University of Saskatchewan

Molecular mechanisms of protection by dietary polyphenols against free fatty acid-induced mitochondrial dysfunction and endoplasmic reticulum stress in an *in vitro* model of non-alcoholic fatty liver disease

A Thesis Submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Pharmacy and Nutrition University of Saskatchewan Saskatoon, SK, Canada

By

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a public health burden. Steatosis as the "first hit", and oxidative stress, inflammation, mitochondrial dysfunction, and endoplasmic reticulum stress as the "second hits" are the main contributors of the progression of fatty liver to non-alcoholic steatohepatitis (NASH). Dietary polyphenols have shown promise in protecting the liver against NAFLD. The relative effectiveness and mechanisms of different polyphenols however is mostly unknown.

In this thesis HepG2 hepatocytes exposed to oleic or palmitic acid were used as a model system to explore the ability of selected polyphenols (resveratrol, quercetin, catechin, cyanidin, cyanidin-3-glucoside, berberine) from different classes to protect against molecular aspects of NAFLD and NASH. In an investigation of the "first hit" (Chapter 3), different polyphenols protected similarly against oleic acid-induced intracellular lipid accumulation, but differed in their effects on the expression of genes and proteins involved in lipogenesis, fatty acid oxidation, mitochondrial biogenesis, and bioenergetics. In an investigation of "second hits" (Chapter 4), most of the polyphenols decreased reactive oxygen species (ROS), prevented the decrease in uncoupling protein 2 (UCP2) mRNA, prevented the increase in tumor necrosis factor alpha (TNFα) mRNA, reversed decreases in mitochondrial biogenesis and increased expression of mitochondrial respiratory complexes and manganese superoxide dismutase (MnSOD). The anthocyanins were unique in decreasing ROS without inducing mitochondrial biogenesis or Mn-SOD mRNA expression.

In investigations with palmitic acid (Chapter 5), exposure of HepG2 cells to palmitic acid induced endoplasmic reticulum (ER) stress evidenced by upregulated mRNA for the ER chaperones glucose-regulated protein 94 and 78 (GRP94, GRP78) and oxygen-regulated protein 150 (ORP150), cochaperone endoplasmic reticulum-localized DnaJ homologue 4 (ERdj4), and proapoptotic CCAAT-enhancer-binding protein homologous protein (CHOP). A few of the polyphenols (quercetin, catechin, cyanidin) protected against these changes.

In a comparison of flavonoids with their phenolic breakdown/digestion products (Chapter 6), the polyphenols 2,4,6-trihydroxybenzaldehyde, protocatechuic acid, and caffeic acid protected similarly to quercetin and cyanidin against oleic and palmitic acid-induced steatosis and ROS

generation. Moreover, in a short-term 1 h exposure (to limit spontaneous degradation in the medium), only breakdown/digestion products prevented an oleic acid-induced decrease of mitochondrial biogenesis.

In conclusion, different classes of dietary polyphenols were all able to protect against steatosis and ROS generation in this *in vitro* model of NAFLD. Part of the mechanism for some polyphenols was through effects on mitochondrial biogenesis and function, bioenergetics, and ER stress. Phenolic breakdown/digestion products of flavonoids were shown to contribute to the protective effects of parent polyphenols.

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DEDICATION

This dissertation is dedicated to my wife, Kosar, who has been a powerful encouragement and support. I am extremely happy to have you in my life and words can not express my true love for you. This work is also dedicated to my parents and sisters who always were supportive to me and helped me to reach this accomplishment.

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LIST OF ABBREVIATIONS

ΔΨm	Mitochondrial membrane potential
4-HNE	4-Hydroxynonenal
ACC	Acetyl CoA carboxylase
ACO	Acyl-CoA oxidase
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
ATP5G1	ATP synthase, mitochondrial Fo complex subunit C1
BSA	Bovine serum albumin
C/EBPs	CCAAT-enhancer-binding proteins
CBG	Cytosolic β-glucosidase
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
cDNA	Complementary DNA
CHOP	CCAAT-enhancer-binding protein homologous protein
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate-responsive element-binding protein
COX6B1	Cytochrome c oxidase subunit 6B1
CPT-1	Carnitine palmitoyltransferase-1
CPT1A1	Carnitine palmitoyltransferase-1 (liver)
CYP2E1	Cytochrome P450 2E1
DCFH-DA	Dichloro-dihydrofluorescein diacetate
ddH_2O	Double-distilled water
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
ER stress	Endoplasmic reticulum stress

ERdj4	Endoplasmic reticulum-localized DnaJ homologue 4
$FADH_2$	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FBS	Fetal bovine serum
FFA	Free fatty acid
FOXO1	Forkhead box protein O1
GADD34	Growth arrest and DNA damage-inducible
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPAT	Glycerophosphate acyltransferase
GPX-1	Glutathione peroxidase 1
GRP58	Glucose-regulated protein 58KD
GRP78	Glucose-regulated protein 78KD
GRP94	Glucose-regulated protein 94KD
GSH	Reduced glutathione
GSH/GSSG	Reduced to oxidized glutathione
HSL	Hormone sensitive lipase
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRE1	Inositol-requiring enzyme 1
IRS-1	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LPH	Lactase phloridzin hydrolase
LXR	Liver X receptor
MDA	Malondialdehyde
MEM	Minimum essential medium
Mn-SOD	Manganese-superoxide dismutase
MTATP6	Mitochondrially encoded ATP synthase F_o subunit 6
MTCO1	Mitochondrially encoded cytochrome c oxidase I
MTCYB	Mitochondrially encoded cytochrome b

mtDNA	Mitochondrial DNA
MTND1	Mitochondrial NADH dehydrogenase subunit 1
NAD	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NDUFS8	NADH: ubiquinone oxidoreductase core subunit S8
NEFA	Non-esterified fatty acid
NFκB	Nuclear factor kappa b
NRF1	Nuclear respiratory factor
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OA	Oleic acid
OAA	Oxaloacetic acid
ORP150	Oxygen-regulated protein 150KD
OXPHOS	Oxidative phosphorylation
PA	Palmitic acid
p-AMPKα	Phospho-AMPKα on threonine 172
p-eIF2α	Phosphorylated eukaryotic translation initiation factor 2
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1
PI3K	Phosphatidylinositol-3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RXR-α	Retinoid X receptor alpha
SCD-1	Stearoyl-CoA desaturase-1
SDHB	Mitochondrial succinate dehydrogenase iron-sulfur subunit
SIRT	Sirtuin
SOD	Superoxide dismutase
SREBP1-c	Sterol regulatory element-binding protein 1-c

TBARS	Thiobarbituric acid reactive substances
TFAM	Mitochondrial transcription factor A
TMRE	Tetramethyl rhodamine ethyl ester
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
UCP2	Uncoupling protein 2
UPR	Unfolded protein response
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1
VLDL	Very low density lipoprotein
XBP1	X-box binding protein 1

CHAPTER 1: INTRODUCTION

1.1.Rationale

Non-alcoholic fatty liver disease (NAFLD) is a public health burden affecting 10-35% of the general population in different communities [1]. NAFLD is particularly rising in Western communities and may result in serious complications such as non-alcoholic steatohepatitis (NASH), cirrhosis, fibrosis, and hepatocellular carcinoma. The leading hypothesis regarding fatty liver disease initiation and development is the ‘‘two-hit’’ hypothesis suggesting that hepatic fat accumulation is the first hit which may predispose the liver to the “second hits” including but not limited to oxidative stress and inflammation [2]. More recently, other “hits” have been suggested to be involved including insulin resistance [3], mitochondrial dysfunction [4-6], and endoplasmic reticulum (ER) stress [7, 8] which interplay in a concerted manner to expedite the progression of simple steatosis to steatohepatitis, cirrhosis, and fibrosis.

The liver cells are rich in mitochondria and approximately 800 mitochondria are available in each hepatocyte. These mitochondria occupy 18% of the volume of each hepatocyte [9]. A myriad of evidence suggests NAFLD/NASH as a mitochondrial disease due to the abnormality in function and morphology of hepatic mitochondria [5, 10, 11]. In fact, mitochondrial dysfunction contributes to the severity of NAFLD and its progression to NASH [12]. This is due to the pivotal role of mitochondria in lipid metabolism, lipid peroxidation, ROS and cytokine production, and apoptosis [13]. Defective mitochondrial oxidative phosphorylation (OXPHOS) and function have been reported in the liver of subjects with NASH [14], as well as in several *in vitro* and *in vivo* models of NAFLD including HepG2 hepatocytes treated with palmitate [15], in the liver of *ob/ob* mice with NAFLD [16], and in the liver of mice fed a high-fat diet [17]. Moreover, it has been shown that mitochondrial biogenesis, mRNA expression of mitochondrial DNA-encoded respiratory complex I-V subunits, and the activity of all mitochondrial complexes are decreased in NAFLD [17]. It was suggested that a decrease in mitochondrial biogenesis (the decreased number of the healthy and functional mitochondria) and impaired expression and assembly of mitochondrial respiratory complex subunits may result in depletion of ATP stores [18], increased ROS

production [19], collapsed mitochondrial membrane potential ($\Delta\Psi m$) [20], and impaired fatty acid oxidation which are some of the major contributors of NAFLD progression to NASH.

Inflammatory mechanisms also play a crucial role in the liver damage and activation of collagen-producing hepatic stellate cells which consequently lead to hepatic fibrosis and NASH. Several studies have shown a positive correlation between serum and hepatic levels of TNFα and other inflammatory cytokines with the severity of necrosis and fibrosis in fatty liver disease [21].

Besides mitochondrial dysfunction, endoplasmic reticulum stress has also been reported by numerous studies to facilitate progression of NAFLD to NASH [22]. The smooth endoplasmic reticulum is associated with the metabolism of proteins, lipids, and carbohydrates and is involved in proper folding and maturation of proteins [23]. Lipogenesis has been shown to be affected by ER stress and they appear to have a direct and positive correlation [24]. In addition, it seems that oxidative stress contributes to perturbation of endoplasmic reticulum homeostasis and therefore brings about the activation of a cellular response called unfolded protein response (UPR). The aim of this response is to decrease the load of unfolded/misfolded proteins to endoplasmic reticulum and to restore endoplasmic reticulum homeostasis [24]. Short term activation of UPR is necessary for homeostasis of metabolism but prolonged UPR may have harmful effects and may result in increased hepatic lipid accumulation, inflammation, insulin resistance, impaired lipid metabolism, and apoptosis. In chronic metabolic diseases, such as obesity, diabetes, and NAFLD, ER stress is increased implicating compromised ability of unfolded protein response (UPR) to resolve ER stress [7]. Initially, it was suggested that ER stress may have a possible contribution to hepatic steatosis and insulin resistance due to the activation of several ER stress markers in dietary (high-fat diet) and genetic (*ob/ob)* models of murine obesity [25]. Since then, several studies have reported an increased ER stress in hepatocytes treated with palmitic acid [26], and in rodents [27, 28] and humans [29, 30] with hepatic steatosis.

Previously, experimental studies (genetic models and high-fat diet) have been designed to study the mechanisms of NAFLD development and its progression to NASH [31, 32]. More recently, *in vitro* models of NAFLD have been developed to study the effects of steatosis in hepatic cell line of human origin [33, 34] and explore the contributing mechanisms. These models include using monounsaturated (oleic acid), and saturated fatty acids (palmitic acid) to induce steatosis in

hepatocyte cell line (HepG2) and primary hepatocytes [33-38]. Although human primary hepatocytes are the closest to human liver [39], human hepatocyte-derived HepG2 cell line showed similar behavior to steatosis and appears to be a promising alternative option [40] particularly for investigating NAFLD [39]. HepG2 cell line was also suggested to be a reliable model for studying hepatic lipid metabolism [37]. In the present study, I used HepG2 cells because this cell line is frequently reported in the literature as an *in vitro* model of NAFLD [15, 36, 41, 42].

Polyphenols are abundant micronutrients found in our daily diet such as in different fruits and vegetables, pulses, green and black tea, coffee, extra virgin olive oil, red wine and chocolate [43]. It has been reported that different polyphenols can prevent fatty liver disease in experimental models by showing strong anti-steatotic [44-46], antioxidant [47, 48], and anti-inflammatory effects [49, 50]. Although an accumulating body of evidence suggests that mitochondrial dysfunction and ER stress are very important contributors of NAFLD progression to NASH, only very few studies evaluated the effects of polyphenols on these outcomes. Also, most studies only evaluated the effect of one polyphenol or food extract on NAFLD or NASH. Although different polyphenols have been shown to ameliorate NAFLD in rodents and humans [51, 52] and could be promising treatments for fatty liver disease in future, it is not known which polyphenols are the most potent and through what molecular mechanisms they function. Due to previous lack of evidence, the main focus of this thesis was on comparison of different classes of polyphenols on different aspects of mitochondrial biogenesis and function, and endoplasmic reticulum stress. Another question is to elucidate whether the effects of polyphenols on protection against NAFLD is due to the parent polyphenols or their breakdown/digestion products. These are unresolved questions that this thesis is attempting to address.

The polyphenols investigated in this thesis have been selected from different classes including:

1. FLAVONOIDS - a) flavonol (quercetin), b) flavanol (catechin), and c) anthocyanins (cyanidin and cyanidin-3-glucoside). These flavonoids share an ortho 3’,4’-dihydroxy structure in the B-ring. Cyanidin-3-glucoside is included because this glycoside has been observed to be absorbed intact to appear in the blood of animals and humans. Previous work in our laboratory suggests that cyanidin may be accumulated by mitochondria to a greater extent than other flavonoids.
2. STILBENOID - resveratrol. Resveratrol is a potent activator of SIRT1 and stimulator of

mitochondrial biogenesis.

3. ALKALOID - berberine. Although berberine (a methoxy alkaloid derivative) is not itself a polyphenol, it can be demethylated by the activity of cytochrome P450 in hepatocytes to produce an ortho dihydroxy polyphenolic compound. It also carries a positive charge so may be actively accumulated by mitochondria.

Several chapters of this thesis are being submitted as manuscripts. The first manuscript (CHAPTER 3) deals with steatosis and lipid metabolism (the "first hit" of fatty liver disease), and the role of polyphenols in protection against it by affecting some aspects of mitochondrial dysfunction. The second manuscript (CHAPTER 4) talks about the "second hits" such as oxidative stress and inflammation, and deals with the comparison of different classes of polyphenols in ameliorating these hits by inducing mitochondrial biogenesis (content) and function. The third manuscript (CHAPTER 5) deals with palmitic acid-induced endoplasmic reticulum stress and mitochondrial dysfunction and how polyphenols protect against these outcomes. Finally, comparison of the parent flavonoids quercetin and cyanidin, and their breakdown/digestion products on steatosis, oxidative stress, and mitochondrial biogenesis and function is discussed in CHAPTER 6.

1.2. Hypotheses

The main hypothesis of my study was that different classes of dietary polyphenols, such as resveratrol, quercetin, catechin, cyanidin, cyanidin-3-glucoside (kuromanin), and berberine will protect against indices of non-alcoholic fatty liver disease in HepG2 cells, with different potencies and mechanisms.

The detailed hypotheses of my study were:

1. Some polyphenols will prevent steatosis, mitochondrial dysfunction, and oxidative stress in HepG2 hepatocytes exposed to oleic or palmitic acid.
2. As part of their mechanism of protection, some polyphenols will improve hepatic mitochondrial function through increasing mitochondrial biogenesis and upregulating gene expression of mitochondrial proteins and mitochondrial respiratory complex I-V subunits.

3. In their inhibition of steatosis and oxidative stress in hepatocytes, some polyphenols will suppress hepatic endoplasmic reticulum (ER) stress and unfolded protein response (UPR).
4. Mitochondriogenic (resveratrol) and mitochondrial antioxidant (cyanidin) polyphenols will together provide synergistic protection against non-alcoholic fatty liver disease.
5. The breakdown/digestion products of polyphenols such as caffeic acid, protocatechuic acid, and 2,4,6 trihydroxybenzaldehyde will contribute to the inhibitory effect of the parent flavonoids (quercetin and cyanidin) against non-alcoholic fatty liver disease.

1.3. Objectives

Specific objectives were:

1. To determine the effect of selected dietary polyphenols, resveratrol, quercetin, catechin, cyanidin, cyanidin 3-glucoside, and berberine on oleic acid-induced changes in HepG2 hepatocytes including:
    - Intracellular lipid accumulation
    - Reactive oxygen species (ROS)
    - Mitochondrial membrane potential ($\Delta\Psi m$)
    - The expression of genes involved in lipogenesis (such as FAS and PPARγ)
    - The expression of genes involved in fatty acid β-oxidation (such as PPARα and CPT1A1)
    - Uncoupling proteins (such as UCP2)
    - Antioxidant enzymes (such as Mn-SOD)
    - Inflammatory cytokines (such as TNFα)
    - Mitochondrial biogenesis (MitoTracker Green staining) and the contributing genes (such as PGC1α, NRF1, TFAM, SIRT1, SIRT3)
    - Activity of SIRT1
    - Mitochondrial dysfunction (gene expression of nuclear DNA-encoded respiratory complex I-V subunits)

- Intracellular energy stores (ATP content) with and without shutting down glycolysis (after 24 and 72 h)
- Protein expression of phosphorylated (Thr172) AMP-activated protein kinase (p-AMPKα^{Thr172}), and deacetylated PGC1α using Western blot methodology.

2. To determine the effects of quercetin and cyanidin breakdown/digestion products such as caffeic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde on oleic acid-induced changes in HepG2 hepatocytes including:
   - Intracellular triglyceride accumulation
   - Reactive oxygen species
   - Mitochondrial content (with 1 h or 24 h incubation with polyphenols and breakdown products)
   - Mitochondrial membrane potential

3. To determine the effect of polyphenols such as resveratrol, quercetin, catechin, cyanidin, cyanidin 3-glucoside, and berberine on palmitic acid-induced changes in HepG2 hepatocytes including:
   - Endoplasmic reticulum stress (measuring gene expression of unfolded protein response markers including ATF4, GRP58, ERdj4, GRP78, EDEM1, ORP150, GADD34, GRP94, and apoptotic CHOP)
   - Mitochondrial function (the expression of both nuclear and mitochondrial DNA-encoded respiratory complex I-V subunits)
   - Mitochondrial biogenesis (MitoTracker Green staining)
   - Intracellular lipid accumulation
   - Reactive oxygen species
   - Mitochondrial membrane potential

The first and second hypotheses (and their specific objectives) are addressed in the first and second manuscripts (CHAPTER 3 & 4). The third hypothesis (and its specific objectives) is addressed in the third manuscript (CHAPTER 5). For the fourth hypothesis, no synergism was found between resveratrol and cyanidin in protection against oleic acid-induced ROS generation. The fifth

hypothesis is addressed in the fourth manuscript (CHAPTER 6) showing breakdown/digestion products of polyphenols contribute to the protective effects of polyphenols against NAFLD.

CHAPTER 2: LITERATURE REVIEW

2.1. Background

Nonalcoholic fatty liver disease (NAFLD) which is identified as a public health problem is defined by ectopic accumulation of lipids >5 % of the liver weight [53]. NAFLD is defined as a range of clinical outcomes and histological abnormalities such as simple steatosis (micro- and macro-vesicular), steatohepatitis, cirrhosis, fibrosis, and necrosis which is often caused by metabolic syndrome [54]. Initially a benign condition, NAFLD may progress to advanced stages such as nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma which can be life-threatening. For the first time in 1980, the term NASH was coined to describe the hepatic histological manifestations similar to alcoholic hepatitis (evidenced by fat accumulation, lobular hepatitis, and inflammatory infiltrates) in 20 patients referred to the Mayo clinic with having no history of alcohol abuse in a 10-year period [55]. They also found a different prevalence based on gender (60% in female). Interestingly, they found that most of the subjects (90%) were obese and had obesity-related comorbidities such as diabetes and hyperlipidemia. Prior to popularity of the NASH term for these patients, other terms had been suggested such as alcohol-like hepatitis, pseudo-alcoholic hepatitis, diabetic hepatitis, and fatty-liver hepatitis [56]. The prevalence of NAFLD and NASH is estimated to be about 20-30% and 2-3% of the general population in Western communities, respectively [57, 58]. The prevalence of NAFLD is estimated to be about 70 % in obese subjects and 90% in diabetic patients [59, 60]. The prevalence of NAFLD in children is about 2.6 % with 53% of obese children diagnosed with NAFLD [61, 62].

Although prevention or treatment of NAFLD/NASH is of critical importance, no medication has been approved by the Food and Drug Administration (FDA) for prevention or treatment of NAFLD. Currently, lifestyle modifications and weight loss are suggested to be the most important treatments and the primary line of intervention, particularly in patients with obesity or metabolic diseases [63]. However, the effectiveness of weight loss and adherence to a successful weight reduction diet over the long term is still debated [64]. Moreover, the most effective weight loss strategy has not yet been determined [60]. Recently, dietary polyphenols have been the subject of

increasing number of studies with showing effectiveness in the amelioration of hepatic steatosis in rodents [48, 51] and could be a promising treatment for fatty liver disease in future. In this literature review, firstly I will talk about the contributing mechanisms in initiation of NAFLD and its progression to NASH, then the effects of dietary polyphenols in the prevention of the disease will be discussed in details.

2.2. The "two-hit" *vs*. "multiple-hit" hypothesis in regard to initiation and progression of NAFLD

As mentioned earlier, simple steatosis can progress to NASH and this aroused the curiosity of scientists to explore the contributing mechanisms leading to this progression. In 1998, Day and James suggested a "two-hit" hypothesis [2]. They suggested that the accumulation of fat (steatosis) is the "first hit" making the liver susceptible to the "second hits". They also stated that although mere accumulation of fat may cause lipid peroxidation, not all patients with steatosis develop necrosis. They suggested that other factors (the "second hits") may also contribute to the progression of simple steatosis to NASH. Accordingly, they proposed oxidative stress as one of the "second hits", which by imposing deleterious effects on cellular DNA and lipids may play a pivotal role in the progression of NAFLD to steatohepatitis, cirrhosis, necrosis, and hepatocellular carcinoma [2]. These authors also suggested that oxidative stress induced by higher activity of cytochrome P450 2E1 (CYP2E1) and by enhanced mitochondrial and peroxisomal fatty acid β-oxidation may result in the production of lipid peroxidation aldehydes which have a key role in the progression of NAFLD to NASH [65]. Lipid peroxidation products may further result in necrosis and megamitochondria. In addition, oxidative stress and lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) may activate hepatic stellate cells which are capable of producing collagen and generating Mallory bodies (a marker of fibrosis) [66].

Although mitochondrial dysfunction has a pivotal role in NAFLD and its progression to NASH, it was not suggested by Day and James (1998) as one of the contributing mechanisms [2]. Twelve years after introducing the "two-hit" hypothesis, Tilg & Moschen developed the "multiple parallel hits" hypothesis for the mechanisms of NAFLD progression to NASH [8]. The "multiple parallel hits" hypothesis suggests that NAFLD is a complex disease and it has other contributors other than just steatosis and oxidative stress. They proposed that inflammatory mediators generated mainly by the gut microflora and adipose tissue may have a central role in NAFLD. In this hypothesis,

hepatic steatosis (particularly lipotoxicity) predisposes the liver to the parallel hits including adipocytokines, endoplasmic reticulum stress, and innate immunity [8]. They believed all these factors act in parallel to develop NASH from simple steatosis. In my opinion, compared to the "two-hits" hypothesis, the ''multiple parallel hits'' hypothesis more appropriately reflects the mechanisms behind progression of simple fatty liver disease to NASH. However, the key role of mitochondrial dysfunction in progression of the disease is missing and is worth investigation. Dietary polyphenols have shown potent antioxidant and anti-inflammatory effects and they may be effective in protection against mitochondrial dysfunction in NAFLD.

Another hypothesis ("distinct-hit") was recently (in 2012) proposed to explain the pathogenesis of NAFLD/NASH [67]. In the "distinct-hit" hypothesis and similar to "multiple parallel hits", insulin resistance is considered to be the main underlying mechanism. "Distinct hit" hypothesis suggests that NASH and simple steatosis arise from two independent conditions based on the evidence that inflammation may precede steatosis and some patients with NASH show a very low degree of steatosis [67]. In simple words, following insulin resistance, independent and distinct pathways are triggered resulting in the manifestation of either simple steatosis or NASH.

In the more recent hypothesis (in 2013), it was suggested that *in utero* programming (prenatal metabolic disturbances) may be involved in the pathogenesis of NAFLD in the subsequent generation [68]. To further support this hypothesis, it was shown that exposure to excess energy during maternal life in mice resulted in the manifestation of NAFLD in their offspring [69].

2.3. The culprits of fatty liver disease development and progression to NASH

Mechanisms that contribute to the progression of NAFLD to NASH are poorly understood. As discussed earlier, different contributors include hepatic lipid accumulation, insulin resistance, oxidative stress (ROS), adipocytokines, inflammation, mitochondrial dysfunction, and ER stress. In order to treat NAFLD and prevent its progression to NASH, these contributing mechanisms and their interrelation should be meticulously evaluated. Unfortunately, temporal order or relative contribution of these factors in the genesis of NAFLD is not clearly elucidated. For instance, in 2004 Özcan et al. suggested a role for ER stress and unfolded protein response (UPR) in NAFLD development, while previously researchers considered no role for ER stress in fatty liver disease [25]. Another example is the lower activity of mitochondrial respiratory complexes in NASH

patients, which was firstly reported by Pérez-Carreras more than a decade ago [14]. Recently, it was suggested that mitochondrial dysfunction may occur due to the lower expression of mitochondrial DNA-encoded respiratory complexes subunits [17]. In summary, further studies are needed to elucidate the association between the intertwined networks of contributors in the development and progression of fatty liver disease. In the following sections, the roles of most important mechanisms involved in NAFLD initiation and progression are discussed in more detail.

2.4. Different sources of fatty acids in the liver

All animals including humans have regulatory mechanisms controlling the equilibrium between energy intake and output. Insulin is the main regulator of this equilibrium and controls the metabolism of fats, carbohydrates, and proteins. Different sources of fatty acids in the liver include: food intake, *de novo* lipogenesis, and lipolysis in adipose tissue. General understanding of these sources is important since the relative importance of these sources is different in the fed and fasted state, and in healthy and in fatty liver disease [70].

2.4.1. Food intake

Following the absorption of fatty acids from the small intestine, chylomicrons are formed which first transfer their fatty acids via the blood stream to adipose tissue, and then the chylomicron remnants are taken up by the liver [71]. Evidence reports that 59% of hepatic fat in NAFLD patients originates from the circulating non-esterified fatty acid (NEFA) pool coming from lipolysis of peripheral fat stored in adipocytes, followed by *de novo* lipogenesis (26%), and dietary intake (15%) [72].

2.4.2. De novo lipogenesis

Since steatosis was suggested as the "first hit" and the main contributor of fatty liver disease [2], triglyceride synthesis may play a key role in fatty liver disease development. Modulating the expression and activity of lipogenic enzymes has been the subject of many studies aiming to prevent fatty liver disease [73]. Two important sources of hepatic triglyceride synthesis are free fatty acids (FFA) released from adipose tissue as well as dietary FFA [74]. Triglyceride synthesis in the liver has different steps. Esterification of free fatty acids and glycerol is driven by glycerol-

3-phosphate acyltransferase (GPAT). Insulin upregulates while glucagon inhibits GPAT expression [75]. It was shown that increased flow of FFA to the liver induces fatty acid esterification by GPAT [76].

Insulin induces the metabolism of glucose to pyruvate and acetyl-CoA which the latter is further converted to fatty acid. Following activation by insulin, the transcription factor sterol regulatory element-binding protein 1-c (SREBP1-c) induces the expression of all enzymes involved in lipogenesis [5]. Enzymes induced by SREBP1-c include pyruvate kinase, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD) [77]. After production by insulin action, pyruvate enters mitochondria to produce acetyl-CoA. Acetyl-CoA further reacts with oxaloacetate (OAA) to produce citrate. After leaving mitochondria for the cytosol, citrate moves to the cytosol and produces acetyl-CoA, which is further converted to malonyl-CoA by the activity of the cytosolic enzyme ACC [74]. Malonyl-CoA is a key regulator of free fatty acid entry toward either esterification or β-oxidation [78]. A high concentration of malonyl-CoA stimulates lipogenesis while inhibiting β-oxidation through the inactivation of carnitine palmitoyltransferase-1 (CPT-1). Malonyl-CoA is then converted to palmitoyl-CoA by the activity of fatty acid synthase and palmitoyl-CoA is further converted to monounsaturated fatty acids, mainly oleate (C18:1) and palmitoleate (C16:1) by stearoyl-CoA desaturase [79]. SCD-1 has been shown to play a key role in the onset of diet-induced hepatic insulin resistance [80]. Higher activity of SCD-1 has been shown in NAFLD subjects (concomitant with elevated levels of hepatic oleate) [81] and in fatty livers of *ob/ob* mice [82]. SCD-1 knock-out mice are resistant to hepatic steatosis and adiposity [83, 84]. Moreover, lowering hepatic *scd-1* gene expression using sequence-specific antisense oligodeoxynucleotide in rats and mice on a high-fat diet was shown to induce insulin sensitivity, improve insulin signaling, and decrease gluconeogenesis [80]. Dietary polyphenols can protect against steatosis by decreasing the expression of genes involved in lipogenesis. For example, epigallocatechin gallate found in green tea decreased mRNA expression of *SCD1* in the liver of diet-induced obese mice suggesting a role in inhibiting lipogenesis in these mice [85]

Although hepatic steatosis is the main feature of NAFLD and NASH, the source of fat (triglyceride or toxic free fatty acid) has a critical role in developing NASH. The final step of triglyceride synthesis in the liver is catalyzed by diacylglycerol acyltransferase (DGAT). For instance, it was shown that DGAT antisense oligonucleotide decreased hepatic triglyceride content but increased

hepatic free fatty acids (FFA) which finally led to oxidative stress and fibrosis in methionine choline-deficient (MCD)-fed mice due to lipotoxicity [86]. Figure 2.1 depicts the glycolysis pathway and how its main product pyruvate generates fatty acids by a concerted action of lipogenic enzymes regulated by insulin and transcription factor SREBP-1c.

Studies report that in patients with NAFLD the capacity of the liver to export fats as very low-density lipoproteins (VLDL) is not increased in line with increased hepatic lipogenesis. Therefore, it appears that hepatic VLDL synthesis and secretion increases linearly with increased hepatic triglyceride content in normal subjects but reaches a plateau when hepatic triglyceride content exceeds 10% in NAFLD [87].

Formation of triglyceride is a temporary strategy for the liver to avoid lipotoxicity of free fatty acids in circulation. Although this strategy is beneficial for a short period, if the liver does not handle this lipid accumulation, metabolic disturbances ensue which lead to further liver damage and NASH [88].

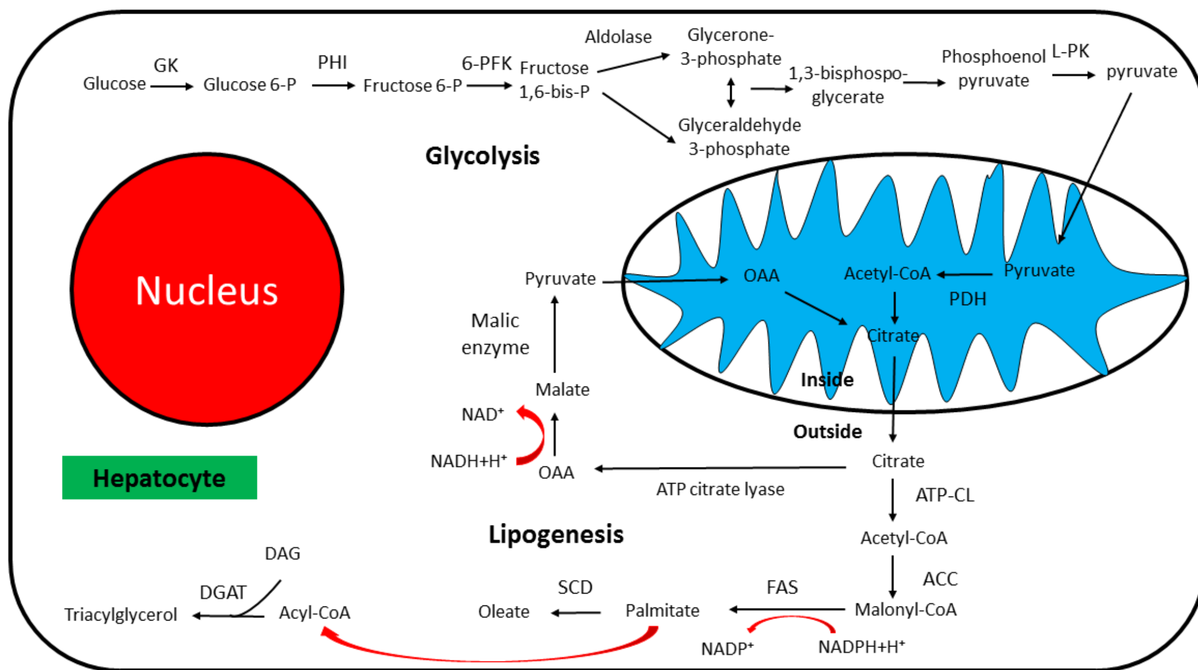

Figure 2. 1. Schematic figure of lipogenesis in hepatocytes.

This figure is adapted from Tessari et al. [79]. GK, glucokinase; PHI, phosphohexose isomerase; 6-PFK, phosphofructokinase; L-PK, liver pyruvate kinase, OAA, oxaloacetic acid; ACC, acetyl-

CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase. Following uptake by hepatocytes, glucose undergoes glycolysis to produce pyruvate. The pyruvate then enters mitochondria to produce acetyl-CoA (by the pyruvate dehydrogenase, PDH) that reacts with OAA to produce citrate which leaves mitochondria to the cytosol to regenerate acetyl-CoA again. Acetyl-CoA then produces malonyl-CoA through the activity of ACC. Malonyl-CoA is the inhibitor of fatty acid β-oxidation and alternatively induces lipogenesis. Under the activity of FAS and SCD, palmitate and oleate are synthesized which are finally re-esterified to produce triglyceride. These reactions and the expression and activity of lipogenic enzymes are regulated by insulin through the transcription factors SREBP-1c and ChREBP.

2.4.3. Adipose tissue lipolysis

Triglycerides stored in adipocytes can be mobilized to the liver by different factors including lower level of insulin and increased level of glucagon, leptin, and TNFα [89]. Lipolysis of peripheral fat stored in adipose tissue is the most important source of the plasma non-esterified fatty acid pool and hepatic lipid accumulation [90]. In patients with NAFLD, insulin is unable to prevent adipose tissue lipolysis [70]. Therefore, due to decreased sensitivity of hormone sensitive lipase (HSL) to insulin, insulin resistance enhances hepatic accumulation of fat.

2.5. The transcription factors controlling lipid metabolism and their contribution to NAFLD

SREBP1-c is an important regulator of lipogenesis which is activated by insulin in a phosphatidylinositol 3-kinase (PI3K)-dependent pathway [5]. SREBP1-c expression is upregulated in NAFLD due to a higher load of FFA to the liver and hyperinsulinemia. Studies in rodents show that any changes in nutritional status results in the regulation of SREBP1-c expression in the liver, adipose tissue, and muscle [91]. Fasting downregulates while refeeding with a high-carbohydrate diet upregulates SREBP1-c expression. In contrast to insulin, glucagon inhibits SREBP1-c transcription through the cyclic adenosine monophosphate (cAMP). Correspondingly, activation of AMP-activated protein kinase (AMPK) by the higher cellular AMP/ATP ratio inhibits SREBP1-c transcription [92, 93]. SREBP1-c has a pivotal role in the pathogenesis of NAFLD in experimental models. It has been reported that the hepatic expression of *SREBP1-c* is increased in obese insulin resistant *ob/ob* mice [94] and in diet-induced mice [95].

Liver X receptor (LXR) is also a member of the nuclear receptor family of transcription factors which has an important role in regulating fatty acid, cholesterol, and glucose metabolism [96, 97].

LXR can bind retinoid X receptor (RXR) and the resultant LXR/RXR heterodimer translocates to the nucleus to promote *de novo* lipogenesis by inducing the expression of transcription factor SREBP-1c and subsequent upregulation of its target lipogenic genes FAS and ACC [5].

SREBP1-c knock-out mice only show 50% reduction in lipogenesis [98]. This shows that other transcription factors such as the carbohydrate-responsive element-binding protein (ChREBP) is also involved in lipogenesis. Following serine residue 196 (Ser-196) dephosphorylation and consequent activation of ChREBP by glucose (by an insulin-independent mechanism), ChREBP translocates from the cytosol into the nucleus inducing lipogenic enzymes by binding to the carbohydrate response elements (ChoRE) [99]. Studies on obese mice models demonstrate the importance of ChREBP in lipogenesis and hepatic steatosis. Decreased expression of lipogenic pyruvate kinase, FAS, and ACC in response to glucose, as well as reduced hepatic lipogenesis, were observed following hepatic ChREBP gene deletion in mice [100, 101]. Nuclear expression of SREBP1-c and ChREBP was increased following the fed state in obese mice, indicating these two transcriptional regulators have very important roles in lipogenesis and hepatic steatosis.

