; Gonzalez-Gallego et al. 2010).

Furthermore, several flavonoids inhibit inducible nitric oxide synthase (iNOS) expression and the production of nitric oxide (NO) (Kim et al. 2005; Gonzalez-Gallego et al. 2010). NO serves as an inflammatory mediator and also leads to the formation of the highly damaging peroxynitrite in conditions of oxidative stress (Garcia-Lafuente et al. 2009). In addition to the inhibition of NO production, flavonoids can scavenge NO (van Acker et al. 1995; Haenen et al. 1999) and peroxynitrite (Haenen et al. 1997). Inhibition of iNOS and COX-2 expression is also found to be related to inhibition of NF-κB and activation of PPARγ (Gonzalez-Gallego et al. 2010).

Recently, it has been reported that flavonoids are able to prevent deterioration of the anti-inflammatory effect of the glucocorticoid cortisol in the presence of oxidative stress (Ruijters et al. 2014). Oxidative stress extinguishes the anti-inflammatory effect of cortisol, leading to cortisol resistance. Flavonoids reduce intracellular oxidative stress as well as the development of cortisol resistance. This further deciphers the enigmatic mechanism of flavonoids by which these bioactives exert their biological effect, and moreover shows that their anti-inflammatory and antioxidant action are intertwined.

Flavonoids in the treatment of NAFLD

Several flavonoids have been studied for the treatment of NAFLD. One of the most investigated flavonoids is silybin, one of the flavonoids in the flavonoid mixture silymarin. Green tea flavonoids and soy isoflavones are also extensively investigated. Together with quercetin and rutin, these are the most studied flavonoids for the treatment of NAFLD. These groups of flavonoids and their effects on NAFLD are reviewed separately.

Animal models

The majority of in vivo studies investigating the use of flavonoids in NAFLD are animal studies. Because NAFLD is a multifactorial disease and the patient population with NAFLD is very heterogenic, it is difficult to imitate all the facets of the disease in one animal model. Furthermore, NAFLD is seen as the hepatic manifestation of the metabolic syndrome: patients do not only show liver abnormalities, but also have obesity, dyslipidemia and insulin resistance. Although many models may succeed to mirror the liver pathology correctly, this does not always reflect the right metabolic context (Larter et al. 2008).

Already many reviews have been devoted to animal models of NAFLD (Nanji 2004; Nagarajan et al. 2012; Takahashi et al. 2012). Therefore, this section will only evaluate the animal models used in the studies investigating the use of flavonoids in NAFLD. Most studies are mice or rat studies. Also one study investigated the development of NAFLD in gerbils. The animals used in

the different studies had various genetic backgrounds and some transgenic animal models were used. Furthermore, different diets were used to induce NAFLD, such as high fat diets, high fructose diets and methionine- and choline deficient diets.

To validate the different models used, it has to be examined which model pictures the disease process most completely. Therefore, the models were compared regarding the development of liver damage (steatosis, inflammation and fibrosis), the presence of the most important pathogenic pathways (metabolic abnormalities, oxidative stress and inflammation) and the presence of other signs of the metabolic syndrome (dyslipidemia, obesity and insulin resistance).

Firstly, the different dietary models are compared. The methionine and choline deficient diet (MCD diet) causes lipid deposition in the liver by interfering with β-oxidation and VLDL secretion. The diet lacks methionine and choline, which are essential for hepatic β-oxidation and the production of VLDL. In addition to liver steatosis and inflammation, oxidative stress and changes in the liver cytokines and adipocytokines are found (Takahashi et al. 2012). The advantage of this model is that liver steatosis and inflammation are found within ten days of the diet. Fibrosis is found after 8-10 weeks (Takahashi et al. 2012). The MCD-model causes the most inflammation, oxidative stress and liver damage compared to other dietary models. However, the extent of damage is dependent on species, gender and strain of the animals used (Kirsch et al. 2003). C57Bl/6 mice are found to develop the most inflammation and necrosis, best approximating the histological features of NASH. Male gender and the strain Wistar rats are associated with the highest degree of steatosis (Kirsch et al. 2003). The disadvantage of the MCD-model is that it lacks the metabolic context of human NAFLD/NASH. Animals on the

MCD-diet are found to lose weight, to have no insulin resistance and unchanged or increased serum adiponectin levels (Kirsch et al. 2003). In some studies this is resolved by using the MCD diet in genetically obese animals, such as ob/ob mice.

Various high fat diets have been used in animal models of NAFLD. Sprague-Dawley rats on a high fat diet are found to develop steatosis, inflammation and oxidative damage in the liver. Also insulin resistance is found after 3 weeks of a high fat diet (Takahashi et al. 2012). However, development of steatohepatitis is dependent on the rodent species and strain used. For example, while Sprague-Dawley rats do develop steatohepatitis, Wistar rats are found to be less susceptible to develop steatohepatitis on a high fat diet (Romestaing et al. 2007). C57Bl/6 mice are found to develop steatosis after 10 weeks of high fat diet. Also insulin resistance, increased plasma cholesterol levels and obesity are found. However, slight inflammatory changes are only found after 35 weeks of high fat diet (Ito et al. 2007). The development of steatohepatitis in animals on a high fat diet is not only dependent on the rodent species and strain, but also on the fat content in the diet, the composition of dietary fat and the duration of the treatment (Takahashi et al. 2012). Although the use of a high fat diet seems to reproduce the metabolic context of human NASH better, liver damage is less severe than with use of the MCD-diet. Among the various high fat diet models, the pathological changes in the intragastric-overfeeding model are found to resemble human NASH best (Ito et al. 2007).

Rats and mice fed a fructose rich diet have been found to be good models for the metabolic syndrome (Takahashi et al. 2012). Liver damage is also found in these models. Wistar rats on a high fructose diet (70%) develop liver steatosis and inflammation (Kawasaki et al. 2009).

However, the distribution pattern of steatosis in the liver is different from that in human NAFLD. While in human NAFLD steatosis is mostly present in zone 3, the steatosis in Wistar rats on a high fructose diet is predominant in zone 1 (Takahashi et al. 2012). Interestingly, inflammation does follow the same pattern as in human NASH: predominantly lobular and not periportal (Kawasaki et al. 2009). Like with the use of a high fat diet, the extent of liver damage developed on a high fructose diet is also dependent on the type and strain of animals used and the fructose content in the diet.

Transgenic animal models in studies investigating flavonoids in NAFLD include db/db mice, ob/ob mice, nSREBP-1c transgenic mice, obsese Zucker fa/fa rats and obese diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

In ob/ob mice, a spontaneous mutation in the leptin gene causes leptin deficiency, leading to hyperphagic, inactive, extremely obese and diabetic mice that develop liver steatosis spontaneously (Nagarajan et al. 2012; Takahashi et al. 2012). However, ob/ob mice do not spontaneously develop steatohepatitis. Therefore, a second 'hit' is needed; such as a MCD diet or high fat diet (Nagarajan et al. 2012; Takahashi et al. 2012).

Db/db mice carry a spontaneous mutation in the leptin-receptor gene. These mice have normal or increased levels of leptin, but are resistant to its effects, which leads to obesity and insulin resistance (Nagarajan et al. 2012; Takahashi et al. 2012). Db/db mice also develop steatosis, but require a second 'hit' for progression to steatohepatitis, such as the MCD diet or high fat diet (Nagarajan et al. 2012; Takahashi et al. 2012). The advantage of both the ob/ob and db/db mice models is that they develop NAFLD in conditions resembling the metabolic syndrome. A

disadvantage is that they need a second hit for progression to steatohepatitis. Db/db mice fed a MCD diet, were found to have higher serum ALT levels and more severe hepatic inflammation and fibrosis than ob/ob mice fed the MCD diet (Takahashi et al. 2012).

Obese Zucker fa/fa rats also have a mutation in the leptin receptor gene leading to leptin resistance. Until four weeks of age, these rats only display increased appetite (Nanji 2004). At four to five weeks of age, the fat mass and the serum level of free fatty acids increase and triglycerides accumulate in various organs, including the liver (Nanji 2004). Hyperinsulinemia also develops, eventually leading to diabetes. Development of steatohepatitis in Zucker fa/fa rats is described after feeding a high fat high cholesterol diet (Matsunami et al. 2010).

In SREBP-1c transgenic mice, SREBP-1c is overexpressed, causing congenital lipodystrophy and severe insulin resistance. At the age of 1 week, liver steatosis is found, which progresses to steatohepatitis within 20 weeks of age without the requirement of a second hit (Takahashi et al. 2012). A disadvantage of this model is that in contrast with human NAFLD/NASH, visceral fat is decreased in this animal model.

The last genetic model used to investigate flavonoids in the treatment of NAFLD is the OLETF rat. This is an established model of the metabolic syndrome, characterized by insulin resistance, abdominal obesity, hypertension and dyslipidemia (Song et al. 2013). Due to a gentic deletion of the cholecystokinin 1 receptor, these rats lack the feeling of satiety. From 8 weeks of age, the OLETF rats develop obesity and hyperinsulinemia. Also liver steatosis develops spontaneously in these rats at 18 weeks of age (Song et al. 2013). However, after 42 weeks of age steatosis declines and inflammation and fibrosis do not develop spontaneously (Song et al. 2013).

From all the studies evaluated, the models using rats/gerbils on a high fat diet (Haddad et al. 2011; Yao et al. 2011; Yao et al. 2013) (table 1), (Yalniz et al. 2007; Ronis et al. 2009; Ji et al. 2011) (table 3), (Kuzu et al. 2008; Xiao et al. 2013) (table 4), (Ying et al. 2013) (table 5) or on a high fat diet combined with high fructose or carbohydrates (Panchal et al. 2011; Panchal et al. 2012) (table 4 and 5), seem to approximate the human conditions of NAFLD best. Also studies using genetic models of the metabolic syndrome combined with a high fat diet or MCD diet are useful (Kim et al. 2012; Salamone et al. 2012; Salamone et al. 2012) (table 1). Studies using the MCD diet only, do not correctly mirror the circumstances of the metabolic syndrome. Studies using mice on a high fat diet, Wistar rats on only a high fat diet, or genetic models of the metabolic syndrome without a special diet or second 'hit', do not seem to provoke sufficient liver damage compared to the human situation and can only be used to evaluate the development of steatosis.

Silymarin and silybin

Silymarin is a flavonoid mix that originates from milk thistle extract. It was already used by doctors and herbalists to treat diverse liver and gallbladder disorders in ancient history (Abenavoli et al. 2012). Nowadays, 65% of the patients with liver diseases take herbal preparations, which are mainly derived from milk thistle (Loguercio et al. 2012). Silymarin contains at least eight different compounds: silybin (A and B), isosilybin (A and B), silichristin, silidianin, dehydrosilybin, taxifolin and others. A small fraction of silymarin consists of polymerized polyphenolics that have not been identified yet (Skottova et al. 2003). About 50-

70% of the silymarin extract consists of the flavonolignan silybin (fig. 7), also known as silibinin, which is extensively studied and is regarded as the most active component of silymarin (Loguercio et al. 2011).