2.6. Fatty acid β-oxidation in NAFLD

As discussed earlier, oxidation of fatty acids occurs in mitochondria and peroxisomes by β-oxidation, and in microsomes by ω-oxidation. Under normal physiologic condition, β-oxidation in mitochondria is the dominant oxidative pathway for the oxidation of fatty acids to produce ATP [9].

While short (< C8) and medium (C8-C12) chain fatty acids can freely enter mitochondria, the story for long chain fatty acids (C12-C20) is different. Carnitine palmitoyltransferase-1 (CPT-1) is the rate-limiting enzyme in mitochondrial β-oxidation. CPT-1 is located in the outer mitochondrial membrane and helps transporting long chain fatty acids into mitochondria. Malonyl CoA which is produced in lipogenesis, allosterically inhibits CPT-1 [78]. By phosphorylation and inactivation of lipogenic ACC, AMPK decreases malonyl CoA leading to increased β-oxidation of fatty acids [92]. Accordingly, it is demonstrated that there is an inverse association between mitochondrial β-oxidation and *de novo* lipogenesis [4]. Metformin is a medication for type 2 diabetes and is shown to decrease lipogenesis and hepatic gluconeogenesis by activating AMPK in hepatocytes [102].

A few studies reported the extent of β-oxidation in NAFLD. With indirect methods, these studies showed increased β-oxidation in fatty liver disease as an adaptive mechanism [103, 104]. One important limitation is that due to the lower expression and activity of mitochondrial complexes, this increased β-oxidation contributes to enhanced production of ROS through electron leakage from complex I and III. This damage to mitochondria further causes mitochondrial DNA (mtDNA) depletion and consequent defective mitochondrial function [105].

Another protein involved in β-oxidation is uncoupling protein 2 (UCP2). Uncoupling proteins are a family of mitochondrial proteins which uncouple oxidative phosphorylation from ATP synthesis. UCP2 decreases mitochondrial membrane potential ($\Delta\Psi$) leading to increased flow of electrons through the mitochondrial respiratory chain. This increased flow of electrons results in the increased re-oxidation of NADH to NAD^+ [10]. By providing substrate (NAD^+) for continuing mitochondrial β-oxidation, UCP2 induces the oxidation of excess fats in fatty liver disease.

2.7. Reactive oxygen species (ROS) and their pivotal role in NAFLD progression to NASH

Oxidative stress is the imbalance between generation of reactive oxygen and nitrogen species (ROS, RNS) and antioxidant enzymes [106]. Physiological levels of ROS have been widely implicated in some important processes including cell signaling [106, 107]. ROS is a term which refers to species such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet-}$). Following leakage of electrons by mitochondrial complex I and III and premature transfer to oxygen, superoxide radicals are generated [105].

Although it was evidenced for a long time that only complex I and III produce ROS, complex II was also shown to have an important role in substantial generation of H_2O_2 [108]. While superoxide radical is moderately active and is considered as a primary ROS, it can generate secondary ROS such as H_2O_2 [109]. However, although the superoxide radical has a very short half-life and can damage the iron-sulfur center of proteins, particularly mitochondrial complexes, it is rapidly dismutated to H_2O_2 by the superoxide dismutases. H_2O_2 can be completely reduced to water by the activity of catalase and glutathione peroxidase, or may be converted to very active hydroxyl radical by the Fenton reaction in the presence of iron [105].

Iron deposition was reported in the liver of 30% of NAFLD patients. It seems that NASH patients have more hepatic iron stores compared to NAFLD patients [110]. The mechanism of increased iron stores in these patients is not clearly understood but insulin resistance, genetic factors, erythrophagocytosis by Kupffer cells, and disturbance in molecules which regulate iron status, may have contribution [110]. This increase in iron stores as the form of ferritin (hyperferritinemia) is shown to be highly associated with metabolic syndrome, insulin resistance, and type 2 diabetes [111]. Accumulated iron may be responsible for a higher production of hydroxyl radicals by the Fenton reaction and consequent hepatic fibrosis and hepatocellular carcinoma in NASH patients. Hydroxyl radical is the most potent and dangerous radical in nature with very short *in vivo* half-life (10^{-9} s) which can attack every macromolecule such as fats, proteins, and DNA [105]. Although antioxidant enzymes help preventing formation of $OH^{\bullet-}$ radicals, once generated its damage is unavoidable.

Increased levels of oxidative stress and lipid peroxidation products, and decreased levels of antioxidant enzymes such as the superoxide dismutase (SOD) and catalase have been reported in patients with NAFLD and NASH compared to healthy subjects [112]. Some studies have looked at several oxidative stress markers to find indicators distinguishing between NAFLD and NASH. Some oxidative stress markers such as serum thioredoxin have been shown to discriminate between NAFLD and NASH and elevated serum thioredoxin is suggested to be an indicator for the severity of NASH [113]. Higher levels in NASH patients of heme oxigenase-1 (HO-1), which protects cells against oxidative stress is also suggested to reflect the severity of NASH (as an adaptive response) compared to NAFLD [114]. Some animal and human studies also showed a correlation between oxidative stress and lipid peroxidation biomarkers such as MDA and 4-HNE with the stage of NAFLD and NASH [112, 115]. Oxidative stress is the major contributor in the progression of simple steatosis to necrosis and fibrosis. Therefore, by inhibiting oxidative stress, progression of NAFLD to NASH may be halted [90]. Figure 2.2 depicts the interrelation between oxidative stress and inflammation in inducing the progression of simple fatty liver to NASH.

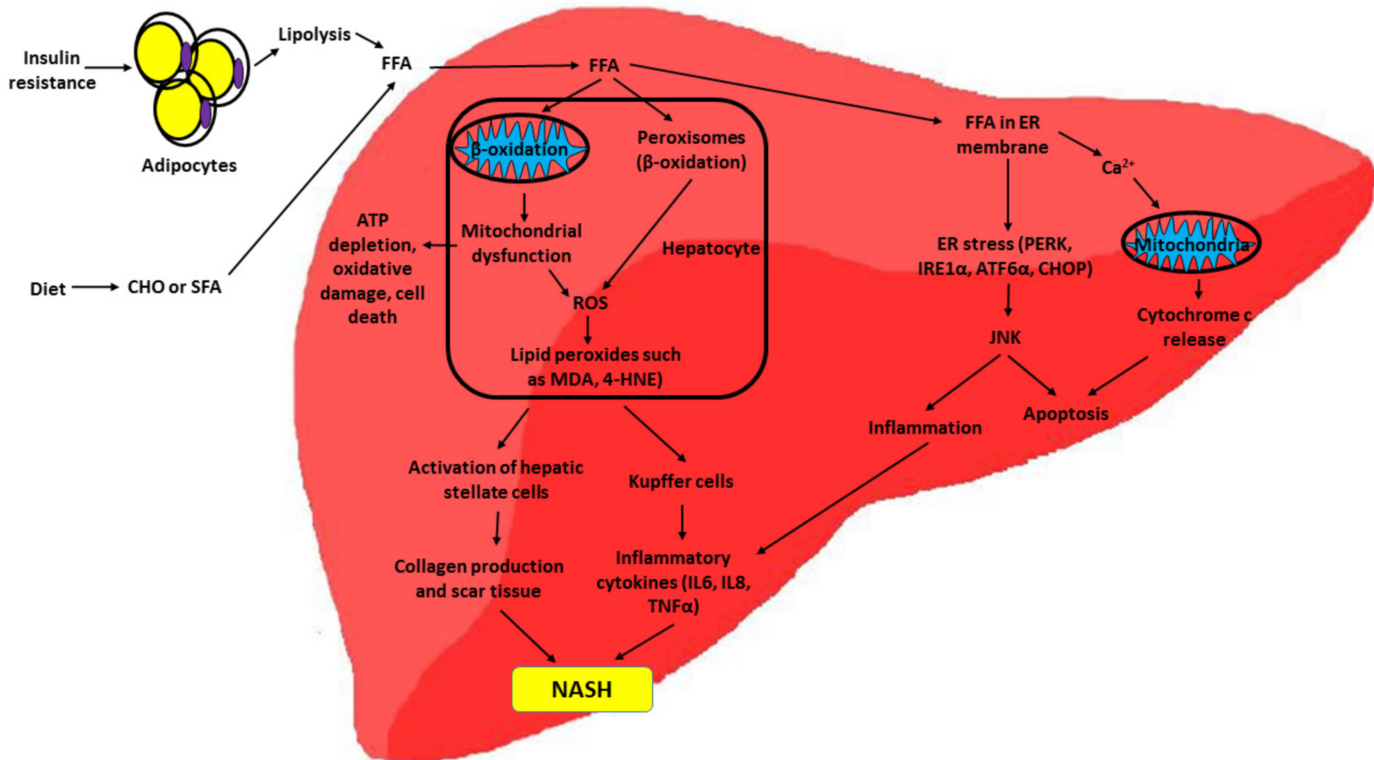

Figure 2.2. Schematic depicts the interrelationship between oxidative stress and inflammation in the progression of NAFLD to NASH

This figure is adapted from Ucar et al. [116]**.** Insulin resistance facilitates lipolysis in adipocytes which along with a high-fat and high-sugar diet increases the accumulation of fat in the liver. The excess fat undergoes β-oxidation in hepatic mitochondria and peroxisomes leading to mitochondrial dysfunction and production of ROS. ROS in turn may damage membrane lipids and produce lipid peroxidation products such as MDA and 4-HNE. Although ROS have a short half-life, lipid peroxides have a longer half-life and can leave hepatocytes and therefore prolong ROS-mediated damage. Lipid peroxides can further induce Kupffer cells (liver residing macrophages) to produce inflammatory cytokines such as TNFα and IL6. Lipid peroxides may also activate hepatic stellate cells which produce collagen and finally leading to scar tissue and fibrosis. Incorporation of saturated fatty acids into the endoplasmic reticulum (ER) membrane can also enhance the stiffness of the membrane leading to ER stress and release of calcium from ER which then accumulate in mitochondrial matrix. The increase in calcium results in opening of mitochondrial permeability transition pores (MPT) and consequent release of cytochrome c which may induce apoptosis. ER stress may also increase phosphorylation and activation of JNK causing inflammation which along with higher ROS produced by defective mitochondria may facilitate progression of simple steatosis to NASH.

The redox state of the mitochondrial respiratory chain mainly determines ROS production. Chronic intake of a high-fat high-sucrose diet (a frequent contributor to obesity and fatty liver) increases the flow of electrons to mitochondria which exceed the capacity of the mitochondrial electron

transport chain. In this case, increased production of NADH and $FADH_2$ in the tricarboxylic acid cycle and higher transfer of electrons to complex I and II leads to over-reduction of mitochondrial complexes (reviewed in [105]). Due to increased entry of electrons to the electron transport chain as well as increased load of protons in the intermembrane space, the dissipation of the proton gradient by complex V (ATP synthase) is not sufficient to overcome this load of electrons. This phenomenon results in over-reduction of mitochondrial complexes, leakage of electrons, and subsequent ROS production. This increased ROS generation cannot be sufficiently scavenged by mitochondrial antioxidant enzymes leading to mtDNA damage (reviewed in [105]). This damage to mtDNA may decrease the synthesis and assembly of mitochondrial complex subunits causing further ROS production. This vicious cycle caused by reactive oxygen and nitrogen species (RNS) in NASH patients may lead to lower activity of respiratory complexes and perturbation of mitochondrial homeostasis.

By activation of enzymes involved in lipid peroxidation such as lipoxygenase, cyclooxygenase, and cytochrome P450, ROS may cause NAFLD progression to NASH [117]. Furthermore, by inducing serine phosphorylation of insulin receptor substrate-1 (IRS-1), ROS may induce insulin resistance which is one of the main contributors of fatty liver disease [48].

2.7.1. Sources of reactive oxygen species in NAFLD

2.7.1.1. Mitochondria

Although several sources of intracellular ROS have been identified including the endoplasmic reticulum, cytosol, and peroxisomes [118, 119], mitochondria are quantitatively the most important source of ROS (by about 90 %) due to the higher oxygen consumption [120]. Under normal physiologic conditions only 1-2 % of oxygen consumed leads to ROS production [120]. This mitochondrial-generated ROS is primarily determined by the metabolic state. Complex I and III produce the highest amount of ROS in mitochondria [118].

2.7.1.2. Peroxisomes

During fatty liver disease both mitochondria and peroxisomes work in concert to metabolize excess fatty acids. However, due to the increased activity of peroxisomal acyl-CoA oxidase, which is a

key enzyme in peroxisomal β-oxidation, excess ROS is produced [121]. Like mitochondria, peroxisomes have a series of antioxidant enzymes including catalase to detoxify ROS generated by peroxisomal fatty acids β-oxidation. It was reported that in some pathological conditions a five-fold increase in peroxisomal β-oxidation results in less than a 10% increase in mRNA expression of peroxisomal antioxidant enzymes. This disequilibrium between peroxisomal ROS generation and removal systems show that peroxisomes can be a potential source of ROS generation during lipid over accumulation states such as steatohepatitis [122].

2.8. Antioxidant enzymes in NAFLD

Oxidative stress can be the consequence of an imbalance between ROS generation and antioxidant enzymes [116]. As discussed earlier, oxidative stress following mitochondrial dysfunction in NAFLD/NASH may damage mitochondrial and nuclear DNA, proteins, fats, and causes apoptosis [5]. ROS generated by mitochondria may be neutralized by antioxidant enzymes. Mitochondria possess a number of antioxidant enzymes and therefore are considered as a sink for ROS removal instead of ROS producing organelles in normal physiologic conditions. The major antioxidant enzymes in the cells include the superoxide dismutase 1 (Cu/Zn-SOD) and superoxide dismutase 2 (Mn-SOD) which are mainly found in the cytosol and mitochondria respectively and can dismutate superoxide radical to H_2O_2 [116].

Deletion of Mn-SOD leads to neonatal death in mice [123]. The lethality is apparently due to the damage mediated by oxidative stress [123]. Moreover, mice deficient in Mn-SOD suffer from metabolic dysfunction and the accumulation of intracellular fat in the liver and muscle [124]. These symptoms are attributed to the reduced activity of aconitase [an iron-sulphur (Fe-S) protein] that is very sensitive to superoxide radicals [125]. Therefore, it was suggested by the authors that one of the main functions of Mn-SOD is to prevent any oxidative damage to aconitase. Besides aconitase, other proteins and complexes in the mitochondrial transport chain contain Fe-S proteins. Accordingly, Mn-SOD activity is very important to keep these complexes functioning to metabolize excess fatty acids in NAFLD. Takami et al. reported higher serum levels of Mn-SOD in NAFLD and NASH patients compared to simple steatosis suggesting this level may be a distinguishing marker of NASH [126].

Besides catalase, mitochondria possess other enzymes to detoxify H_2O_2. These enzymes include mitochondrial glutathione peroxidase (GPx) and glutathione reductase, which utilize glutathione as an electron donor, and mitochondrial thioredoxin peroxidase and thioredoxin reductase, which use thioredoxin as an electron donor, to detoxify H_2O_2 [120]. It has been reported that GPx activity is unchanged or decreased in the serum of NASH patients compared to the control group [127-129].

In other studies related to NASH, decreased liver glutathione and lower activity of antioxidant enzymes GPx, SOD, and catalase was reported and this decrease was in association with the severity and stage of NASH [105, 130]. One explanation for decreased activity of GPx in NASH is due to a decrease in reduced mitochondrial glutathione which is needed for GPx activity. Lower import of cytosolic glutathione to mitochondria may contribute to the decline in mitochondrial glutathione levels in NASH [131].

2.9. Effect of inflammatory cytokines in NAFLD

Cytokines are soluble molecules produced by different cells such as hepatocytes that have important roles in intercellular communications. Under normal circumstances, the expression of cytokines in the liver is absent or very low. Cytokines play a central role in the initiation and progression of NAFLD by the activation of liver inflammation, necrosis, fibrosis, and apoptosis [132].

Increased ROS production in NAFLD induces the expression of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) which are able to perturb insulin signaling and consequently contribute to the lipid and glucose metabolism disruption [133]. Besides ROS, lipid peroxidation aldehydes also induce the expression of inflammatory cytokines leading to inflammation, neutrophil chemotaxis, and necrosis in NASH (reviewed in [2]).

TNFα can be secreted by any of hepatocytes, Kupffer cells, or visceral fat adipocytes [134]. The expression of TNFα in adipocytes and hepatocytes of NASH patients was shown to be higher compared to the normal group and this was associated with hepatic fibrosis [135]. Some studies demonstrated the association between TNFα expression and insulin resistance in NASH [136]. In addition, an accumulating body of evidence shows the central role of TNFα and other

inflammatory cytokines in the progression of simple steatosis to NASH. There is a strong association between circulating level of inflammatory cytokines and the severity of fatty liver disease and fibrosis [21].

Moreover, by the activation of nuclear factor kappa B (NF-κB), increased load of fatty acids in hepatocytes brings about hepatic insulin resistance [137]. Once activated, transcription factor NF-κB translocates to the nucleus and induces the expression of several pro-inflammatory genes such as TNFα, IL6, IL8, transforming growth factor-β (TGF-β) and Fas ligand. By inducing the expression of TGF- β, Kupffer cells activate hepatic stellate cells to produce collagen resulting in fibrosis in NASH. Lipid peroxidation aldehydes and ROS can activate hepatic stellate cells to produce collagen [137].

2.10. Mitochondria and their contribution to fatty liver disease

2.10.1. Mitochondria are powerhouse of the cells

Liver is rich in mitochondria and approximately 800 mitochondria are available in each hepatocyte. These mitochondria occupy 18% of the volume of each hepatocyte [9]. Mitochondria are involved in both free fatty acid β-oxidation and ROS generation [3].

The mitochondrial respiratory chain consists of five complexes (I-V) which transfer electrons from NADH and $FADH_2$ to oxygen to produce water. During this process NADH and $FADH_2$ are converted to oxidized NAD^+ and FAD. According to the chemiosmotic theory of Mitchel, during this transfer of electrons, protons (H^+) are pumped out of the mitochondrial matrix into the intermembrane space by complex I, III, and IV generating higher mitochondrial membrane potential (ΔΨ) [138]. When energy is needed, protons enter mitochondria through the F_o subunit of ATP synthase (complex V) and this drives phosphorylation of ADP to ATP [139]. If electron flow through mitochondrial complexes is interrupted at any point, electrons transfer to oxygen prematurely and this causes generation of reactive oxygen species such as superoxide radicals or hydrogen peroxide [140].

2.10.2. Mitochondrial biogenesis helps to fuel hepatocytes with healthy and functional mitochondria

Mitochondrial biogenesis involves two processes: mitochondrial replication and differentiation. Mitochondrial replication is defined as synthesizing mtDNA and new biomolecules, while differentiation affects pre-existing mitochondria and improves mitochondrial function by increasing the mitochondrial inner membrane with more cristae and overexpression of mitochondrial proteins such as the respiratory complex subunits [141]. Fatty liver has been associated with reduced PGC1α mRNA or activity in the muscle and liver of rodents, and therefore reduced mitochondrial biogenesis [142, 143]. PGC-1α is a transcriptional factor which is very important for regulating the expression of genes involved in mitochondrial biogenesis, function, and energy metabolism [144]. PGC-1α co-activates nuclear respiratory factors (NRF1, NRF2) leading to increased synthesis of nuclear DNA-encoded proteins involved in the electron transport chain. Moreover, NRFs induce mitochondrial transcription factor A (TFAM) which in turn induces the expression of mitochondrial DNA-encoded subunits of the electron transport chain [10]. TFAM also regulates stability, transcription, and replication of mtDNA [143]. Activation of PGC1α induces the expression of genes involved in fatty acid β-oxidation, antioxidant enzymes, lipid transport, and gluconeogenesis [144-147]. For instance, PGC-1α cooperates with PPARα to induce the expression of CPT1 and medium chain acyl-CoA dehydrogenase [10]. The result of these concerted mechanisms is the increased number of healthy and functional mitochondria and consequent metabolism of excess fat [10].

2.10.3. NAFLD/NASH: a mitochondrial disease

An accumulating body of evidence suggests NAFLD as a mitochondrial disease due to the abnormality in function and morphology of hepatic mitochondria [5, 9, 10]. This is due to the pivotal role of mitochondria in lipid metabolism, ROS generation, lipid peroxidation, cytokine production, and apoptosis [4, 13].

One of the mechanisms by which mitochondrial dysfunction exacerbates NAFLD is due to the higher ROS production by defective mitochondria. ROS and RNS may damage mitochondrial DNA leading to mitochondrial DNA depletion and consequent downregulation of mitochondrial complex subunits expression [15, 17]. Moreover, as mentioned earlier, ROS can damage the iron-

sulfur cluster enzymes [148] in the mitochondrial electron transport chain leading to mitochondrial dysfunction. Figure 2.3 depicts the mechanisms by which a high-fat high-sugar diet may cause mitochondrial dysfunction, oxidative stress, and the depletion of ATP stores in hepatocytes.

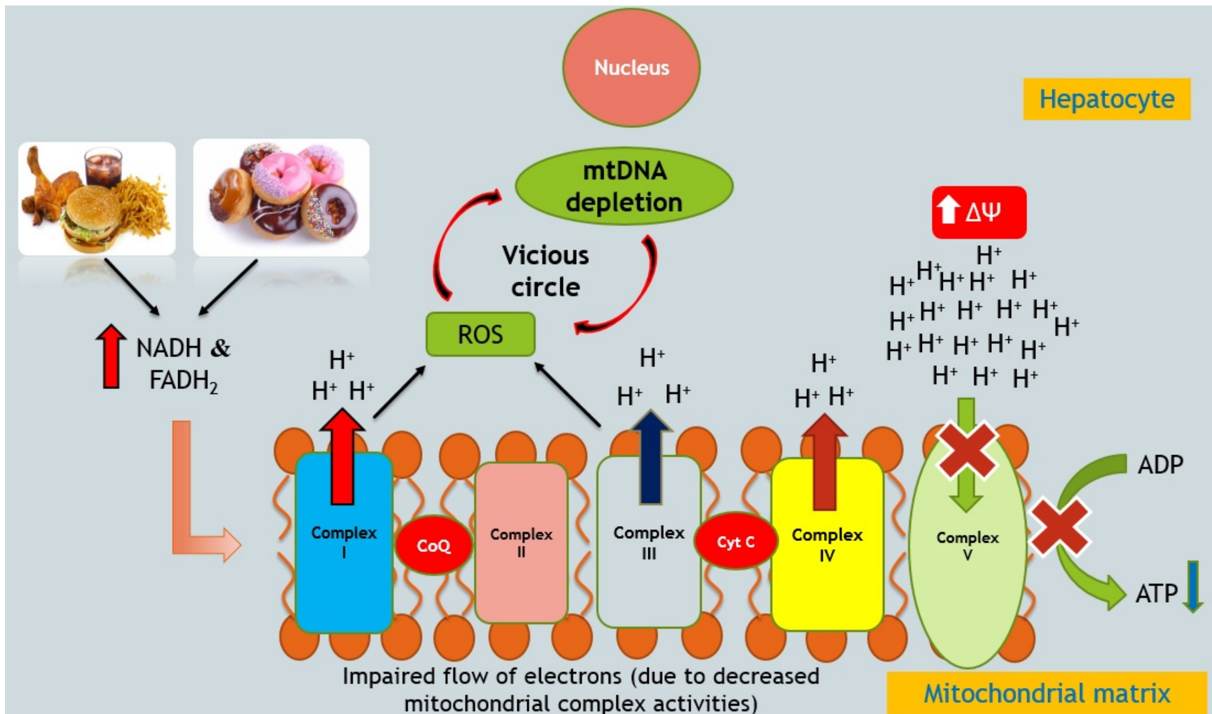

Figure 2. 3. Mechanisms of the vicious cycle of mitochondrial dysfunction and damage induced by nutrient excess.

Higher intake of a high-fat high-sugar diet produces more electron donors (NADH and $FADH_2$) to the mitochondrial electron transport chain which causes more protons to be pumped out to the intermembrane space through complex I, III, and IV. Due to reduced activity of all mitochondrial complexes in NAFLD, electron flow slows down in mitochondria leading to premature transfer of electrons to oxygen to produce ROS which can damage mitochondrial DNA to produce more ROS. Moreover, due to the lower activity of ATP synthase, less protons can enter mitochondria through this complex leading to the depletion of ATP stores in NASH which makes hepatocytes susceptible to oxidative damage and necrosis.

Mitochondrial DNA depletion which is very difficult to be repaired causes reduced expression of complex I, III, IV, and V subunits (due to encoding 13 polypeptides involved in the electron transport chain by mitochondrial DNA) [3]. Pérez-Carreras et al. for the first time reported the lower activity of all the mitochondrial respiratory complexes in the liver of patients with NASH [14]. Following this finding, studies were conducted to explore the mechanisms of mitochondrial dysfunction in animal models of NASH. It was later shown that increased TNFα and inducible

nitric oxide synthase (iNOS) and tyrosine-nitrated proteins in the liver of *ob/ob* mice [16] are some of the contributors for mitochondrial complex dysfunction. For instance, it was reported that TNFα induces the mitochondrial release of cytochrome c leading to a decreased flow of electrons in mitochondria [16].

Cellular ATP levels are also important in maintaining the integrity of hepatic tissue and ATP depletion may result in hepatocellular injury. Evidence shows that the hepatic ATP stores become depleted in experimental models of NAFLD using methionine-choline deficient diet [149]. One study comparing hepatic ATP stores between normal and NASH subjects showed similar depletion of hepatic ATP stores but delayed recovery in ATP stores in NASH [150]. This inability in restoring ATP stores after a stress such as oxidative stress may make hepatic cells vulnerable to hepatocellular injury and cirrhosis in NASH. Mitochondrial injury is considered as an important contributor in depleted hepatic ATP stores in NASH [151].

The lower hepatic ATP levels in NASH may also be due to the increased expression of UCP2. UCP2 which is discussed in details in section 2.12 is a mitochondrial membrane protein which uncouples oxidative phosphorylation from ATP generation. It is shown that UCP2 expression increases in experimental models of NAFLD [48]. This enhanced expression of UCP2 may contribute to the dissipation of energy which in turn reduces ATP synthesis in NAFLD. During uncoupling, each mitochondrion may generate a lower amount of ATP for a given amount of oxygen consumed [151].

Interestingly, several studies report morphological abnormality of mitochondria in patients with fatty liver disease. These mitochondria are enlarged, rounded and swollen (megamitochondria), scarce in number, hypodense in matrix, with loss of cristae which impairs mitochondrial function in NAFLD/NASH patients [9, 103, 152, 153].

2.10.4. Evidence regarding impaired mitochondrial oxidative phosphorylation (OXPHOS) capacity in NASH

There have been conflicting findings about OXPHOS dysfunction in different tissues in patients with NASH. For the first time, Caldwell et al. showed normal activity of complex I and III in the platelets of subjects with NASH [152]. Sanyal et al. also could not show any defect in the muscle

mitochondrial respiratory chain in a patient with NASH [103]. Perez-Carreras et al. however showed decreased activity of all of the five mitochondrial complexes in liver tissues of patients with NASH [14]. Researchers also showed that mitochondrial DNA is severely depleted in the muscle of patients with alcoholic fatty liver disease and this in turn resulted in impairment of mitochondrial function [154].

Dietary studies in laboratory animals have also shown differing results. Chavin et al. [155] showed increased activity of mitochondrial complexes in the liver of obese mice, suggesting an adaptive mechanism to metabolize increased load of fatty acids [139]. In contrast, García-Ruiz et al. demonstrated reduced assembly and activity of all mitochondrial complexes by 50-60% in mice fed a high-fat diet [17]. They found reduced mRNA expression of mitochondrial DNA-encoded complex subunits but not nuclear DNA-encoded ones. In line with this result, they found oxidative damage to mitochondrial DNA but not nuclear DNA. They concluded that the high-fat diet decreased OXPHOS complexes activity due to the reduced expression of their subunits which results in impaired full assembly of mitochondrial complexes. Decreased mitochondrial complex activity in obese *ob/ob* mice is partially due to an increased oxidative stress, and treatment with antioxidants prevented the decrease [16].

2.11. Role of PPARs in metabolism

PPARs are members of a nuclear receptor subfamily. There are three isoforms of PPARs: PPARα, PPARβ/δ, and PPARγ. Fatty acids are natural ligands of PPARs. Synthetic activators of PPARα and PPARγ are fibrates and thiazolidinediones (TZDs), respectively [156]. PPARs have a pivotal role in metabolism since it was shown that the activation of PPARα and PPARγ by fibrates and TZDs alleviates diabetes type 2 and dyslipidemia [157]. Furthermore, transcriptional control of genes by PPARs plays a key role in lipid metabolism (particularly hepatic steatosis), inflammation, and fibrosis [156].

2.11.1. The importance of PPARα in protection against NAFLD

PPARα is highly expressed in metabolically active tissues such as the liver and skeletal muscles. Higher PPARα expression plays a central role in inducing hepatic fatty acid β-oxidation in NAFLD [156]. Once activated, PPARα binds to retinoid X receptor alpha (RXR-α) and induces fatty acid

oxidation in mitochondria, peroxisomes and endoplasmic reticulum, as well as inducing lipoprotein lipase, and gluconeogenesis [158]. These effects will together result in more availability of fatty acids for β-oxidation and consequently alleviation of hepatic fatty liver. It is shown that the expression of PPARα is decreased in experimental models of steatosis [95].

2.11.2. The controversial role of hepatic PPARγ in NAFLD

Although PPARγ is a regulator of adipogenesis in adipocytes, its expression in hepatocytes is very low except in pathological conditions, in which its expression increases and regulates metabolic homeostasis [157]. The role of hepatic PPARγ in fatty liver disease is controversial. Many studies show that hepatic PPARγ expression increases in diabetic or obesity models of NAFLD which results in increased hepatic lipogenesis [44, 95, 159]. In contrast, adenovirus-mediated over-expression of PPARγ in rodents with NASH decreased hepatic steatosis, inflammation, and necrosis [160]. In line with this study, heterozygous mice deficient for PPARγ (PPARγ$^{+/-}$) showed lower hepatic mRNA and protein for PPARγ concomitant with more severe hepatic steatosis with a methionine- and choline-deficient diet. The effects were alleviated after adenovirus-mediated over-expression of PPARγ [161]. Interestingly, by reducing the proliferation of hepatic stellate cells, PPARγ over-expression in the liver by adenovirus-expressing PPARγ was shown to prevent hepatic fibrosis in mice [162]. One of the mechanisms of protective effect of PPARγ against NAFLD is due to increased insulin sensitivity in the adipose tissue and skeletal muscles [163, 164] and consequent reduction of adipocyte lipolysis. While it appears that enhanced level of PPARγ in the liver may promote lipogenesis, its expression in the adipose tissue contributes to adipocyte differentiation, promotes fat storage in adipocytes, and increases insulin sensitivity [165], all favoring reduced adipocyte lipolysis and amelioration of NAFLD. In conclusion, evidence about PPARγ effects on the hepatic steatosis is controversial and needs to be further investigated.

2.12. Uncoupling proteins (UCPs)

As discussed earlier, uncoupling proteins are mitochondrial inner-membrane proteins that uncouple mitochondrial respiration from oxidative phosphorylation and therefore reduce ATP synthesis [166]. There are several isoforms of UCPs such as UCP1 (brown adipose tissue), UCP2 (ubiquitous), UCP3 (brown adipose tissue and skeletal muscle), UCP4 and UCP5 (brain) [167].

Although UCP2 is ubiquitous, its expression is very low in hepatocytes and mostly localized in the Kupffer cells [166].

2.12.1. UCP2 impact on hepatic reactive oxygen species and ATP stores

UCP2 is localized in the inner mitochondrial membrane and was shown to increase the flow of electrons through the mitochondrial respiratory chain inhibiting premature transfer of electrons to oxygen through complexes I and III. This higher flow of electrons leads to less production of ROS in mitochondria [168]. Moreover, by uncoupling the electron transport chain from ATP production, UCP2 decreases the electrochemical potential ($\Delta\Psi$) [90, 169]. Studies report that even small changes in $\Delta\Psi$ can have an order of magnitude effect on mitochondrial ROS generation [168]. This decrease in $\Delta\Psi$ by increased UCP2 expression is responsible for a decline in ROS generation.

As discussed earlier one of the limitations of increased UCP2 expression is dissipating energy and therefore decreasing ATP stores. ATP stores are very important in NAFLD as evidence shows that impaired ATP homeostasis is observed in NAFLD and NASH [18, 170]. It is reported that a decline in ATP stores makes the liver vulnerable to inflammation, fibrosis and necrosis and helps progression of simple steatosis to steatohepatitis (NASH) [150]. Interestingly, it is suggested that this decline in energy is not always the case in hepatic steatosis but it can manifest when the liver is in an acute need for energy at the advanced stages of NAFLD [139].

2.12.2. UCP2 and amelioration of fatty liver disease

An accumulating body of evidence reports increased expression of UCP2 in fatty liver disease [48, 171]. This up-regulation of hepatic UCP2 may induce fatty acid β-oxidation and alleviate fatty liver disease and diabetes [166, 168]. The mechanisms which were suggested include: 1) Proton leak through UCP2 may be a contributing factor in the induction of β-oxidation of fatty acids in mitochondria. This induction in β-oxidation is mediated through higher re-oxidation of NADH/NAD^+, leading to increased ratio of NAD^+/NADH which is needed for ongoing β-oxidation [10]. 2) It seems that UCP2 prevents intra-mitochondrial accumulation of free fatty acid and therefore facilitates the export of FFA from the mitochondrial matrix to the cytosol, leading

to decreased mitochondrial lipotoxicity and subsequently prevents mitochondrial dysfunction [171].

Moreover, by preventing apoptosis [172, 173] possibly through reducing ROS [174], UCP2 is suggested to be beneficial against NASH [166]. However, this decrease in apoptosis may increase the chance of hepatocellular carcinoma. Therefore, UCP2 is considered as a double-edge sword since it can exert both beneficial and deleterious effects and its role in fatty liver disease should be further evaluated. In total, UCPs are considered as a beneficial mechanism for cells to adapt with high amount of intracellular fatty acid accumulation [166].

2.13. Effect of sirtuins and AMP-activated protein kinase (AMPK) in NAFLD

Silent information regulator 2 proteins (sirtuins) are a group of NAD^+-dependent deacetylases. There are different isoforms of sirtuins: SIRT1-SIRT7. SIRT1 (localized in cytoplasm and nucleus), SIRT3 (mitochondria, nucleus), and SIRT6 (nucleus) have important roles in metabolism. Mitochondria are one of the main targets of sirtuins action (particularly SIRT1, SIRT3, SIRT4, and SIRT5) leading to the regulation of metabolism [175].

SIRT1 is the most studied sirtuin which has a pivotal role in the metabolic pathways such as lipid and glucose metabolism [176, 177]. SIRT1 induces mitochondrial biogenesis through activating AMPK and PGC1α and therefore increases fatty acid β-oxidation that might be of great importance for alleviating NAFLD [178]. It is shown that prolonged fasting results in SIRT1-mediated deacetylation and consequent activation of PGC1α and PPARα leading to increased β-oxidation of fatty acids and the regulation of glucose metabolism [179, 180].

SIRT1 and AMPK can activate each other. Excess calorie intake was shown to increase the $NADH/NAD^+$ ratio which decreases deacetylase activity of SIRT1. Reduced activity of SIRT1 in turn reduces phosphorylation of AMPK by its upstream serine/threonine liver kinase (LKB1) leading to higher lipogenesis and decreasing fatty acid β-oxidation. Vice versa, during starvation or exercise, increased $NAD^+/NADH$ ratio activates SIRT1 which in turn deacetylates LKB1 leading to increased phosphorylation of threonine 172 (Thr^{172}) on AMPK catalytic α subunit and its subsequent activation (reviewed in [181]). AMPK which is an important regulator of energy homeostasis and is sensitive to increased levels of intracellular cAMP, increases during starvation

causing inhibition of all the ATP-consuming pathways such as lipogenesis, and induces the ATP-producing pathways such as β-oxidation. (reviewed in[181]). The major effect of SIRT1 on hepatic lipid metabolism was demonstrated by SIRT1 knock-out mice. In one study, knocking-out hepatic SIRT1 resulted in reduced β-oxidation of fatty acids due to the impaired PPARα signaling. Therefore, these mice were more susceptible to a high-fat diet-induced hepatic steatosis [182]. In addition to the effects on mitochondrial biogenesis, fatty acid oxidation, and cholesterol metabolism, SIRT1 inhibits the transcription of all genes involved in lipogenesis by direct deacetylation of SREBP [183, 184].

SIRT3 is mostly localized in mitochondria but also has a very important role in energy metabolism including fatty acid β-oxidation, ketogenesis, tricarboxylic acid cycle (TCA), and the mitochondrial respiratory chain [185]. For instance, several studies have shown a pivotal role of SIRT3 in hepatic fatty acid β-oxidation of experimental animals fed a high-fat diet. $SIRT3^{-/-}$ mice develop hepatic lipid dysregulation due to disrupted β-oxidation of fatty acids [186]. Moreover, SIRT3 regulates mitochondrial electron flow and oxidative stress [185]. Evidence shows that over-expression of SIRT3 induces PGC-1α-mediated mitochondrial gene expression and subsequent mitochondrial electron transport chain activity while ROS generation was suppressed [187]. SIRT3 has a major role in mitochondrial energy metabolism following the observation that SIRT3 knock-out mice develop 50% less ATP content in the liver, kidney, and heart [188]. One of the mechanisms by which SIRT3 increases ATP generation is through the activation of AMPK [185].

2.14. Endoplasmic reticulum (ER) stress

Ozcan et al. for the first time suggested that ER stress may be a missing link in hepatic steatosis and insulin resistance due to the activation of ER stress arms in obese rats fed a high-fat diet [25]. Since then, several studies reported increased ER stress in animals [27, 28] and humans [29, 30] with hepatic steatosis.

The smooth endoplasmic reticulum is involved in the metabolism of proteins, lipids, and carbohydrates, and in protein folding and maturation [23]. Lipogenesis is shown to be affected by ER stress and the two processes appear to have a direct and positive correlation. Moreover, it seems that oxidative stress results in the disruption of ER homeostasis and therefore brings about the activation of a cellular response called the unfolded protein response (UPR). The aim of this

response is to decrease the load of proteins to endoplasmic reticulum and to restore endoplasmic reticulum homeostasis [24].

2.14.1. Different arms of ER stress and their role in NAFLD and insulin resistance

Unfolded protein response (UPR) has three signaling pathways (3 arms) initiated by the activation of endoplasmic reticulum transmembrane proteins: 1) protein kinase RNA-like endoplasmic reticulum kinase (PERK), 2) inositol requiring enzyme 1 (IRE1) and 3) activating transcription factor 6 (ATF6) [189]. Bound to the chaperone glucose-regulated protein 78 kDa (GRP78), all of these three arms remain inactive. It was shown that over-expression of GRP78 improves hepatic steatosis due to suppressing SREBP-1c activity [27]. Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), a downstream target of PERK causes increased expression of CCAAT enhancer-binding-proteins (C/EBPs), PPARγ, and SREBP-1c leading to hepatic steatosis by a high-fat diet [190]. Therefore, the PERK-eIF2α pathway induces lipogenesis in the liver and other tissues.

Regarding the 2nd arm, phosphorylation of IRE1 results in splicing and activation of its downstream effector, X-box binding protein 1 (XBP1), which controls lipogenesis. Therefore, XBP1s is also steatotic and causes the activation of lipogenic enzymes [24]. Regarding the 3rd arm, once being dissociated from GRP78, ATF6 translocates to the Golgi apparatus to be activated. ATF6 causes protection against steatosis and leads to increased β-oxidation (Reviewed in [190]).

Evidence also shows that insulin resistance (one of the main factors implicated in the pathophysiology of NAFLD) is closely associated with ER stress [191]. Insulin resistance can be directly affected by the activation of ER stress in adipocytes. Indeed, elevated ER stress evidenced by IRE1 activation was shown to activate JNK which induces insulin resistance [191]. It is revealed that treatment of obese and diabetic *ob/ob* mice which develop ER stress with active chemical chaperones such as 4-phenyl butyric acid (4-PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) alleviates ER stress and consequently resolves hyperglycemia, insulin resistance, and fatty liver disease [192]. Chemical chaperones also decrease ER stress and inflammation in adipose tissue, and reduce hepatic lipid content of mice fed a high-fat diet for 14 weeks [193]. Figure 2.4 depicts the interrelationship between different arms of UPR and how they affect hepatic lipid metabolism to finally induce hepatic steatosis.

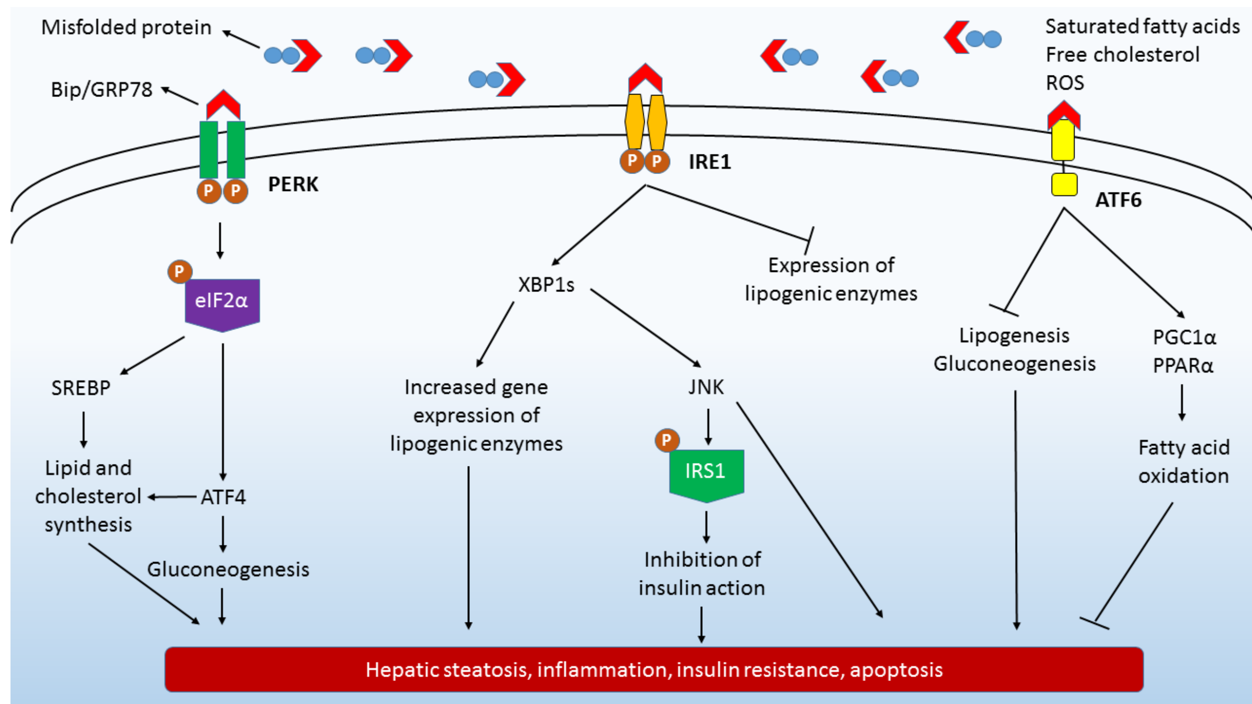

Figure 2. 4. Crosstalk between ER stress arms and lipogenesis

This Figure is adapted from Basseri & Austin [190]**.** Higher intake of saturated fatty acids and enhanced ROS generation increases the load of misfolded proteins to ER. Accordingly, the chaperone GRP78 dissociates from different arms of ER (PERK, IRE1, ATF6) in order to help proper folding of the misfolded proteins. While attached to GRP78, these arms are inactive and following dissociation become activated. Following dissociation from GRP78, PERK induces phosphorylation of eIF2α which can further increase lipogenesis and gluconeogenesis through increased expression of ATF4 and SREBP. Although IRE1 inhibits the expression of lipogenic enzymes, its downstream effector (XBP1s) activates JNK leading to impaired insulin signaling, inflammation, and apoptosis. XBP1s can also increase the expression of lipogenic enzymes further exacerbating hepatic steatosis. Moreover, following cleavage and activation by the Golgi apparatus, ATF6 inhibits lipogenesis and gluconeogenesis, and induces fatty acid oxidation through activating PGC1α and higher expression of PPARα.

2.15. Models for studying NAFLD and the molecular mechanisms underlying the disease

2.15.1. Experimental models of NAFLD/NASH

An ideal animal model should represent the pathophysiology of both human NAFLD and NASH. Accordingly, along with hepatic steatosis, inflammation, hepatocellular ballooning and fibrosis, an ideal animal model should reflect metabolic abnormalities such as obesity, insulin resistance, dyslipidemia, and aberrant adipokines profile [194]. Generally, available models are categorized

into genetic models including *ob/ob* and *db/db* as well as KK-A^y mice, and dietary models including methionine- and choline-deficient diet (MCD), and high-fat diet [194].

2.15.1.1. Genetic models of NAFLD

2.15.1.1.1. *Ob/ob* and *db/db* mice

Ob/ob mice have a mutation in the leptin gene (leptin deficient) while *db/db* mice have a mutation in the leptin receptor (leptin resistant) and these mice show obesity, insulin resistance, and NAFLD [195]. Leptin is an adipokine produced by the white adipose tissue that has anorexic effects on the hypothalamus which is the center for satiety and hunger [196]. *Ob/ob* mice are hyperphagic, inactive, and extremely obese while showing metabolic abnormalities such as insulin resistance, dyslipidemia, and hyperglycemia [195]. Although hepatic steatosis is a main feature in these mice, it can not spontaneously progress to NASH and some triggers are essential including a high-fat or MCD diet to develop NASH [197]. *Ob/ob* mice are protected against hepatic fibrosis which led to elucidating a major role for leptin in hepatic fibrogenesis.

Db/db mice have normal level of leptin but they are resistant to it. These mice are obese and insulin resistant and develop hepatic steatosis. Along with the administration of a second insult such as MCD diet, these mice develop hepatic fibrosis [198]. One of the main advantages of *ob/ob* and *db/db* mice is that they show metabolic abnormalities similar to human metabolic syndrome while the disadvantage of using them is that they do not spontaneously develop hepatic fibrosis [194].

2.15.1.2. Dietary models of NASH

2.15.1.2.1. Methionine- and choline-deficient diet

This diet lacks methionine and choline which are essential for fatty acid β-oxidation and VLDL synthesis [196]. Therefore, excess fat accumulates in the liver and along with oxidative stress and increased adipocytokines result in fibrosis [199]. The main advantage of this model is that it can lead to accumulation of large amount of fat in the liver, and they may develop fibrogenesis compared to other models. However, the disadvantage of them is that rodents on this model show reduced body weight (20%) and food intake, and they do not show metabolic abnormalities similar

to human metabolic syndrome and they have low leptin and insulin levels with low fasting blood glucose [194]. In order to cope with these disadvantages, genetically obese animals are chosen to put on a MCD diet.

2.15.1.2.2. High-fat diet

A high-fat diet is used by a wide range of experimental studies to simulate the obesity and fatty liver disease induced by a Western diet in humans [194]. According to fat content and composition, rodent strains, and duration, a high-fat diet result is variable results regarding hepatic steatosis, oxidative stress, inflammation, and fibrogenesis [194]. For example, Sprague-Dawley rats are specifically more susceptible to a high-fat diet-induced obesity and steatohepatitis compared to Wistar rats [199, 200].

2.15.2. *In vitro* models

Previously, experimental studies (genetic models and high-fat diet) have been designed to study NAFLD development and its progression to NASH [31, 32]. More recently, *in vitro* models of NAFLD have been developed to study the effects of steatosis in hepatic cell lines of human origin [33, 34] and to explore the molecular mechanisms underlying NAFLD. These models include using monounsaturated (oleic), and saturated (palmitic) fatty acids to induce steatosis in a hepatocyte cell line (HepG2) and in primary hepatocytes [33-38]. Oleic and palmitic acids are the most abundant fatty acids in the hepatic triglycerides of healthy subjects and patients with fatty liver disease [201]. Palmitic acid is the most prevalent saturated fatty acid found in triglycerides of meats and dairy products, as well as in palm oils that are widely used by the food industry. Upon consumption of saturated fats, the liver is exposed to high levels of palmitic acid that can contribute to NAFLD. The liver can also convert palmitic acid to monounsaturated fatty acids, mainly oleic acid (C18:1) by the activity of stearoyl-CoA desaturase (SCD-1) [79]. SCD-1 has been shown to play a key role in the onset of diet-induced hepatic insulin resistance [80]. Higher activity of SCD-1 has been shown in NAFLD subjects, concomitant with elevated levels of hepatic oleic acid [81], and in fatty livers of *ob/ob* mice [82]. SCD-1 knock-out mice are resistant to hepatic steatosis and adiposity [83, 84]. Moreover, lowering hepatic scd-1 gene expression using sequence-specific antisense oligodeoxynucleotide in rats and mice on a high-fat diet was shown to induce insulin

sensitivity, improve insulin signaling, and decrease gluconeogenesis [80]. Therefore, it seems that accumulation of both palmitic and oleic acid in the liver can induce NAFLD and accordingly these free fatty acids are widely used *in vitro* studies to investigate the molecular mechanisms underlying NAFLD.

2.16. Polyphenols as important components in diet

Polyphenols are considered as the plants' secondary metabolites which have important roles in protection against ultraviolet light and pathogens [202]. After reports of beneficial effects on human health by different studies in the 1990s, polyphenols have attracted great attention in research. Polyphenols are found in our daily diet in fruits and vegetables, pulses, green and black tea, coffee, extra virgin olive oil, and chocolate [43]. Although widespread in our diet, they have been the subject of many studies only in the last few decades [203].

Polyphenols may be effective in prevention of cancer, and cardiovascular diseases [43, 204]. Polyphenols are potent antioxidants *in vitro* and their dietary intake is related to lower rate of morbidity and mortality due to coronary heart diseases [205]. More than 8000 polyphenols are found in plants [206].

2.16.1. Classification of polyphenols

Flavonoids, phenolic acids, lignans, and stilbenes are the major groups of polyphenols [203]. The structure of some of the polyphenols of interest in this thesis is shown in Figure 2.5.

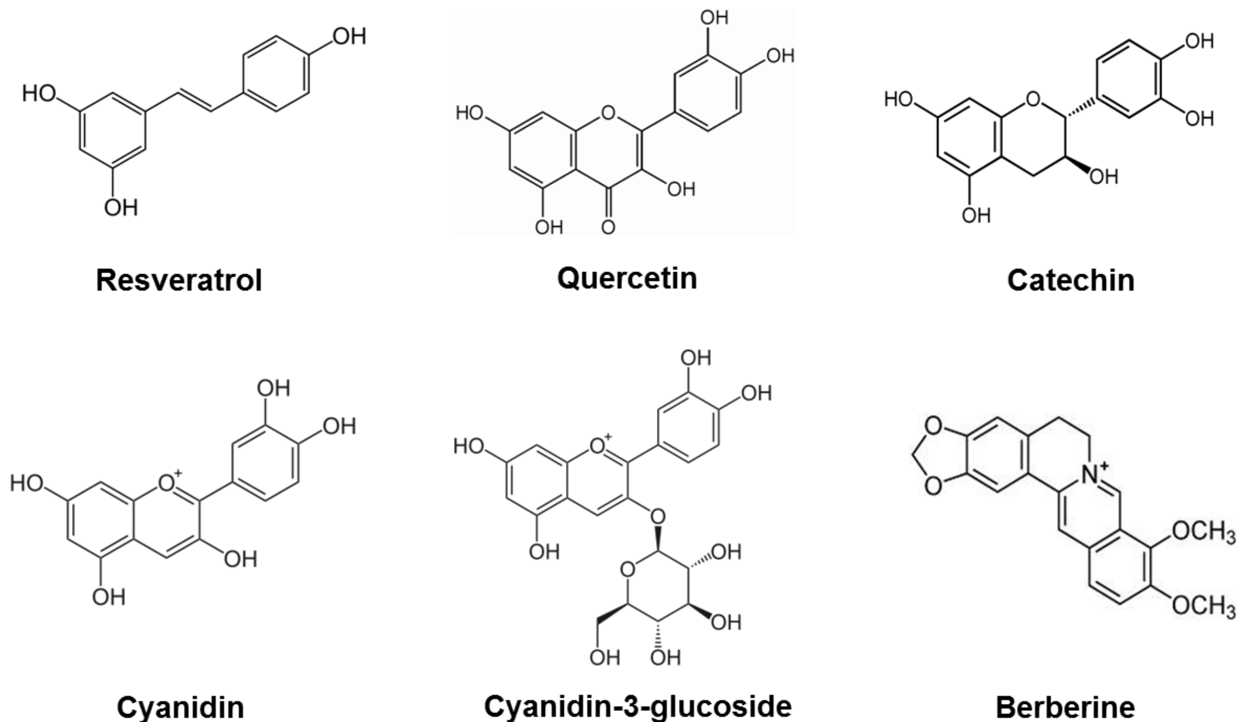

Figure 2. 5. The structure of some of the main polyphenols and a methoxy alkaloid derivative (berberine)

2.16.1.1. Flavonoids

The structure of flavonoids consists of two benzene rings attached together by a pyrone ring. Flavonoids are usually found as glycosidic forms rather than aglycones. The six major subclasses of flavonoids include: flavan-3-ols (e.g., epicatechin, gallocatechin), flavonols (e.g., quercetin, myricetin), flavones (e.g., apigenin, luteolin), flavanones (e.g., naringenin, hesperidin), isoflavones (e.g., genistein, daidzein), and anthocyanins (e.g., cyanidin, pelargonidin) based on differences in the pyrone ring structure. The most abundant flavonoids include quercetin, kaempferol, isorhamnetin, and myricetin that are usually found in edible plants as glycosides. Although there are only a few aglycones, almost 200 sugar conjugates were identified for kaempferol [207].

One of the richest sources of flavonols is onion which contains high amounts of quercetin [207]. Flavanones are generally found in citrus fruits and grapefruit.

Anthocyanins are natural pigments in plants which have red, blue, or a purplish color [203]. Anthocyanins are found in different berries, pomegranates, purple grapes, apple skins, cherries [208], red wine, and some types of vegetables including purple onions, radishes, and cabbages [203]. Some of the most important anthocyanins are cyanidin, malvidin, delphinidin, petunidin, pelargonidin, and peonidin [209]. Anthocyanins were shown to have antioxidant [210] and anti-inflammatory effects and can prevent obesity, diabetes, and insulin resistance [46].

2.16.1.2. Phenolic acids

There are two classes of phenolic acids: a) derivatives of benzoic acid, and b) derivatives of cinnamic acid. Some foods such as onions, black radish, and some red fruits have a high content of hydroxybenzoic acid derivatives [202]. Tea leaves are very good source of gallic acid (3,4,5-trihydroxybenzoic acid), containing 4.5 g gallic acid/kg dry weight [202].

Hydroxycinnamic acids are more ubiquitous in foods compared to hydroxybenzoic acids. Hydroxycinnamic acids consist of caffeic acid, ferulic acid, *p*-coumaric acid, and sinapic acid. The highest content of hydroxycinnamic acids can be found in cherries, blueberries, apples, plums, and kiwis [211]. The most common form of hydroxycinnamic acid is caffeic acid which consists 75-100% of hydroxycinnamic acid in most fruits. Moreover, the most abundant phenolic acid in cereals is ferulic acid which consists 90 % of the total polyphenols in wheat [202].

2.16.1.3. Stilbenes

Stilbenes are produced in plants in response to stress, disease and injury [212] and they are available in our diet from some sources. Resveratrol is the main stilbene in our diet which is available in red grapes and red wine [207]. Red wine contains several stilbene derivatives although their concentration is low compared to other polyphenolic components [213].

2.16.2. Bioavailability, metabolism, and absorption of flavonoids

Different factors including food processing, sex, age, and the composition of intestinal microflora are associated with the bioavailability of polyphenols [214]. The bioavailability of flavonoids is quite low [209]. It is estimated that approximately 5-10% of the total ingested polyphenols is

absorbed in the small intestine and the remaining (90-95%) will accumulate in the colon where they can produce breakdown/digestion products by colonic microbiota [215].

Except flavanols, all flavonoids are found in foods as glycosylated form. Most glycosides escape acid hydrolysis in the stomach and therefore can enter the small intestine in the intact form [203]. Studies in rodents show that some polyphenols can be absorbed in the stomach, such as quercetin (not in the glycosylated form) and anthocyanins [203, 216].

As mentioned earlier, most polyphenols are often found in foods as glycosylated, ester, or polymer forms. Polyphenols generally cannot be absorbed in these forms (except some glucosides) and need to be hydrolyzed by intestinal enzymes or the microflora to form aglycones [43]. Exceptionally, anthocyanins can be absorbed and found in the blood as glycoside forms [217].

Flavonoid glycosides can be cleaved to aglycones by lactase phloridzin hydrolase (LPH) in the brush border of small intestine cells. LPH has broad specificity for flavonoid glycosides and due to their lipophilic properties, resultant aglycones can be absorbed by passive diffusion [207]. One of the alternatives of this pathway is metabolizing flavonoid glycosides by the cytosolic-β-glucosidase (CBG) within the intestinal epithelial cells. In order to be metabolized by this pathway, glycosides must be transported to the epithelial cells by active sodium-dependant glucose transporter 1 (SGLT1) [218]. After metabolism by LPH and cytosolic β-glucosidase and before transporting to the blood stream, flavonoid aglycones may be conjugated by phase II enzymes to sulfate, glucuronide, and methylated metabolites by the action of sulfotransferases (SULT), uridine-5′-diphosphate glucuronosyltransferases (UGT), and catechol-*O*-methyltransferases (COMTs) [207].

Flavonoids are rarely found in the plasma as the parent aglycones, and tend to be conjugated as glucuronides, and methyl or sulphate esters in the small intestine, colon and later in the liver. After conjugation in the small intestine and liver to glucuronides and sulphate products, flavonoids are deconjugated by the vascular and tissue β-glucuronidases [219]. The "flavonoid paradox" suggests that conjugation of flavonoids may be a reversible process and these conjugated flavonoids (e.g. quercetin) can be deconjugated to active aglycones at the tissue cells. Interestingly, since vessel walls can deconjugate flavonoid glucuronides prior to their uptake by cells, aglycones are expected to be found inside the tissue cells even without the non-vascular β-glucuronidases activity [219].

Substantial quantities of polyphenol glycosides which are resistant to hydrolysis by LPH/CBG will enter the colon in which they are metabolized by colonic microflora. The resultant aglycones can undergo ring fission by colonic microbiota producing small molecules including phenolic acids and hydroxycinnamic acids [207]. These breakdown/digestion products (phenolic acids) can be absorbed and metabolized in the liver and may contribute to the protective effects of the parent polyphenols against NAFLD (discussed in CHAPTER 6).

2.16.3. Circulation and concentration of polyphenols in the plasma

Polyphenol metabolites circulate in the plasma bound to proteins, mainly albumin. Albumin is the primary protein for the plasma transportation of polyphenols and depending on chemical structures, different polyphenols have different affinity to albumin [220]. Polyphenol-albumin complexes may have consequences on cellular and tissue uptake of polyphenols. It may be possible that the unbound concentration of polyphenols determines their cellular uptake [203]. In order to keep high plasma concentration of flavonoids, one must regularly ingest sources of flavonoids [221]. For most polyphenols, the highest plasma concentration is reached 1-2 h after their ingestion [222]. However, determining the concentration and bioavailability of polyphenols in tissues may be more important than their plasma concentration [203]. Flavonoids may accumulate in tissues (especially lipid soluble forms), so the clearance can be substantially delayed. Unfortunately, evidence regarding tissue uptake and accumulation of polyphenols is very scarce, not only in humans but even in animals [203]. For instance, it was evidenced that after treatment with quercetin, it is metabolized in the intestine and liver and can gradually accumulate in the liver. Therefore, it is suggested that the liver can be a major tissue for the accumulation and metabolism of quercetin after ingesting a quercetin-rich diet [48].

2.16.4. Polyphenols as potent antioxidants

Polyphenols may be acting through their antioxidant properties or may synergistically enhance the effect of other antioxidants. Several mechanisms have been proposed as their antioxidant activity including: a) inhibiting generation of ROS b) scavenging ROS and c) protecting or enhancing antioxidant enzymes [223]. Flavonoids have been shown to fulfill most of the criteria mentioned above. In addition to radical scavenging, flavonoids are also known as metal chelators. Chelation

of metals such as Fe^{2+} can directly decrease the rate of the Fenton reaction, thus resulting in the prevention of highly reactive hydroxyl radicals [224]. In addition, flavonoids do not act alone. Therefore, as was indicated in the study by Bandy & Bechara, flavonoids can really function as a co-antioxidant, and are involved in the regeneration of essential vitamins [225].

2.17. Beneficial effects of polyphenols

2.17.1. Beneficial effects of polyphenols on oxidative stress and antioxidant enzymes

An accumulating body of evidence shows that polyphenols possess strong antioxidant activities and may have a therapeutic effect in NAFLD and NASH. In one study, oral supplementation of resveratrol at 15 and 45 mg/kgbw/d for 6 weeks decreased hepatic thiobarbituric acid reactive substances (TBARS) formation in obese Zucker rats showing its antioxidant activities. In this study, resveratrol also attenuated hepatic steatosis and increased the reduced to oxidized glutathione ratio (GSH/GSSG) which is a reliable indicator of oxidative stress [226]. Beside resveratrol, other polyphenols also have been shown to exert antioxidant effects. For instance, it is shown that due to their gallate ester moiety, catechins such as epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) have very strong antioxidant properties and can effectively prevent lipid peroxidation [227] which may be beneficial in protecting against NASH. Other studies also point to the beneficial effects of green tea polyphenols in steatohepatitis and fibrosis in regard to ameliorating oxidative stress. Nakamoto et al. [228] investigated the effects of fermented green tea (*Camellia sinensis*) extract (100 and 300 mg/kg/d) for 6 weeks in rats fed a choline-deficient high-fat diet. For inducing NASH, oxidative stress was induced by intraperitoneal injection of nitrite. Fermented green tea extract (300 mg/kgbw/d) attenuated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (which are two reliable markers of liver damage and necrosis), hepatic lipid accumulation, lipid peroxidation products (MDA and 4-HNE) and mitochondrial ROS production, and increased plasma SOD activity. Fermented green tea extract also inhibited cirrhosis development showing it can be effective against progression of NASH. The authors concluded green tea extract prevents NASH, and liver cirrhosis via attenuation of oxidative stress.

In vitro studies also show protective effects of polyphenols against oxidative stress. For instance, quercetin treatment at 10 μM was effective to reduce lipid accumulation, TNF-α, IL-8, lipid

peroxides, and increase activity of antioxidant defense such as catalase, SOD, and GPx, and increased intracellular GSH concentration in oleic acid-induced fatty liver condition in HepG2 cells [35]. Quercetin contains several phenolic hydroxyl groups which are the reason behind its potent antioxidant activity. Similar to catechin that we discussed previously, quercetin possess anti-inflammatory and anti-fibrosis effect in mice with non-alcoholic steatohepatitis [229] showing these effects may be attributed to its potent antioxidant effect.

Experimental studies in rodents also support *in vitro* studies in regard to antioxidant effect of quercetin. Quercetin dietary treatment (0.05%) to mice fed a high-fat diet for 4, 8, and 20 weeks decreased hepatic lipid peroxidation (TBARS), increased hepatic total glutathione level, and hepatic mRNA expression of glutathione peroxidase 1 and catalase, and decreased hepatic gene expression of UCP2. These concerted effects of quercetin reversed high-fat diet-induced oxidative stress [48]. The antioxidant effects of polyphenols are sometimes conflicting however, as Marcolin et al. showed that 4-week dietary quercetin treatment to rats fed a methionine- and choline-deficient diet (MCD) reversed increasing TBARS, but had no effect on SOD and catalase activity [230]. Similar to quercetin, kaempferol which is found in different fruits and vegetables including tea and broccoli, and taxifolin (at 5, 25, and 50 μM) was shown to inhibit inflammatory cytokine mixture-induced production of nitric oxide, superoxide anions and lipid peroxidation in cultured Chang liver cells resulting in improved cellular oxidative stress [231].

Another factor which has an important role in progression of NAFLD to NASH is over-expression of hepatic cytochrome P450 (CYP2E1). As discussed earlier, this is a less efficient pathway to metabolize excess fatty acids in endoplasmic reticulum while producing high amount of ROS. Epigallocatechin has been demonstrated to inhibit oxidative stress (as was evidenced with lower plasma and liver MDA, and higher GSH level) by inhibiting high-fat diet-induced hepatic CYP2E1 over-expression in NASH and therefore inhibiting oxidative stress [232]. There is over-expression of CYP2E1 in NASH which results in damage to cells due to higher ROS production [233]. It is shown that ketone bodies and fatty acids are substrates for CYP2E1 and increase its expression [32]. Therefore, it appears that polyphenols can inhibit CYP2E1 expression.

Similar to lipogenesis and fatty acid oxidation, polyphenols can also affect the expression of transcription factors involved in oxidative stress. For instance, oral administration of resveratrol at 10 mg/kg/day for 8 weeks was shown to increase Nuclear factor E2-related factor 2 (Nrf2) in

hepatic nuclear protein extract which resulted in increasing antioxidant enzymes activity such as SOD, catalase, GSH and consequently protected against fructose-induced oxidative stress in rats [47]. Nrf2 is an important transcription factor for protecting against oxidative stress. Through increasing the gene expression of downstream antioxidant enzymes, the transcription factor Nrf2 results in maintaining cellular redox status [234]. Sequestered in the cytoplasm, Nrf2 is inactive and during oxidative stress it translocates to the nucleus and binds to antioxidant response elements (AREs) in regulatory regions of targets genes and upregulates the expression of antioxidant enzymes [235]. The pivotal role of Nrf2 in NAFLD has been revealed by genetic studies showing Nrf2 knockout mice ($Nrf2^{-/-}$) develop more severe oxidative stress, steatosis (evidenced by reduction of fatty acid oxidation and induction of lipogenesis), and inflammation on a high-fat diet compared to wild type mice [236, 237]. This shows a key role of oxidative stress (second hit) in inducing fatty liver disease and inflammation while polyphenols can protect against it.

Coffee polyphenols extract (4.2 mg of polyphenols/d corresponding to 6 cups of espresso coffee or approximately 2 cups of filtered coffee for a 70kg person) for 8 weeks also reduced hepatic fat, systemic and liver oxidative stress, and inflammation in Wistar rats fed a high-fat diet for 12 weeks [238]. Coffee polyphenols extract attenuated oxidative stress by increasing GSH/GSSG ratio and ferric reducing antioxidant power (FRAP) in serum, and decreasing serum MDA. Cyanidin-3-*O*-β-glucoside (C3G) has also been shown to protect against hepatic steatosis, neutrophil infiltration and hepatocyte apoptosis in *db/db* mice and high glucose-treated HepG2 cells in which the effects were attributed to the prevention of oxidative stress, ROS production, and induction of glutamate-cysteine ligase expression and subsequently glutathione synthesis [239].

In addition to antioxidant properties some polyphenols chelate metal ions (Fe^{3+}, Cu^{2+}) bringing about prevention of oxidative stress. Studies show that strong iron-binding properties of quercetin and other polyphenols such as rutin are due to their iron-binding motifs in their chemical structure [240].

2.17.2. Beneficial effects of polyphenols on intracellular triglyceride and enzymes involved in β-oxidation and lipogenesis

As discussed earlier, adipocyte lipolysis, hepatic *de novo* lipogenesis, and fatty acid β-oxidation are three important pathways which have pivotal roles in hepatic lipid accumulation. Therefore,

modulating these pathways can prevent NAFLD. Since hepatic lipid accumulation is recognized as the "first hit" of NAFLD, quite a lot of studies evaluated the preventive effects of polyphenols on hepatic lipid accumulation and the enzymes involved in lipogenesis and β-oxidation.

Some polyphenols were shown to be effective in decreasing hepatic accumulation of fatty acids. For instance, in an *in vitro* model of steatosis it was shown that 40 μM resveratrol decreased palmitate-induced accumulation of intracellular lipids to basal levels mainly by inhibiting the expression and activity of the transcription factor SREBP-1c [241]. One of the main limitations of this study was using 40 μM resveratrol which is higher than the relevant physiological dose (≤10 μM).

Alberdi et al. [45] reported the same effect of resveratrol on decreasing hepatic steatosis *in vivo*. The authors showed that six-week administration of 30 mg/kg bw resveratrol to Sprague Dawley rats taking an obesogenic diet for 6 week increases the phosphorylated ACC (inactivated) and AMPK (activated) compared to total ACC and AMPK protein. Moreover, the authors found resveratrol treatment increased activities of liver proteins involved in fatty acid oxidation (mitochondrial CPT1A and peroxisomal acyl-CoA oxidase) while having no effect on lipogenic FAS.

Preventive effects on hepatic lipid accumulation have also been shown using anthocyanins. For instance, purple sweet potato anthocyanins (anthocyanin fraction 200 mg/kg/d) was shown to phosphorylate hepatic AMPK and ACC and consequently decrease hepatic lipid accumulation in mice taking a high-fat diet for 4 weeks [46]. AMPK activation was shown to indirectly inhibit FAS and ACC expression through inhibition of the transcription factor SREBPs [242]. In one study in HepG2 cells and obese mice it was shown that using anti-AMPK treatment inhibits the effect of anthocyanin rich purple sweet potato on lipid accumulation and expression of FAS and SREBP-1 protein [46].

In addition to modulating lipogenesis, polyphenols are also able to induce enzymes involved in fatty acid β-oxidation. Açaí berry aqueous extract treatment (3g/kgbw/d for 6 weeks) of mice fed a high-fat diet increased hepatic mRNA for adiponectin receptor protein 2 (AdipoR2), CPT-1A1 (the specific isoform found in the liver), and PPARα, showing increased β-oxidation via adiponectin-mediated up-regulation of PPARα [243]. Moreover, resveratrol delivered orally at 30

mg/kgbw for 6 weeks enhanced hepatic activity of acyl-CoA oxidase and CPT1A in rats fed a high-fat diet [45].

Polyphenol treatment does not always result in increased CPT-1 expression. For instance, treatment of Wistar rats fed a high-fat diet with grape polyphenol extract (2g/kg diet) for 6-week was shown to decrease muscle CPT-1 protein expression. This was explained by the authors to be due to the decreased protein expression of muscle fatty acid translocase (FAT/CD36). FAT/CD36 is an integral protein found on the surface of different cells responsible for the uptake of fatty acids. Therefore, decreased expression of FAT/CD36 leads to inhibition of fatty acid translocation to cells resulting in decreased lipid accumulation and consequent decline of CPT-1 protein expression [51].

Anthocyanins may also act as PPARα agonists to induce genes involved in β-oxidation. In a model of insulin resistance and hyperlipidemia with Dahl rats, feeding tart cherries at 1% of the diet for 90 days induced mRNA expression and activity of hepatic PPARα [244]. Anthocyanins may have a direct agonist effect on PPARα, since cyanidin (but not the degradation products protocatechuic acid or phloroglucinaldehyde) was shown to bind to and transactivate purified PPAR proteins, especially PPARα, as well as induce PPAR responsive genes in HepG2 cells [245].

Quercetin was also found to alter the gene expression of transcription factors involved in β-oxidation or lipogenesis. In the previously mentioned study by Kobori et al., gene expression of lipogenic transcription factors such as PPARγ and SREBP1-c was decreased by dietary quercetin in the liver of mice fed a high-fat diet for 20 weeks [48].

Polyphenols also can be beneficial in decreasing adipogenesis. Apigenin, a flavone which is found in foods such as celery and parsley, at 10 and 50 μM inhibited lipolysis by decreasing hormone-sensitive lipase mRNA and inducing AMPK phosphorylation in 3T3-L1 adipocytes [246]. This can be beneficial for hepatic steatosis since the most important source of hepatic fatty acids (59%) are fatty acids generated from adipocyte lipolysis. Moreover, apigenin decreased mRNA and protein expression of PPARγ and stearoyl-CoA desaturase (SCD) both involved in lipogenesis.

UCP2 is another enzyme influencing lipid metabolism that can be affected by polyphenols. Oral administration of epigallocatechin gallate purified from green tea (supplemented 0.5-1% in the

diet) to New Zealand black mice fed a high-fat diet for 4 weeks also increased hepatic UCP2 mRNA which explains higher metabolism of fatty acids in these mice. Epigallocatechin gallate also decreased hepatic mRNA expression of *SCD1* and glucokinase in the liver suggesting a role in inhibiting lipogenesis in these mice [85]. Resveratrol was also found to increase hepatic UCP2 mRNA which suggests it can inhibit mitochondrial dysfunction in high-fat diet-induced hepatic steatosis [171].

2.17.3. Beneficial effects of dietary polyphenols on insulin resistance

Polyphenols can also prevent fatty liver by affecting insulin resistance. By inducing hepatic insulin signalling (IRS/PI3K/Akt), resveratrol (2-4 g/kg diet) delivered orally for 12 weeks increased insulin sensitivity and decreased serum insulin and hepatic glycogen in obese insulin-resistant KK-A^y mice fed a high-fat diet [247, 248]. The authors observed increased hepatic SIRT1, phosphorylated Akt (p-Akt), p-IRS1, and p-AMPK, all favoring improved insulin sensitivity and signaling.

Dietary supplementation of cyanidin 3-glucoside (0.2% in diet) for 5 weeks was also shown to improve hepatic steatosis via regulation of forkhead box protein 1 (FoxO1) in adipose tissue of both C57BL/6J mice fed a high-fat diet and leptin-resistant *db/db* mice [249]. The authors found that by increasing pAkt/Akt protein expression after refeeding, cyanidin 3-glucoside phosphorylates and inactivates FoxO1 in adipocytes leading to reduced hepatic steatosis and insulin resistance in these mice. Moreover, insulin resistance in these mice was mediated by increased phosphorylated JNK (p-JNK) which was inhibited by treatment with cyanidin 3-glucoside.