Silymarin flavanolignans have a limited bioavailability that was reported to be 0.45% in human volunteers (Calani et al. 2012). Similarly, the bioavailability of silybin in rats was calculated to be 0.95% (Wu et al. 2009). Extensive phase II metabolism, low permeability across intestinal epithelial cells, low solubility in water and rapid excretion in bile and urine are the major causes of this limited bioavailability (Javed et al. 2011). To improve bioavailibility, derivatives of silybin have been synthesized, such as silybin-phosphatidylcholine (Javed et al. 2011; Loguercio et al. 2011). The silymarin that is not absorbed in the gastrointestinal tract will be subjected to metabolism by bacteria in the colon. In the biological effect of polyphenolic compounds, the effect of colonic metabolites plays a key role (Mateo Anson et al. 2011). However, the contribution of the colonic metabolites to the health effect of silymarin has not been examined.

In human studies daily doses up to 800 mg silymarin appeared to be safe. Only few and minor side effects of silymarin, primarily on the gastrointestinal tract, have been reported (Jacobs et al. 2002; Gazak et al. 2007).

The results of ten identified in vivo studies investigating the use of silymarin/silybin in NAFLD models are summarized in table 1 (Serviddio et al. 2010; Shetty et al. 2010; Haddad et al. 2011; Yao et al. 2011; Kim et al. 2012; Qin et al. 2012; Salamone et al. 2012; Salamone et al. 2012; Grattagliano et al. 2013; Yao et al. 2013). The molecular pathways implicated in the therapeutic effect of silymarin in NAFLD, are elaborated in detail in table 1, clustered in metabolic abnormalities, oxidative stress and inflammation. An improvement of lipid metabolism is demonstrated by a decrease in serum and hepatic lipid values in numerous studies. This is caused by a stimulation of free fatty acid oxidation and positive effects on coordinating factors of lipid metabolism, such as adiponectin and PPARα. Also insulin resistance is reduced in most studies. Antioxidant effects of silymarin represented by a decrease in lipid peroxidation and other oxidative stress markers might be caused by stimulation of endogenous antioxidants such as GSH and SOD. The anti-inflammatory effects of silymarin in NAFLD models include a decrease in the pro-inflammatory cytokine TNF-α and inhibition of NF-κB. Together, the metabolic, antioxidant and anti-inflammatory effects of silymarin lead to a marked improvement of steatosis and liver inflammation in in vivo models of NAFLD (table 1).

In a well-designed, randomized controlled trial (RCT), 138 patients with histologically proven NAFLD were treated with a silybin-phosphatidylcholine complex or placebo for 12 months (table 2) (Loguercio et al. 2012). Treatment showed positive effects on serum ALT, AST and γGT levels, suggesting an improvement of hepatic damage. In addition, positive effects on insulin resistance and body mass index were found. Patients of the treatment group that agreed to

liver biopsy showed significant improvements in steatosis, lobular inflammation, ballooning and fibrosis, while no improvements were seen in the biopsies of patients in the placebo group (Loguercio et al. 2012).

Taken together, the data indicate that silymarin and silybin show inhibitory effects on NAFLD progression. The influence of silymarin/silybin on NAFLD seems to be in line with the multifactorial mode of action of flavonoids; not one single mechanism of action, but multiple mechanisms that reinforce each other, emerge from the studies. The exact value of silymarin in the treatment of NAFLD still has to be established, but results so far are relatively consistent and encouraging.

Soy isoflavones

Unlike most flavonoids, isoflavones are not commonly found in a Western diet (Messina 2010). In fact, soybeans and products derived from soybeans are the only relevant sources of isoflavones (Messina 2010). Isoflavone intake in Western countries does not exceed 1 mg per day, whereas consumption of isoflavones in Japan and China can be as high as 40 mg/day (van Erp-Baart et al. 2003; Messina et al. 2006). The isoflavones in soy are genistein (fig. 7), daidzein (fig. 7) and glycitein. Generally, the content of genistein in soy is larger than the content of daidzein and glycetein (Larkin et al. 2008).

Five to seven hours after intake, genistein and daidzein plasma concentrations reach their maximum (Vitale et al. 2013). Plasma half-lives of genistein and daidzein aglycones are found to be 7 and 9 hours respectively (Larkin et al. 2008). Due to extensive first-pass metabolism, isoflavone bioavailability is low (Larkin et al. 2008). Plasma genistein concentrations around 40 nM are measured in people consuming a Western diet, while concentrations of approximately 4 µM are measured in people consuming a traditional Japanese soybean rich diet (Mann et al. 2009).

Isoflavones are generally regarded as safe (Qin et al. 2013). Most clinical trials do not report any adverse effects. Side effects that have been reported include abdominal bloating, constipation and hot flushes (Qin et al. 2013). Isoflavones have estrogen-like activity in women with low endogenous estrogen levels (Andres et al. 2011). Due to the estrogen-like activity of isoflavones, concerns were raised that isoflavones could stimulate breast cancer development in postmenopausal women. This is an issue of major concern; however, the limited studies in humans that addressed this subject did not corroborate this serious side effect (Andres et al. 2011).

Thirteen in vivo studies investigating the use of soy isoflavones in animal models of NAFLD were found (Ae Park et al. 2006; Gudbrandsen et al. 2006; Lee et al. 2006; Davis et al. 2007; Ustundag et al. 2007; Yalniz et al. 2007; Gudbrandsen et al. 2009; Mohamed Salih et al. 2009; Ronis et al. 2009; Kim et al. 2010; Crespillo et al. 2011; Ji et al. 2011; Kim et al. 2011). The

various effects of soy isoflavones on metabolic abnormalities, oxidative stress and inflammation are shown in detail in table 3. Improvement of lipid metabolism, evidenced by a decrease in hepatic and serum lipid values, has been attributed to stimulation of free fatty acid oxidation, inhibition of lipogenesis and interaction of soy isoflavones with coordinating factors of lipid metabolism, such as adiponectin, leptin, PPARα, PPARγ and others (table 3). Protection against oxidative stress is demonstrated by a decrease in lipid peroxidation and protein carbonyl levels. The mechanism proposed for this protection is stimulation of endogenous antioxidant levels. Inflammation is mitigated by inhibition of NF-κB activation and reduced production of pro-inflammatory cytokines (table 3). All these subtle effects combined can lead to a decrease in steatosis. The few studies that have investigated histological signs of inflammation corroborate the anti-inflammatory effect of isoflavones.

Since isoflavones inhibit many of the pathogenic mechanisms, they are promising compounds for the treatment of NAFLD. Although several animal studies have found positive effects of the use of soy isoflavones in the treatment of NAFLD, no clinical trials have been conducted on the use of soy isoflavones in NAFLD patients.

Green tea flavonoids

Green tea, similar to oolong tea and black tea, is derived from the plant Camellia Sinensis (Masterjohn et al. 2012). The main flavonoids in green tea are the catechins (30-42% of solid

weight). They comprise epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin-gallate (EGCG) (Paquay et al. 2000; Masterjohn et al. 2012). The highest percentage of catechins in green tea consists of EGCG (50-75%) (fig. 7). Other flavonoids that can be found in small amounts in green tea are quercetin and myricitin (Masterjohn et al. 2012).

One to three hours after ingestion of green tea, maximal catechin plasma concentrations (0.1-4.4 µM) are reached (Masterjohn et al. 2012). Plasma half-lives of green tea catechins range from 1.5 to 5.7 hours (Masterjohn et al. 2012). Similar values were obtained in studies investigating pure EGCG (Manach et al. 2005). Because of the rapid elimination of catechins from the body, intake of tea catechins might be beneficial only when they are consumed several times a day (Masterjohn et al. 2012).

In some studies, concerns were raised regarding the possible hepatoxicity of green tea extract (GTE) (Sarma et al. 2008). Nevertheless, a review of several clinical trials showed that the use of GTE/EGCG up to doses of 800 mg/kg/day is safe and well tolerated in humans (Sarma et al. 2008). The only side effects that were reported were mild headache and fatigue. In a recent clinical trial, daily consumption of 714 mg GTE/day for 3 weeks did not lead to liver toxicity in healthy males (Frank et al. 2009).

Ten studies investigating the use of GTE or EGCG in animal models of NAFLD were found (Bose et al. 2008; Bruno et al. 2008; Kuzu et al. 2008; Nakamoto et al. 2009; Ueno et al. 2009; Chen et al. 2011; Park et al. 2011; Chung et al. 2012; Park et al. 2012; Xiao et al. 2013). The effects of GTE/EGCG on metabolic abnormalities, oxidative stress and inflammation are presented in detail in table 4. Improvement of lipid metabolism, represented by a decrease in serum and hepatic lipid levels, might be caused by interaction of green tea catechins with coordinating factors of lipid metabolism or a decrease in lipogenesis (table 4). Stimulation of antioxidants and inhibition of ROS production by green tea catechins leads to an attenuation of oxidative stress, demonstrated by a decrease in lipid peroxidation (table 4). Also inflammation is reduced by green tea catechins by inhibition of NF-κB activity and a decrease in pro-inflammatory cytokines (table 4). In the majority of studies these effects led to a decrease in steatosis. Only one study did not find a reduction of steatosis (Nakamoto et al. 2009). Histological inflammation was only investigated in two studies (Chung et al. 2012; Xiao et al. 2013). Both studies found a diminution of inflammation.

Unfortunately, no studies investigating GTE/EGCG in NAFLD patients were found. In addition, most of the investigated animal studies focused on steatosis only. Although in general promising effects of GTE/EGCG on steatosis are reported, the effects of GTE/EGCG on inflammation in NAFLD models are not conclusive. Clinical trials are lacking to substantiate the therapeutic potential of green tea catechins in NAFLD. Furthermore, the rapid elimination from the body seriously questions the prospect of the use of green tea in NAFLD treatment.

Quercetin

Quercetin (fig. 7) is a flavonol that is one of the most abundant flavonoids in the human diet. It is found in various fruits and vegetables, such as onions, apples and tomatoes. The average intake of quercetin is estimated to be 5-40 mg/day (Hertog et al. 1995). In the diet quercetin is often bound by sugars (quercetin glycosides).

Quercetin has a relatively high bioavailability compared to other flavonoids (Russo et al. 2012). The bioavailability depends on the type of quercetin glycoside. It has been reported that glucosides of quercetin (quercetin bound by glucose) are absorbed in the gastro-intestinal tract better than the aglycon (Hollman et al. 1995; Graefe et al. 2001). Fifty-two percent of quercetin glucosides from onions are absorbed in the gastro-intestinal tract, versus 24% of the quercetin aglycone (Hollman et al. 1995). Quercetin and its metabolites have long plasma half-lives, i.e. 11-28h (Manach et al. 2005). Therefore, plasma concentrations can increase significantly upon frequent intake. Quercetin is normally found in the human plasma in low nanomolar concentrations, but upon supplementation, this can increase to high nanomolar or low micromolar concentrations (Hollman et al. 1996; Conquer et al. 1998).

No adverse effects of quercetin were reported in studies investigating oral intake of quercetin or quercetin glucosides in doses of 3-1000 mg/day for periods up to 12 weeks (Harwood et al.

2007). Also for intravenous doses of ±10.8 mg/kg body weight no adverse effects were reported. For higher intravenous doses up to 51.3 mg/kg body weight, pronounced pain at the injection site, dyspnea, emesis and transient nephrotoxicity were reported (Harwood et al. 2007). Clinical symptoms lasted for a period of time after each injection (Harwood et al. 2007).

Although in vitro toxicity studies have reported mutagenic effects of quercetin and two in vivo animal studies reported quercetin carcinogenicity, many other animal studies fail to show increased tumor incidence related to quercetin administration (Harwood et al. 2007). Quercetin toxicity is assumed to be related to the formation of the quercetin-quinone, which is produced when quercetin is oxidized by radicals (Boots et al. 2007; Boots et al. 2008). The quercetin-quinone binds to thiols and causes toxic effects like increased membrane permeability and altered function of enzymes with a critical sulfhydryl –group (Boots et al. 2007; Boots et al. 2008).

Six animal studies investigating the use of quercetin in animal models of NAFLD were identified (Kobori et al. 2011; Marcolin et al. 2012; Panchal et al. 2012; Jung et al. 2013; Marcolin et al. 2013; Ying et al. 2013). Effects of quercetin on lipid metabolism, oxidative stress and inflammation are summarized in table 5. Quercetin improves lipid metabolism by affecting coordinating factors of lipid metabolism, such as PPARα, PPARγ and adiponectin. Inhibition of lipogenesis and stimulation of fatty acid oxidation by quercetin also contribute to the improvement of lipid metabolism (table 5). Reduction of oxidative stress, demonstrated by a

decrease in lipid peroxidation, is caused by a potent direct antioxidant effect, induction of endogenous antioxidant defences and inhibition of iNOS expression in the liver (table 5). In only two studies inflammatory markers, such as TNF-α and Il-6, were investigated and found to be decreased by quercetin (table 5). In all studies the quercetin administration led to a decrease in steatosis. In general, histological signs of liver inflammation were also reduced. Two studies that investigated fibrosis, found a reduction in fibrosis as well.

In the in vivo studies mainly positive effects of quercetin on NAFLD were demonstrated. A randomized clinical trial on the use of quercetin in NAFLD patients is lacking, whereas various clinical trials have investigated the use of quercetin in other diseases (Valensi et al. 2005; Edwards et al. 2007; Egert et al. 2009; Heinz et al. 2010; Boots et al. 2011). To substantiate the therapeutic effect of quercetin in NAFLD, clinical trials are mandatory. A concern for approval of such studies will be the potential carcinogenic effect of quercetin, though the relatively high intake of quercetin in the normal diet as well as the widely applied supplementation of quercetin have not pinpointed this as a problem. In fact, epidemiological studies reveal the opposite: flavonoid intake, which for the substantial part is quercetin, has an inverse relationship with the incidence of cancer.

Rutin

Rutin is quercetin with the disaccharide rutinose covalently bound to the 3-OH group (quercetin-3-O-β-rutinoside) (fig. 7). It is abundantly found in plants such as buckwheat (Fagopyrum Esculentum) and citrus fruits such as oranges (Citrus Sinensis) and grapefruits (Citrus Paradisi) (Sharma et al. 2013). The uptake of rutin in the gastro-intestinal tract is less than that of quercetin and quercetin mono-glucosides and lower peak plasma concentrations of rutin are reached after intake of an equivalent dose compared to that of quercetin (Hollman et al. 1995; Graefe et al. 2001). The main reason for rutins poor bioavailability is its poor solubility in aqueous media (Sharma et al. 2013) and the resulting low bioaccessibility. However, rutin also has advantages over other flavonoids. It is relatively stable and does not display prominent pro-oxidant activity. While some flavonoids are labeled as mutagenic and relatively cytotoxic, rutin is neither (Sharma et al. 2013).

Four studies examining the use of rutin in animal models of NAFLD were found (Hsu et al. 2009; Ziaee et al. 2009; Panchal et al. 2011; Gao et al. 2013). The effects of rutin on metabolism, oxidative stress and inflammation are presented in table 6. Hepatic and/or serum lipid values were decreased in all studies, demonstrating an improvement in lipid metabolism. The mechanism of action was not investigated extensively in the studies. Inhibition of leptin and SREBP-1c was suggested to play a role (table 6). Only two studies examined the effects of rutin on oxidative stress. Both studies concluded that rutin administration reduced oxidative stress and implied as mechanism stimulation of endogenous antioxidant levels and inhibition of the production of ROS (table 6). One study reported that rutin administration reduced inflammatory

markers, such as TNF-α (table 6) (Gao et al. 2013). The combined effects of rutin on metabolism, oxidative stress and inflammation, can explain the decrease in steatosis found in all studies. Liver inflammation was investigated in two studies and found to be reduced in both studies (Ziaee et al. 2009; Panchal et al. 2011). The only study that examined the effect of rutin on liver fibrosis, did find a reduction in fibrosis (Panchal et al. 2011).

Rutin is not extensively investigated in animal models of NAFLD. Also no clinical trials have been performed. The promising results for the use of rutin against NAFLD summarized in table 6 show the potential of rutin and indicate that further studies are warranted. A disadvantage of rutin is its poor bioavailability. Semisynthetic analogues of rutin with increased bioavailability might be more promising. In MonoHER, a hydroxyethyl group is attached to the oxygen to the 7-OH group, which increases the water solubility. MonoHER is proven to be a very potent antioxidant (Haenen et al. 1993; van Acker et al. 1995; Haenen et al. 1997; van Acker et al. 2000) with anti-inflammatory characteristics (Abou El Hassan et al. 2003).

Other flavonoids

A wide spectrum of flavonoids has been investigated in animal models of NAFLD. However, the number of studies on many flavonoids is limited to one or two studies. These flavonoids are presented in table 7.

Naringenin, a flavanone found in grapefruit, completely reverses steatosis in LDLr$^{-/-}$ mice fed a high fat diet. It also reduces dyslipidemia, hyperglycemia, hyperinsulinemia and body weight (Mulvihill et al. 2009; Mulvihill et al. 2010).

Cyanidin 3-O-β-D-glucoside, an anthocyanin found in various plants and fruits that gives them a purple color, reduces hepatic steatosis, oxidative stress and inflammation in diabetic db/db mice (Guo et al. 2012; Zhu et al. 2012). Furthermore, it reduces hyperglycemia and insulin resistance in db/db mice as well as C57Bl/6J mice fed a high fat diet (Guo et al. 2012). Cyanidin 3-O-β-D-glucoside also decreases body weight and hepatic lipid content in C57Bl/6J mice (Tsuda et al. 2003).

Xanthohumol, a chalcone from the hop plant (Humulus Lupulus), decreases steatosis, inflammation and fibrosis in murine models of NAFLD (Dorn et al. 2010; Doddapattar et al. 2013). Also the other flavonoids noted in table 7 were found to have positive effects on NAFLD and other factors contributing to NAFLD, such as dyslipidemia, body weight and insulin resistance (Guo et al. 2009; Zheng et al. 2009; Lee et al. 2012; Mei et al. 2012; Lee et al. 2013).

Since the number of different flavonoids known is huge, up to more than 5,000 different chemical entities, it is mandatory to make a selection in order to keep clinical research feasible form a practical point of view. Although the miscellaneous flavonoids mentioned in this

paragraph might have merit in the treatment of NAFLD, their biochemical profile is not that different form the more extensively investigated flavonoids. Therefore, we focus on the latter group of more extensively studied flavonoids in this review.

Summary and perspective

The contemporary pathophysiological model of NAFLD consists of multiple parallel pathways with a dynamic cross talk that cumulate in steatosis and inflammation and ultimately fibrosis, cirrhosis, liver failure and hepatocellular carcinoma. The multitude of pathways involved in the pathogenesis underpins the need for treatments that address these various pathways.

Flavonoids are compounds derived from plants with subtle effects on multiple targets that finally accumulate in a substantial health benefit. Interestingly, flavonoids have been found to have positive effects on lipid metabolism, insulin resistance, inflammation and oxidative stress, the most important pathological processes in the etiology of NAFLD. This puts flavonoids in the spotlight for the treatment of NAFLD. In this review the existing evidence for the use of flavonoids in the treatment of NAFLD is evaluated.

Flavonoids and flavonoid mixtures that have been widely investigated in animal models of NAFLD include silymarin, silybin, soy isoflavones, green tea flavonoids, quercetin and rutin. The protective biochemical profile of these flavonoids on lipid metabolism, insulin resistance,

oxidative stress and inflammation as well as their beneficial therapeutic effect on steatosis and liver inflammation in most of the studies form the scientific fundament for the use of flavonoids in the treatment of NAFLD. However, further clinical studies are needed to examine the exact value of flavonoids in the treatment of NAFLD patients.

Further studies examining the use of flavonoids in NAFLD should include double-blinded randomized clinical trials. In designing and interpreting clinical studies, it is of importance to carefully consider the heterogeneity of the NAFLD patient group. A caveat is that the high heterogeneity will negatively affect the power of the study. Moreover, NAFLD patients with a very different biochemical profile might benefit form flavonoids that display a biochemical profile that fits these different profiles the best. This means that it is unlikely that a uniform treatment for all types of NAFLD will be found. Personalized treatment with close monitoring of the therapeutic effect seems warranted to come to optimal treatment. To reach this stage, the most promising flavonoids should be tested first. The spectrum of flavonoids includes over 5,000 different compounds with their own unique profile, illustrating that it is impossible to study all the flavonoids. Therefore, the compounds best suitable for further investigation have to be identified. In identifying the most promising compound in a large series, structure-activity relationships are mandatory, although these relationships should always be critically evaluated (Haenen et al. 2006). Criteria that can be used to evaluate the therapeutic potential and to form a rational basis for selection of flavonoids for further investigation are their molecular mechanism of action and clinical evidence, bioavailability and safety.

Regarding their molecular mechanism of action, none of the flavonoids seems to protrude from the animal studies. All flavonoids have been found to improve lipid metabolism, insulin resistance, oxidative stress and inflammatory markers. However, in structure activity relationship studies, quercetin, rutin and its derivates are found to belong to the most potent antioxidants (Heijnen et al. 2002). Most clinical evidence is found for silymarin and silybin because these compounds are widely investigated in animal models of NAFLD and silybin was also examined in a randomized clinical trial.

Regarding bioavailability, quercetin has the best characteristics. Bioavailability of rutin is slightly lower, but can be improved by the use of semi-synthetic derivates, such as MonoHER, which is more water-soluble. Bioavailability of silybin and silymarin is considerably lower and can be improved by conjugation to polar and hydrophilic moieties, e.g. as in silybin-phosphatidylcholine. Due to the rapid elimination from the body and consequent necessity of frequent administration, green tea flavonoids are not likely to get a place in NAFLD treatment.