2.17.4. Beneficial effects of polyphenols on inflammation

Polyphenols are effective against inflammation in NAFLD and NASH. It is reported that a high-fat diet induces a low-grade inflammation characterized with obesity and the induction of the NF-κB/iNOS pathway. Once activated this pathway induces transcription of TNFα and IL6 which are the main pro-inflammatory cytokines to damage tissues in metabolic diseases such as obesity, diabetes, and fatty liver disease [136].

Several polyphenols have been reported to inhibit inflammation and inflammatory signaling pathways in models of NAFLD and NASH. It is observed that dietary quercetin (30-60 mg/kg bw) in gerbils fed a high-fat diet for 14 days inhibited the NF-κB/iNOS pathway preventing inflammation in hepatocytes, along with up-regulating the expression of SIRT1 [250]. In this study quercetin reversed the high-fat diet hepatic steatosis and induction of serum TNFα and IL6, and decreased NF-κB p65 protein expression and collagen deposition in hepatocytes preventing hepatic fibrosis. Quercetin (0.05% of diet) delivered orally was also shown to decrease hepatic thiobarbituric acid reactive substances and plasma TNFα and increased plasma adiponectin level in Wistar rats fed a high-fat high-sucrose diet for 20 weeks [48]. Taken collectively, it seems that polyphenols can alleviate NAFLD/NASH by modulating genes or transcription factors involved in inflammation or inflammatory signalling pathways.

2.17.5. Beneficial effects of polyphenols on mitochondrial biogenesis and function in NAFLD

The effect of polyphenols on hepatic mitochondrial biogenesis in fatty liver disease has been looked at in only a few studies. Liver and skeletal muscle are the main tissues studied for the effect of polyphenols on mitochondrial biogenesis in NAFLD since inducing mitochondrial biogenesis and subsequent lipid metabolism in these tissues protect against lipid accumulation. Studies on resveratrol have given conflicting results. One study reported that oral administration of resveratrol (30 mg/kg bw/d) had no effect on the gene expression of PGC1α, SIRT1, mitochondrial transcription factor A (TFAM), and cytochrome oxidase subunit II (complex IV) in the liver of rats fed a high-fat diet for 6 weeks [45]. Lagouge et al. [251] however reported that resveratrol (56-80 mg/kg bw/d) induces the activity of citrate synthase and mRNA expression of SIRT1, PGC-1α, estrogen-related receptor α (ERRα), NRF1, and TFAM in the muscle of mice fed a high-fat diet for 15 weeks. Consequently, it was suggested that resveratrol could prevent metabolic diseases, obesity, and insulin resistance by improving mitochondrial biogenesis and function. Compared to the Lagouge study, the Alberdi study treated the animals with a high-fat diet and resveratrol for a shorter period (6 *vs.* 15 wk), twice the amount of resveratrol and evaluated different tissues (liver *vs.* muscle) which may partly explain the difference between the results on mitochondrial biogenesis.

In one other study relevant to NAFLD, treating rats with a high-fat high-sucrose diet for 6 weeks reduced mRNA expression of the muscle genes involved in mitochondrial biogenesis including PGC1α and NRF1, but had no effect on TFAM [51]. The authors found that grape polyphenol extract induced NRF1 mRNA expression but had no effect on TFAM and PGC1α. Moreover, mitochondrial respiratory complex II, II+III, and IV activity was unchanged in the muscle tissue after using either a high-fat diet or grape polyphenols alone.

One study has looked at the effect of berberine. Gomes et al. reported that treatment of Sprague Dawley rats with a high-fat diet for 16 weeks decreases mitochondrial function and ATP content in the skeletal muscle, while treatment with berberine (100 mg/kg bw/d) improved mitochondrial function and increased the succinate dehydrogenase (complex II), cytochrome oxidase (complex IV), and ATPase (complex V) activity as well as TFAM and PGC1α mRNA expression [252]. In the same study, the authors evaluated the effect of 5 μM berberine on hyperglycemia-induced (25 mM glucose) mitochondrial dysfunction in C2C12 myotubes. Berberine reversed hyperglycemia-induced decrease of mitochondrial DNA content and mitochondrial mass. Interestingly, berberine had no effect on mitochondrial DNA content and mass in cells knocked-down for SIRT1. In addition, while hyperglycemia in myotubes prevented the expression of genes involved in mitochondrial biogenesis such as PGC1α, NRF1, NRF2, and TFAM, berberine reversed this prevention. Berberine also protected against hyperglycemia-induced decrease of nuclear DNA-encoded complex subunits (NDUFS8 and COX5b), and mitochondrial DNA-encoded subunits (MTND1, MTCO1) mRNA expression.

Anthocyanins were also shown to induce mitochondrial biogenesis *in vivo*. Peng [253] observed that due to their partially positive charge, anthocyanins can accumulate within mitochondria which have negative charge inside their matrix. It was revealed that treatment with purified anthocyanins from black currant and bilberry to Wistar rats fed a choline- and methionine-deficient diet protected against NASH due to increasing mitochondrial biogenesis evidenced by elevated PGC1α, NRF1, NRF2, and TFAM hepatic gene expression in a PGC1α/p-AMPK mediated pathway [254]. Purified anthocyanins from black currant and bilberry also increased mitochondrial complex I and IV protein expression in the liver, and along with increased mitochondrial biogenesis they increased the expression of proteins involved in fatty acid β-oxidation such as PPARα, CPT-1, and MCAD.

Curcumin also showed promising results in improving mitochondrial function. In one study, treatment with 1-3 % w/w curcumin along with a high-fat diet protected against the decrease in hepatic mtDNA, ATP content, and complex I activity leading to attenuation of hepatic fat content in genetically obese mice [18]. It was shown by the authors that a decrease in hepatic protein expression of TNFα, and IL-6, and NF-κB activity is responsible for the protective effect of curcumin on mitochondrial function.

In vitro studies also support the notion that polyphenols can improve mitochondrial function. In one study, eriocitrin (eriodictyol 7-rutinoside) which is a major flavonoid in lemon (*citrus limon)* inhibited palmitate-induced (400 μM) lipid accumulation in HepG2 cells by increasing the expression of genes involved in mitochondrial biogenesis (NRF1, TFAM), and ATP synthesis (ATP5J, COX4I1) [255]. Eriocitrin Also increased mitochondrial size, mtDNA copy number, and increased intracellular ATP content showing improved function of OXPHOS complexes.

2.17.6. Beneficial effects of dietary polyphenols in protection against ER stress

Currently, only very few studies have assessed the effect of polyphenols on hepatic ER stress [256-258]. What is interesting is that all studies that have evaluated the effect of polyphenols on ER stress have been published recently (after 2010). Available studies suggest that treatment with polyphenols may alleviate ER stress. In one study, it was observed that treatment of rats fed a high-fat diet for 12 weeks with 100 mg/kg per day resveratrol (for 8 weeks) decreased protein expression of ER stress markers such as GRP78, ATF4, p-PERK, and CCAAT-enhancer-binding protein homologous protein (CHOP) in the liver [256]. Resveratrol reduced ER stress markers protein expression along with attenuating hepatic lipid accumulation, lobular inflammation and ballooning score (fibrosis), and inhibiting expression of genes involved in inflammation such as suppressor of cytokine signaling-3 (SOCS-3), TNFα, and IL-1β. Suppression of ER stress was suggested by the authors to be one of the mechanisms by which resveratrol protected against high-fat diet-induced hepatic steatosis in rodents. Resveratrol was also shown to alleviate palmitate-induced ER stress in HepG2 hepatocytes by decreasing gene expression of the apoptotic CHOP, and protein expression of p-PERK and p-eIF2α as well as increasing 150-kDa oxygen-regulated protein (ORP150) gene expression in a SIRT1-dependant mechanism [257].

In a recent study, berberine has also been shown to prevent progression of simple hepatic steatosis to NASH by suppressing ER stress [258]. The authors showed that beneficial effects of berberine (200 mg/kg/d) in prevention of hepatic steatosis, inflammation, and fibrosis in *db/db* mice and methionine choline-deficient mice is due to the protective effect of berberine against hepatic ER stress. They found decreased mRNA for ATF6, ATF4, XBP1, and CHOP, and protein expression of p-PERK, p-eIF2α, GRP78, in the liver of *ob/ob* mice following treatment with berberine. This was concomitant with reduced expression of genes involved in lipogenesis (SREBP1, ChREBP, FAS, C/EBPβ), inflammation (IL-6, TNFα, IL-1β, and ICAM-1), fibrosis, and oxidative stress (catalase, CYP2E1, CYP4A10). In another experiment and in an *in vitro* model of ER stress using free fatty acids (mixture of oleic and palmitic acid at 0.5-1 mM) the same authors showed potential chaperone activity of polyphenols and showed that berberine exhibits chaperone activity and consequently decreases accumulation of unfolded proteins and consequently intracellular lipid in primary hepatocytes and HepG2 cells. They reported for the first time that berberine has potent chaperone activity which reduces ER stress by its protein-stabilizing activity and therefore decreases unfolded/aggregated proteins responsible for initiation of ER stress. Interestingly, the chaperone activity of berberine in that study was comparable to chemical chaperone 4-PBA representing potential effect of polyphenols in alleviating ER stress [258].

Some *in vitro* studies also use tunicamycin instead of palmitic acid to induce ER stress. The results of these studies further elucidate the mechanisms by which ER stress impairs insulin signaling. For instance, in one study, Wang et al. reported the protective effect of berberine against tunicamycin-induced ER stress in HepG2 cells [259]. They found that while treatment of HepG2 cells with tunicamycin increases gene and protein expression of chaperone ORP150, activates JNK, and increases phosphorylation of PERK and eIF2α, pre-treatment with 10-20 µM berberine for 14 h protected against all of these changes. Pre-treatment of HepG2 cells with berberine also decreased phosphorylation of IRS-1 Ser^{307}, whereas increased IRS-1 tyrosine phosphorylation showing it can alleviate tunicamycin-induced impaired insulin signaling in HepG2 cells. Accordingly, berberine decreased ER stress-mediated insulin resistance and glucose production in these cells in the presence of insulin. The authors concluded that antidiabetic effects of berberine may be mediated through the decrease in ER stress and improvement in insulin signaling transduction [259].

More recently, it was shown that maternal quercetin (50-200 mg/kg bw) administration along with a high-fat diet during gestation and lactation decreased birth weight, postnatal weight gain, insulin resistance, inflammation, and improved lipid metabolism via suppressing ER stress in the liver and adipose tissue of mature rat offspring of obese rats [260]. The maternal high-fat diet increased protein expression of ER stress markers such as Bip/GRP78, p-PERK, p-IRE1 in the liver and adipose tissue of the offspring while treatment with quercetin could protect against these changes. Therefore, by alleviating impaired early programming and ER stress, polyphenols such as quercetin may have the potential to decrease the risk for chronic metabolic diseases in adult offspring [260].

These beneficial effects of polyphenols against ER stress have also been investigated in the skeletal muscle tissue. Increased ER stress in the skeletal muscle results in impaired insulin sensitivity and therefore enhances lipid accumulation and inflammation in the tissue further exacerbating metabolic homeostasis in obesity, diabetes, and fatty liver disease. Rodriguez et al. for instance, demonstrated that while a high-fat diet for 20 weeks increases mRNA expression of ER stress markers such as BiP, ATF4, XBP1s (spliced) and XBP1u (unspliced) in the skeletal muscle of mice, administration of pomegranate and green tea extracts could protect against these increases [261].

In conclusion, very few studies are available that have investigated the effect of polyphenols on hepatic, adipose tissue, and skeletal muscle ER stress and most of them show promising results. Therefore, due to the lack of evidence in this field, more studies are needed in order to find the effect of polyphenols on ER stress in metabolic diseases and to explore the contributing mechanisms.

CHAPTER 3: DIETARY POLYPHENOLS PROTECT AGAINST OLEIC ACID-INDUCED STEATOSIS BY MODULATING LIPID METABOLISM AND MITOCHONDRIAL DYSFUNCTION

Abbreviations: ATP, Adenosine triphosphate; **CPT1A1**, Carnitine palmitoyltransferase-1 (liver); **FAS**, Fatty acid synthase; **GAPDH**, Glyceraldehyde-3-phosphate dehydrogenase; mRNA, Messenger ribonucleic acid; **NAFLD**, Non-alcoholic fatty liver disease; **NASH**, Nonalcoholic steatohepatitis; **OXPHOS**, Oxidative phosphorylation; **p-AMPK**, Phospho-AMP-activated protein kinase; **PGC1α**, Peroxisome proliferator-activated receptor gamma coactivator 1; **PPARα**, Peroxisome proliferator-activated receptor alpha; **PPARγ**, Peroxisome proliferator-activated receptor gamma; **SIRT1**, Sirtuin 1; **SIRT3**, Sirtuin 3; **ΔΨm**, Mitochondrial membrane potential

Abstract

Scope: Dietary polyphenols have been shown to protect against NAFLD, but the mechanisms of action and their relative effectiveness on mitochondrial metabolism and cellular bioenergetics are largely unknown.

Methods and results: Treatment of HepG2 cells with 1.5 mM oleic acid increased intracellular lipid, distorted HepG2 cell morphology, and decreased the mitochondrial membrane potential. Co-treatment with 10 μM of selected polyphenols all strongly protected against these changes. In investigations into lipid metabolism, oleic acid induced mRNA for FAS and CPT1A, and decreased mRNA for PPARγ. Polyphenols, except cyanidin inhibited the effects on FAS and PPARγ, and further increased the expression of CPT1A1, suggesting a shift toward increased β-oxidation. Oleic acid also depleted ATP stores in the cells grown on galactose but not glucose, while all polyphenols except kuromanin and berberine maintained the ATP stores. While oleic acid had no effect on the expression and activity of SIRT1 or on the formation of p-AMPKα^{Thr172}, resveratrol, catechin and berberine increased these factors. Catechin was unique in increasing the expression of SIRT3.

Conclusion: While different polyphenols protected similarly against intracellular lipid accumulation, they differed in their effects on the expression of genes and proteins involved in lipogenesis, fatty acid oxidation, mitochondrial biogenesis, and bioenergetics, showing that they act through different mechanisms.

Keywords: NAFLD, polyphenols, steatosis, mitochondrial dysfunction, ATP stores.

3.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) occurs following ectopic accumulation of intracellular fat in the liver (>5% of the liver weight) which is the manifestation of an imbalance between lipid influx and removal mechanisms [78]. Initially a benign condition, it can progress to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma [262]. The "two-hit" hypothesis suggests steatosis as the "first hit" making the liver susceptible to the "second hits" including oxidative stress, gut-derived endotoxins, or pro-inflammatory cytokines [2].

Hepatic lipid metabolism is delicately regulated by the interaction among different transcription factors including the peroxisome proliferator-activated receptors (PPARs) and nuclear receptors, and genes involved in lipogenesis and fatty acid β-oxidation [71]. Any disturbances in these intertwined and precisely regulated pathways may initiate or promote NAFLD progression to NASH and cirrhosis. Although mitochondrial fatty acid β-oxidation has been shown to increase in high-fat diet-induced obesity and insulin resistance [263, 264], it cannot proportionately metabolize an increasing load of free fatty acids and its capacity may be disrupted by mitochondrial dysfunction and OXPHOS incompetence [105].

More recently, mitochondrial dysfunction was suggested to contribute to the progression of simple steatosis to NASH [4-6]. Mitochondrial dysfunction has a pivotal role in progression of NAFLD. Due to the major roles of mitochondria in energy and lipid metabolism, ROS production, and apoptosis [13], mitochondrial dysfunction is suggested to be linked with the severity of NAFLD/NASH [12]. This mitochondrial dysfunction may also result in collapse of the mitochondrial membrane potential [265], ending up with exacerbation of NAFLD. Due to the impaired oxidative phosphorylation, hepatic ATP stores which are critical in maintaining the integrity of hepatic tissue, become depleted in NAFLD and NASH making the condition worse [18, 266]. Moreover, mitochondrial biogenesis decreases in NAFLD due to a higher ROS production and mitochondrial DNA damage, meaning that fewer functional mitochondria are available in hepatocytes (reviewed in [19]). Therapies to induce mitochondrial biogenesis therefore, may facilitate metabolizing excess fat and have a major role in the prevention of NAFLD progression to NASH [267].

Found in foods such as fruits and vegetables, green and black tea, chocolate, coffee, pulses, and extra virgin olive oil [43], polyphenols have been the subject of many studies only in the last few decades [203]. As well as being potent antioxidants [43, 47], polyphenols have previously been observed to protect against intracellular lipid accumulation [37, 48] which make them candidates for prevention and treatment of NAFLD. However, very few studies have investigated the effect of different polyphenols on aspects of mitochondrial biogenesis and dysfunction, and lipid metabolism. It is not known which polyphenols are the most potent in prevention of NAFLD and by what molecular mechanisms. The aim of the present study was to compare different classes of polyphenols for their abilities to prevent oleic acid-induced steatosis and mitochondrial dysfunction, induce mitochondrial biogenesis, and preserve HepG2 cell morphology. The contributing mechanisms were investigated by evaluating mitochondrial biogenesis, the mitochondrial membrane potential, intracellular ATP stores, and the expression of proteins involved in mitochondrial biogenesis, lipogenesis and fatty acid β-oxidation.

3.2. Materials and Methods

3.2.1. Materials

Eagle's minimum essential medium (MEM) for growing HepG2 cells was purchased from Hyclone, Logan, USA. Fluorescent Nile Red dye was from MP Biomedical, USA. Fetal bovine serum (FBS), mixture of antibiotic (penicillin/streptomycin), fatty acid-free bovine serum albumin (BSA), oleic acid, resveratrol, catechin, berberine, and quercetin were from Sigma-Aldrich, USA. Cyanidin, and cyanidin-3-glucoside (kuromanin) were purchased from Extrasynthese, France. RNAeasy mini kit for isolating RNA was from Qiagen. VILO cDNA synthesis kit, TRIzol reagent, Power SYBR Green PCR master mix for polymerase chain reaction, and tetramethlyrhodamine ethyl ester (TMRE) were purchased from Invitrogen, USA. CellTiter-Glo Luminescent Cell Viability Assay kit for quantification of intracellular ATP was from Promega, USA. SIRT1 direct fluorescent screening assay kit was from Cayman, USA. Manufacturers of materials used for Western blot are mentioned in section 3.2.10.

3.2.2. HepG2 cell culture conditions

HepG2 cells were cultured in T75 flasks using minimum essential medium with Earle's balanced salts (MEM/EBSS) which contained 2 mM glutamine, 1 g/L glucose (5.5 mM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (100 units/mL penicillin, and 100 mg/mL streptomycin) in a humidified incubator with 5% CO_2 at a temperature of 37ºC. When 70-80% confluent, HepG2 cells were detached with trypsin, harvested and used for different experiments.

3.2.3. Treatment with oleic acid/BSA and polyphenols

Oleic acid was dissolved in dimethyl sulfoxide (DMSO) in order to prepare a 300 mM stock solution and kept at -20°C for further use. HepG2 cells at 3×10^4 cells/well were cultured in the 96-well optical-bottom plates for 24 h. Old medium was then replaced with fresh medium containing 10% FBS and 1% fatty acid-free bovine serum albumin (BSA). One percent BSA was used to ensure the solubility of oleic acid in the medium. HepG2 cells were then pre-treated with different polyphenols at 10 μM for 2 h. After treatment of the cells with polyphenols for 2 h (polyphenols were not removed thereafter and the cells were incubated with them for the next 24 h), the cells were treated with or without 1.5 mM oleic acid for 24 h.

3.2.4. Measuring intracellular lipid content

Intracellular triglyceride was measured using the lipophilic fluorescent dye Nile Red (MP Biomedicals, USA) according to the manufacturer’s protocol, which stains the cytosolic neutral lipids [268]. Intracellular lipid content is therefore proportional to the Nile Red fluorescence. HepG2 hepatocytes at 3×10^4 were cultured in black 96-well clear-bottom plates. After 24 h, the medium was replaced with fresh medium containing 10% FBS and 1% BSA and hepatocytes were pre-treated with different polyphenols at 10 μM concentration for 2 h. To avoid a toxic effect of dimethyl sulfoxide (DMSO) in which the polyphenols were solubilized, the final concentration of DMSO in the medium was set at 0.5% and to control for any possible effect of DMSO, the same volume was added to untreated and control cells. After 2 h of treatment with polyphenols, the cells were treated with or without 1.5 mM oleic acid for 24h. After 24 h the old medium was replaced with fresh medium containing 1 μg/mL Nile Red and the cells were covered and incubated for 20 min at 37ºC. The cells were then washed carefully 2-3 times with phosphate buffered saline (PBS)

and the fluorescence of each well was read at 9 different spots using a microplate reader at excitation 488 nm and emission 585 nm. Fluorescence images were then captured using a ZOE Fluorescent Cell Imager (BioRad, USA). Each experiment was repeated in triplicate with 3 replicate wells per experiment.

3.2.5. HepG2 cell forward and side scattering using flow cytometry

To get a measure of cell size and granularity, forward and side scattering of HepG2 cells was measured by flow cytometry. After seeding $5x10^5$ cells in each well (in 6-well plates) for 24 h, the cells were pretreated with 10 µM polyphenols for 2 h. The cells were then treated with oleic acid at 1.5 mM dissolved in the medium containing 1% BSA. After 24h, the cells were washed twice with PBS and harvested by trypsinization. After adding 2 ml medium and centrifugation at 1200 rpm for 5 min, the supernatant was discarded and the pellet was suspended in PBS. Then, 0.5 ml of the cell suspension was transferred to a flow cytometry tube and the forward and side scattering was determined using a FACS system. Data were collected for 10,000 cells per sample.

3.2.6. RT-qPCR

3.2.6.1. Total RNA isolation

For determining mRNA expression, total RNA was isolated from HepG2 cells using TRIzol reagent and RNAeasy mini kit according to the manufacturer's instructions (Qiagen, USA). For isolating RNA, $1x10^6$ cells per well were plated in a 6-well plate for the first 48 h to grow. On the third day, the medium was replaced with fresh medium and the cells were pre-treated with 10 µM polyphenols for 2 h followed by treatment with 1.5 µM oleic acid for 24 h. After 24 h, the cells were washed twice with PBS and 1 ml of TRIzol was added to each well for 2 min to lyse the cells. Then, the cell lysates were transferred to a clean RNase-free 1.5 ml Eppendorf tube and 200 µl chloroform was added to each sample lysate followed by vigorous shaking for 10 seconds. After incubation at room temperature for 3 min, the lysates were vortexed again and centrifuged at 12,000 g for 10 min at 4 ^oC. Then, the clear aqueous upper layer was carefully separated and transferred to a new Eppendorf tube and 500 µl isopropanol was added to each microtube. After incubating for 5 min at room temperature, each sample was applied to a spin column and

centrifuged at 8000 g for 15s at 4 ºC. Then, the flow-through was discarded and 350 µl RW1 buffer (provided with the kit) was added to each sample and centrifuged again at 8000 g for 15s and the flow-through was discarded. To remove any DNA contamination, the samples were treated with 80 µl DNase 1x (Invitrogen, USA) and incubated at room temperature for 15 min. Then, the samples were washed with 350 µl of RW1 buffer and centrifuged at 8000 g for 15s at 4°C. After discarding the flow-through, the samples were washed with RPE buffer (provided with the kit), spin-dried for 1 min and the spin columns were transferred to a fresh RNase-free Eppendorf tube. Finally, 50 µl nuclease free water (Invitrogen, USA) was added to each spin column which was then centrifuged at 8000 g for 1 min and the flow-through containing purified total RNA was stored at -80 for later use.

3.2.6.2. Synthesis of complementary DNA (cDNA)

Frozen RNA samples were thawed and RNA concentration and purity were measured using a Nano Drop spectrophotometer (BioRad, USA) and the A260/A280 absorbance ratio. The A260/A280 absorbance ratio for all extracted RNAs was between 2-2.1 showing the total RNA was highly pure (within the acceptable ratio range of 1.8-2.1). Each $1x10^6$ HepG2 cells yielded approximately 18 µg total RNA. Using a VILO cDNA synthesis kit (Invitrogen, USA) and a Thermocycler (BioRad, USA), reverse transcription of 2 µg mRNA to cDNA was performed in 20 µl reactions according to the manufacturer's instructions. For cDNA synthesis, thermal cycling was adjusted as follows: one cycle at 25ºC for 10 min, one cycle at 42ºC for 60 min (optimal for synthesis of cDNA), and one cycle at 85ºC for 5 min (to inactivate DNA polymerase). The cDNA samples were kept in a -80 ºC freezer and were diluted 20 times before use according to the VILO cDNA synthesis kit protocol to remove the PCR inhibitory effects of reverse transcription. All primers were provided by Integrated DNA Technologies (IDT, Canada).

3.2.6.3. Polymerase Chain Reaction (PCR)

The PCR was performed using a PCR system (ABI 7300; Applied Biosystem) using the Power SYBR Green real-time PCR master mix (Life Technologies, USA) in 20 µl reactions according to the manufacturer's protocol. The thermal cycling used for the PCR was as follows: one cycle at 95ºC for 5 min (for enzyme activation), followed by 40 cycles at 95ºC for 15s and 60ºC for 30s.

The melting curves of all PCR products were evaluated to confirm amplification and primer quality. A comparative method ($2^{-\Delta\Delta CT}$) was used to analyze the relative expression of genes of interest using GAPDH as the reference gene. Since the expression of β-actin did not show the same trend in all groups in this study, GAPDH was used as the reference gene which showed similar expression in different groups and is routinely used as reference gene in studies on fatty liver disease. All results were normalized to the GAPDH. Each experiment was repeated in duplicate with 3 replicate wells per experiment. Sequences of the primers of interest are shown in appendix Table 7.1.

3.2.7. Culturing the cells in galactose medium

Cells which are grown in glucose medium can compensate for their mitochondrial dysfunction by higher production of ATP from glycolysis and may be resistant to mitochondrial defects. In order to bypass glycolysis, HepG2 cells were grown in DMEM medium (Gibco, USA) without glucose supplemented with 25 mM galactose, 2 mM glutamine, 10% FBS, and 1% penicillin/streptomycin (100 units/mL penicillin, and 100 mg/mL streptomycin) in a humidified incubator with 5% CO_2 at a temperature of 37ºC.

3.2.8. Quantification of intracellular ATP

ATP content was quantified using the CellTiter-Glo Luminescent Cell Viability Assay kit based on the manufacturer's instructions. This kit results in lysis of the cells and generation of a "glow-type" luminescent signal proportional to the amount of ATP present. Therefore, if there are equal number of cells in different wells, the signals may be interpreted as relative ATP content. Briefly, $1x10^4$ cells per well were seeded in white 96-well clear-bottom plates with either glucose or galactose. The next day, the cells were pre-treated with polyphenols for 2 h and treated with oleic acid for 24 h or 72 h. Rotenone (inhibitor of mitochondrial complex I) and antimycin A (inhibitor of complex III) were used as positive controls. Intracellular ATP content was then measured using the kit based on the manufacturer's instructions. Briefly, the cells were lysed using the reagent included in the kit and after 3 min of vigorous shaking and 10 min incubating in room temperature while covered (to stabilize the luminescence signals), the luminescence generated from the ATP-mediated chemical reaction was read using a microplate reader under standard luminescence

settings. Each experiment was repeated in triplicate with 3 replicate wells per experiment.

3.2.9. SIRT1 deacetylase activity

SIRT1 activity was measured using a SIRT1 direct fluorescence screening assay kit (Caymen, USA) according to manufacturer's protocol. In this method, deacetylation of acetylated p53 peptide by SIRT1 in the unknown sample sensitizes its binding to a fluorometric probe and the resultant fluorescence is proportional to the SIRT1 activity in the sample. The assay was performed by incubation of 5 µl of protein lysates with 15 µl substrate solution containing the fluorogenic acetylated p53 peptide (Arg-His-Lys-Lys(ε-acetyl)-AMC) and co-substrate NAD+, and 25 µl assay buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl2). The plate was covered and incubated on a shaker at room temperature for 45 min. After 45 min, 50 µl of the developer was added to each well and plate was incubated at room temperature for 30 min to release AMC from the deacetylated peptide. Using a fluorescence reader, the intensity of fluorescence was measured at excitation 360 nm and emission detection at 460 nm. Protein concentration in samples was quantified using a bicinchoninic acid (BCA) protein assay kit and the results of SIRT1 activity were normalized to protein concentrations. Each experiment was repeated in triplicate with 3 replicate wells per experiment.

3.2.10. Protein extraction, quantification, and Western blot

HepG2 cells were seeded in T25 flasks and exposed to polyphenols at 10 µM for 2 h followed by treatment with 1.5 mM oleic acid for 24 h. For total protein extraction, the cells were homogenized using ice-cold RIPA buffer 1x (10x solution was diluted to 1x with ddH_2O) (Millipore, USA) for 30 min on ice, supplemented with Halt protease and phosphatase inhibitor and EDTA (Thermo Fisher Scientific, USA). After centrifugation at 14,000 rpm for 15 minutes at 4°C, the supernatant containing protein was gently obtained, aliquoted and used for protein quantification and western blot. Protein content was quantified using a BCA protein assay kit (Thermo Fisher Scientific). Protein samples were then normalized, mixed with the loading buffer (2x Laemmli buffer supplemented with 10% β-mercaptoethanol), and denatured by heating at 70 °C for 10 min. Equal amount of proteins (40 µg in 40 µl volume) were loaded into each well of 8% or 4-12% Novex WedgeWell Tris-Glycine Pre-Cast gels (Invitrogen, USA) and then transferred to a 0.45 µm nitrocellulose membrane by the wet transfer method using Tris-Glycine running buffer. Following

transfer, nonspecific protein binding sites in the membrane were blocked with Tris-buffered saline containing Tween-20 (TBST) and 5% bovine serum albumin (BSA) for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with rabbit primary monoclonal antibodies for phospho-AMPKαThr172 (1:500) and β-actin (1:500) (both from Cell Signaling Technology, Danvers, MA, USA), and PGC-1α (1:500, Abcam, USA). The membranes were then washed with Tris-buffered saline (TBS, PH:7.6) with 0.1% Tween-20 (TBST) three times (5 minutes each) and then incubated for 1 h with secondary anti-rabbit antibody (Horseradish Peroxidase 1:2000, Santa Cruz Biotech, CA, USA). After several washings with TBST and TBS, the membrane was incubated with the enhanced chemiluminescence western blotting detection kit (Thermo Fisher Scientific) for 3 min and the bands were observed and quantified using an Alpha Imager Gel Imaging System (Alpha Innotech). The results were finally normalized to their corresponding control β-actin.

3.2.11. Mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential was measured using the fluorescent probe, tetramethlyrhodamine ethyl ester (TMRE) according to the manufacturer's protocol (Invitrogen, USA). TMRE (a cationic fluorescent probe) is driven into the negatively-charged mitochondrial matrix due to its lipophilic feature and positive charge. Thus, the mitochondrial membrane potential is directly proportional to the intensity of red fluorescence signals [269]. HepG2 hepatocytes at 3×10^4 per well were seeded in the black 96-well optical-bottom plates for 24 h in the medium containing 10% FBS. After 24 h, the old medium was replaced with fresh medium containing 10% FBS and 1% fatty acid-free bovine serum albumin and the cells were pre-treated with 10 µM polyphenols for 2 h followed by treatment with or without 1.5 mM oleic acid for 24 h. After 24 h, the old medium was replaced with fresh medium (without FBS) containing TMRE at 500 nM final concentration and the cells were covered and incubated at 37 °C for 20 min. Excess dye was then removed and the cells were washed carefully 2-3 times with PBS and the mean fluorescence of 9 different spots of each well was measured using a microplate reader at an excitation 549 nm and emission 575 nm. The uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 50 µM which collapses the mitochondrial membrane potential was used as a positive control and added 10 min before the cells being exposed to TMRE. The fluorescence images showing the mitochondrial membrane potential in HepG2 cells were captured

using a ZOE Fluorescent Cell Imager. Each experiment was repeated in triplicate with 3 replicate wells per experiment.

3.2.12. Statistical analysis

Results were expressed as mean values with their standard error of means (SEM) for the number of experiments indicated. Results were analyzed using the Statistical Package for the Social Science (SPSS 22) (IBM, USA). One-way ANOVA was used initially, and when any significance detected, Dunnett's post hoc test was used to compare the mean of each group with the oleic acid alone condition. The level of significance was $P<0.05$. Correlation was determined by the Pearson's R.

3.3. Results

3.3.1. Effects on HepG2 cell morphology

By evaluating the cells with an inverted microscope, we found that untreated HepG2 hepatocytes have a healthy eye-shaped morphology (Figure 3.1). After treatment with oleic acid, some cells show large droplets of fat (macro-vesicular steatosis). However, most cells show micro-vesicular steatosis with numerous small droplets of fat (sometimes more than 13-14 small fat droplets).

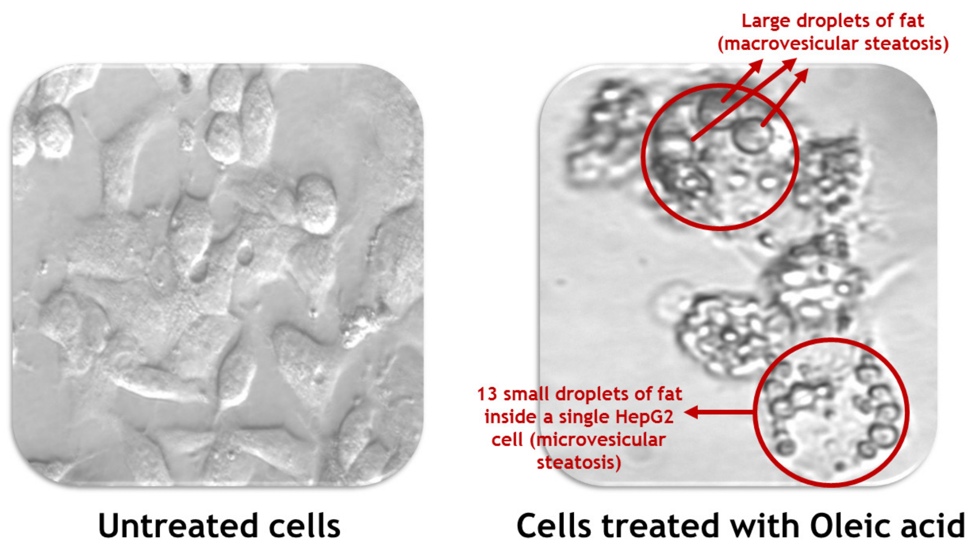

Figure 3. 1. Brightfield images (600x magnification) representative of the morphology of HepG2 cells before and after treatment with oleic acid.

Figure 3.2 shows representative images of the effect of polyphenols on HepG2 cell morphology. All of the polyphenols were effective in inhibiting oleic acid-induced lipid accumulation. HepG2 cells pre-treated with different polyphenols were less steatotic and apparently healthier compared to the oleic acid alone condition. Fat droplets were smaller and more dispersed after treatment with polyphenols (fewer macro- and micro-vesicular droplets). Another important finding is that HepG2 cells pre-treated with polyphenols kept their eye-shaped morphology almost intact compared to the oleic acid alone condition in which the cells were rounded, swollen or distorted in morphology.

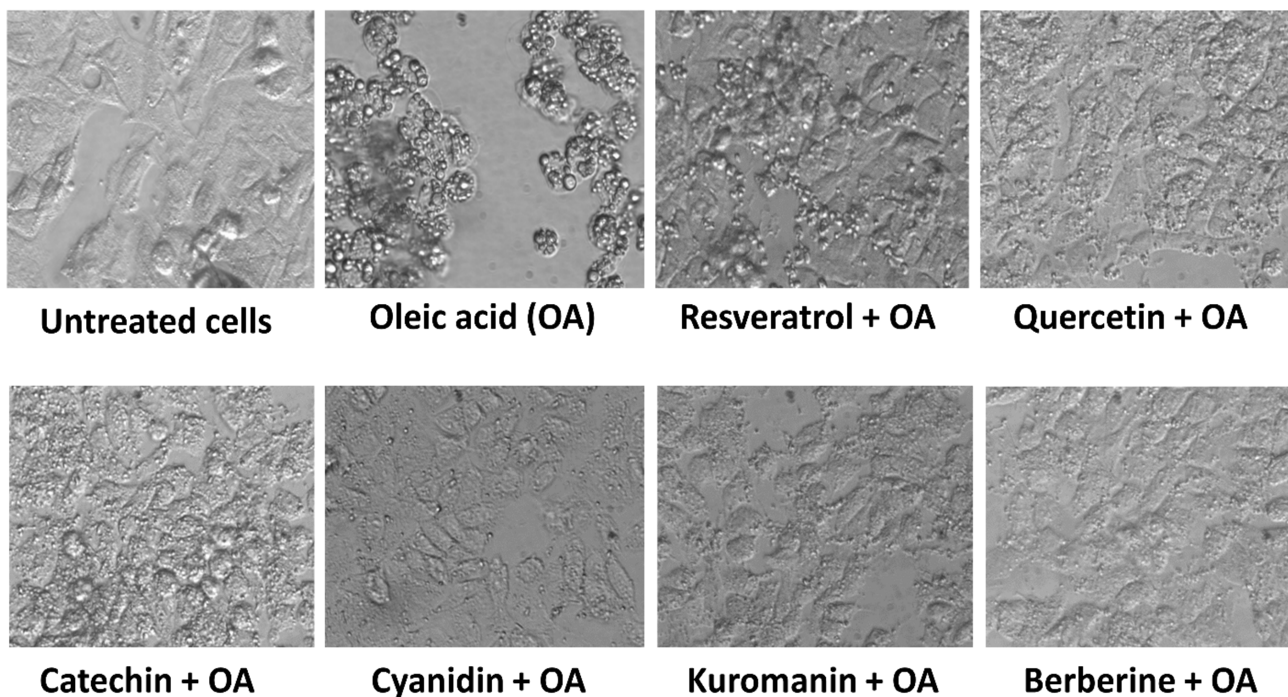

Figure 3. 2. Brightfield images (400x magnification) representative of the morphology of hepatocytes after treatment with 10 µM polyphenols for 2 h followed by treatment with 1.5 mM oleic acid for 24 h.