Although quercetin appears to possess superior antioxidant potential and bioavailability, quercetins safety is still debated due to its potential carcinogenicity. Rutin, which seems to be devoid of carcinogenic properties but displays similar antioxidant potential and only slightly lower bioavailability as quercetin, seems to be a better option. Rutin derivates, developed to

increase bioavailability, seem to be even more appealing compounds for further investigation. For example MonoHER, rutin with a hydroxyethyl group attached to the oxygen on the 7-position, has shown to be a very potent antioxidant (Lemmens et al. ; Haenen et al. 1993).

In conclusion, with their multifaceted actions flavonoids seem to suit perfectly in the pathophysiological model of NAFLD. The heterogeneity of the disease should be carefully considered in the design of clinical studies investigating a treatment for NAFLD. Already, multiple in vivo studies show encouraging results for the use of flavonoids in the treatment of NAFLD, which calls for additional research. Silybin has the advantage that it is the most studied flavonoid. Nevertheless, rutin and its derivatives, such as MonoHER, seem to be the most appealing flavonoids for further investigation due to their high antioxidant potential, bioavailability and safety.

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Tables

Table 1.

Study	Species (n/group)	Model	Intervention Dose (mg/kg/day) or (% of diet)	Effects Silybin/Silymarin																Conclusion Effect
				Lipid Metabolism				Oxidative Stress			Inflammation		Other	ALT	Histology			Weight	IR	
				Lipids liver/serum	Coordinating factors	FA markers	Lipogenesis	Antioxidants	O.S. markers	Lipid Peroxidation	Anti	Pro			Steatosis	Inflammation	Fibrosis			
Intraperitoneal administration																				
Salamone et al. 2012	**Mice** (n=8)	Db/db mice + MCD diet	**20 Sb** dihydrogen succinate 4 wks		↑[1]	↑[2,3]			↓[1,2,3]	↓			↓[1]	↓	↓	↓		↔[bw]	↓	+++
Salamone et al. 2012 (2)	**Mice** (n=6)	Db/db mice + MCD diet	**20 Sb** dihydrogen succinate 4 wks	↓[4]				↑[4]	↓[5,6]	↓	↑[1]	↓[2]	↑[2]↓[3]	↓	↓	↓		↔[bw]	↓	+++
Intragastrical administration																				
Yao et al. 2013	**Rats** (n=15)	HFD	**26.25 Sb** 6 wks	↓[5,6]									↓[4,5,6]	↓	↓	↓		↓[bw] ↓[vf] ↓[vf/bw] ↔[scf]	↓	+++
Yao	**Rats**	HFD	**26.25 Sb**	↓[5]	↑[8]↓[9]			↑[4]		↓			↓[7]	↓	↓	↓			↓	+++

64

Study	Animal (n)	Diet	Dose / Duration															
et al. 2011	(n=15)		6 wks	↔[6,7]				↑[7]										
Qin et al. 2012 *	**Rats** (n=13)	HFD + alcohol i.g.	**23 Sm** 10 wks	↓[7,10-12] ↑[13] ↔[4-6]	↑[14]				↓			↓[8] ↔[9]	↓			↔bw ↓lw/bw		+
Oral administration																		
Shetty et al. 2010 *	**Rats** (n=6)	HFD 6 wks	**50 Sm** last 4 wks of diet	↓[5,6,15] ↔[13]									↓					+
Grattagliano et al. 2013	**Rats** (n=5)	CD diet	**47 mg Sb/day** 7/14/30 days					↑[4,12]	↓				↓	↓		↔bw ↔lw		+
		HFD	**47 mg Sb/day** 14/30/60 days					↔[4,12]	↓				↓	↓		↔bw ↓lw		+
Dietary intervention																		
Haddad et al. 2011	**Rats** (n=6-8)	HFD 12 wks	**0.02% Sb-phosphatidylcholine** last 5 wks of diet	↓[7] ↓[13] ↔[5]	↔[14]			↓[4$]	↓[8]	↓		↓[2§]	↓[10,11] ↔[9,12]	↔	↓	↓	↔bw ↓lw ↓lw/bw	↓ ↓	++
Serviddio et al. 2010	**Rats** (n=5)	HF MCD diet	**0.04% Sb-phospholipid**					↔[4]	↓[9-11]	↓			↓[13] ↑[14,15]	↓	↓				+

| Kim et al. 2012 | Rats (n=10) | OLEFTs + MCD diet | complex 7/14 wks 0.5% Sm 8 wks | | | | ↔[16] | | | | ↓[2] | ↑[16] ↓[17-19] | ↔ ↓ | | ↔ bw ↔ lw ↔ lw/bw | | + |

Effects of silymarin/silybin are observed in the treatment group compared to the model group. Green = inhibiting effect on the development of NAFLD, blue = unknown or no effect on development of NAFLD, orange = stimulating effect on development NAFLD. $ = theoretically a stimulating effect on the development/progression of NAFLD, however the effect is opposite from the effect induced by the NAFLD model. *= silymarin used as control group for comparison other compounds, therefore limited description of effects silymarin.

Specific markers related to lipid metabolism are categorized in four categories: lipids in liver or serum, general coordinating factors of lipid metabolism, markers involved in fatty acids oxidation, and markers involved in lipogenesis. Markers related to oxidative stress are divided in antioxidants, oxidative stress markers (O.S. markers) and lipid peroxidation. Specific markers related to inflammation are categorized in 2 categories: pro = pro-inflammatory factors, anti = anti-inflammatory factors. ALT = alanine aminotransferase levels serum. Histology is divided in steatosis, inflammation and fibrosis. IR = insulin resistance. Markers of inflammation and oxidative stress are measured in the liver, unless mentioned by § = measured in serum.

Abbreviations: MCD diet = methionine choline deficient diet, CD diet = choline deficient diet, HFD = high fat diet, HF MCD diet = high fat MCD diet, bw = body weight, vf = visceral fat weight, bw/vf = body weight/visceral fat weight-ratio, scf =

subcutaneous fat weight, lw = liver weight, lw/bw = liver weight/body weight-ratio, OLEFTs= Otsuka Long-Evans Tokushima Fatty rats with type 2 diabetes.

Markers:

Lipid metabolism: 1 = SCD-1 (g.ex + act), 2= Acyl-CoA oxidase (g.ex + act), 3 = L-FABP (g.ex), 4 = TG liver, 5 = TG serum, 6 = TC serum, 7 = LDL-C serum, 8 = Adiponectin (p.ex + g.ex), 9= Resistin (p.ex + g.ex), 10 = FFA serum, 11= FFA liver, 12 = TC liver, 13= HDL-C serum , 14 = PPARα (p.ex + g.ex), 15 = total lipid content liver, 16 = SREBP-1c (g.ex)

Oxidative stress: 1 = ROS, 2 = iNOS (p.ex), 3 = protein nitration, 4 = GSH, 5 = DNA-damage, 6 = liver nitrites/nitrates, 7 = SOD, 8= superoxide, 9 = GSSG, 10 = GSSG/GSH-ratio, 11 = H_2O_2 production , 12 = GPx activity

Inflammation: 1 = Il-6, 2 = tnf-α

Other: 1 = NF-κB binding activity, 2 = MRC-complexes activity, 3 = phosphorylated JNK (p.ex), 4 = FoxO1 (g.ex), 5 = PEPCK (g.ex), 6 = G-6-Pase (g.ex), 7 = mitochondrial microviscosity, 8 = hepatocyte apoptosis, 9=cyp2e1 (p.ex), 10 = LDH serum, 11 = serum insulin, 12 = serum glucose, 13 = mitochondrial proton leakage, 14 = mitochondrial respiration, 15 = ATP liver, 16 = nuclear nrf2 (p.ex), 17 = α1-procollagen (g.ex), 18 = α-SMA (g.ex), 19 = pERK1/2 in HSCs

Table 2.

Study	Model	Intervention	Effects of Silymarin found on							EffectConcl
			Histology			Weight	IR	Liver enzymes	Other	
			S	I	F					
Oral administration										
Loguercio et al. 2012	Double-blind RCT: ▪ Placebo (n=69) ▪ Realsil. (n=69) All patients received recommendations for life style modifications and an individually designed diet.	Realsil = 94 mg silybin complexed with 194 mg phosphatidylcholine + 89.28 mg vitamin E acetate, 2x/day p.o	↓#	↓#	↓#	Normalisation BMI*	↓	Normalisation ALT, AST, γGT*	↓Blood glucose + insulin	++

Effects of Realsil (silybin-phosphatidylcholine complex with vitamin E) in a randomized controlled clinical trial. *= Higher

percentage of patients with normalization of ALT, AST, γGT and BMI than in placebo group. #= Only 35 patients of the study

agreed to liver biopsy at the end of the study. In the silymarin group there was an improvement in liver biopsy, while in the

placebo group there was no change.

68

Table 3.

Study	Species (n/group)	Model	Intervention Dose (mg/kg/day) or (% of diet)	Lipids liver/serum	Coordinating factors	Markers FA oxidation Markers	Lipogenesis	Antioxidants	O.S. markers	Lipid Peroxidation	Anti	Pro	Other	ALT	Steatosis	Inflammt	Fibrosis	Weight	IR	Conclusion Effect
Subcutaneous administration																				
Yalniz et al. 2007	Rats (n=12)	HFD	**0.2 G** 6 wks	↓1 ↔2						↓↓§	↔3§	↓1§	↔1*	↓	↓	↓	↔**	↔bw*, lw*, bw/lw		+
Intraperitoneal administration																				
Crespillo et al. 2011	Rats (n=8)	HFD 12 wks	**50 D** Last 2 wks of diet	↔1*, 2*,9* ↓11	↓3,17$, 18$, 20# ↑4↓24 ↔16, 16#, 18# 23, 23# ↓21#	↔27 ↓27#$	↔29 ↑30$						↑1* ↔2*	↔*	↓			↓bw		+/-
Intragastrical administration																				
Ji et al. 2011	Rats (n=9)	HFD	**4/8 G** 12 wks							↓↓§	↔3 ↔3§	↓1, 2 ↓1, 2§	↓3-5↑6 ↔7, 8	↓		↓				+
Oral administration																				
Salih	Rats	HFrD	**1 G**	↓1,2,5-				↑1,2,5	↓3, 4,	↓↓§		↓1,2§	↓1, 2, 9	↓	↓			↓bw↓1	↓	++

et al. 2009	(n=12)		8.6 wks	8,12-14 ↑9 ↑15				-7 ↑5-7§	8		↑10-12			w↓1w/bw		

Dietary intervention

Kim et al. 2010	Mice (n=18)	HFD	0.1/0.2/0.4% G 12 wks	↓1,2,5,7,11-13 ↑9	↑16#, 20#, 31# ↓18#$ ↓21#	↑32#	↓25#, 33#, 35-37#			↓1#		↓	↓	↓bw ↓vfw		+
Lee et al. 2006	Mice (n=15)	HFD	0.1/0.2/0.4% G 12 wks	↓1, 2, 12, 13	↔16, 18, 22 ↑38	↔39 ↑40		↑7 ↔9-11	↔				↓	↓bw↓1w↓efw		+
Park et al. 2006	Mice (n=10)	Db/db mice	0.2% G/D 6 wks	↓1, 2, 5, 12 ↑9 ↔13	↔4	↓42, 43	↑41$ ↓30				↓1, 15, 16, 20, 21, ↔2 ↑13↑14			↔b w	↓	+/-
Kim et al. 2011	Mice (n=18)	HFD	0.01/0.05/0.1/0.2% D 12 wks	↓1,2, 10-13	↑4,49# ↓3 ↑18, 50#, 31# ↓46↓47 ↔16,18#	↑51#,52#	↑33 ↔29 ↓48	↑12 ↑13		↓1 ↓1#	↓1,2,18 ↑17	↓	↓	↓bw ↓efw ↔vfw	↓	+/-
Gudbrandsen et al. 2006	Rats (n=6)	Obese Zucker fa/fa rats	(0.045% D + 0.04% G) / (0.45% D + 0.4% G) † 6 wks	↑1, 5 ↓12, 54 ↓55	↓18, 22, 56 ↑57, 60 ↓58, 59 ↔61	↑53, 42, 44, 28, ↔45	↑26, 30, 33					↓	↓			+/-
Gudb	Rats	Obese	0.45% D	↓12					↓6	↓1	↓22↓23			↓bw		+

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Study	n	Model	Dose																
rands en et al. 2009	(n=6)	Zucker fa/fa rats	+ 0.4% G) 6 wks						↔[7]	↓[1,4,5§]									
Ustu ndag et al. 2007	Rats (n=7)	MCD diet	0.002% G + 0.005% D + 0.003% Gl 8 weeks	↓[1,2] ↑[9] ↔[7] ↑[8]			↑[14]	↓↓[§]				↓	↓	↓	↓			++
Ronis et al. 2009 **	Rats (n=7-10)	HFD	0.03% G + 0.02% D 5.7 wks	↓[2,12] ↔[1,13]	↓[23]	↔[33] ↓[25,29,34]					↓[1] ↔[2]	↓				↓[bw] ↓[fw] ↓[lw/bw]	↓	++
Davis et al. 2007	Rats (n=10)	Obese Zucker fa/fa rats	0.1% SI 11 wks	↓[1,2,12]	↑[19#]	↓[30,30#,62#] ↔[62]					↓[1] ↑[19\] ↔[2]					↔[bw,fw] ↓[lw]		+

Effects of soy isoflavones are observed in the treatment group compared to the model group. Green = inhibiting effect on the development of NAFLD, blue = unknown or no effect on development of NAFLD, orange = stimulating effect on development NAFLD. $ = theoretically a stimulating effect on the development/progression of NAFLD, however the effect is opposite from the effect induced by the NAFLD model. * no change induced by model, ** = only second experiment of study included, # expression in adipose tissue, § = measured in plasma, † = effects shown only found with high dose intervention.

Specific markers related to lipid metabolism are categorized in four categories: lipids in liver or serum, general coordinating factors of lipid metabolism, markers involved in fatty acids oxidation, and markers involved in lipogenesis. Markers related to oxidative stress are divided in antioxidants, oxidative stress markers (O.S. markers) and lipid peroxidation. Specific markers related to inflammation are categorized in 2 categories: pro = pro-inflammatory factors, anti = anti-inflammatory factors. ALT = alanine aminotransferase levels serum. Histology is divided in steatosis, inflammation and fibrosis. IR = insulin resistance. Markers of inflammation and oxidative stress are measured in the liver, unless mentioned by § = measured in serum.

Abbreviations: HFD = high fat diet, MCD diet = methionine choline deficient diet, HFrD = high fructose diet, wks = weeks, D = daidzein, G = genestein, Gl = glycitin, SI = soy isoflavones

Markers:

Lipid metabolism: 1 = TG plasma, 2 = TC plasma, 3 = adiponectin plasma, 4 = leptin plasma, 5 = FFA plasma, 6 = phospholipids plasma, 7 = LDL-C plasma, 8 = VLDL-C plasma, 9 = HDL-C plasma, 10 = total lipids plasma, 11 = total lipid content liver, 12 =TG liver, 13 = TC liver, 14 = FFA liver, 15 = phospholipids liver, 16 = PPARa (g.ex) 17 = PPARa (p.ex),18 = PPARg (g.ex), 19 = PPARg (p.ex,), 20 = adiponectin (g.ex), 21 = leptin (g.ex), 22 = PPARb/d (g.ex), 23 = SCD-1 (g.ex), 24 = SCD-1 (p.ex), 25 = ACC (g.ex), 26 = ACC (p.ex/act), 27 = ACOX (g.ex), 28 = ACOX (p.ex/act), 29 = FAS (g.ex), 30 = FAS (p.ex/act), 31 = AMPK (g.ex), 32 = VLACAD (g.ex), 33 = SREBP-1c (g.ex), 34 = SREBP-1c (p.ex), 35 = LXRα (g.ex), 36 = C/EBPβ (g.ex), 37 = RXR-α (g.ex), 38 = PPARγ coactivator (PGC-1) (g.ex), 39 = peroxisomal acyl-CoA oxidase

(ACOX) (g.ex), 40 = medium chain acyl-CoA dehydrogenase (MCAD), 41 = glucokinase (p.ex/act), 42 = b-oxidation activity liver, 43 = CPT activity liver, 44 = CPTII activity, 45 = CPTI activity, 46 = ghrelin plasma, 47 = AGPAT2 (g.ex), 48 = ChREBP (g.ex), 49 = leptin (g.ex), 50 = adiponectin (g.ex), 51 = ACAD (g.ex), 52 = ACAS (g.ex), 53 = ACAS (act), 54 = ApoB (g.ex), 55 = VLDL-receptor (g.ex), 56 = AADA (g.ex), 57 = elongase (g.ex) 58 = Δ5-desaturase (g.ex), 59 = lipoprotein lipase (g.ex), 60 = GPAT (act), 61 = HMG-CoA synthase activity, 62 = GPDH (p.ex).

Inflammation: 1 = tnf-α, 2 = Il-6, 3 = tgf-β, 4 = Il-1β, 5 = MCP-1, 6 = IL-10, 7 = Il-2

Oxidative stress: 1 = GPx activity, 2 = GR activity, 3 = lipid hydroperoxide, 4 = protein carbonyl level, 5 = vitamin E, 6 = vitamin C, 7 = GSH, 8 = 3-NT immunoreactivity, 9 = SOD level, 10 = GPx level, 11 = GR level, 12 = SOD (g.ex), 13 = GST (g.ex), 14 = PON1,

Other: 1 = plasma glucose, 2 = plasma insulin, 3 = pJNK (p.ex), 4 = Ncl NF-κB p65 (p.ex), 5 = p-IκB-α (p.ex), 6 = IκB-α (p.ex), 7 = pERK (p.ex), 8 = p-p38MAPK (p.ex), 9 = LDH activity liver, 10 = glyoxalase I activity liver, 11 = glyoxalase II activity liver, 12 = arginase activity liver, 13 = glycogen liver, 14 = glucokinase activity liver, 15 = G6Pase activity liver, 16 = PEPCK activity liver, 17 = lipids feces, 18 = JNK1 (g.ex) liver, 19 = GLUT4 (p.ex) adipose tissue, 20 = glucagon (plasma), 21 = HbA$_{1c}$ plasma, 22 = food intake, 23 = UCP-2 (g.ex)

Table 4.

Study	Species (n/group)	Model	Intervention Dose (mg/kg/day) or (% of diet)	Lipid Metabolism				Oxidative Stress			Inflammation		Other	ALT	Histology			Weight	IR	Conclusion Effect
				Lipids liver/serum	Coordinating factors Markers	FA Markers	Lipogenesis	Antioxidants	O.S. Markers	Lipid peroxidation	Anti	Pro			Steatosis	Inflammt	Fibrosis			
Intraperitoneal administration																				
Xiao et al. 2013	Rats (n=6)	HFD	**50 EGCG (3x/wk) 8 wks**					↔[3,4,5] ↑	↓[1,2]			↓[1-3]	↓[1-9,12] ↑[10,11]	↓	↓	↓	↓	↓bw		++
Oral administration																				
Nakamoto et al. 2009	Rats (n=6)	CD HFD (4 wks) + nitrite i.p (6 wks)	**100/300 FGTE 6 wks**	↓[1]				↑[6§]	↓[7]	↓	↓[4-7§]			↓	↔		↓	↔bw ↑lw ↑lw/bw		+/-
Dietary intervention																				
Chen et al. 2011	Mice (n=21)	HFWD	**0.32% EGCG 17 wks**	↓[1,2]									↓[13,14] ↑[15]	↓	↓			↓bw ↓vf w ↓lw/bw	↓	+
Bose et al. 2008	Mice (n=11-22)	HFD	**0.32% EGCG 16 wks**	↓[1,2]							↓[6§]		↓[13,14]	↓	↓			↓bw ↓vf w ↓lw/bw	↓	+

Study	Animal	Model	Treatment	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Kuzu et al. 2008	Rats (n=7)	HFD	0.1% EGCG in drnk water 6 wks	↔[2] ↓[3]				↑[8]		↓		↓[4,13,14,16]	↓	↓		↔[bw] ↓[lw]	↓	+
Ueno et al. 2009	Mice (n=6)	nSREBP-1c transgenic mice	0.05/0,1% ECGG + 0.005% ascorbic acid in drnk water 12 wks	↓[2-4] ↔[5]								↔[13] ↓[11] ↑[17,18]	↓	↓		↔[bw] ↓[lw/bw]		+
Bruno et al. 2008	Mice (n=8)	Ob/ob mice	1/2% GTE 6 wks	↓[1-3, 6] ↔[7]	↔[8]			↓[9] ↔[10]						↓		↓[bw] ↓[fw]		+
Park et al 2011	Mice (n=12)	Ob/ob mice	0.5/1.0% GTE 6 wks	↓[1, 2, 6, 7, 9]	↔[12] ↓[12, 13#]		↔[10,11] ↓[10,11#]	↑[8, 13-20] ↔[21]		↓	↓[2]			↓		↓[bw] ↓[lw] ↓[fw]		+
Chung et al. 2012	Mice (n=12)	Ob/ob mice	0.5/1.0% GTE 6 wks						↓[1,2, 11,12] ↓		↓[10]			↓	↓	↓	↓[bw] ↓[lw]	+
Park et al. 2012	Rats (n=15-16)	HFD	1/2% GTE 8 wks	↓[1, 6]	↓[14]			↑[8]			↓[2, 3, 6, 11]			↓	↓	↓[bw] ↓[fw]		+

Effects of green tea extract/epigallocatechin-3-gallate are observed in the treatment group compared to the model group. Green = inhibiting effect on the development of NAFLD, blue = unknown or no effect on development of NAFLD, orange = stimulating effect on development NAFLD.