3.3.2. The effect of polyphenols on oleic acid-induced intracellular lipid accumulation

Figure 3.3 shows the Nile Red fluorescence captured by a fluorescence microscope representing intracellular triglyceride content. Higher red fluorescence in HepG2 cells exposed to oleic acid shows higher intracellular fat compared to untreated cells. Different polyphenols reduced the

fluorescence intensity, representing inhibition of oleic acid-induced lipid accumulation. These results are in accordance with the results shown in Figure 3.2.

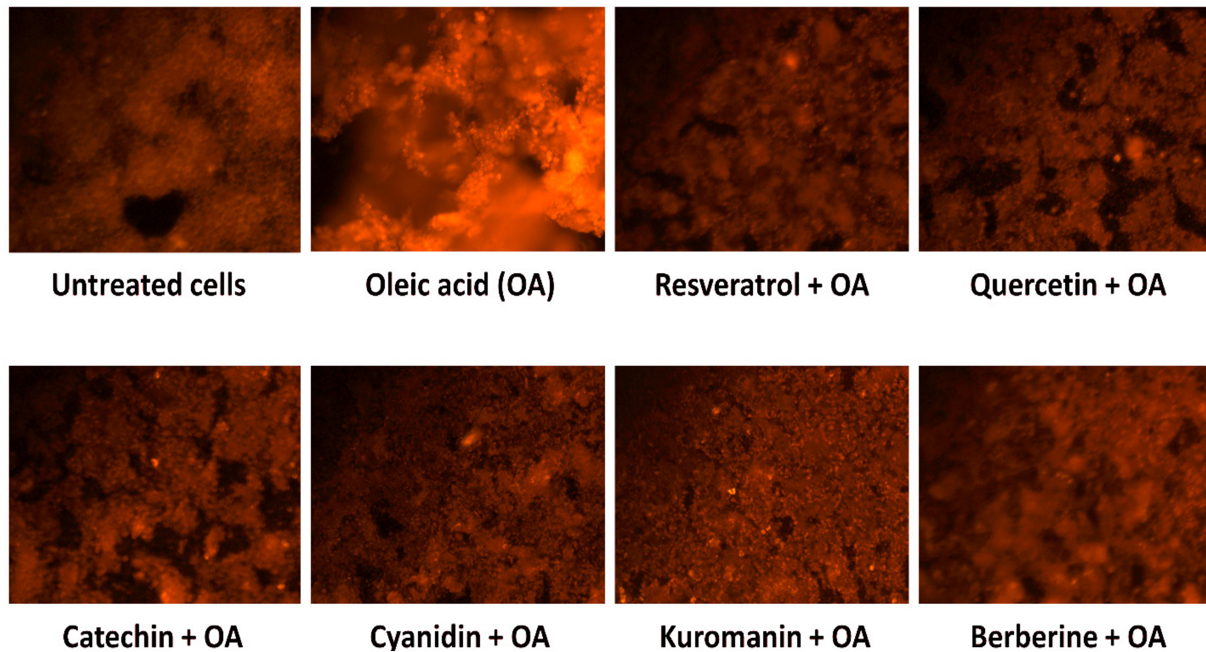

Figure 3. 3. Fluorescence images (400x magnification) using the Nile Red fluorescence probe for intracellular lipid content. Higher red fluorescence represents higher lipid content in HepG2 cells.

Intracellular lipid content was quantified using the Nile Red staining and a microplate reader. Treatment with oleic acid alone increased intracellular lipid content in HepG2 cells by 150% while treatment with 10 µM polyphenols inhibited accumulation of lipids by 42-58% (Figure 3.4).

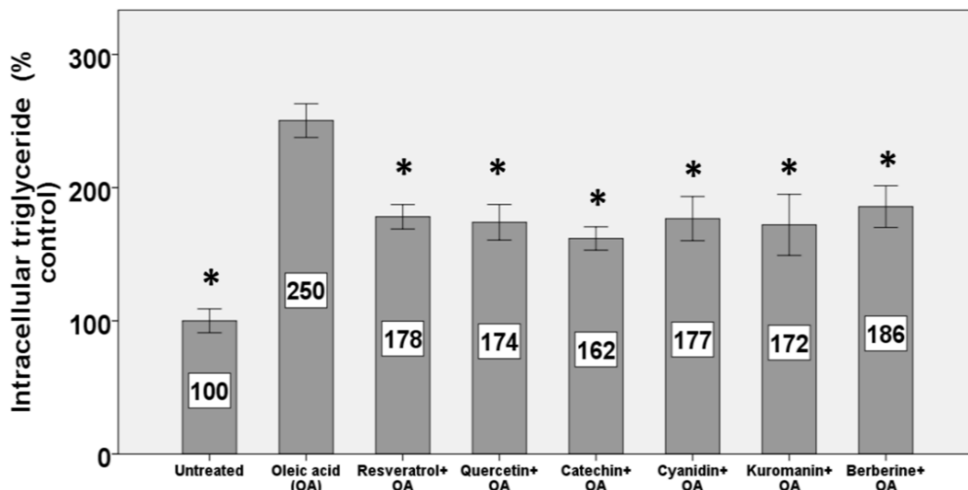

Figure 3. 4. Intracellular triglyceride content after quantification of the Nile Red fluorescence

HepG2 cells were pretreated with 10 µM polyphenols for 2 h followed by treatment with 1.5 mM oleic acid for 24 h. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$. The significant difference between groups was tested with one-way ANOVA followed by Dunnett's post-hoc test.

3.3.3. Flow cytometry of HepG2 cells

In order to find the effect of oleic acid and polyphenols on the size and morphology of the cells, HepG2 cells were evaluated by a flow cytometer. Compared to untreated cells, oleic acid produced less forward and higher side scattering (Figure 3.5), showing the cells have abnormal size, and distorted shape and morphology. Treatment with all polyphenols at 10 µM increased forward and decreased side scattering of HepG2 hepatocytes to be more similar to untreated cells. These results are consistent with the results shown in Figure 3.2 in which polyphenols preserved oleic acid-induced distortion of HepG2 cell morphology.

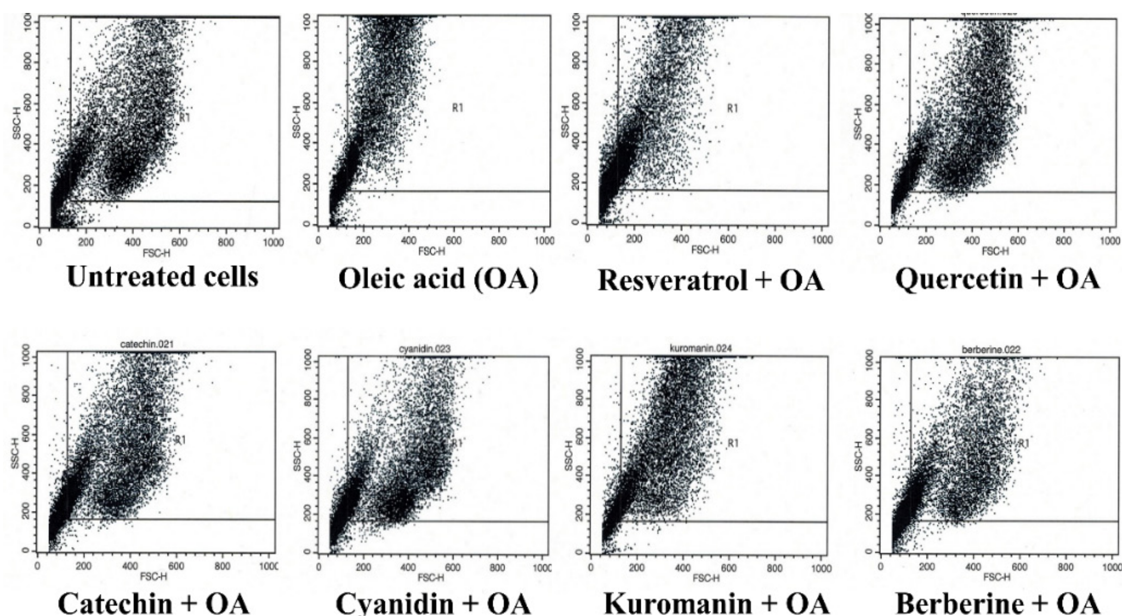

Figure 3. 5. Representative plots of forward and side scattering of 10,000 cells using flow cytometry after treatment with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h

3.3.4. Effects of oleic acid and polyphenols on the mitochondrial membrane potential (ΔΨm)

Fluorescence images captured by a ZOE Fluorescent Cell Imager represent how treatment with oleic acid or the uncoupler CCCP (used as a positive control) dissipated the mitochondrial membrane potential in HepG2 cells evidenced by decreased intensity of TMRE red fluorescence (Figure 3.6.A). Treatment with catechin, resveratrol, quercetin, and cyanidin prior to treatment with oleic acid protected against collapse of the mitochondrial membrane potential evidenced by higher intensity of the red fluorescence (Figure 3.6.A). Enlarged images of both healthy untreated hepatocytes and hepatocytes treated with oleic acid alone show the mitochondrial membrane potential dissipation in enlarged fat-laden hepatocytes (Figure 3.6.B).

Quantification of TMRE fluorescence using a microplate reader shows that treatment of HepG2 hepatocytes with 1.5 mM oleic acid decreased the mitochondrial membrane potential by 48%, even more than a mitochondrial uncoupler (CCCP) that was used as a positive control (Figure 3.6.C). Consistent with the images shown in Figure 3.6.A, treatment with polyphenols such as cyanidin, catechin, quercetin, and resveratrol prior to treatment with oleic acid protected against mitochondrial membrane potential collapse by 44-60%. Berberine and kuromanin did not give any significant protection.

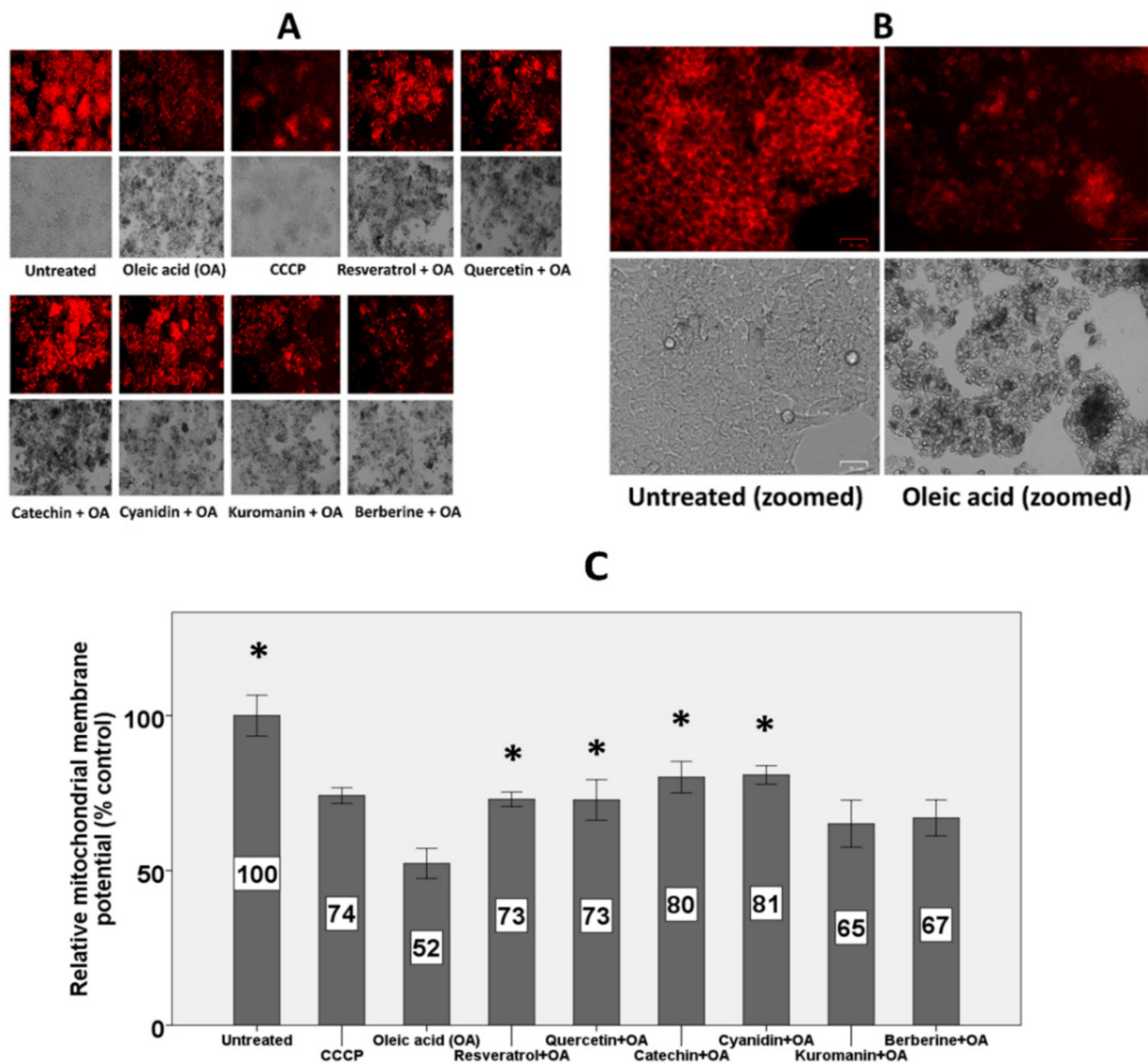

Figure 3. 6. Effects of oleic acid and polyphenols on the mitochondrial membrane potential.

(A) Fluorescence images (with their corresponding brightfield images) showing the effect of oleic acid and polyphenols on the mitochondrial membrane potential after TMRE staining. Images at 20x magnification were captured using a ZOE Fluorescent Cell Imager 30 min after staining the cells with TMRE. (B) Enlarged images (digital zoom at 60x magnification) of HepG2 cells exposed to oleic acid alone (droplets of fat are obvious in the cells in corresponding brightfield images) compared to healthy untreated cells. (C) The mitochondrial membrane potential in HepG2 cells after treatment with 10 µM polyphenols for 2 h and treatment with 1.5 mM oleic acid for 24 h was measured by quantification of TMRE fluorescence using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents

means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from control group at P<0.05.

3.3.5. Effects of oleic acid and polyphenols on genes involved in fatty acid β-oxidation (*CPT1A1* and *PPARα*)

Oleic acid significantly induced mRNA for carnitine palmitoyltransferase 1 (*CPT1A1*) by 200%, which appears to be an adaptive mechanism (Figure 3.7). Treatment of HepG2 cells with berberine, resveratrol, quercetin, catechin, and kuromanin further significantly increased *CPT1A1* expression by 118, 87, 54, 52, and 50 %, respectively. Cyanidin had no significant effect on *CPT1A1* expression.

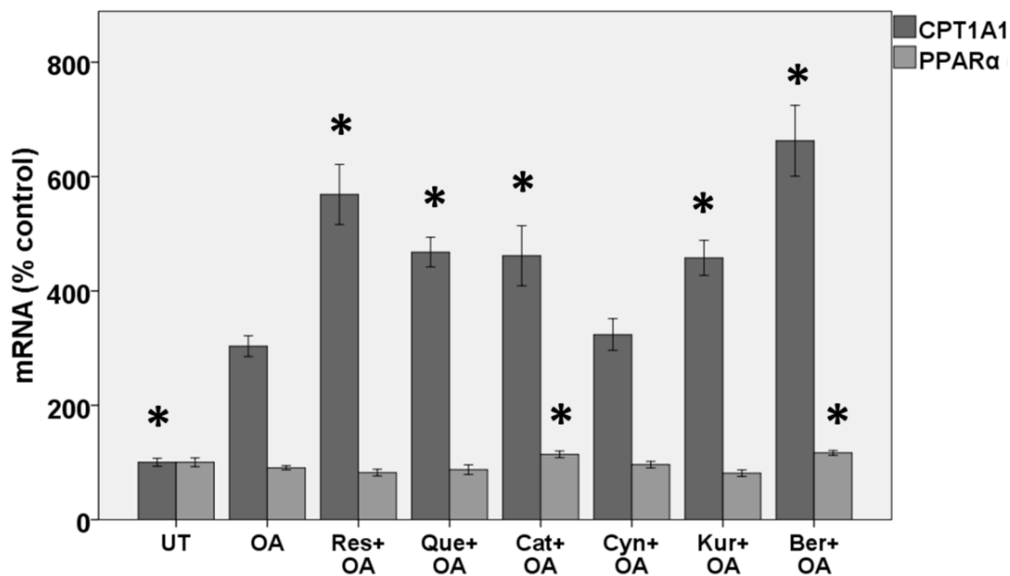

Figure 3. 7. Expression of mRNA for carnitine palmitoyltransferase 1 (CPT1A1) and peroxisome proliferator-activated receptor alpha (PPARα).

UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine. HepG2 cells were pretreated with 10 µM polyphenols for 2 h followed by treatment with 1.5 mM oleic acid for 24 h. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at P<0.05.

Oleic acid did not significantly affect the peroxisome proliferator-activated receptor alpha mRNA (*PPARα*) expression, while treatment with berberine and catechin significantly increased *PPARα* expression by 29, and 25%, respectively (Figure 3.7). Resveratrol, quercetin, and anthocyanins

had no significant effect on *PPARα* expression.

3.3.6. The effect of oleic acid and polyphenols on expression of genes involved in lipogenesis (*FAS* and *PPARγ*)

Figure 3.8 shows mRNA for fatty acid synthase (*FAS*) after treatment with polyphenols and oleic acid. Oleic acid significantly increased *FAS* by 43%. Resveratrol, quercetin, and kuromanin completely prevented the increase in *FAS* expression. Catechin, cyanidin, and berberine however, did not inhibit *FAS* mRNA.

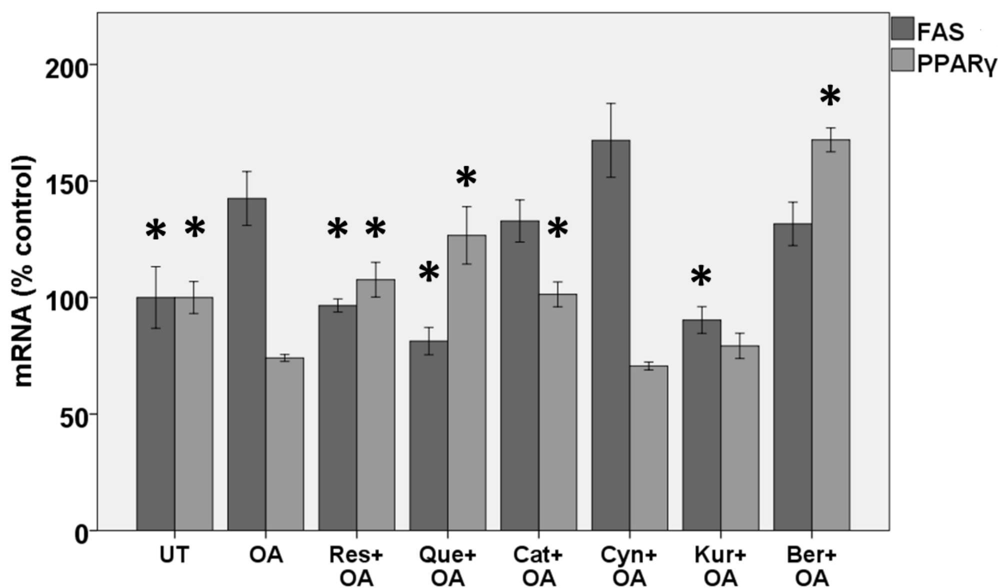

Figure 3. 8. mRNA expression for fatty acid synthase (FAS) and peroxisome proliferator-activated receptor gamma (PPARγ).

UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine. HepG2 cells were pretreated with 10 μM polyphenols for 2 h followed by treatment with 1.5 mM oleic acid for 24 h. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

Figure 3.8 shows the peroxisome proliferator-activated receptor gamma mRNA (*PPARγ*) expression in HepG2 hepatocytes. Oleic acid significantly inhibited *PPARγ* expression by 26% while berberine, quercetin, resveratrol, and catechin significantly reversed the expression by more than 100%. Anthocyanins had no significant effect on *PPARγ* expression. Berberine showed the strongest effect, increasing *PPARγ* expression by 68% over that of untreated cells.

3.3.7. Effects of oleic acid and polyphenols on the gene expression and activity of sirtuins

Shown in Figure 3.9.A, oleic acid had no significant effect on the sirtuin 1 mRNA (*SIRT1*) expression while catechin, berberine, and resveratrol significantly induced *SIRT1* mRNA by 46%, 44%, and 35% respectively. Quercetin did not significantly induce *SIRT1* expression (P=0.15). Anthocyanins also had no significant effect on *SIRT1* mRNA expression.

As with mRNA expression, oleic acid had no effect on SIRT1 activity, while resveratrol, catechin, and berberine significantly increased SIRT1 activity by 25, 19, and 17%, respectively (Figure 3.9.B). Consistent with no effect on mRNA expression, quercetin and kuromanin had no effect on SIRT1 activity. An exception for a different effect on the mRNA expression and SIRT1 activity was cyanidin, which though it had no effect on the SIRT1 expression, it increased SIRT1 activity by 19%.

Expression of mRNA for the mitochondrial sirtuin3 (*SIRT3*) was also investigated. Treatment with oleic acid alone had no significant effect on *SIRT3* mRNA while only catechin increased its expression by 57% compared to the oleic acid alone condition (Figure 3.9.C).

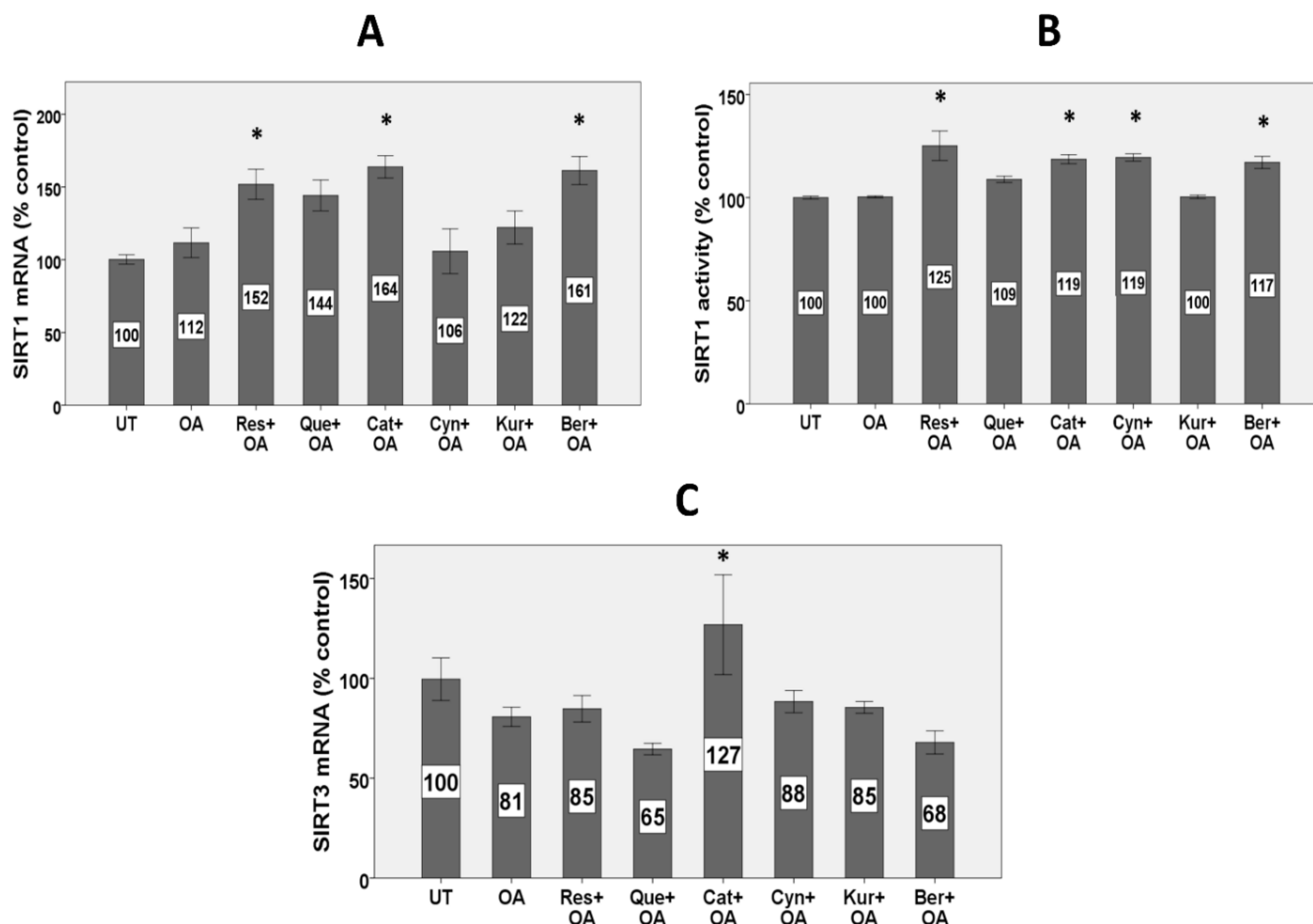

Figure 3. 9. (A) SIRT1 mRNA, (B) SIRT1 activity and (C) SIRT3 mRNA.

Experimental conditions were as described in Figure 3.4. UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine. HepG2 cells were pre-treated with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h. For SIRT1 activity, the data are normalized to protein concentration. Untreated cells were set at 100% and data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments for SIRT1 and SIRT3 expression and 3 independent experiments for SIRT1 activity with 3 replicate wells in each experiment. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

3.3.8. Effects on the expression of proteins involved in mitochondrial metabolism and biogenesis (phosphorylated AMPKα and deacetylated PGC1α)

Treatment of HepG2 cells with oleic acid did not change the phosphorylation of 5' AMP-activated protein kinase alpha (AMPKα) on threonine 172 (p-AMPKα^{Thr172}) while some polyphenols such as resveratrol, berberine, and catechin induced the phosphorylation by 35, 32, and 28%,

respectively (Figure 3.10). Quercetin, cyanidin, and kuromanin (cyanidin-3-glucoside) had no significant effect on the AMPKα phosphorylation.

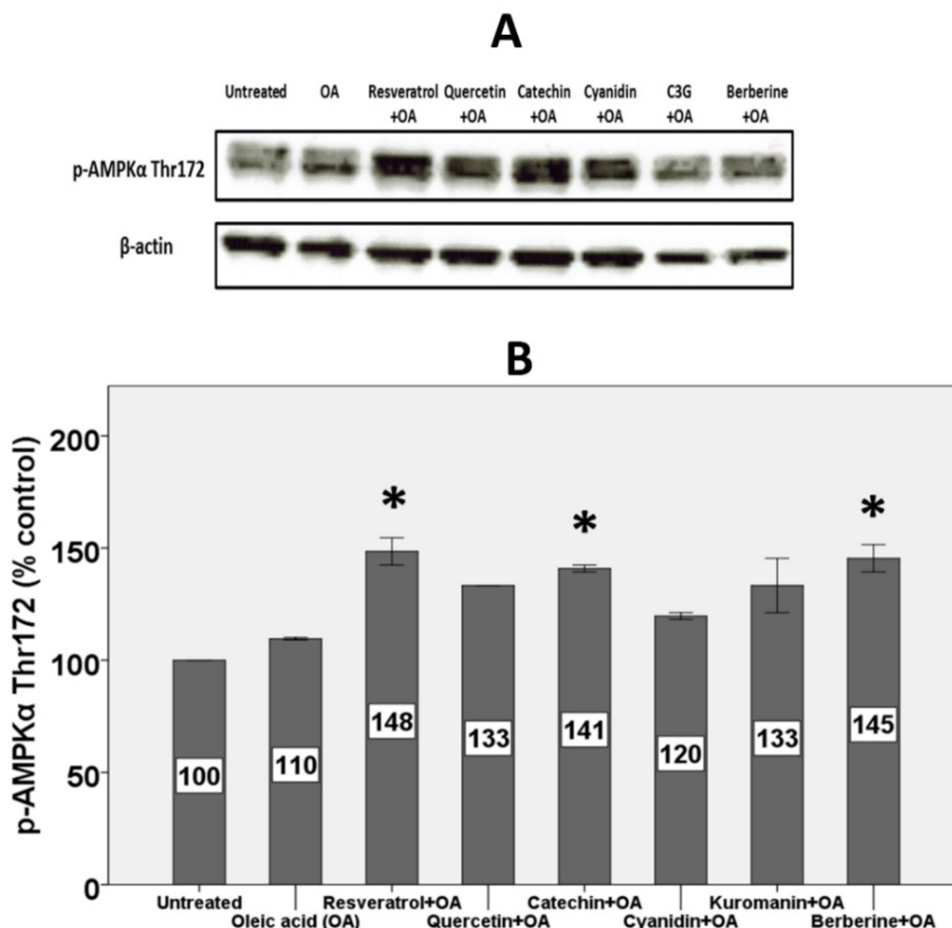

Figure 3. 10. Protein expression of p-AMPKαThr172 in HepG2 cells pre-treated with 10 μM polyphenols for 2 h and 1.5 mM oleic acid for 24 h measured by Western blot.

Experimental conditions were as described in Figure 3.4. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 2 independent experiments. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

Regarding the deacetylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), oleic acid had no effect while only kuromanin and berberine increased protein expression of deacetylated PGC1α by 54 and 51%, respectively (Figure 3.11). Other polyphenols had no effect on the deacetylation of PGC1α.

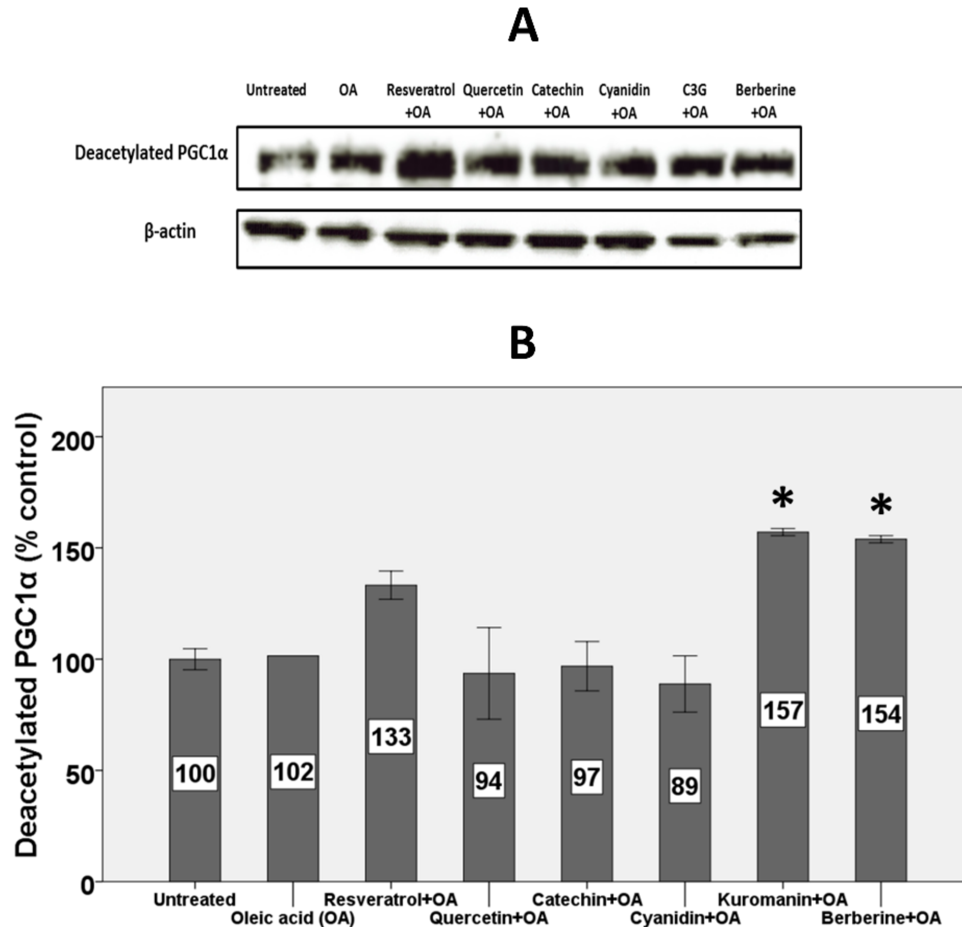

Figure 3. 11. Protein expression of deacetylated PGC1α in HepG2 cells pre-treated with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h measured by Western blot.

Experimental conditions were as described in Figure 3.4. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 2 independent experiments. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

3.3.9. Effects of oleic acid and polyphenols on intracellular ATP stores

Intracellular ATP stores in HepG2 cells were determined after growing the cells in either glucose or galactose medium. In 5.5 mM glucose medium after 24 h, treatment of HepG2 cells with oleic acid and polyphenols had no effect on ATP stores except for slight (12 and 7%) but significant decreases by kuromanin and berberine (Figure 3.12). In glucose medium after 72 h, oleic acid increased ATP stores by 23%, and treatment with polyphenols had no effect on ATP stores except a slight (9%) but significant decrease by berberine (Figure 3.12).

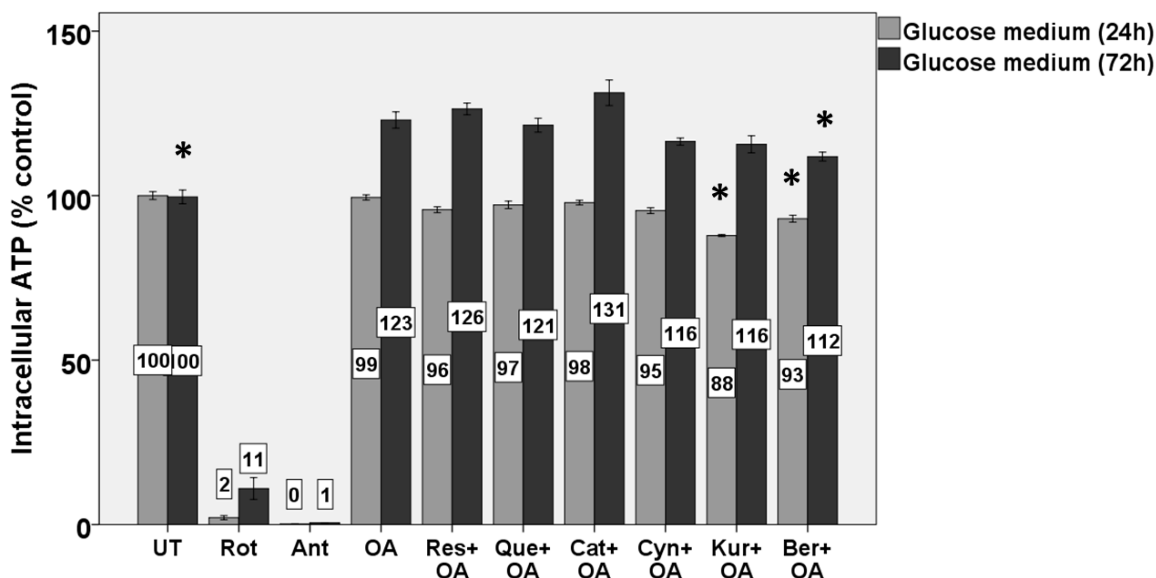

Figure 3. 12. Intracellular ATP stores in HepG2 cells grown in glucose medium.

UT: untreated, Rot: rotenone, Ant: antimycin, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine. (A) HepG2 cells were grown in glucose medium (5.5 mM) and pre-treated with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h. Rotenone and antimycin (for 24 or 72 h) were used as positive controls to prevent the complex I and III activity, respectively. Untreated cells were set at 100% and the data are presented as % of untreated cells. The bars represent means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

When the cells were grown in galactose medium (to supress glycolysis) for 24h, treatment with oleic acid alone significantly decreased intracellular ATP stores by 11% while no polyphenol could protect against the inhibition (Figure 3.13). Berberine exacerbated the effect of oleic acid and together decreased ATP stores by 73% compared to untreated cells. In galactose medium for 72 h, oleic acid depleted intracellular ATP by 57% (Figure 3.13). Compared to the oleic acid alone condition, treatment with the polyphenols cyanidin, catechin, resveratrol, quercetin, increased ATP stores by 95, 88, 84, and 81%, respectively. Berberine and kuromanin did not give any protection.