Specific markers related to lipid metabolism are categorized in four categories: lipids in liver or serum, general coordinating factors of lipid metabolism, markers involved in fatty acids oxidation, and markers involved in lipogenesis. Markers related to lipid metabolism are measured in liver or serum unless mentioned by # = measured in adipose tissue. Markers related to oxidative stress are divided in antioxidants, oxidative stress markers (O.S. markers) and lipid peroxidation. Specific markers related to inflammation are categorized in 2 categories: pro = pro-inflammatory factors, anti = anti-inflammatory factors. ALT = alanine aminotransferase levels serum. Histology is divided in steatosis, inflammation and fibrosis. IR = insulin resistance. Markers of inflammation and oxidative stress are measured in the liver, unless mentioned by § = measured in serum.

Abbreviations: HFD = high fat diet, CD HFD = choline deficient high fat diet, FGTE = fermented green tea extract (10.2% EGC, 6.9% GC, 1.1% ECG, 0.6% EGCG), GTE = green tea extract GTE: 30% total catechins (48% EGCG, 31% EGC, 13% ECG, 8% EC), HFWD = high fat western diet, drnk water = drinking water, bw = body weight, lw = liver weight, lw/bw = liver/body weight-ratio, vfw = visceral fat weight, BAT = brown adipose tissue, ob/ob mice = leptin deficient obese mice.

Markers:

76

Lipid metabolism: 1 = TG liver, 2 = TC plasma, 3 = TG plasma, 4 = phospholipids plasma, 5 = free fatty acids plasma, 6 = total hepatic lipid content, 7 = TC liver, 8 = serum adiponectin, 9 = NEFA plasma, 10 = SREBP-1c (g.ex) liver, 11. FAS (g.ex) liver, 12 = SCD-1 (g.ex) liver, 13 = hormone-sensitive lipase (HSL) (g.ex) adipose tissue, 14 = serum leptin

Inflammation: 1 = COX2 (g.ex/p.ex), 2 = tnf-α (g.ex/p.ex), 3 = NF-κB activity, 4 = CRP plasma, 5 = Il-6 plasma, 6 = MCP-1 plasma, 7 = G-CSF plasma, 8 = phosphorylated IKKβ (p.ex), 9 = phosphorylated NF-κB (p.ex), 10 = MPO (p.ex), 11 = phosphorylated IκBα

Oxidative stress: 1 = iNOS (g.ex/p.ex), 2 = nitrotyrosine formation, 3 = SOD (g.ex), 4 = GPx (g.ex), 5 = CAT (g.ex), 6 = SOD like activity plasma, 7 = mitochondrial ROS production, 8 =GSH, 9 = vitamin E, 10 = ascorbic acid, 11 = NADPH oxidase activity, 12 = NO_x concentration, 13 = GSSG, 14 = GCLC (p.ex), 15 = GCLM (p.ex), 16 = SOD 1 activity, 17 = SOD2 activity, 18 = CAT activity, 19 = GPx activity, 20 = GST activity, 21 = GR activity

Other: 1 = food intake, 2 = hepatic stellate cell activation, 3 = tgf-β (p.ex), 4 = α-SMA (p.ex), 5 = MMP-2 activity, 6 = TIMP-2 (p.ex), 7 = SMAD2 (p.ex), 8 = SMAD4 (p.ex), 9 = phosphorylated FoxO1 (p.ex), 10 = phosphorylated P13K (p.ex), 11 = phosphorylated akt (p.ex), 12 = phosphorylated $P27^{kip1}$ (p.ex), 13 = glucose plasma, 14 = insulin plasma, 15 = fecal lipids, 16 = CYP2E1 liver, 17 = insulin receptor (p.ex), 18 = insulin receptor substrate (p.ex).

Table 5.

Study	Species (n/group)	Model	Intervention Dose (mg/kg/day) or (% of diet)	Lipids liver/serum	Coordinating factors	Markers FA	Markers Lipogenese	Antioxidants	O.S. markers	Lipid Peroxidation	Anti	Pro	Other	ALT	Steatosis	Inflammt	Fibrosis	Weight	IR	Conclusion Effect
				Lipid Metabolism				**Oxidative Stress**			**Inflammation**		**Other**		**Histology**			**Weight**	**IR**	
Intragastrical administration																				
Marc olin et al. 2013	Mice (n=16)	MCD diet	**50 Q** 4 wks	↔[1, 2, 3]				↔[1,2]	↓[3]	↓			↔[1]	↓	↓	↓				+
Oral administration																				
Marc olin et al. 2012	Mice (n=8)	MCD diet	**50 Q** 2/4 wks						↓		↓[1-5]	↓[1-5]		↓	↓	↓		↔[bw] ↔[lw]		++
Ying et al. 2013	Gerbils (n=8)	HFD 28 days	**15/30/60 Q** Last 14 days of diet	↓[1, 2, 4] ↔[3]					↓[4]		↓[1§, 2§] ↓[5]	↓[1, 6]↑[7]		↓	↓	↓	↓			+
Dietary intervention																				
Kobo ri et al. 2011	Mice (n=6)	HS HFD	**0.05% Q** 4/8/20 wks	↓[1, 2, 5, 7, 8]	↑[6, 9] ↓[11, 13]		↓[10, 12]	↑[5-7]		↓		↓[1§]	↓[1, 8] ↔[9] ↑[10]↓[11]	↓				↓[bw] ↓[vfw]		+
Jung	Mice	HFD	**0.025%**	↓[1, 2]	↑[9]		↓[12]	↓[8§]	↓[§]					↓			↓[bw]↓[1]		+

et al. 2013	(n=10)		**Q** 9 wks	↔[3]	↓[11, 13]										w ↓ efw	
Panc hal et al. 2012	Rats (n=10)	HC HFD 16 wks	**0.08% Q** Last 8 wks of diet	↑[1] ↔[2, 5]		↑[14]		↑[9]		↓[5]	↓[1, 12] ↑[13, 14]	↓	↓	↓	↔ bw ↓ lw ↓ vfw	++

Effects of quercetin are observed in the treatment group compared to the model group. Green = inhibiting effect on the development of NAFLD, blue = unknown or no effect on development of NAFLD, orange = stimulating effect on development NAFLD.

Specific markers related to lipid metabolism are categorized in four categories: lipids in liver or serum, general coordinating factors of lipid metabolism, markers involved in fatty acids oxidation, and markers involved in lipogenesis. Markers related to lipid metabolism are measured in liver or serum unless mentioned by # = measured in adipose tissue. Markers related to oxidative stress are divided in antioxidants, oxidative stress markers (O.S. markers) and lipid peroxidation. Specific markers related to inflammation are categorized in 2 categories: pro = pro-inflammatory factors, anti = anti-inflammatory factors. ALT = alanine aminotransferase levels serum. Histology is divided in steatosis, inflammation and fibrosis. IR = insulin resistance. Markers of inflammation and oxidative stress are measured in the liver, unless mentioned by § = measured in serum. Abbreviations: wks = weeks, MCD = methionine choline deficient diet, HS HFD = high sucrose high fat diet, HC HFD = high carbohydrate high fat diet, bw = body weight, vfw = visceral fat weight, lw = liver weight, efw = epididymal fat weight.

Markers:

Lipid metabolism: 1 = TC plasma, 2= TG plasma, 3 = HDL-C plasma, 4 = LDL-C plasma, 5 = NEFA plasma, 6 = adiponectin plasma, 7 = TG liver, 8 = TC liver, 9 = PPAR-α (g.ex) liver, 10 = SREBP-1c (g.ex) liver, 11 = CD36 (g.ex) liver, 12 = fatty acid synthase (FASN) (g.ex) liver, 13 = PPARγ (g.ex) liver, 14 = CPT-1 (p. ex) liver.

Inflammation: 1 = tnf-α (g.ex), 2 = Il-6 (g.ex) 3 = Hmgb1 (g.ex), 4 = ptgs2 (g.ex), 5 = NF-κB p65 (p.ex).

Oxidative stress: 1 = SOD activity, 2 = CAT activity, 3 = DNA damage, 4 = iNOS (p.ex), 5 = catalase (g.ex), 6 = GPx1 (g.ex), 7 = total glutathione liver, 8 = total antioxidant status, 9 = HO-1 (p.ex).

Others: 1 = plasma glucose, 2 = α-SMA staining (fibrosis), 3 = fibrosis related genes: Col1a1and 3a1, Plod3, tgf-β, Smad 7 and 3, Pdgfb, Areg, Ctgf, Mmp9 and Timp 1 (g.ex), 4= phosphorylated JNK (p.ex), 5= TLR-4 (p.ex), 6 = fibrosis markers serum (HA, LN, collagen I, collagen III, HYP), 7 = sirt1 (p.ex), 8= plasma insulin, 9 = mitochondrial complex I activity liver, 10 = Pck1 (g.ex) liver, 11 = Ucp2 (g.ex) liver, 12 = abdominal circumference, 13 = nrf 2 (p.ex), 14 = cleaved caspase 3.

Table 6.

Study	Species (n/group)	Model	Intervention Dose (mg/kg/day) or (% of diet)	Lipids liver/serum	Coordinating factors / Markers	FA markers	Lipogenesis	Antioxidants	O.S. markers	Lipid Peroxidation	Anti	Pro	Other	ALT	Steatosis	Inflammt	Fibrosis	Weight	IR	Conclusion Effect
Intraperitoneal administration																				
Gao et al. 2013	Mice (n=5)	HFD	**50** mg/kg 2x/wk 8 wks	↓[1]	↓[2]		↓[3] ↔[4,5]					↓[1-3]	↓[1-3,5] ↑[4]	↓	↓			↓bw ↓lw ↓fw ↓efw	↓	++
Oral administration																				
Ziaee et al. 2009	Rats (n=10)	High Chol. Cocktail	**10/100** by gavage 28 days	↓[6,7]									↓	↓	↓				+
Dietary intervention																				
Panchal et al. 2011	Rats (n=12)	HC HFD 16 wks	**0.16%** Last 8 wks of diet	↓[6,8,9]				↑[2§,3§]	↓[1§]	↓[§]			↓[1,2,6] ↑[7-9]	↓	↓	↓	↓	↓bw ↓vfw	↓	+++
Hsu et al. 2009	Rats (n=6)	HFD 12 wks	**0.005/ 0.01%** Last 8 wks of diet	↓[1,6-8,11,12]	↓[10]			↑[2,5-9]	↓[4]	↓			↓[2,10]		↓			↓bw ↓lw ↓fw		++

81

Effects of rutin are observed in the treatment group compared to the model group. Green = inhibiting effect on the development of NAFLD, blue = unknown or no effect on development of NAFLD, orange = stimulating effect on development NAFLD.

Specific markers related to lipid metabolism are categorized in four categories: lipids in liver or serum, general coordinating factors of lipid metabolism, markers involved in fatty acids oxidation, and markers involved in lipogenesis.Markers related to oxidative stress are divided in antioxidants, oxidative stress markers (O.S. markers) and lipid peroxidation. Specific markers related to inflammation are categorized in 2 categories: pro = pro-inflammatory factors, anti = anti-inflammatory factors. ALT = alanine aminotransferase levels serum. Histology is divided in steatosis, inflammation and fibrosis. IR = insulin resistance. Markers of inflammation and oxidative stress are measured in the liver, unless mentioned by § = measured in serum.

Abbreviations: wks = weeks, HFD = high fat diet, HC HFD = high carbohydrate high fat diet, chol = cholesterol, bw = body weight, vfw = visceral fat weight, lw = liver weight, efw = epididymal fat weight, fw = fat weight.

Markers:

Lipid metabolism: 1 = TG liver, 2 = CD 36 (g.ex), 3 = SREBP-1c (g.ex), 4 = ACC (g.ex), 5 = FAS (g.ex), 6 = TC plasma, 7 = LDL-C plasma, 8 = TG plasma, 9 = NEFA plasma, 10 = leptin plasma, 11 = TC liver, 12 = phospholipids plasma

Inflammation: 1 = tnf-α (g.ex), 2 = Ifn-γ (g.ex), 4 = F4/80 (g.ex),

<u>Oxidative stress</u>: 1= Superoxide production erythrocytes, 2 = glutathione peroxidase activity, 3 = plasma total antioxidant capacity, 4 = GSSG, 5 = TEAC, 6 = glutathione reductase, 7 = SOD, 8 = GSH, 9 = GSH/GSSG-ratio

<u>Other:</u> 1 = plasma glucose, 2 = plasma insulin, 3 = PEPCK (g.ex), 4 = Pdk4 (g.ex), 5 = G-6-Pase (g.ex), 6 = abdominal circumference, 7 = caspase-3 (p.ex), 8 = Hsp 70 (p.ex), 9 = ERK 1/2, 10 = GPDH activity liver and adipose tissue.

Table 7

Other flavonoids investigated in animal models of NAFLD
Flavanones
• Naringenin (Wood 2004; Mulvihill et al. 2009; Mulvihill et al. 2010)
Anthocyanins
• Cyanidin-3-O-β-D-glucoside (Tsuda et al. 2003; Guo et al. 2012; Zhu et al. 2012)
Chalcones
• Xanthohumol (Dorn et al. 2010; Doddapattar et al. 2013)
Flavones
• Baicalin (Guo et al. 2009)
• Nobiletin (Lee et al. 2013)
Isoflavones
• Puerin (Zheng et al. 2009)
Isoflavanes
• Glabridin (Lee et al. 2012)

Other flavonoids investigated in animal models of NAFLD.

Figure legends
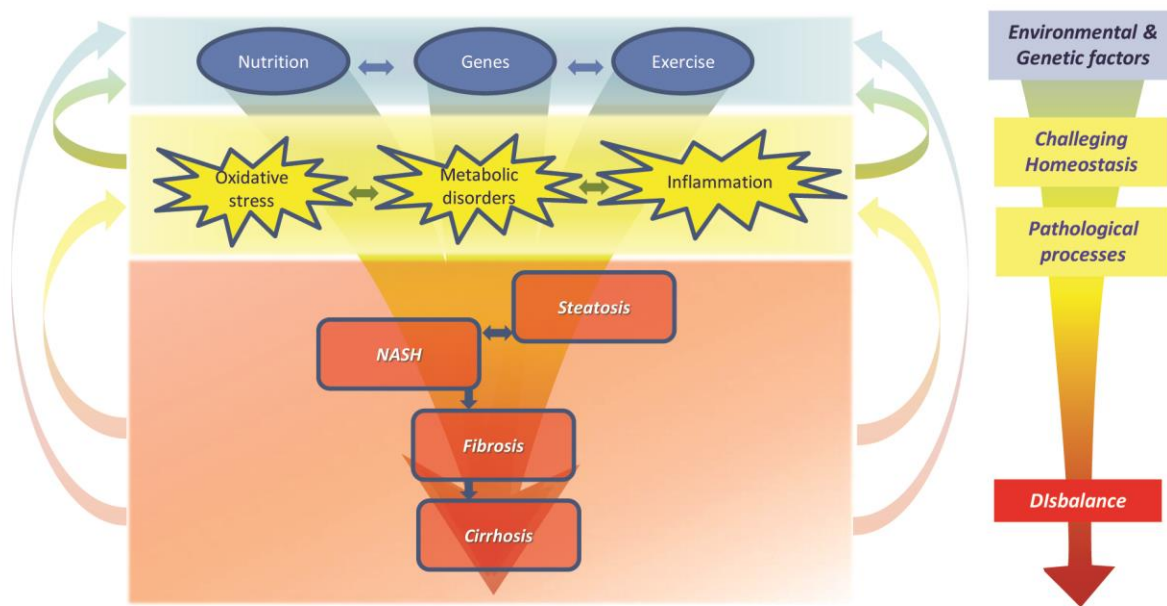
Figure 1: Contemporary pathophysiological model of NAFLD.

Environmental and genetic factors challenge homeostasis and lead to several pathological

processes: metabolic disorders, oxidative stress and inflammation. These processes also fortify

each other and cause a disbalance, leading to the development of steatosis and non-alcoholic

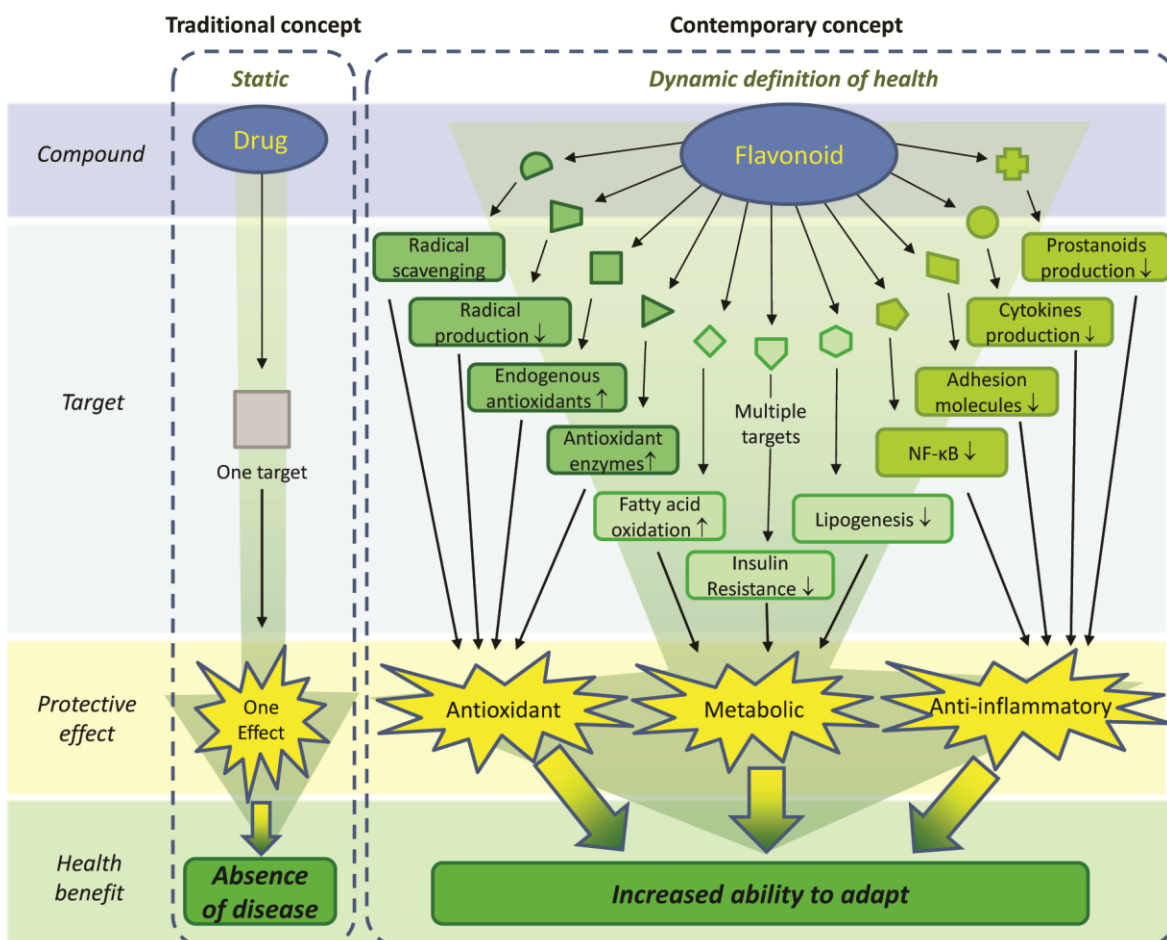steatohepatitis (NASH). NASH can eventually progress to fibrosis and cirrhosis.

Figure 2: 'Traditional' concept of action of drugs versus the contemporary concept of action of bioactives, such as flavonoids.

While traditional drugs are developed to act on one target, leading to absence of a disease, flavonoids act on multiple targets, affecting diverse pathological processes, leading to increased ability to adapt. This fits seamlessly in the pathophysiologic model of NAFLD, since diverse pathological processes are involved (fig. 1).
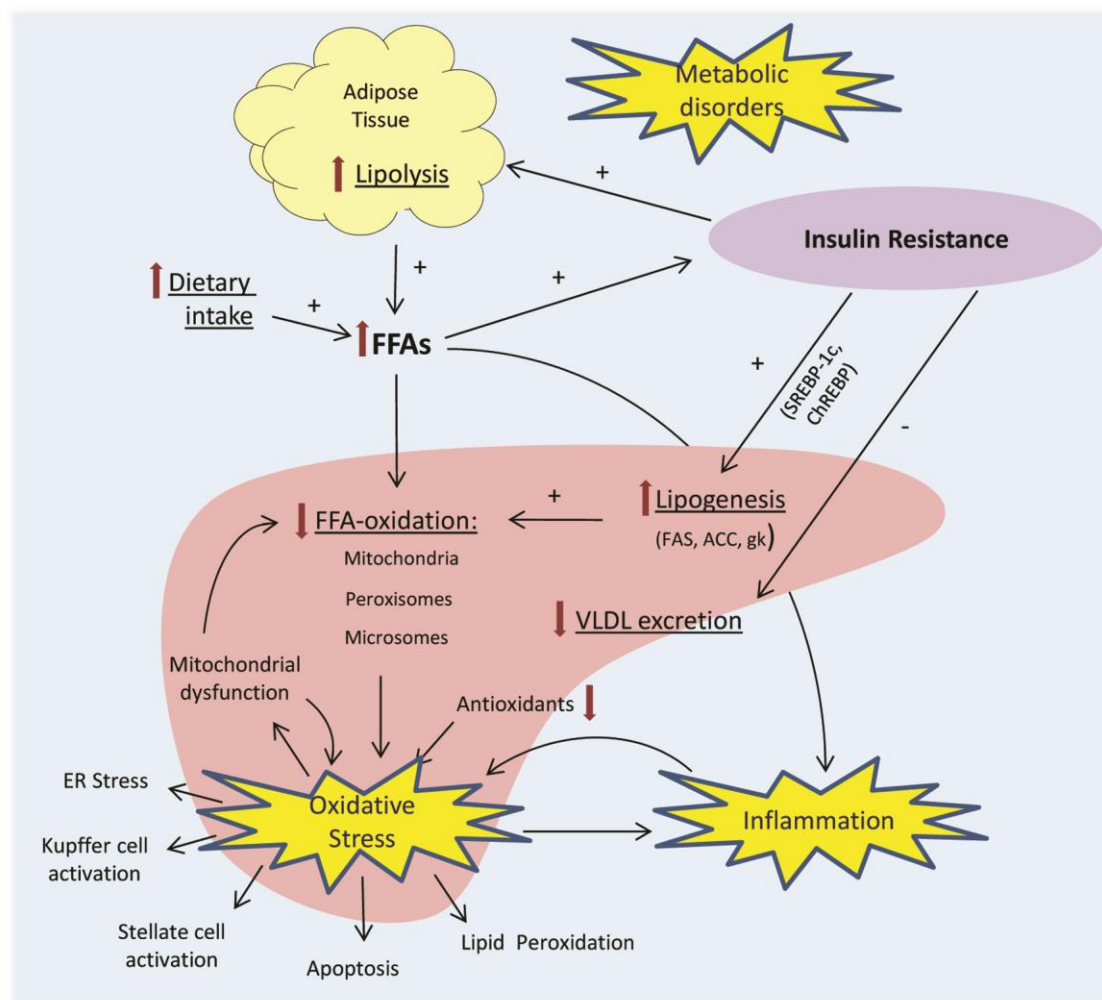
Figure 3: Schematic view of the pathophysiological pathways involved in NAFLD.