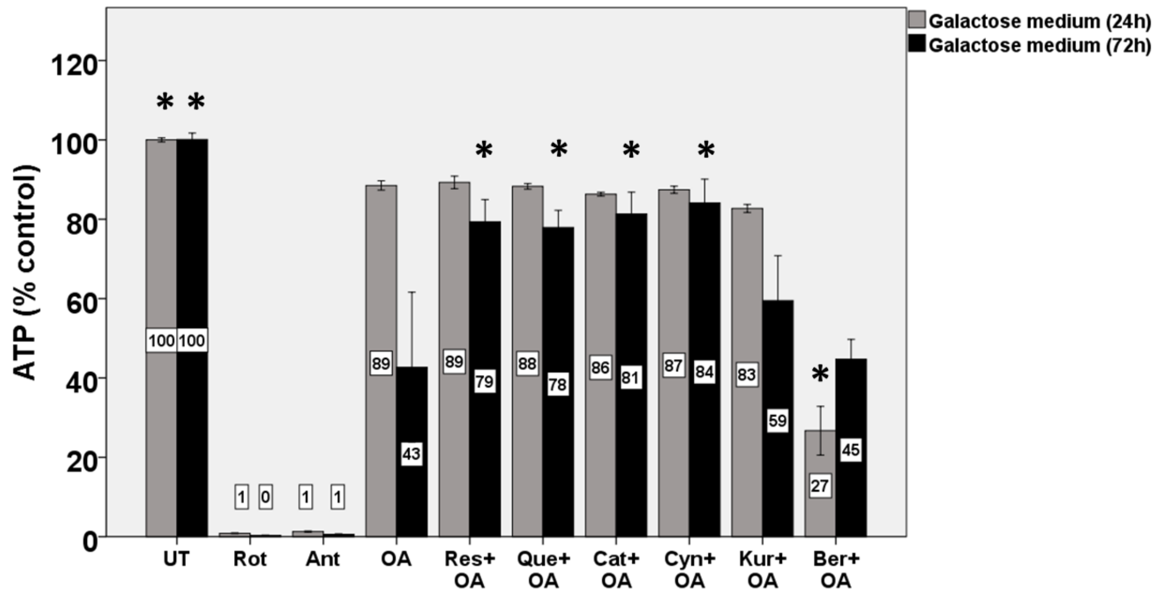

Figure 3. 13. Intracellular ATP stores in HepG2 cells grown in galactose medium.

UT: untreated, Rot: rotenone, Ant: antimycin, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine. HepG2 cells were grown in galactose medium (25 mM) and pre-treated with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h. Rotenone and antimycin (for 24 or 72 h) were used as positive controls to prevent the activity of complex I and III, respectively. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

3.4. Discussion

This study was designed to investigate and compare the effects of different classes of polyphenols on steatosis (the "first hit"), lipid metabolism, and mitochondrial biogenesis and membrane potential in an *in vitro* model of non-alcoholic fatty liver disease. In contrast to many *in vitro* studies using high doses of polyphenols, a relatively low dose of polyphenols (10 µM) was used in the current study. In measurements of steatosis (cellular morphology, Nile Red staining, and flow cytometry), treatment of the HepG2 cells with oleic acid increased intracellular lipid accumulation, while different classes of polyphenols gave similar protection. Although the results from these measurements did not distinguish the different polyphenols, they are consistent with previous studies showing individual polyphenols to inhibit intracellular lipid accumulation in HepG2 cells treated with oleic acid [35-37] or palmitic acid [42].

In investigations on cell size and morphology, we found that oleic acid induced less forward scattering and higher side scattering. Forward and side scattering usually represent the cell size and granularity, respectively. Accordingly, higher side scattering in hepatocytes treated with oleic acid may be due to increased cytoplasmic granules (fatty acid droplets). Increased side scattering in these cells showed that the cells have distorted shape, morphology, and size. Consistent with the results on intracellular lipids, all the polyphenols showed similar protection and produced higher forward and lower side scattering in the cells. These results show that the polyphenols produced more normal cell size and morphology.

The anti-steatotic effect of polyphenols in the current study may be partly explained by increased expression of enzymes involved in fatty acid β-oxidation such as the hepatic enzyme carnitine palmitoyltransferase 1 (*CPT1A1*), and decreased expression of enzymes involved in lipogenesis such as fatty acid synthase (*FAS*). However, this mechanism was not the case for cyanidin, which had no effect on these genes. Inhibition of steatosis by cyanidin in the current study may be partially explained by the higher expression of uncoupling protein 2 (*UCP2*) by this polyphenol, as shown in CHAPTER 4, which induces fatty acid metabolism. However, cyanidin may also affect other genes or proteins involved in lipogenesis or fatty acid oxidation that were not investigated in the current study [245].

In expression of *CPT1A1*, oleic acid and polyphenols acted in the same direction to increase the expression. Increased expression of *CPT1A1* by oleic acid in the current study may be an adaptive mechanism to induce β-oxidation due to increased load of fatty acids which need to be metabolized in mitochondria. Consistent with our results, other studies show hepatic fatty acid oxidation and CPT1 expression or activity is often enhanced in rodents and patients with NAFLD and obesity (reviewed in [4]). We also found that all polyphenols except cyanidin further upregulated *CPT1A1* mRNA. These results are consistent with a few previous studies where individual flavonoids or flavonoid-rich extracts were shown to increase mRNA for CPT1 dose-dependently in HepG2 cells treated with free fatty acids [270], or CPT1 expression or activity in genetically obese or high-fat fed rats [95, 271].

To gain further insights into the effect on lipid metabolism by polyphenols we measured effects on the expression of PPARs (PPARα and PPARγ) and FAS. The transcriptional control of genes by PPARs has critical roles in lipid metabolism (particularly hepatic steatosis), inflammation, and

fibrosis [156]. PPARα, once activated, induces the expression of genes involved in β-oxidation such as CPT1A1 in NAFLD [156]. Although numerous studies report increased expression of PPARα in fatty liver disease, others are in accordance with our results observing unchanged or even less expression of PPARα in NAFLD [4, 48]. Polyphenols also had little effect on PPARα expression in the current study, in contrast to some studies in rodents fed polyphenols with a high-fat diet [95, 243]. Some polyphenols such as cyanidin may be an agonist and a natural ligand for different PPARs [245], and therefore may affect PPAR activity without affecting expression.

In the current study, treatment of HepG2 hepatocytes with oleic acid significantly decreased PPARγ expression while several polyphenols except anthocyanins reversed this inhibition. PPARγ induces genes involved in lipid uptake and adipogenesis [157]. While most evidence shows that PPARγ expression increases in diabetic or obesity models of NAFLD [159] and suggest a deleterious and lipogenic role for hepatic PPARγ in NAFLD [272, 273], others suggest a beneficial role for PPARγ [160, 161] . While some studies show that treatment of rodents on an obesogenic high-fat diet with polyphenols decreases PPARγ expression in NAFLD [48, 188], others are consistent with our study and showed higher hepatic expression of PPARγ by polyphenols favoring improved hepatic insulin signaling and decreased hepatic steatosis [274]. Therefore, enhanced expression of PPARγ in hepatocytes by polyphenols in our study may be a beneficial outcome in protection against NAFLD. However, more studies are needed to elucidate the controversial role of PPARγ in NAFLD.

In the current study, oleic acid induced *FAS*. Fatty acid synthase (FAS) is an important enzyme in *de novo* lipogenesis which catalyzes the production of palmitic acid from malonyl-CoA [275]. In agreement with our findings, others found increased *FAS* in the liver of high fat-fed mice [276]. It was also shown by several researchers that the gene expression of FAS was increased in NAFLD patients [277], and FAS was suggested to be an intriguing therapeutic target for NAFLD [275]. We observed that several polyphenols prevented the increase in expression of *FAS*. Polyphenols have previously been reported to inhibit steatosis by decreasing mRNA and protein expression of FAS in HepG2 cells treated with oleic [37] or palmitic acid [270]. Although cyanidin decreased intracellular triglyceride in our study, it was unique in having no effect on both CPT1A1 and FAS, which suggests it may act through other mechanisms.

Other aspects of NAFLD and lipid metabolism that we investigated in the current study are those of mitochondrial biogenesis and energy metabolism. Numerous studies have shown that mitochondrial biogenesis is decreased in fatty liver disease and fewer mitochondria are available in hepatocytes to metabolize excess fat [19]. Increasing mitochondrial biogenesis can therefore be a promising approach in prevention or treatment of NAFLD/NASH. Accordingly, the effects of oleic acid and polyphenols were investigated on several factors involved in mitochondrial biogenesis. The results showed that the expression of SIRT1 and SIRT3 and the activity of SIRT1 were not changed following treatment of HepG2 cells with oleic acid. In contrast to our findings, lower protein expression of SIRT1 was reported in rodents with NAFLD [250, 278]. However, resveratrol, catechin, and berberine increased both gene expression and activity of SIRT1 in our study. Different polyphenols such as berberine [252] and resveratrol [248] have been shown to induce SIRT1 expression in different models of NAFLD and insulin resistance. Mitochondria are one of the main targets of sirtuins' action (including SIRT1 and SIRT3) leading to the regulation of metabolism. SIRT1 induces mitochondrial biogenesis through the deacetylation of PGC1α and activation of AMPK (through the deacetylation of serine/threonine liver kinase (LKB1) which phosphorylates AMPK) and therefore increases fatty acid β-oxidation that may help alleviate NAFLD [178]. In addition to the induction of mitochondrial biogenesis and fatty acid β-oxidation, SIRT1 inhibits the transcription of all genes involved in lipogenesis [183, 184]. SIRT1-knockout mice showed lower fatty acid oxidation and higher hepatic steatosis [182]. Among polyphenols, resveratrol has been widely studied with regard to SIRT1 since resveratrol is a potent activator of SIRT1 [279]. In agreement with this property, among all the polyphenols of interest in our study, resveratrol showed the strongest effect on SIRT1 activity. Consistent with the results on SIRT1 expression and activity, we found higher phosphorylation of threonine 172 on AMPK catalytic α subunit by the same polyphenols (resveratrol, catechin, and berberine). AMPK activation induces mitochondriogenesis in hepatocytes and leads to increased ATP-producing pathways such as β-oxidation and glycolysis but reduces energy-consuming pathways such as lipogenesis and gluconeogenesis [280]. Interestingly, the same polyphenols (resveratrol, catechin, and berberine) as well as quercetin induced several genes involved in mitochondrial biogenesis (shown in CHAPTER 4). However, only kuromanin and berberine induced the deacetylation of PGC1α in the current study. PGC1α is a key protein downstream of AMPK and SIRT1 which is involved in mitochondrial biogenesis and is a promising factor in prevention of NAFLD [178]. PGC1α protein

can be activated by either phosphorylation by kinases (such as AMPK) or deacetylation by SIRT1. Since some polyphenols such as catechin had no effect on deacetylation of PGC1α but increased AMPK phosphorylation in our study, it may be that such polyphenols activate PGC1α by phosphorylation but not deacetylation.

Treatment with oleic acid in the current study depolarized the mitochondrial membrane potential in HepG2 cells by approximately 50%. Recent investigations show collapsed mitochondrial membrane potential in NASH compared to fatty liver and it was suggested that a lower activity of the mitochondrial respiratory chain could play a major role in this collapse [4]. Depolarization of mitochondria has a critical role in initiation of apoptosis and cell death particularly in fatty liver disease [281]. The results of the current study show that polyphenols differ in preserving the mitochondrial membrane potential. Resveratrol, catechin, quercetin, and cyanidin protected against oleic acid-induced collapse of the mitochondrial membrane potential, while kuromanin and berberine were not effective. Consistent with our results, others have shown that different polyphenols such as curcumin [20] or punicalagin polyphenols found in pomegranate [265] can protect against free fatty acid-induced dissipation of the mitochondrial membrane potential. This protection was also shown by flavonoids from *Rosa laevigata Michx* fruit in rodents fed a high-fat diet [282]. Therefore, it appears that preserving the mitochondrial membrane potential may be one of the mechanisms by which polyphenols prevent progression of simple steatosis to NASH, since it is one of the hallmarks of cell survival and functionality of mitochondria.

In order to further compare the effectiveness of different classes of polyphenols for protection against mitochondrial dysfunction, intracellular ATP stores were determined. Hepatic ATP stores become depleted in NASH due to impaired or reduced activity of mitochondrial respiratory complexes [149]. The cellular ATP levels are important in maintaining the integrity of hepatic tissue, and ATP depletion may result in hepatocellular injury [266]. Mitochondrial injury and dysfunction is considered as an important contributor of low hepatic ATP stores in NASH [151]. Although mitochondrial function in our study was affected by oleic acid due to collapsed mitochondrial membrane potential, and lower mitochondrial biogenesis and complex activity (discussed in CHAPTER 4), we could not observe any depletion of ATP stores in HepG2 cells grown in glucose medium for 24 and 72 h. Numerous studies have shown that when mitochondrial oxidative phosphorylation is impaired in the cells (particularly cancer cells), they rely on

glycolysis to compensate for their reduced mitochondrial-mediated ATP production [283]. Replacing glucose with galactose is a way to shift metabolic pathways toward oxidative phosphorylation which increases the sensitivity and responsiveness to mitochondrial dysfunction [283, 284]. Therefore, in order to bypass glycolysis and more appropriately elucidate the effect of mitochondrial dysfunction on ATP production, HepG2 cells were grown in galactose medium. In this medium, treatment with oleic acid for 72 h depleted ATP stores by more than 50% while treatment with most polyphenols maintained ATP stores. These results are consistent with other studies showing treatment of hepatocytes with free fatty acids depletes while polyphenols preserve ATP stores [20, 255]. Interestingly, the results on ATP are consistent with the results of the mitochondrial membrane potential in which resveratrol, quercetin, catechin, and cyanidin showed significant effects while kuromanin and berberine had no effect on preserving both mitochondrial membrane potential and ATP stores. Also, in both experiments oleic acid showed approximately 50% inhibition. It appears that when grown on galactose, ATP stores are dependant on the mitochondrial membrane potential and by inhibiting oleic acid-induced dissipation of $\Delta\Psi m$ and mitochondrial dysfunction, the effect of polyphenols in this study allowed mitochondria to maintain intracellular ATP stores.

Another interesting finding of the current study was decreased ATP stores by berberine in both glucose and especially galactose medium after 24h, in contrast with most polyphenols which increased ATP stores. A previous study [285] showed that treatment of 3T3-L1 adipocytes or L6 myotubes with 5 μM berberine for 0.5 to 16 h, increased the AMP/ATP ratio concomitant with lower oxygen consumption and inhibition of mitochondrial function, which induced the phosphorylation and activation of AMPK and eventually lead to higher ATP contents. In support of this mechanism, 10 μM berberine in our study in HepG2 cells grown on glucose increased p-$AMPK\alpha^{Thr172}$, concomitant with a decline of ATP stores. Also, while most polyphenols protected against the oleic acid-induced decline in the mitochondrial membrane potential, berberine did not. When grown in galactose medium for 24 h, berberine worsened the depletion of ATP stores compared to oleic acid alone, while after 72 h this worsening was not evident (although it still did not protect). The sharp depletion of ATP stores by berberine in galactose medium after 24 h was not evident in glucose medium after the same time point since it is reported that berberine can increase glucose metabolism through glycolysis to produce ATP [285], which may have partially compensated.

Another interesting finding is the difference of the ATP stores between 24 h and 72 h incubation in glucose or galactose medium. In the cells grown in glucose medium, ATP stores increased in the cells exposed to oleic acid after 72 h compared to untreated cells, and treatment with polyphenols had no effect. One possible explanation is that the cells adapted to glucose metabolism for producing ATP after 72 h to compensate for mitochondrial dysfunction. In galactose medium however, ATP stores were depleted after 72 h of exposure to oleic acid showing that the cells cannot produce enough ATP due to the mitochondrial defect that was evident at this time point (since both galactose and oleic acid require mitochondrial metabolism for net ATP production).

In conclusion, oleic acid induced steatosis, distorted HepG2 cell morphology, modulated the expression of genes involved in lipogenesis and fatty acid oxidation, and induced mitochondrial dysfunction evidenced by the mitochondrial membrane potential collapse and depleted ATP stores. Treatment with different polyphenols prevented these changes. Although they differed in the strength with which they affected different mechanistic pathways, the different polyphenols showed similar protection against intracellular steatosis and mitochondrial dysfunction.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interests.

3.5. Transition to CHAPTER 4

In CHAPTER 3, we focused on steatosis as the “first hit” of non-alcoholic fatty liver disease and how mitochondrial dysfunction may affect steatosis. We showed that oleic acid can induce steatosis and distort the morphology of HepG2 hepatocytes while all polyphenols of interest

showed similar protection. It was also shown that polyphenols can inhibit steatosis by modulating the expression of genes involved in lipogenesis and fatty acid oxidation. We also showed that oleic acid can induce mitochondrial dysfunction evidenced by collapse of the mitochondrial membrane potential and depletion of intracellular ATP stores while several polyphenols rescued these effects. Finally, the effects on different genes and proteins involved in metabolism such as SIRT1, SIRT3, AMPK, and PGC1α were evaluated. In the next chapter, we focus on oxidative stress and inflammation as the "second hits" of NAFLD, and their association with mitochondrial biogenesis and function and uncoupling protein 2. The expression of nuclear DNA-encoded mitochondrial complex I-V subunits has also been investigated.

CHAPTER 4: COMPARISON OF DIETARY POLYPHENOLS FOR PROTECTION AGAINST MOLECULAR MECHANISMS UNDERLYING NON-ALCOHOLIC FATTY LIVER DISEASE IN A CELL MODEL OF STEATOSIS

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Abbreviations: 4-HNE, 4-hydroxynonenal; **AMPK,** AMP-activated protein kinase; **ATP5F1,** ATP synthase, mitochondrial Fo complex, subunit B1; **ATP5G1,** ATP synthase, mitochondrial Fo complex, subunit C1 (subunit 9); **BSA,** bovine serum albumin; **COX6B1,** cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous); **DMSO,** dimethyl sulfoxide; **ETC,** electron transport chain; **GAPDH,** Glyceraldehyde 3-phosphate dehydrogenase; **MDA,** malondialdehyde; **Mn-SOD,** manganese superoxide dismutase; **mtDNA**, mitochondrial DNA; **NAFLD,** non-alcoholic fatty liver disease; **NASH,** non-alcoholic steatohepatitis; **NDUFS8,** NADH dehydrogenase (ubiquinone) Fe-S protein 8; **NFκB,** nuclear factor kappa b; **NRF,** nuclear respiratory factor; **OA,** oleic acid; **OXPHOS,** oxidative phosphorylation; **PGC1α,** peroxisome proliferator-activated receptor gamma coactivator 1-alpha; **ROS,** reactive oxygen species; **SDHb,** succinate dehydrogenase complex, subunit B, iron sulfur; **SOD,** superoxide dismutase; **TFAM,** mitochondrial transcription factor A; **TNFα,** tumor necrosis factor alpha; **UCP2,** uncoupling protein 2; **UQCRC1,** ubiquinol-cytochrome c reductase core protein I; **ΔΨ,** mitochondrial membrane potential.

Abstract

Scope: Dietary polyphenols have shown promise in protecting the liver against non-alcoholic fatty liver disease. The relative effectiveness and mechanisms of different polyphenols however is mostly unknown.

Methods and results: In a cell model of steatosis using HepG2 hepatocytes, we evaluated the protective effects of different classes of polyphenols and the contributing mechanisms. Treatment of the cells with oleic acid increased ROS generation and mRNA expression of TNFα, decreased mRNA expression of UCP2 and decreased mitochondrial content and biogenesis (evidenced by MitoTracker Green and mRNA for PGC1α, NRF1 and mitochondrial complex I subunit NDUFS8). Treatment with 1-10 μM polyphenols (resveratrol, quercetin, catechin, cyanidin, kuromanin, berberine) all protected by more than 50% against the oleic acid-induced increase in ROS. Most of the polyphenols prevented the decrease in UCP2 mRNA, while several prevented the increase in TNFα mRNA, reversed decreases in mitochondrial biogenesis and increased expression of mitochondrial respiratory complexes and MnSOD. The anthocyanins were unique in decreasing ROS generation without inducing mitochondrial biogenesis or Mn-SOD mRNA expression.

Conclusion: While different polyphenols similarly decreased cellular ROS in this model of steatosis, they differed in their ability to suppress TNFα expression and induce mitochondrial biogenesis and content.

Keywords: non-alcoholic fatty liver disease, polyphenols, oxidative stress, mitochondrial biogenesis, respiratory proteins

4.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a prevalent liver disease, affecting 10-35% of the general population in different communities [1] and 75% of obese patients with type 2 diabetes [286, 287]. NAFLD may result in serious complications such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Among possible treatments, dietary polyphenols have been identified to offer a potential therapy for NAFLD and its progression to NASH [288, 289].

The leading hypothesis regarding development and progression of fatty liver disease is a ‘‘two-hit’’ hypothesis [2]. This hypothesis suggests that hepatic accumulation of fats containing oleic acid [81] is the first hit which predisposes the liver to second hits such as oxidative stress and inflammation. Recently other second hits have been suggested to be involved, including insulin resistance and mitochondrial dysfunction [6, 290], which interplay in a concerted manner to exacerbate simple steatosis to steatohepatitis, cirrhosis, and fibrosis.

Oxidative stress and inflammation are two “second-hits” that may be targeted by polyphenols. Oxidative stress has been demonstrated by numerous studies to have a central role in the liver damage and the progression of NAFLD to NASH and has been associated with depleted antioxidant activities [10]. Accordingly, maintaining redox homeostasis by polyphenol supplementation can be a possible strategy to prevent reactive oxygen species (ROS) production in NAFLD [250]. Inflammatory processes also have a major role in the liver damage and activation of collagen-producing hepatic stellate cells leading to fibrosis [21], and dietary polyphenols often show anti-inflammatory effects [289].

Mitochondrial dysfunction is another aspect that could be targeted in therapies involving polyphenols. An accumulating body of evidence suggests NAFLD as a mitochondrial disease due to the abnormality in function and morphology of hepatic mitochondria [10, 11, 103, 152]. Correspondingly, mitochondrial dysfunction is correlated with the severity of the disease and its progression to NASH [12]. This involvement is due to the pivotal role of mitochondria in lipid metabolism, lipid peroxidation, ROS generation, cytokine production and apoptosis [13]. Polyphenols such as resveratrol have been observed to stimulate mitochondrial biogenesis in different tissues in a variety of dietary studies [251, 291].

There are many studies on NAFLD in animal models or *in vitro* showing beneficial effects of polyphenols, including effects on oxidative stress [48, 226], inflammation [48, 270], steatosis [45, 46, 241, 292], and mitochondrial biogenesis [20, 291]. However, most are with a single polyphenol or food source, so a major question might be which polyphenols are more effective and by what mechanisms. Therefore, in an *in vitro* model of steatosis we compared the effects of different classes of polyphenols on ROS generation, TNFα production, and mitochondrial biogenesis to explore the molecular mechanisms underlying NAFLD.

4.2. Materials and Methods

4.2.1. Materials

Dichloro-dihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich, USA. Malvidin and delphinidin were from Extrasynthese, France. MitoTracker Green was from Invitrogen, USA. For the rest of materials see section 3.2.1.

4.2.2. HepG2 cells culture condition

See section 3.2.2.

4.2.3. Treatment with oleic acid/BSA and polyphenols

See section 3.2.3.

4.2.4. Measurement of intracellular total ROS

Intracellular ROS was determined using 2’,7’-dichloro-dihydrofluorescein diacetate (DCFH-DA) according to the manufacturer’s protocol (Sigma-Aldrich, USA). DCFH-DA is a cell-permeant non-fluorescent probe. Following cleavage of the acetate moieties of DCFH-DA by intracellular deacetylases and consequently being trapped inside the cells, reactive oxygen species oxidize DCFH to DCF (2’,7’-dichlorofluorescein) which is highly fluorescent. Therefore, the intensity of DCF fluorescence is proportional to intracellular total ROS. HepG2 cells at 3×10^4 per well were cultured in black 96-well optical-bottom plates for 24 h in MEM medium containing 10% FBS. After 24 h, the old medium was replaced with fresh medium containing 10% FBS and 1% fatty

acid-free bovine serum albumin and the cells were pre-treated with different concentrations of polyphenols (1, 5, 10 μM) for 2 h (polyphenols were not removed thereafter and the cells were incubated with them for the next 24 h) followed by treatment with 1.5 mM oleic acid for 24 h. After 24 h, the old medium was removed and the cells were exposed to fresh medium (without FBS) containing DCFH-DA at 25 μM final concentration. H_2O_2 at 100 μM final concentration was used as positive control. After incubating with DCFH-DA for 20-30 min at 37ºC, mean fluorescence of 9 different spots of each well was measured using a microplate reader at excitation 485 nm and emission 528 nm. Fluorescence images were captured using a ZOE Fluorescent Imager.

4.2.5. RT-qPCR

See section 3.2.6. Sequences of primers of interest are shown in appendix (Table 7.2).

4.2.6. Measurement of mitochondrial content using MitoTracker Green

To determine mitochondrial content (mitochondrial biogenesis), HepG2 hepatocytes were labelled with a mitochondria-specific dye, MitoTracker Green according to the manufacturer's protocol (Invitrogen, USA). Initially non-fluorescent in aqueous solutions, and insensitive to the mitochondrial membrane potential (ΔΨ), MitoTracker Green becomes highly fluorescent following accumulation in the mitochondrial lipid compartment [293]. Following passive diffusion across mitochondrial membrane, MitoTracker Green accumulates inside metabolically active mitochondria and labels mitochondria due to its mildly thiol-reactive chloromethyl moiety. Accordingly, resultant fluorescence is proportional to a cell's mitochondrial number. Briefly, 3×10^4 HepG2 cells were seeded per well. After 24 h, the old medium was replaced with fresh medium containing 1% fatty acid free bovine serum albumin and cells were pre-treated with 10 μM polyphenols for 2 h followed by treatment with oleic acid for 24 h. After 24 h, the old medium was removed and cells were exposed to 100 nM final concentration of MitoTracker Green dissolved in medium (without FBS) and the plate was covered and incubated in 37ºC for 20-30 min. After incubation, the medium containing probe was removed and the cells were carefully washed 2-3 times with PBS and mean fluorescence of 9 different spots of each well was measured using a microplate reader at excitation 485 nm and emission 528 nm. Fluorescence images were

captured using a ZOE Fluorescent Cell Imager.

4.2.7. Statistical analysis

See section 3.2.12.

4.3. Results

4.3.1. Effects of oleic acid and polyphenols on ROS generation in HepG2 cells

Treatment of HepG2 cells with 1.5 mM oleic acid for 24 h significantly increased ROS generation by 127% (Figure 4.1). All polyphenols at different concentrations (1, 5, 10 μM) except delphinidin (5, 10 μM) significantly decreased ROS generation. Compared to the oleic acid alone condition, cyanidin-3-glucoside (kuromanin) was the strongest polyphenol and at 10 μM decreased ROS by approximately 77%. Delphinidin inhibited significantly at 1 μM, but at 5 and 10 μM was unable to significantly prevent ROS generation.

Fluorescence images of intracellular ROS captured by a ZOE Fluorescent Cell Imager are shown in the Appendix (Figure 7.1).

Figure 4. 1. Effects of polyphenols on intracellular ROS production.

UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Mal: malvidin, Del: delphinidin, Ber: berberine. Comparison of different classes of dietary polyphenols at 1, 5, and 10 μM on oleic acid-induced ROS generation. H_2O_2 at 100 μM was used as positive control. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition ($P<0.05$).

4.3.2. Effects of oleic acid and polyphenols on mitochondrial content

Evaluating mitochondrial number by MitoTracker Green shows that oleic acid significantly decreased mitochondrial content in hepatocytes by 21 % and some of the polyphenols protected against this reduction (Figure 4.2 and 4.3). The strongest compound was berberine followed by

quercetin and resveratrol which reversed oleic acid-induced inhibition of mitochondrial content to levels higher than control cells. Anthocyanins along with catechin had no significant effect on mitochondrial content.

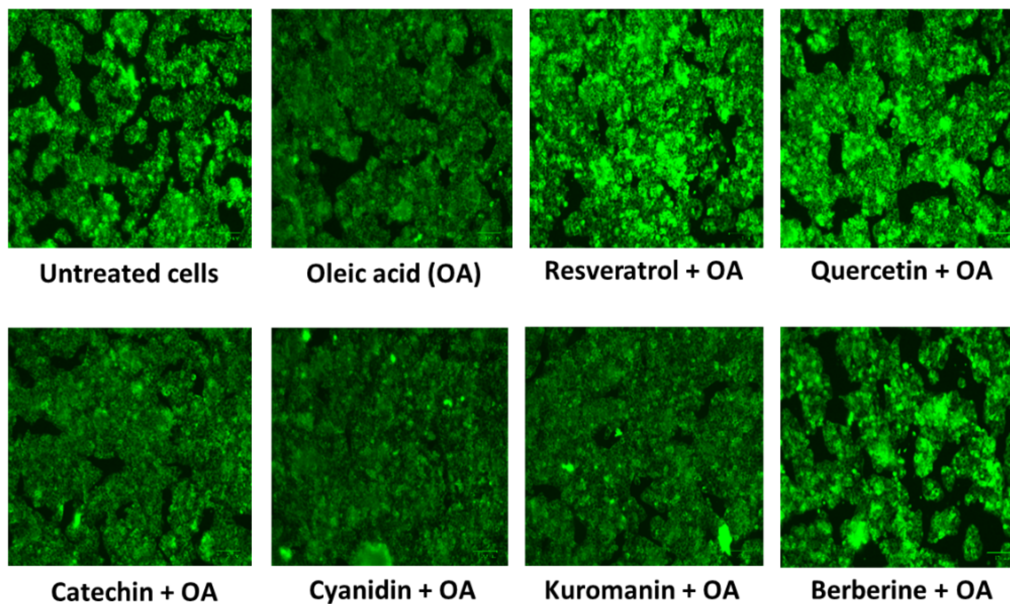

Figure 4. 2. Fluorescence images of HepG2 cells showing mitochondrial content.

The images were captured using a ZOE Fluorescent Cell Imager at 20x magnification after staining the cells with MitoTracker Green. Higher green fluorescence represents a higher number of mitochondria.

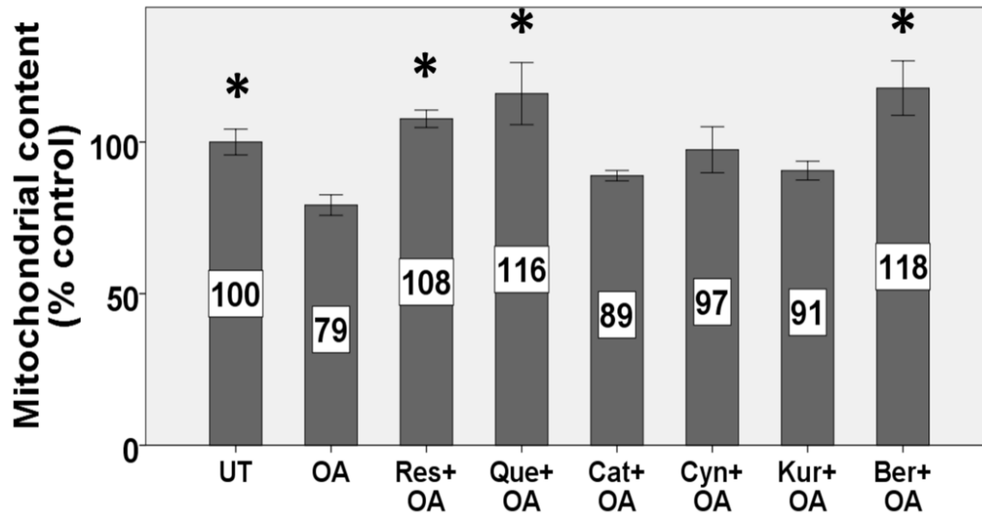

Figure 4. 3. Comparison of polyphenols on mitochondrial content after quantification of MitoTracker Green fluorescence using a microplate reader.

(UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

4.3.3. The effect of oleic acid and polyphenols on the expression of genes of interest

4.3.3.1. The effect on uncoupling protein 2 (UCP2)

Figure 4.4a shows *UCP2* mRNA after treatment with 10 μM different polyphenols for 2 h and 1.5 mM oleic acid for 24 h. Oleic acid significantly inhibited *UCP2* mRNA expression by 37%. Catechin, quercetin, cyanidin and kuromanin significantly reversed oleic acid-induced inhibition of *UCP2* expression by more than 100%. Resveratrol and berberine did not rescue *UCP2* expression.

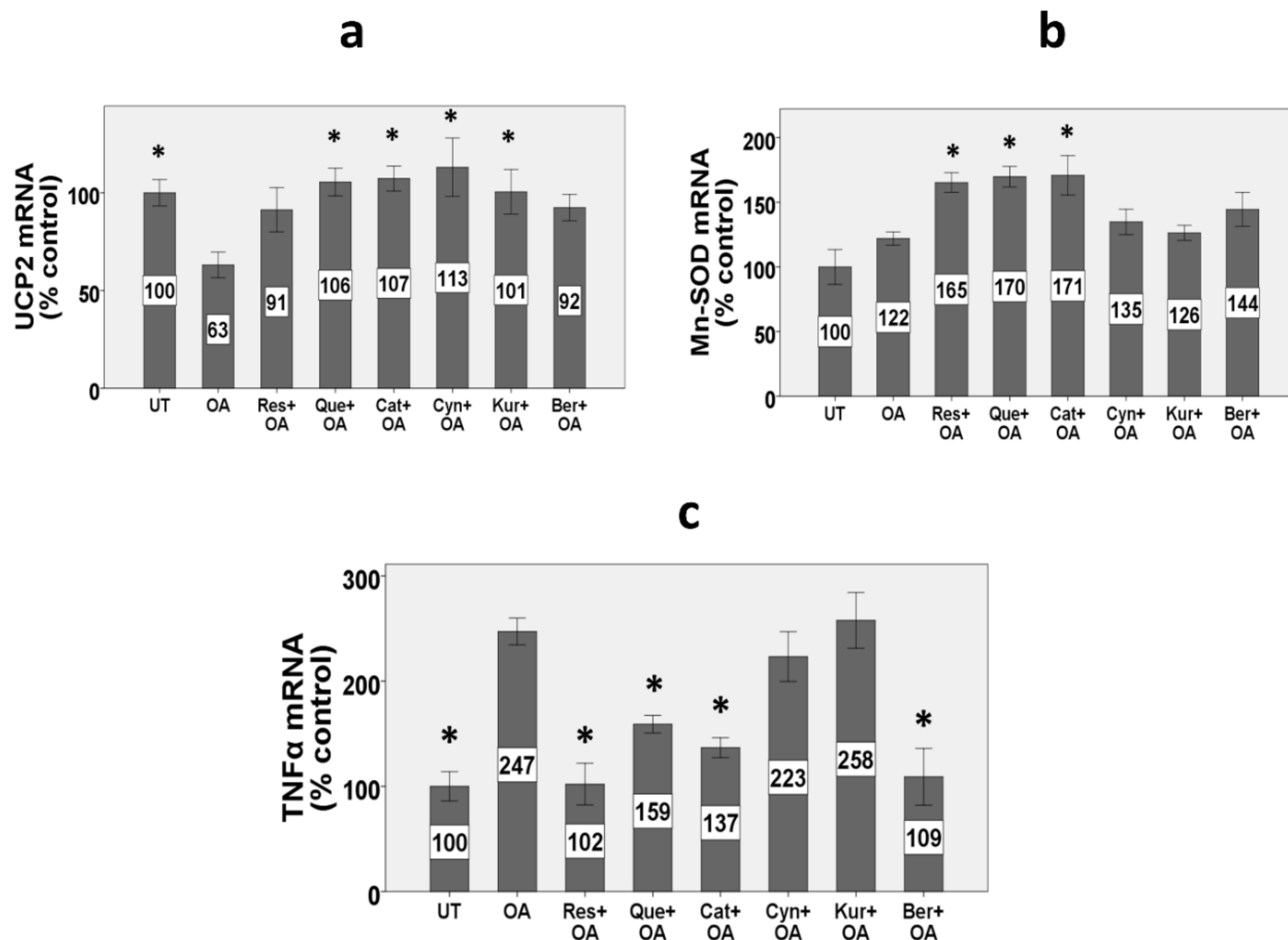

Figure 4. 4. Comparison of dietary polyphenols on mRNA expression of UCP2 (a), Mn-SOD (b), and TNFα (c) after treatment of HepG2 hepatocytes with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h.
(UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different ($P<0.05$) from the oleic acid alone condition.

4.3.3.2. The effect on mitochondrial manganese superoxide dismutase (*Mn-SOD*)

Oleic acid alone had no significant effect on *Mn-SOD* expression (Figure 4.4b). Catechin, quercetin and resveratrol significantly increased *Mn-SOD* expression by 40, 39, and 35%, respectively compared to oleic acid alone. The anthocyanins and berberine had no significant effect on *Mn-SOD* expression.