Overview of the processes in liver, adipose and other tissues that are responsible for the metabolic disturbances, oxidative stress and inflammation in NAFLD. Abbreviations: FFA = free fatty acids, SREBP-1c = sterol regulatory element-binding protein 1c, ChREBP = carbohydrate-responsive element-binding protein, FAS = fatty acid synthase, ACC = acetyl-CoA carboxylase, gk = glucokinase, VLDL = very low-density lipoprotein, ER = endoplasmic reticulum.

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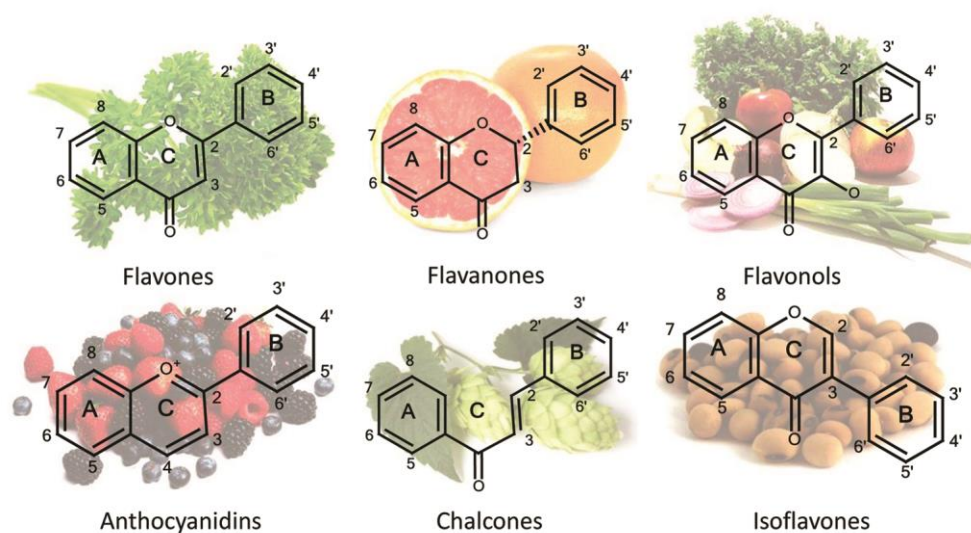
Figure 4: Major subclasses of flavonoids found in significant amounts in the pictured fruits and vegetables.
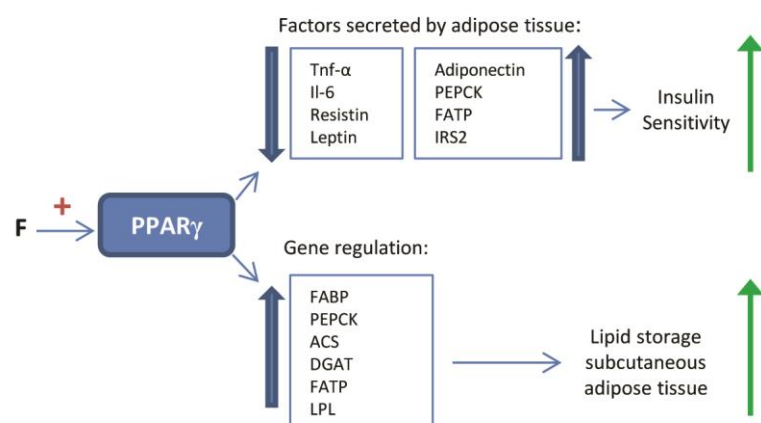
Figure 5: Effects of PPARγ stimulation by flavonoids.

PPARγ stimulation by flavonoids (F) leads to changes in the factors secreted by adipose tissue, stimulating insulin sensitivity. Furthermore, it leads to the upregulation of specific genes, stimulating lipid storage in subcutaneous adipose tissue instead of the liver. Abbreviations: Tnf-α = tumor necrosis factor α, Il-6 = interleukin 6, PEPCK = phospoenolpyruvate carboxykinase, FATP = fatty acid transport protein, IRS2 = insulin receptor substrate 2, FABP = fatty acid-binding protein, ACS = acetyl-CoA synthase, DGAT = diglyceride acyltransferase, LPL = lipoprotein lipase.
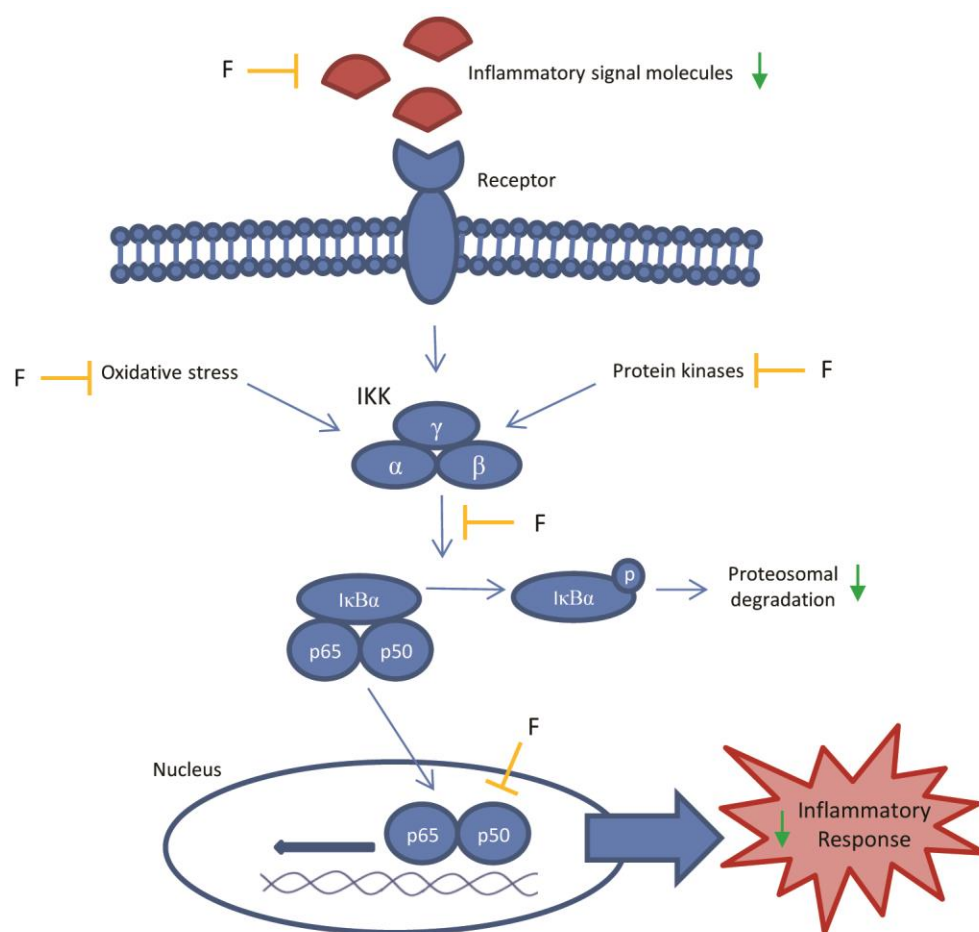
Figure 6: Effects of flavonoids on the NFκB-pathway.

Inhibitory effects (—|) of flavonoids (F) on different processes in the canonical NF-κB

pathway, leading to a decreased inflammatory response. Abbreviations: IKK = IκB kinase, IκBα

= NF-κB inhibitor-α, p65 = NF-κB complex subunit p65, p50 = NF-κB complex subunit p50.
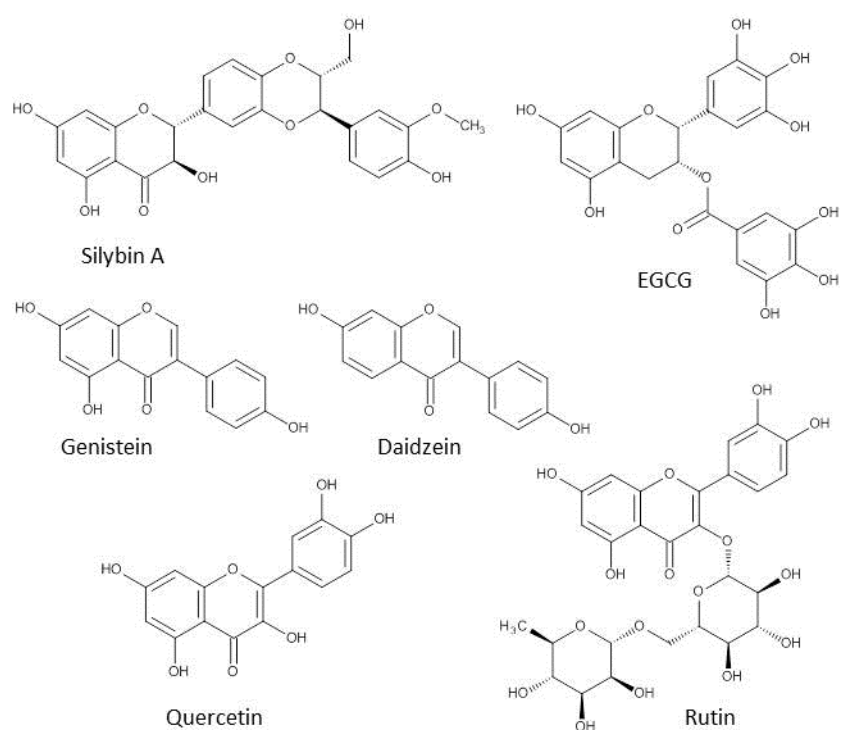
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Figure 7: Most studied flavonoids in the treatment of NAFLD.

Molecular structures of the most investigated flavonoids in the treatment of NAFLD: Silybin (mixture of two diateromers, of which one is pictured), genistein, daidzein, epigallocatechin-3-gallate (ECGC), quercetin and rutin.