4.3.3.3. The effect on the expression of *TNFα*

Oleic acid elevated mRNA expression of *TNFα* by 147% while polyphenols could prevent this induction (Figure 4.4c). Resveratrol, berberine, catechin, and quercetin inhibited oleic acid-induced *TNFα* expression by 98, 94, 75, and 60%, respectively. Anthocyanins (cyanidin and kuromanin) did not give any inhibition.

4.3.3.4. The effects on genes involved in mitochondrial biogenesis (PGC1α, NRF1, TFAM)

Oleic acid alone significantly inhibited *PGC1α* mRNA expression by 21% while berberine, resveratrol, quercetin, and catechin induced its expression by 84, 63, 42, and 37% compared to control cells, respectively (Figure 4.5). Moreover, oleic acid significantly decreased *NRF1* mRNA expression by 26% and different polyphenols such as catechin, berberine, and quercetin significantly increased *NRF1* expression by 66, 43, and 40% respectively compared to oleic acid alone. Additionally, while treatment with oleic alone had no significant effect on *TFAM* mRNA expression, berberine, catechin, and resveratrol significantly induced *TFAM* expression by 77%, 71%, and 71% compared to oleic acid alone. Anthocyanins such as cyanidin and kuromanin were unique in having no significant effect on the expression of genes involved in mitochondrial biogenesis.

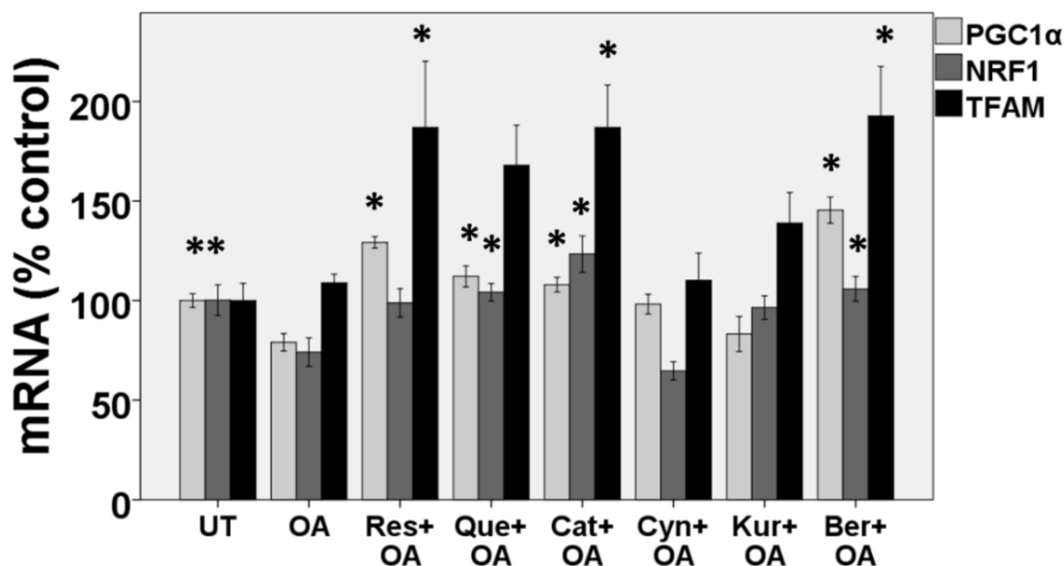

Figure 4. 5. Comparison of dietary polyphenols on mRNA expression of genes involved in mitochondrial biogenesis.

(UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

4.3.3.5. The effects on mRNA for nuclear DNA-encoded mitochondrial respiratory complex subunits

The effect of oleic acid and polyphenols on mRNA expression of nuclear DNA-encoded mitochondrial complex subunits is shown in Figure 4.6. While oleic acid alone significantly decreased mRNA expression of mitochondrial respiratory complex I subunit (*NDUFS8*) by 49 % compared to untreated cells, all polyphenols except cyanidin protected against this inhibition and in most cases increased the expression to the level more than untreated cells. Oleic acid alone had no significant effect on mRNA expression of *SDHb* (complex II subunit), mitochondrial complex III (*UQCRC1*), or IV (*COX6B1*) mRNA expression. Among polyphenols, only berberine increased expression of *SDHb* (by 81 %), while treatment with catechin, resveratrol and cyanidin significantly increased *UQCRC1* expression (by 58, 46, and 45%, respectively), and berberine and catechin were able to significantly increase *COX6B1* expression (by 34 and 31%, respectively).

While oleic acid alone significantly inhibited the expression of complex V subunit (*ATP5G1*) by 29%, no polyphenol protected against this inhibition (Figure 4.6).

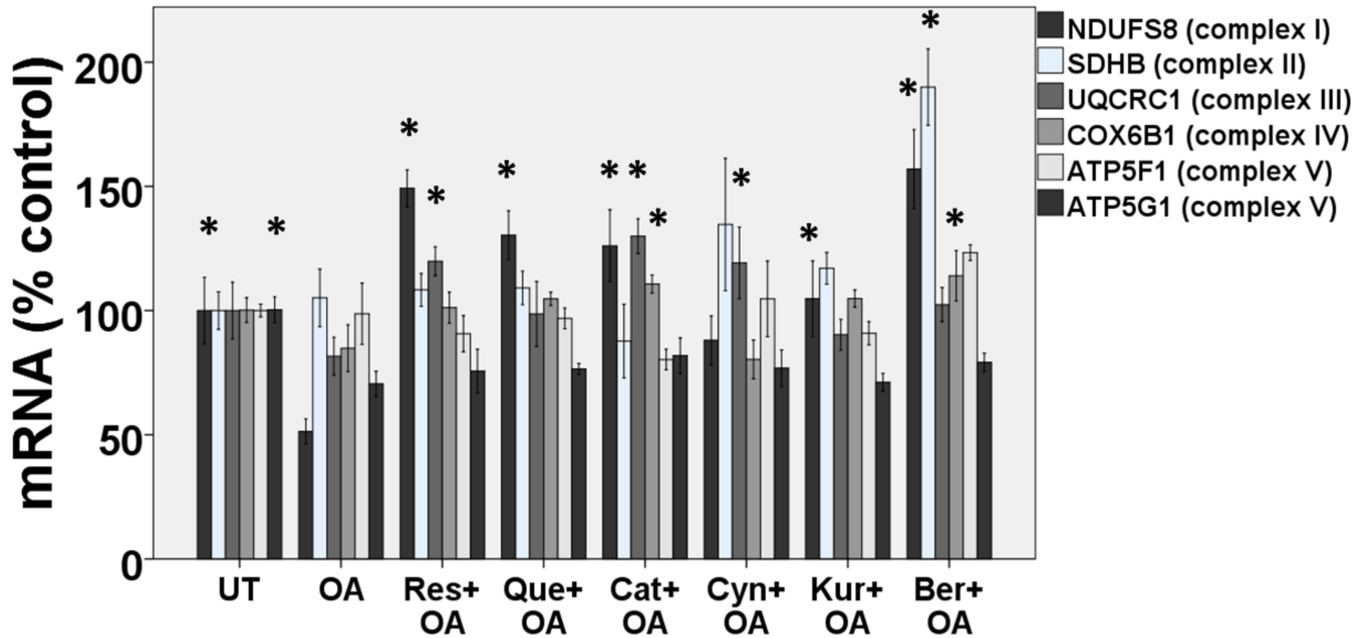

Figure 4. 6. Comparison of dietary polyphenols on mRNA of nuclear DNA-encoded mitochondrial respiratory complex subunits after treatment of HepG2 hepatocytes with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h.

(UT: untreated, OA: oleic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from oleic acid alone condition at P<0.05.

4.3.4. Correlation results

In order to evaluate the relationship between different parameters involved in the progression of fatty liver disease, a set of correlation studies have been performed by Pearson's R. Intracellular triglyceride had a high inverse correlation with mitochondrial membrane potential (r=-0.6, P<0.001) (Figure 4.7A). A fairly high inverse correlation was also found between ROS and TNFα with the mitochondrial membrane potential (r=-0.5, P<0.001; r=-0.43, P=0.003) representing along with accumulation of fat, higher oxidative stress and inflammation may result in mitochondrial dysfunction. A fairly high positive correlation was found between the expression of mitochondrial biogenesis markers such as *SIRT1, PGC1α,* and *TFAM* with *CPT1A1* (r=0.48, r=0.5, and r=0.48, respectively) suggesting increased fatty acid oxidation concomitant with increased

mitochondrial number (Figure 4.7B). The gene expression of *NDUFS8* (mitochondrial complex I subunit) was also positively correlated with the expression of *CPT1A1* (r=0.56, P<0.001) showing higher mitochondrial activity leads to increased oxidation of excess fat in mitochondria (Figure 4.7C). *PGC1α* expression was positively correlated with the expression of mitochondrial superoxide dismutase (*Mn-SOD*) (r=0.4, P=0.005) suggesting higher antioxidant defense along with elevated mitochondrial biogenesis (Figure 4.7D). An inverse correlation was also found between mRNA for mitochondrial biogenesis markers (*PGC1α* and *NRF1*) and TNFα (r=-0.54, r=-0.48, respectively) suggesting decreased inflammation along with elevated mitochondrial biogenesis (Figure 4.7E). There was also an inverse correlation between mRNA expression of *UCP2* and ROS generation (r=-0.42, P=0.003) showing reduced level of ROS along with elevated uncoupling effect of UCP2 (4.7F). Finally, gene expression of complex I and III subunits (*NDUFS8* and *UQCRC1*) was negatively correlated with expression of *TNFα* (r=-0.44, P=0.003, and r=-0.45, P=0.002 respectively) showing reduced inflammation along with increased mitochondrial activity (Figure 4.7G & H).

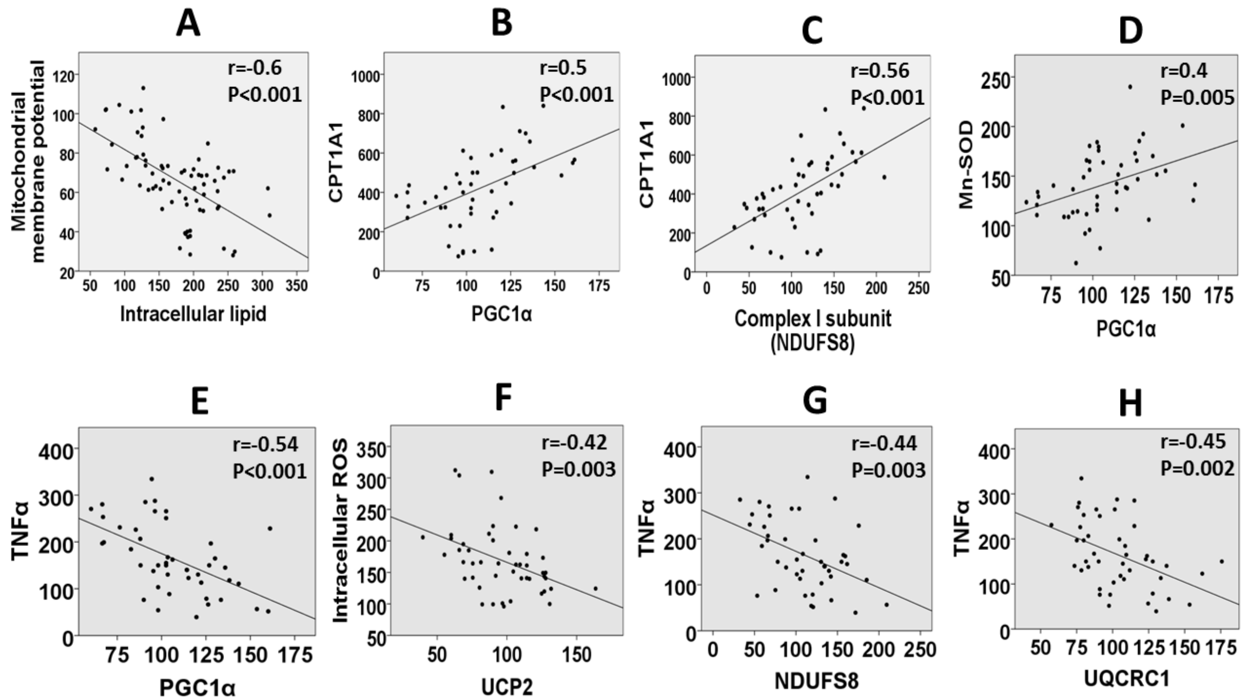

Figure 4. 7. Correlations between different parameters of interest.

These correlations are based on the results previously shown in figure 3.4 to 4.6.

4.4. Discussion

Oxidative stress and inflammation have been suggested to accelerate the progression of simple steatosis to NASH [2]. Therefore, we compared the preventive effects of different classes of polyphenols on these stressors (hits) in an *in vitro* model of steatosis. We showed that 1.5 mM oleic acid significantly induced ROS generation in HepG2 cells (by 127%). Zhang et al. previously showed that oleic acid at 1 mM could increase ROS generation in HepG2 cells by 80% making it an effective stress to simulate oxidative stress found in fatty liver disease [292]. The higher concentration of oleic acid (1.5 mM) in our study gave a slightly greater increase in ROS generation (127%) compared to the previous study. Moreover, we found that oleic acid increased the expression of the inflammatory cytokine TNFα. In line with this finding, Liu et al. observed that 1.5 mM oleic acid, but not lower doses increased release of the liver inflammation markers ALT and AST, as well as ROS levels in HepG2 cells [88]. Together the results suggest that this model can be an effective *in vitro* model for studying molecular mechanisms underlying NAFLD, due to increased oxidative stress and inflammation.

All of the tested polyphenols strongly inhibited oleic acid-induced ROS generation. One reason that they decreased ROS generation may have been direct antioxidant activities [227]. However, we also found increased expression of the antioxidant enzyme MnSOD by some polyphenols (resveratrol, catechin, and quercetin) and of the ROS-decreasing enzyme UCP2 in hepatocytes which may further explain the ROS-reducing effect of these compounds. Together these mechanisms resulted in decreased intracellular ROS, which by contribution to the cellular oxidative stress as one of the "second-hit" mechanisms may facilitate to progression of NAFLD to NASH [2]. In NAFLD, excess fatty acids are oxidized resulting in increased ROS generation. This increased ROS can result in mitochondrial dysfunction which increases hepatic steatosis in the liver, and this vicious circle finally leads to cirrhosis and hepatocellular carcinoma [294]. Therefore, our results show that by suppressing ROS generation in hepatocytes, polyphenols have the potential to break this vicious circle and to prevent mitochondrial dysfunction. Another study [292] was in agreement with our results showing polyphenol inhibition of ROS generation in HepG2 hepatocytes. In this study, different flavonoids had different potencies in inhibiting oleic acid-induced ROS generation and triglyceride accumulation. The potency was not clearly related to the extracellular radical-reducing potential of the flavonoid. This previous study included two

of the flavonoids in the current study (catechin and quercetin), but not anthocyanins or other polyphenols such as resveratrol described herein.

Delphinidin was unique in showing lower protection with increasing concentration from 1 to 10 μM. This effect may be due to higher concentrations producing more prooxidant activity [295]. Due to having three hydroxyl groups in the B-ring, delphinidin would have a lower reduction potential than the other flavonoids and be more easily oxidized by molecular oxygen (catalyzed by metals in the cell culture medium) to produce ROS.

Another "second-hit" that we investigated in HepG2 cells was inflammation. The results showed strong oleic acid-induced TNFα expression and inhibition by the polyphenols (except cyanidin and kuromanin). An accumulating body of evidence shows that NAFLD-associated ROS and lipid peroxidation aldehydes (MDA and 4-HNE) result in increased release of cytokines such as TNFα and Fas ligand which play a major role in inflammation, cell death and fibrosis [13, 105]. Increased release of TNFα by hepatocytes, adipocytes and Kupffer cells (residing macrophages in liver) results through the activation of NFκB by ROS (reviewed in [296]) and may contribute to the severity of mitochondrial dysfunction seen in NASH patients [105]. Elevated levels of hepatic TNFα expression was reported in obese patients with NAFLD, and the level of TNFα was correlated with advanced hepatic fibrosis in these patients [135]. Other studies in experimental models of NAFLD have shown benefits of polyphenols against inflammation. In line with our result, treating HepG2 cells with oleic acid was previously shown to induce mRNA expression of TNFα by more than 2.5 fold, and quercetin at 10 μM was effective in its inhibition [35]. In studies on animals fed a high-fat diet, dietary quercetin inhibited hepatic steatosis and decreased the liver and plasma levels of the pro-inflammatory cytokines TNFα and IL-6 [48, 250].

Because mitochondrial dysfunction is a major factor in NAFLD and progression to NASH [6], we investigated the effects of oleic acid and polyphenols on mitochondrial expression in the HepG2 cell model. In the present study, we observed decreased mitochondrial biogenesis in HepG2 cells exposed to oleic acid, shown by decreased MitoTracker fluorescence, decreased expression of the mitochondrial biogenesis transcription factors PGC1α and NRF1, and decreased expression of the respiratory complex I and V subunits NDUFS8 and ATP5G1. An accumulating body of evidence reports that mitochondrial number and function are decreased in subjects with obesity and diabetes [19, 297] which are the major contributors of NAFLD/NASH. In line with our results, Koliaki et

al. also reported decreased hepatic mRNA expression of PGC1α, NRF1, and TFAM in patients with NAFLD and NASH [298]. Reduced number of hepatic and muscle healthy mitochondria result in reduced β-oxidation of fatty acids while producing more ROS.

Other studies support that a high-fat diet decreases expression of mitochondrial respiratory complex proteins. In one study [299], feeding an isocaloric high-fat diet decreased expression of nuclear-encoded mitochondrial complex subunits in the skeletal muscle of rodents and humans. In a previous study on non-alcoholic steatohepatitis in mice [17], the authors found that the activity of all of the mitochondrial respiratory complexes in the liver was reduced by 50-60% by a high-fat diet and this decline was particularly due to the decreased gene expression of mitochondrial DNA-encoded subunits. Those authors also found that due to the protection of vulnerable mitochondrial DNA against ROS, administration of antioxidants such as MnTBAP (a superoxide dismutase mimetic) and uric acid was effective to reverse these effects induced by high-fat diet.

In the present study, we also show that most polyphenols increased mitochondrial content along with increased expression of genes involved in mitochondrial biogenesis (PGC1α, NRF1, and TFAM) to levels more than in control cells. The anthocyanins (cyanidin and kuromanin) however had no effect on mitochondrial biogenesis. This could be because anthocyanins, unlike other polyphenols such as resveratrol, reportedly have no direct effect on SIRT1 activity [279], which is shown to be one of the most important regulators of mitochondrial biogenesis by activating AMPK and PGC1α [178]. In agreement with our findings, resveratrol has been found to reverse the inhibition of mitochondrial biogenesis in the liver of mice fed a high-calorie high-fat diet [291]. Gomes et al. also observed that berberine could protect against high fat diet-induced inhibition of PGC1α and TFAM gene expression in the muscle of rats [252].

Interestingly, catechin induced the expression of genes involved in mitochondrial biogenesis without an effect on mitochondrial mass. One explanation may be that catechin increased mitochondrial differentiation more than mitochondrial replication. Mitochondrial differentiation affects pre-existing mitochondria and improves mitochondrial function by expanding the mitochondrial inner membrane with more cristae and increasing expression of mitochondrial proteins such as the respiratory complex subunits [141, 300]. Although having no effect on mitochondrial mass, catechin induced the expression of mitochondrial respiratory complex I, III,

and IV subunits. Accordingly, catechin appeared to have a more distinct effect on mitochondrial differentiation.

Defective mitochondrial oxidative phosphorylation (OXPHOS) is reported in the liver of subjects with NASH [14], as well as in several models of NAFLD, including HepG2 hepatocytes treated with palmitate [15], obese ob/ob mice with NAFLD [16], and mice fed a high-fat diet [17]. It was suggested that a decrease in mitochondrial biogenesis (decreased number of healthy and functional mitochondria) and impaired expression and assembly of mitochondrial respiratory complex subunits may result in increased ROS production which is one of the main contributors to NAFLD [19]. Therefore, improving mitochondrial function and electron flow in the mitochondrial electron transport chain may be of vital importance to prevent ROS generation in NAFLD. We found that oleic acid inhibited complex I and V subunits (NDUFS8 and ATP5G1) expression. Importantly complex I is the entry point of electrons to the mitochondrial respiratory chain. Most polyphenols could protect against this inhibition and in many cases increased the expression of NDUFS8 to significantly more than control cells. Our present study suggests that in opposition to increased load of NADH and electrons to enter the mitochondrial electron transport chain (ETC) in fatty liver disease, polyphenols induce the expression of subunits of mitochondrial complexes to increase the flow of electrons, which may lead to increased fat oxidation and decreased ROS generation.

We found a significant positive correlation between PGC1α and Mn-SOD mRNA expression, suggesting that this effect could be due to increased mitochondrial biogenesis and number by PGC1α. Additionally, PGC1α has been shown to have a major role in regulating ROS removal by inducing antioxidant defense such as glutathione peroxidase, SOD, and catalase [145, 301].

The mitochondrial enzyme UCP2 is another enzyme implicated in mitochondrial ROS generation and fatty acid β-oxidation. In the present study, we found 37% decreased expression of UCP2 by oleic acid, while most polyphenols prevented this decrease. UCP2 is localized in the inner mitochondrial membrane and uncouples oxidative phosphorylation from ATP production [302], resulting in decreased electrochemical potential ($\Delta\Psi$) and decreased production of mitochondrial ROS [90, 303]. Studies report that even small changes in $\Delta\Psi$ can have an order of magnitude effect in decreasing mitochondrial ROS generation [304]. In agreement with this connection we found an inverse correlation between UCP2 expression and ROS generation.

In line with our results with polyphenols, other evidence suggests that dietary polyphenols (such as resveratrol, and the flavonoid naringenin) can induce the hepatic expression of UCP2 in rats fed a high-fat or high-sucrose diet, alleviating NAFLD [171, 305]. In addition to decreasing ROS, induction of UCP2 expression by polyphenols can protect against NAFLD by increasing fatty acid β-oxidation [166] and export of toxic hydroperoxy fatty acids out of mitochondria [306]. Accordingly, due to these beneficial outcomes it is suggested that pharmacological induction of UCP2 can be a novel treatment for NAFLD [171].

We also found a significant inverse correlation between PGC1α and TNFα in our study, suggesting that increased mitochondrial biogenesis is paralleled with decreased inflammation. In agreement with our findings other studies also show that PGC1α is inversely correlated with the expression of inflammatory markers. For instance, muscle-specific PGC1α knock-out animals show increased gene expression of TNFα, IL6 and other inflammatory markers in their muscle tissue [307]. Moreover, it was shown that genetic ablation of PGC1α in primary muscle cells induces mRNA expression of TNFα and IL6 while adenovirus-mediated expression of PGC1α in cultured myotubes reduced the expression of these inflammatory genes [307]. Although the molecular mechanisms by which PGC1α inhibits inflammation have not been identified, it is suggested that suppressed ROS generation by PGC1α may have a key role since oxidative stress and inflammation go together in pathological conditions such as NASH [308]. Interestingly, the gene expression of complex I and III subunits NDUFS8 and UQCRC1 was also negatively correlated with the expression of TNFα. It can be interpreted that increased flow of electrons in the mitochondrial electron transport chain due to increased activity of complexes I and III decreases the leak of electrons from these complexes which finally inhibits ROS production and inflammation.

In conclusion, dietary polyphenols of different classes were similarly effective in decreasing oleic acid-induced ROS generation in human hepatocytes. As part of the mechanism all polyphenols except anthocyanins increased mitochondrial biogenesis and the expression of mitochondrial respiratory complex subunits. Figure 4.8 schematically represents how polyphenols may inhibit ROS production, decrease inflammation, and improve mitochondrial biogenesis and function in HepG2 cells exposed to oleic acid.

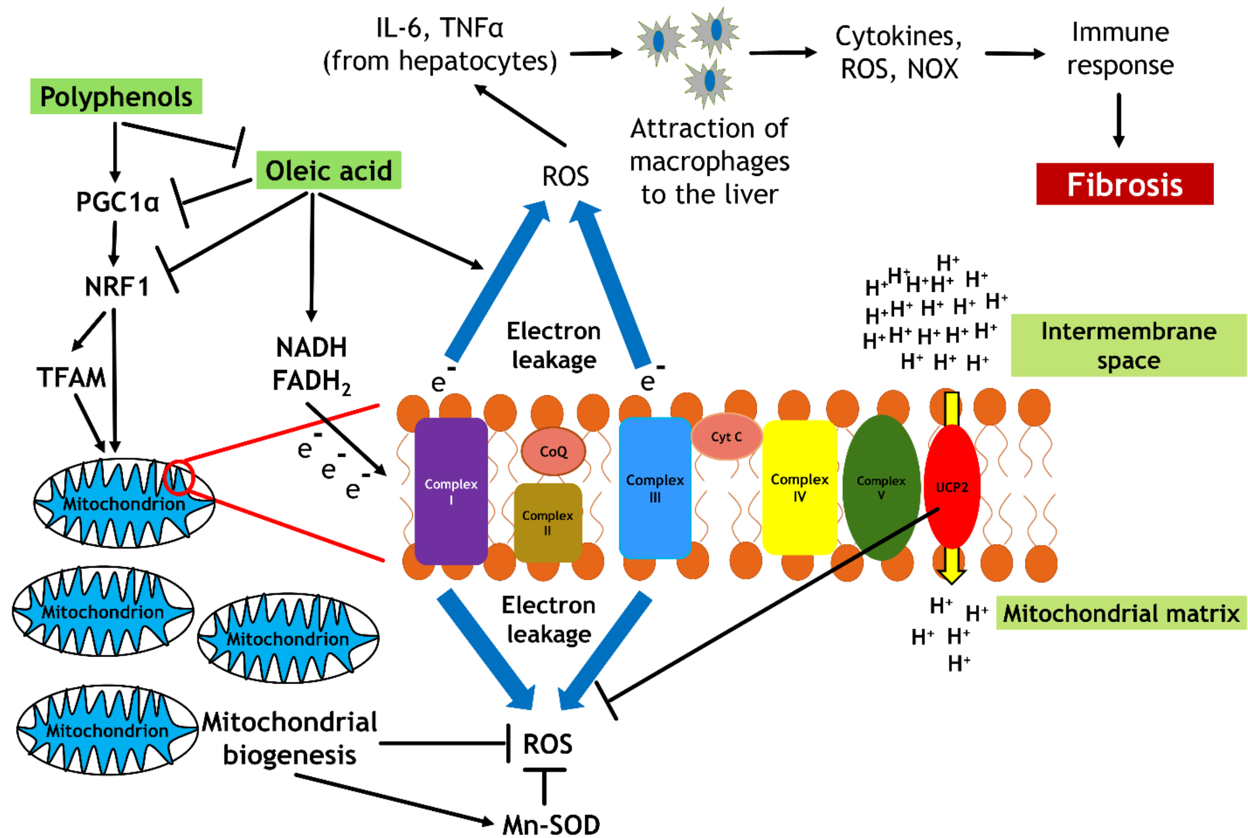

Figure 4. 8. Schematic representing the mechanisms by which polyphenols inhibit ROS production, inflammation, and improve mitochondrial biogenesis and function.

Oleic acid inhibited mitochondrial biogenesis by blunting the expression of PGC1α and NRF1. However, polyphenols induced mitochondrial biogenesis in HepG2 cells leading to overexpression of the antioxidant enzyme Mn-SOD which further suppresses ROS production. Reduced ROS production by polyphenols in turn decreases TNFα expression and prevents inflammation. Polyphenols also protected against oleic acid-induced decrease of mitochondrial complex I subunit expression and increased the expression of complex III and IV subunits which may help flow of electrons. This flow of electrons most likely inhibits the leakage of electrons from complex I and III resulting in decreased ROS production. Polyphenols also reversed oleic acid-induced decrease of UCP2 expression which by facilitating import of H^+ to the mitochondrial matrix suppresses ROS production and may improve flow of electrons in the electron transport chain.

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Conflict of interests

The authors declare no conflicts of interests.

4.5. Transition to CHAPTER 5

So far, the effects of polyphenols were investigated in HepG2 cells on oleic acid-induced steatosis, oxidative stress, inflammation, and mitochondrial biogenesis and dysfunction (Chapters 3 and 4). Since endoplasmic reticulum stress is also involved in progression of fatty liver disease to NASH, we hypothesised that some polyphenols protect against endoplasmic reticulum stress (3rd hypothesis). In line with other studies however, oleic acid did not induce ER stress in HepG2 cells (Appendix, Figure 7.2). But my preliminary studies showed that the saturated fatty acid palmitic acid can induce ER stress in HepG2 cells. Therefore, in the next chapter the effects of polyphenols were investigated on concomitant ER stress (3rd hypothesis) and mitochondrial dysfunction induced by palmitic acid.

CHAPTER 5: PROTECTION BY DIFFERENT CLASSES OF DIETARY POLYPHENOLS AGAINST PALMITATE-INDUCED MITOCHONDRIAL DYSFUNCTION AND ENDOPLASMIC RETICULUM STRESS IN HEPG2 HEPATOCYTES

Abbreviations

AMPK, AMP-activated protein kinase; **ATP5G1,** ATP synthase, mitochondrial Fo complex, subunit C1 (subunit 9); **CCCP,** carbonyl cyanide 3-chlorophenylhydrazone; **CHOP,** CCAAT-enhancer-binding protein homologous protein; **COX6B1,** cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous); **EDEM1,** ER degradation enhancing alpha-mannosidase like protein 1; **ER,** endoplasmic reticulum; **ERdj4,** endoplasmic reticulum DnaJ homolog 4; **GADD34,** growth arrest and DNA damage-inducible protein; **GRP78,** 78 kDa glucose-regulated protein; **iNOS,** inducible nitric oxide synthase; **MTATP6,** mitochondrial DNA-encoded ATP synthase Fo subunit 6; **MTCO1,** mitochondrially encoded cytochrome c oxidase I; **MTCYB,** mitochondrial DNA-encoded subunit of respiratory Complex III; **MTND1,** mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1; NADH dehydrogenase subunit 1; **NAFLD,** non-alcoholic fatty liver disease; **NASH,** non-alcoholic steatohepatitis; **NDUFS8,** NADH dehydrogenase (ubiquinone) Fe-S protein 8; **NFκB,** nuclear factor kappa b; **ORP150,** 150-kDa oxygen-regulated protein; **p-eIF2α,** phosphorylated eukaryotic translation initiation factor 2; **PGC1α,** peroxisome proliferator-activated receptor gamma coactivator 1-alpha; **p-PERK,** phosphorylated PKR-like ER-resident kinase; **SIRT1,** sirtuin 1; **UPR,** unfolded protein response; **UQCRC1,** ubiquinol-cytochrome c reductase core protein I.

Abstract

Scope: Mitochondrial dysfunction and endoplasmic reticulum stress induced by dietary saturated fats have recently been implicated in the initiation and progression of NAFLD. Dietary polyphenols hold promise for the amelioration of NAFLD based on preventing steatosis and oxidative stress yet the mechanisms and effectiveness of different classes of polyphenols are largely unknown. Since, oleic acid was unable to induce ER stress (which is the main focus of this chapter), saturated palmitic acid was used as the stressor to investigate the protective effects of polyphenols on ER stress.

Methods and results: In an *in vitro* model of steatosis using HepG2 hepatocytes, we investigated the effect of selected polyphenols (resveratrol, quercetin, catechin, cyanidin, kuromanin, and berberine) to abrogate the development of palmitic acid-induced steatosis, ROS production, mitochondrial impairments, and endoplasmic reticulum stress. Palmitic acid increased steatosis, increased intracellular ROS production, induced expression of iNOS and markers of endoplasmic reticulum stress, and decreased the mitochondrial membrane potential and markers of mitochondrial biogenesis. Treatment with different polyphenols prior to palmitic acid exposure protected against these effects, induced mRNA for nuclear and mitochondrial DNA-encoded respiratory complex subunits, and decreased expression of iNOS and genes responding to endoplasmic reticulum stress.

Conclusion: Together the results show that different polyphenols all similarly protected against steatosis and oxidative stress induced by palmitic acid in HepG2 cells, but differed in mitigating adverse effects of palmitic acid on ER stress, the mitochondrial membrane potential, and mitochondrial biogenesis.

Keywords: Non-alcoholic fatty liver disease, polyphenols, endoplasmic reticulum stress, ER chaperones, mitochondrial defect

5.1. Introduction

Diets high in saturated fats have been widely implicated in the development of nonalcoholic fatty liver disease (NAFLD) [309, 310]. Palmitic acid is the most prevalent saturated fatty acid found in triglycerides of meats and dairy products, as well as in palm oils that are widely used by the food industry. Upon consumption of saturated fats, the liver is exposed to high levels of palmitic acid that can contribute to NAFLD.

NAFLD is a public health burden with clinical and histological abnormalities that can progress to non-alcoholic steatohepatitis (NASH), cirrhosis, and fibrosis [54]. Besides hepatic fat accumulation, other hits such as oxidative stress and inflammation [2], mitochondrial dysfunction [4-6] and endoplasmic reticulum stress [7, 8] have been suggested to interplay in a concerted manner to cause progression of simple steatosis to NASH. A higher ratio of saturated to unsaturated fatty acids stored in the liver may contribute to the progression of NAFLD to NASH [311]. Although the molecular mechanisms by which saturated fats contribute to this effect are still unclear, reactive oxygen species, inflammatory cytokines, and endoplasmic reticulum stress have been implicated. Therefore, it is suggested that saturated fatty acids may produce intrinsic second hits which cause progression to NASH [311].

Excess hepatic fats undergo β-oxidation in the mitochondria and peroxisomes of hepatocytes leading to mitochondrial dysfunction and production of reactive oxygen species (ROS) [116]. ROS are major contributors to the progression of simple steatosis to necroinflammation [90]. Mitochondria are a major source of ROS in cells [120], particularly when they are dysfunctional such as in NAFLD [10]. An increased load of electron donors such as NADH and $FADH_2$ to the mitochondrial electron transport chain during NAFLD can be beyond the capacity of the mitochondrial respiratory complexes (particularly due to reduced activity of complexes). This increased load in turn brings about the leakage of electrons from complex I and III and consequently elevates ROS production [105]. The increased mitochondrial ROS also impairs mitochondrial function and biogenesis and consequently decreases the number of healthy and functional mitochondria [19]. Therefore inducing mitochondrial biogenesis may be a therapeutic strategy in prevention and treatment of NAFLD by inducing fatty acid β-oxidation [267], and suppressing ROS production [301] and inflammation [307].

Besides ROS, reactive nitrogen species (RNS) produced by the activity of inducible nitric oxide synthase (iNOS) may also contribute to the pathogenesis of NAFLD [312]. Higher mRNA and protein expression of iNOS has been demonstrated in different models of NAFLD and NASH [313, 314]. Increased iNOS and tyrosine nitrated proteins in the liver of *ob/ob* mice [16] are suggested as some of the contributors to mitochondrial respiratory complex dysfunction.

Endoplasmic reticulum (ER) stress has also been suggested to be one of the "multiple hits" involved in the progression of NAFLD to NASH [22]. Several studies have reported increased ER stress in hepatocytes treated with palmitic acid [26], or in animals [27, 28] and humans [29, 30] with hepatic steatosis. Lipogenesis and insulin resistance have been shown to be affected by ER stress [24, 191]. While short term activation of ER stress and the unfolded protein response (UPR) is essential for homeostasis of metabolism, prolonged ER stress and UPR have deleterious effects on metabolism and may result in increased hepatic lipid accumulation, inflammation, insulin resistance, impaired lipid metabolism, and apoptosis.

Polyphenols found in various fruits and vegetables have been shown to protect against NAFLD. Dietary polyphenols have been shown to protect against hepatic steatosis [95], oxidative stress [47], inflammation [50], insulin resistance [315], and mitochondrial dysfunction [255] leading to protection against NAFLD. These features of polyphenols make them promising candidates for prevention and treatment of NAFLD. Although polyphenols have been the subject of many studies on prevention and treatment of NAFLD, it is not known which polyphenols are the most potent in improving mitochondrial function and by what mechanisms. Moreover, due to the lack of evidence, the effects of polyphenols on hepatic endoplasmic reticulum stress and the UPR in NAFLD are largely unknown.

Using HepG2 hepatocytes treated with palmitic acid, we compared different classes of polyphenols concomitantly on mitochondrial dysfunction and ER stress and explored the contributing mechanisms of protection. Decreased expression of mitochondrial DNA-encoded respiratory complex subunits in HepG2 cells treated with palmitic acid has recently been observed [15]. Here we show that different classes of polyphenols similarly inhibit steatosis and ROS generation but differ in protection against palmitic acid-induced mitochondrial dysfunction and ER stress.

5.2. Materials and Methods

5.2.1. Materials

Palmitic acid was from Sigma-Aldrich, USA. The rest of the materials were as indicated in sections 3.2.1 and 4.2.1.

5.2.2. HepG2 cell culture conditions

See section 3.2.2.

5.2.3. Treatment with palmitic acid/BSA and polyphenols

Palmitic acid was dissolved in dimethyl sulfoxide (DMSO) in order to prepare 300 mM stock and kept at -20°C for further use. HepG2 cells at 3×10^4 cells/well were cultured in 96-well optical-bottom plates for 24 h. The old medium was then replaced with fresh medium containing 10% FBS and 1% fatty acid-free bovine serum albumin (BSA). One percent BSA was used to ensure the solubility of palmitic acid in the medium. HepG2 cells were then pre-treated with different polyphenols at 10 μM for 2 h. After treatment of the cells with polyphenols for 2 h (polyphenols were not removed thereafter and the cells were incubated with them for the next 24 h), cells were treated with 500 μM palmitic acid for 24 h.

5.2.4. Measuring intracellular lipid content

See section 3.2.4. The only difference is using 500 μM palmitic acid instead of oleic acid.

5.2.5. Measurement of intracellular ROS

See section 4.2.4. However, only one dose of polyphenols (10 μM) was used and oleic acid was replaced with 500 μM palmitic acid.

5.2.6. Measurement of mitochondrial content using MitoTracker Green

See section 4.2.6. The only difference is using 500 μM palmitic acid instead of oleic acid.

5.2.7. Measuring the mitochondrial membrane potential (ΔΨm)

See section 3.2.11. The only difference is using 500 μM palmitic acid instead of oleic acid.

5.2.8. Measuring mRNA expression using reverse transcriptase RT-qPCR

See section 3.2.6. The only difference is using 500 μM palmitic acid instead of 1.5 mM oleic acid. Sequences of primers of interest are shown in the Appendix (Table 7.3).

5.2.9. Statistical analysis

Results were expressed as mean values with their standard error of means (SEM) for the number of experiments indicated. Results were analyzed using the Statistical Package for the Social Sciences (SPSS 22) (IBM, USA). One-way ANOVA was used initially, and when any significance detected, Dunnett's post hoc test was used to compare the mean of each group with the palmitic acid alone condition. The level of significance was $P<0.05$. Correlation was determined by Pearson's R.

5.3. Results

5.3.1. Intracellular lipid accumulation

Fluorescence images of intracellular lipids using Nile Red staining are shown in Figure 5.1A. HepG2 cells treated with palmitic acid alone show higher red fluorescence compared to untreated cells representing higher intracellular lipids. The images also show that compared to the cells treated with palmitic acid alone, the intensity of fluorescence is lower in HepG2 cells treated with different polyphenols, showing that polyphenols protected against steatosis.

Figure 5.1B shows quantitation of the fluorescence due to intracellular triglyceride accumulation measured by a microplate reader. Treating HepG2 cells with 500 μM palmitic acid increased intracellular triglyceride by 39%. All of the polyphenols significantly protected against lipid accumulation ranging from 70-150% inhibition. Cyanidin was the strongest polyphenol with 150% protection.

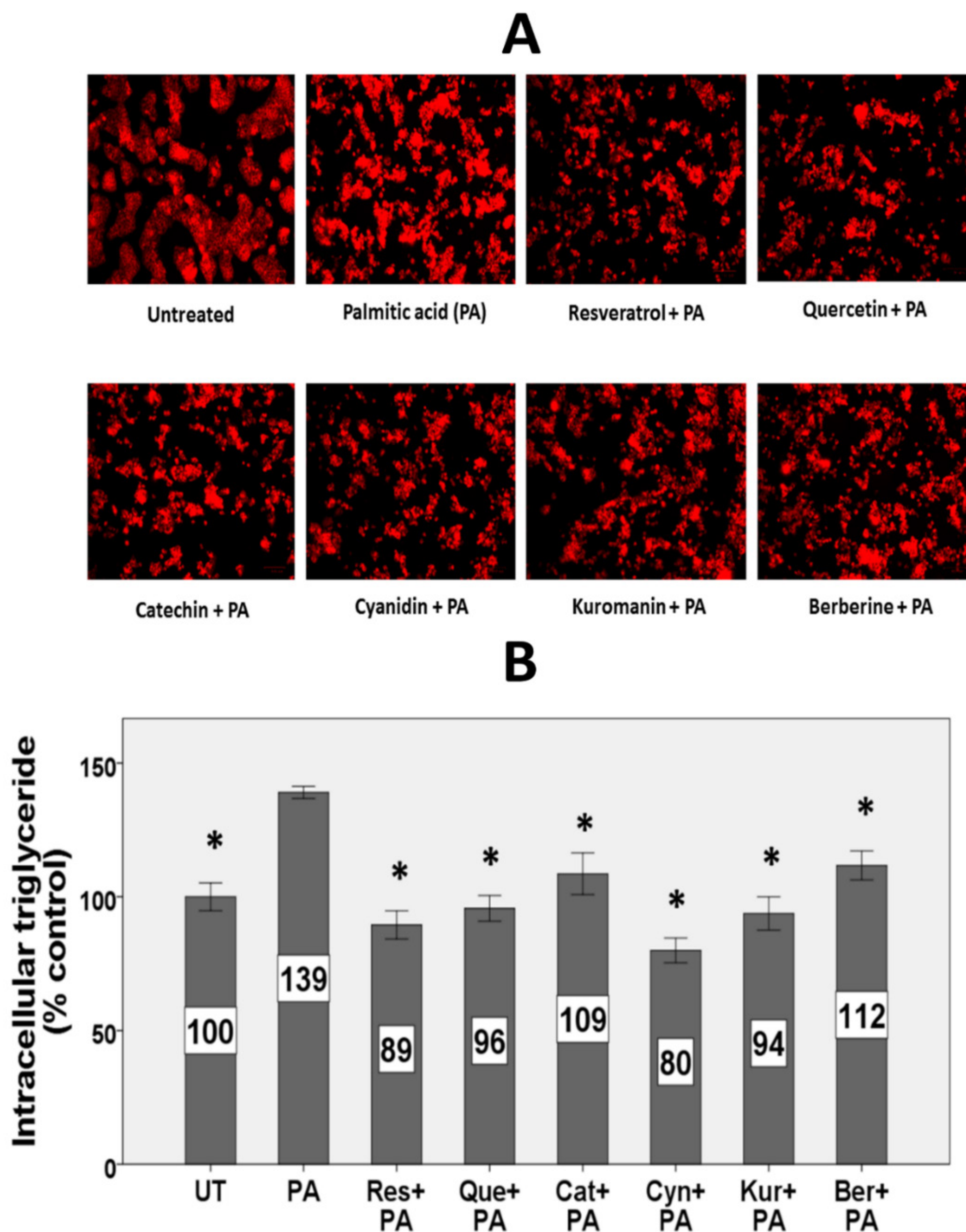

Figure 5. 1. The effect of polyphenols on palmitic acid-induced intracellular lipid accumulation.

(A) Fluorescence images were captured using a ZOE Fluorescent Cell Imager at 20x magnification after staining HepG2 cells with Nile Red fluorescence probe. Higher red fluorescence represents higher fat content. (B) Intracellular lipids were quantified from Nile Red fluorescence using a microplate reader. (UT: untreated, PA: palmitic acid, Res: resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. In each separate experiment, 9 different spots for each well were read by the microplate reader in order to control for the number of cells in each well. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

5.3.2. Intracellular ROS generation

Fluorescence images representative of intracellular ROS stained with the fluorogenic probe DCFH-DA along with their brightfield images are shown in Figure 5.2A. Compared to untreated cells, higher green fluorescence in HepG2 hepatocytes exposed to palmitic acid showed higher ROS in these cells. Treatment with polyphenols starting prior to treatment with palmitic acid decreased the intensity of fluorescence representative of decreased intracellular ROS.

When the fluorescence signals were quantified using a microplate reader, it was revealed that treatment of HepG2 cells with palmitic acid increased intracellular ROS by 42% while treatment with all polyphenols prevented this increase ranging from 70-160% inhibition (Figure 5.2B). The most potent polyphenol was berberine with 160% inhibition, to a level less than untreated cells, and the weakest polyphenol was kuromanin which protected against ROS generation by 70%.

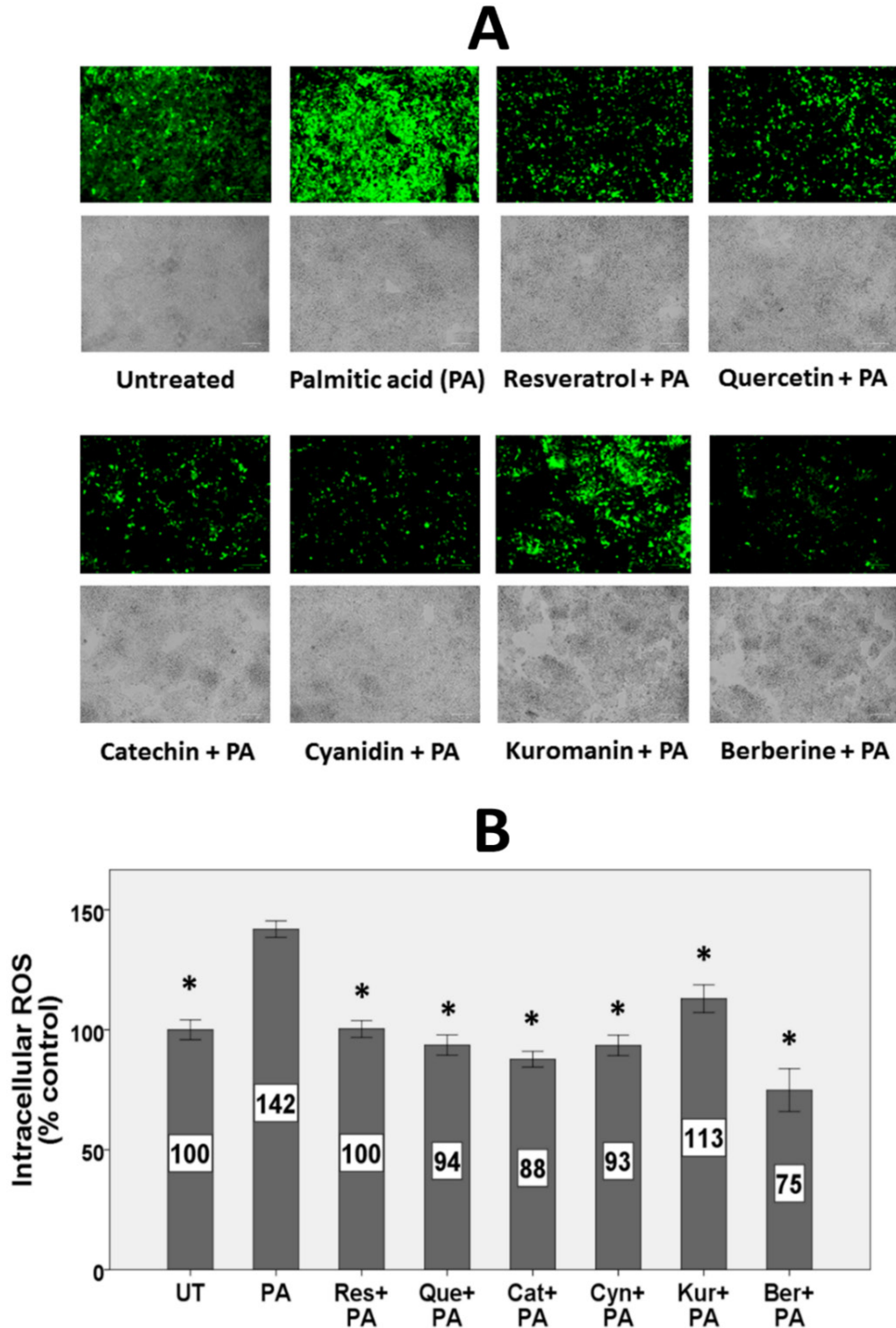

Figure 5. 2. The effect of polyphenols on palmitic acid-induced ROS generation.

HepG2 hepatocytes were pre-treated with different polyphenols at 10 μM for 2 h and then treated with 500 μM palmitic acid for 24 h, and intracellular ROS was measured after staining with the fluorogenic probe DCFH-DA. (A) Fluorescence images along with their corresponding brightfield images were captured using a ZOE Fluorescent Cell Imager at 20x magnification. **(B)** DCF fluorescence was quantified using a microplate reader. (UT: untreated, PA: palmitic acid, Res:

resveratrol, Que: quercetin, Cat: catechin, Cyn: cyanidin, Kur: kuromanin, Ber: berberine). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. In each separate experiment, 9 different spots for each well were read by the microplate reader in order to control for the number of cells in each well. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

5.3.3. Mitochondrial membrane potential

Figure 5.3A shows fluorescence images of HepG2 cells stained with a fluorescence probe (TMRE) specific for the mitochondrial membrane potential. The results show that the mitochondrial membrane potential is much lower in the cells treated with palmitic acid and increased in the cells treated with resveratrol, quercetin, catechin, and cyanidin.

When fluorescence signals were quantified using a microplate reader it was revealed that treatment of HepG2 hepatocytes with palmitic acid alone significantly decreased the mitochondrial membrane potential by 22% (Figure 5.3B). When the cells were treated with 10 µM catechin, quercetin, cyanidin, or resveratrol, the decrease in the mitochondrial membrane potential was inhibited by 100, 73, 60, and 50%, respectively. Berberine and kuromanin did not protect against the palmitic acid-induced mitochondrial membrane potential decrease.

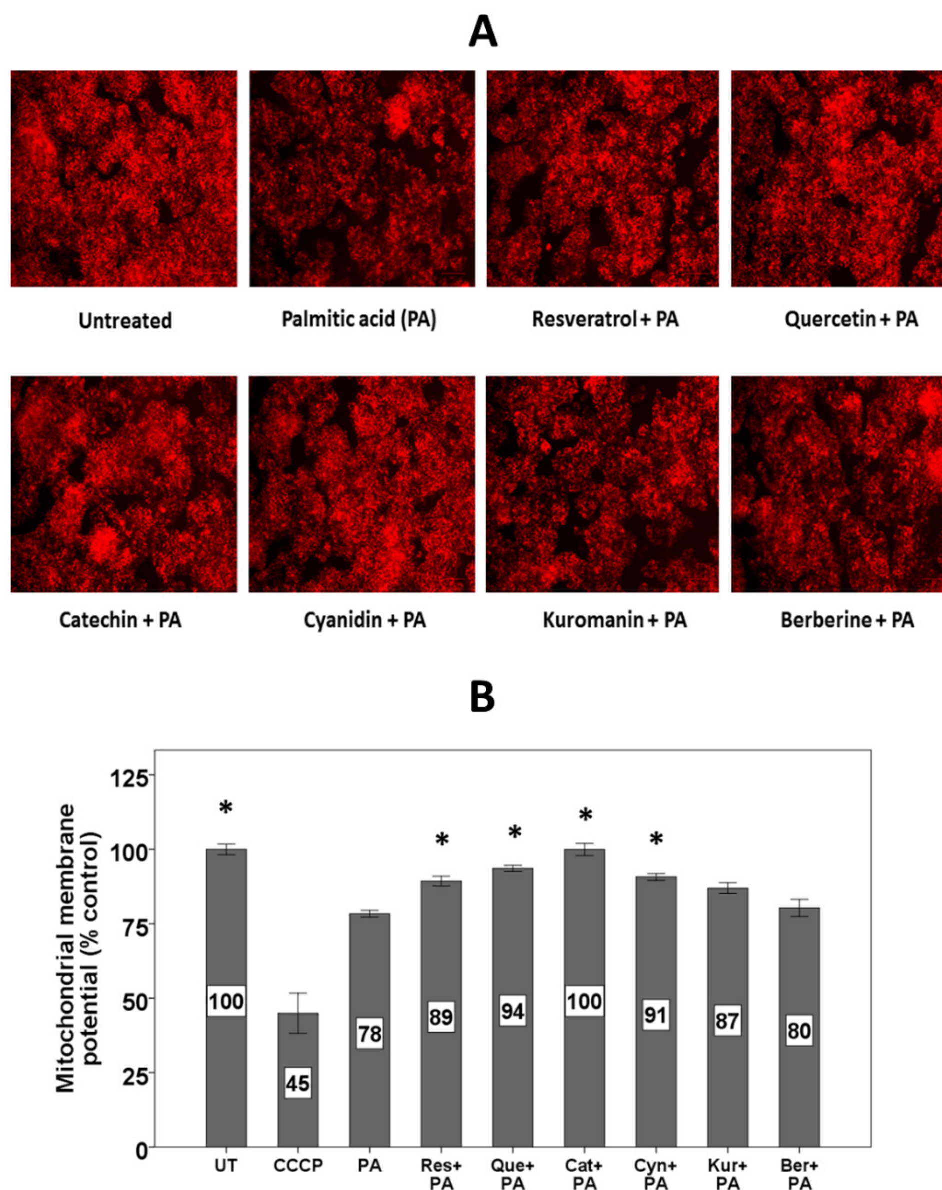

Figure 5. 3. Effects of palmitic acid and polyphenols on the mitochondrial membrane potential in HepG2 cells.

Cells were treated with 10 μM polyphenols starting 2 h prior to the treatment with 500 μM palmitic acid for 24 h. (A) Fluorescence images representative of the mitochondrial membrane potential were captured using a ZOE Fluorescent Cell Imager at 20x magnification after staining HepG2 cells with TMRE red. (B) The quantification of TMRE fluorescence using a microplate reader. Untreated cells were set at 100% and the data are presented as % of untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. In each separate experiment, 9 different spots for each well were read by the microplate reader in order to control for the number of cells in each well. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

5.3.4. Mitochondrial content

As represented in Figure 5.4A, treatment with palmitic acid alone decreased the intensity of MitoTracker Green fluorescence (specific for mitochondrial content) representing fewer mitochondria in HepG2 cells. Treatment with all polyphenols at 10 μM however, increased mitochondrial content compared to palmitic acid alone.

Quantification of the MitoTracker results with a microplate reader showed that treatment with palmitic acid alone for 24 h significantly decreased mitochondrial content in HepG2 cells by 33% while treatment with 10 μM cyanidin, catechin, berberine, and kuromanin protected against the inhibition by 76, 60, 58, and 55%, respectively (Figure 5.4B). Although higher green fluorescence has been shown in Figure 5.4A for resveratrol and quercetin, they did not reach significance when fluorescence was measured using a microplate reader.

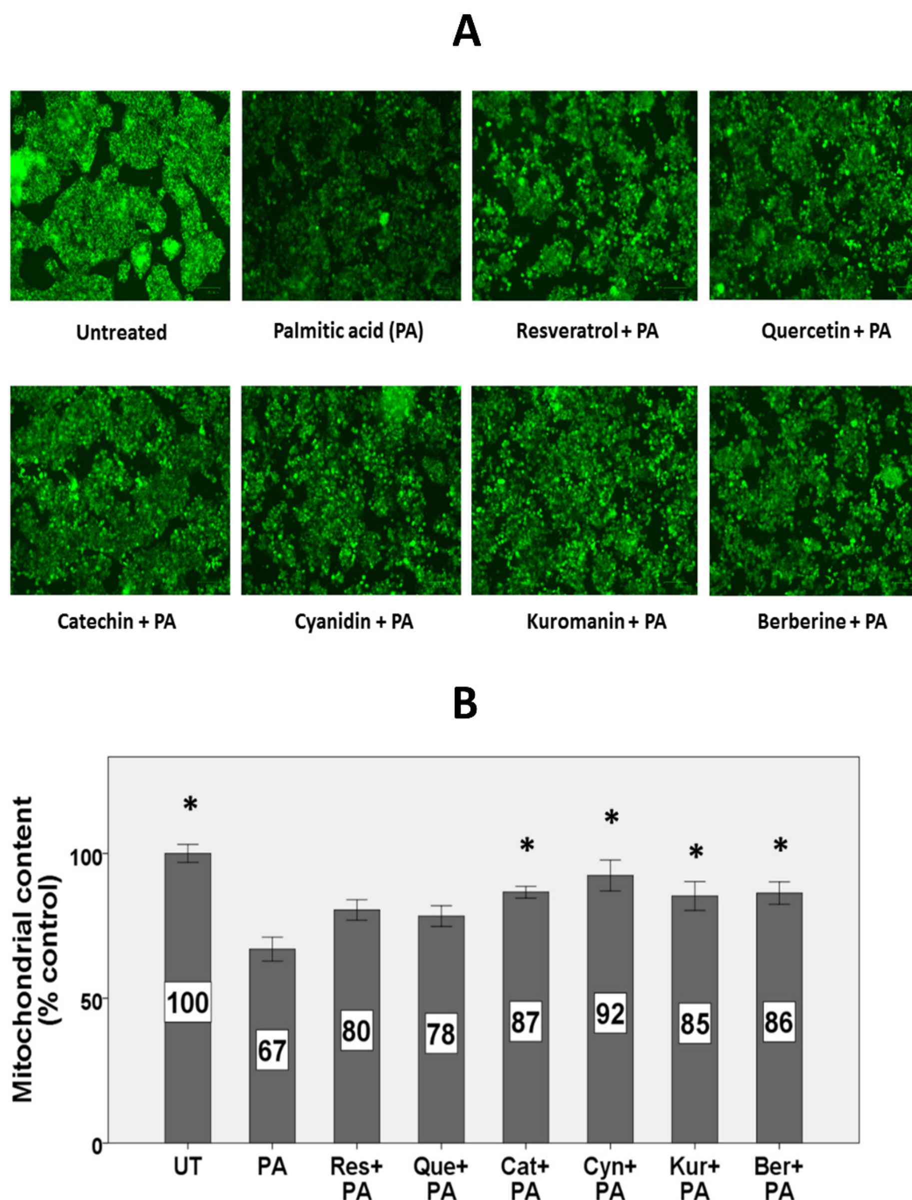

Figure 5. 4. Effects of palmitic acid and polyphenols on mitochondrial content.

(A) Fluorescence images were captured using a ZOE Fluorescent Cell Imager at 20x magnification representative of the mitochondrial content in HepG2 cells stained with MitoTracker Green. (B) Mitochondrial content was measured by quantification of the MitoTracker Green fluorescence using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. In each separate experiment, 9 different spots for each well were read by the microplate reader in order to control for the number of cells in each well. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

5.3.5. Expression of mRNA for nuclear and mitochondrial DNA-encoded respiratory complex subunits

In measurements of mRNA for nuclear DNA-encoded mitochondrial respiratory complex subunits, treatment of HepG2 hepatocytes with palmitic acid alone for 24 h significantly decreased the expression of a subunit of complex V (ATP5G1), while having no effect on the expression of subunits for complex I (NDUFS8), III (UQCRC1), and IV (COX6B1) (Figure 5.5). Treatment with berberine increased the expression of all of the subunits (by 66, 35, 30, and 45% relative to palmitic acid alone). Catechin and kuromanin increased the expression of two of the subunits, and cyanidin increased one.

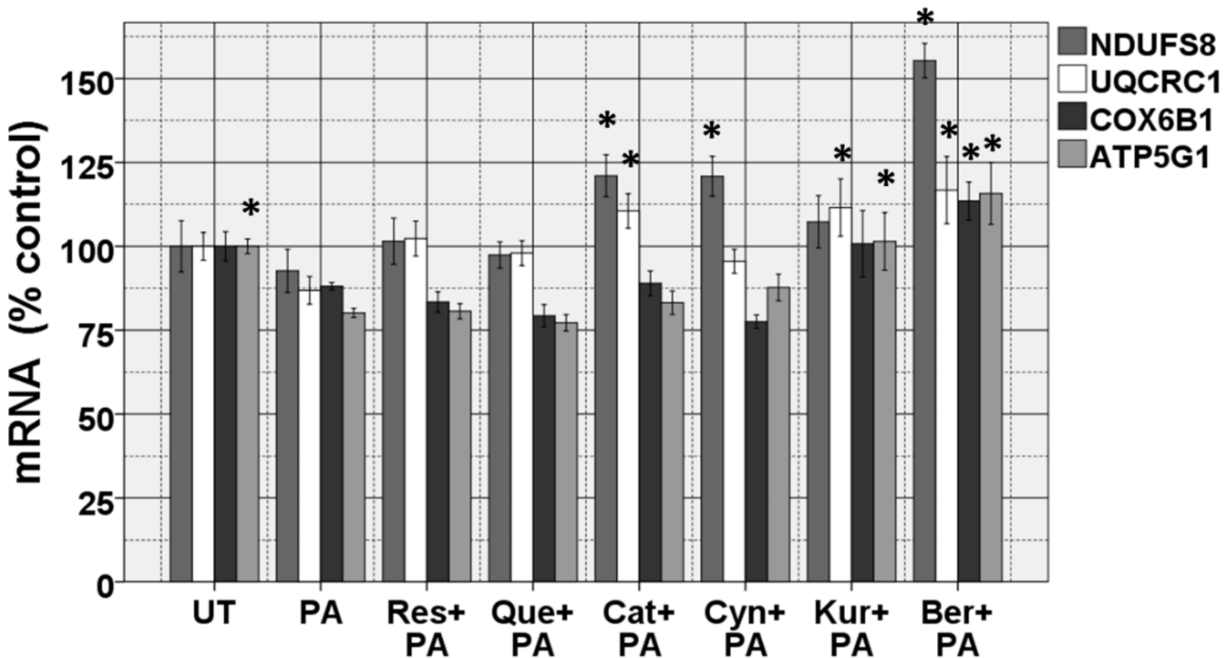

Figure 5. 5. Effects of palmitic acid and polyphenols on the expression of nuclear DNA-encoded mRNA for mitochondrial respiratory complex subunits.

Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at P<0.05.

Treatment of HepG2 cells with palmitic acid alone had no significant effect on the mRNA for mitochondrial DNA-encoded subunits (Figure 5.6). Compared to palmitic acid alone, treatment with resveratrol increased the expression of three of the subunits (MTND1, MTCYB, and MTCO1

by 115, 40 and 28% respectively). Quercetin, cyanidin and berberine increased the expression of two of the subunits, and catechin increased one.

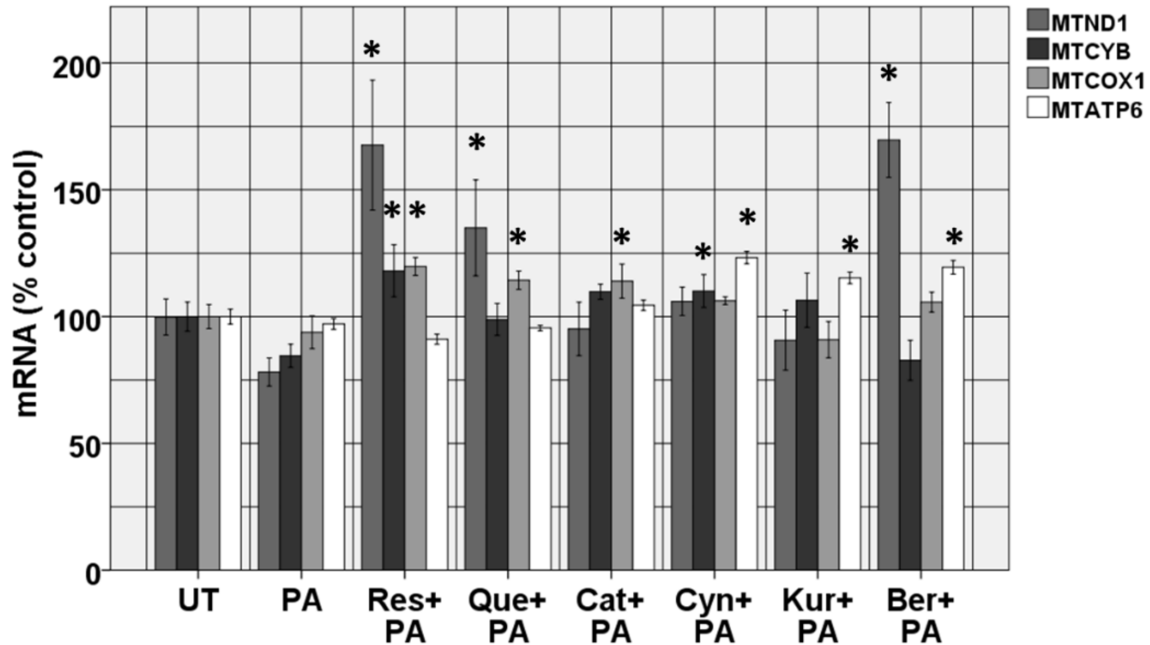

Figure 5. 6. Effects of palmitic acid and polyphenols on mitochondrial DNA-encoded mRNA for the mitochondrial respiratory complex subunits.

Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at P<0.05.

5.3.6. The expression of mRNA for inducible nitric oxide synthase (iNOS)

Shown in Figure 5.7 palmitic acid increased mRNA for inducible nitric oxide synthase (iNOS) by 245% while all polyphenols except berberine prevented this increase by 82-110%. Kuromanin was the strongest (110% protection) while berberine was the weakest polyphenol with no significant effect on iNOS mRNA expression.

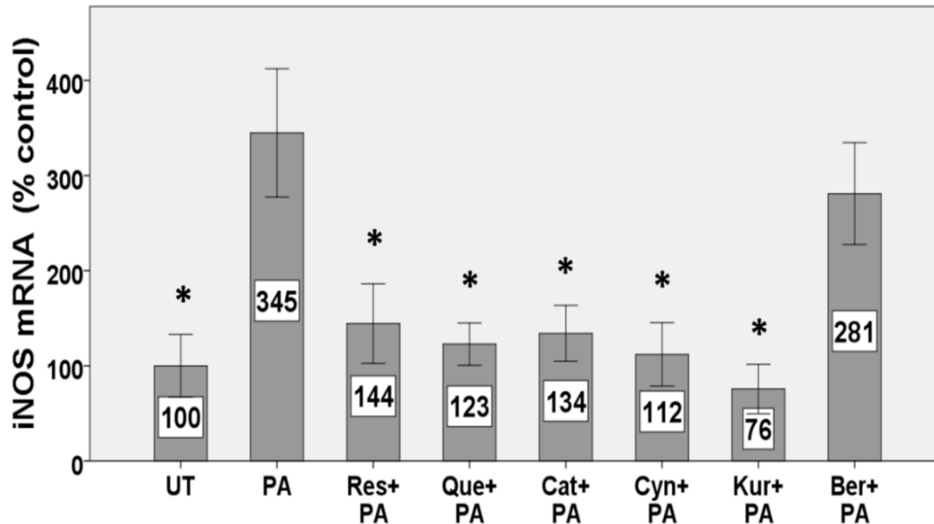

Figure 5. 7. Effects of palmitic acid and polyphenols on mRNA expression of inducible nitric oxide synthase.

Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at P<0.05.

5.3.7. ER stress and unfolded protein response

5.3.7.1. ER chaperones

Palmitic acid significantly increased mRNA for the endoplasmic reticulum chaperones GRP94, GRP78, ORP150, and co-chaperone ERdj4 by 60, 147, 184, and 170%, respectively (Figure 5.8). Compared to palmitic acid (PA) alone, quercetin decreased expression of four of the chaperones, catechin and cyanidin three, and kuromanin and berberine one. Resveratrol did not significantly affect expression of any of the chaperones.

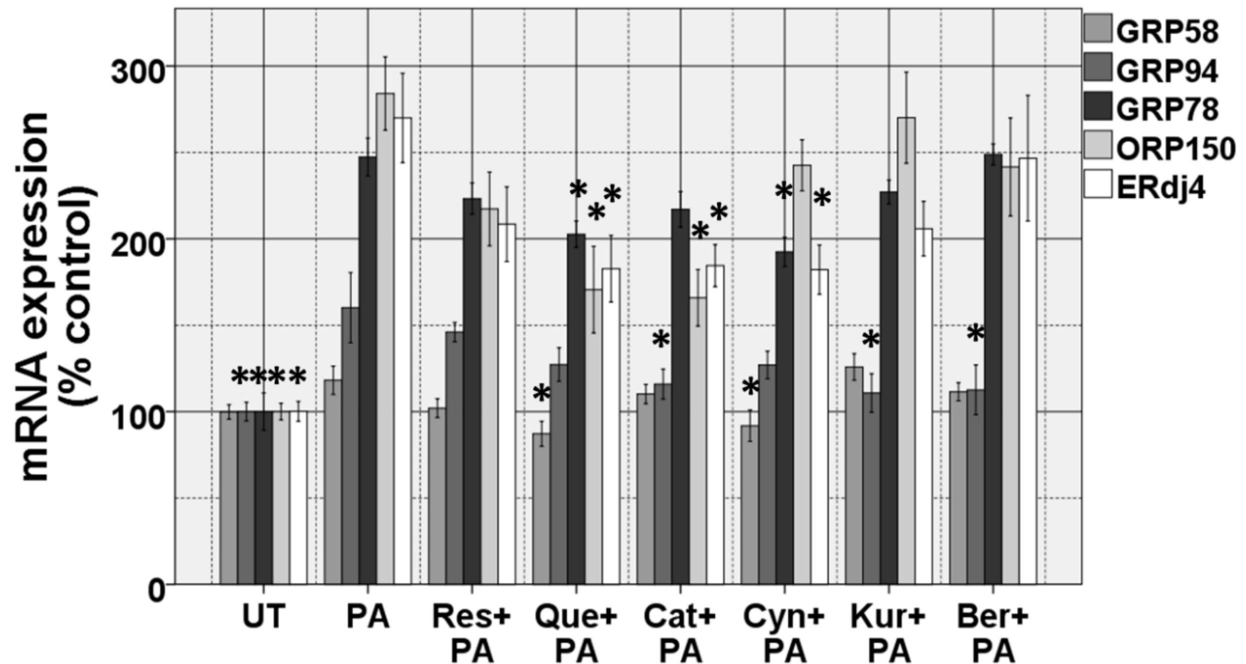

Figure 5. 8. Effects of palmitic acid and polyphenols on the expression of mRNA for ER chaperones.

Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

5.3.7.2. Other endoplasmic reticulum stress markers and apoptotic CHOP

While treatment of HepG2 cells with 500 µM palmitic acid alone for 24 h had no significant effect on the expression of ATF4, it significantly increased the expression of the other ER stress markers EDEM1 and GADD34 as well as the ER stress-mediated apoptotic marker CCAAT-enhancer-binding protein homologous protein (CHOP) by 77, 140, and 171%, respectively (Figure 5.9). Quercetin decreased the expression of three of the markers, catechin two, and resveratrol and cyanidin one. Only the polyphenols catechin, quercetin, and cyanidin which strongly inhibited the increase in expression of several endoplasmic reticulum chaperones (Figure 5.8), showed protection against the increase in CHOP.

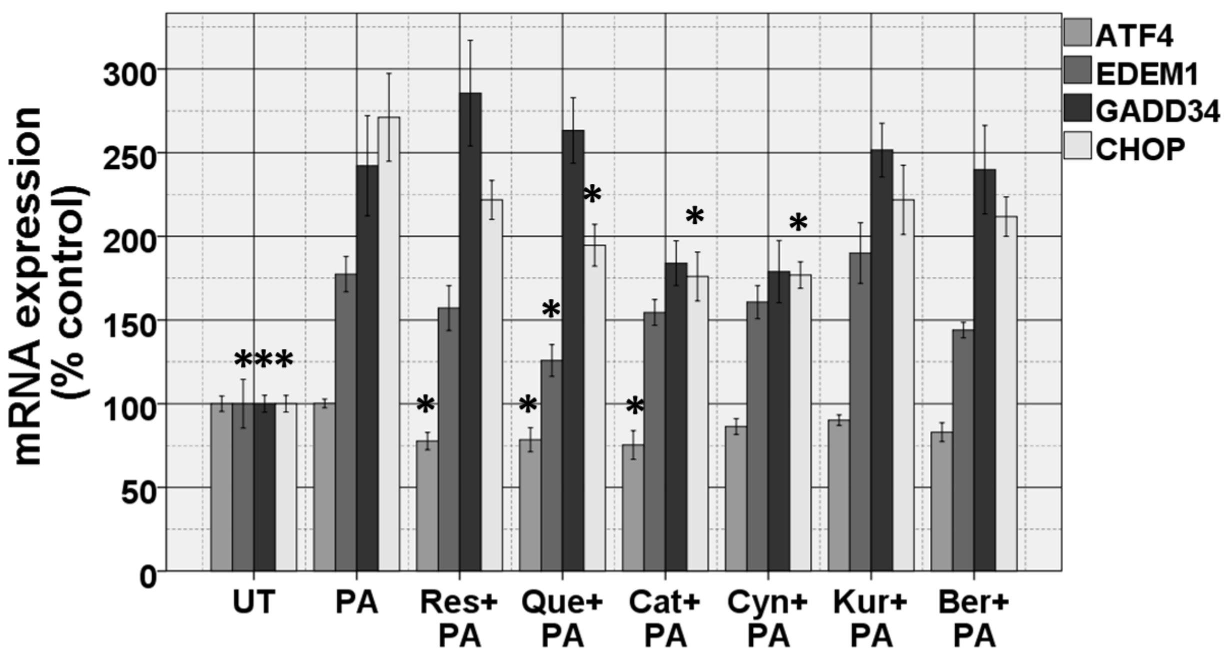

Figure 5. 9. Effects of palmitic acid and polyphenols on expression of mRNA for some ER stress markers as well as apoptotic CHOP.

Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 2 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at P<0.05.

5.3.8. Correlation results

In order to find the association between different parameters, we evaluated the correlation between several variables using Pearson's R. Intracellular triglyceride in HepG2 hepatocytes was positively correlated with iNOS mRNA expression (r=0.55, P<0.001) (Figure 5.10A) and ER stress markers such as GRP78 and the apoptotic CHOP (r=0.40, P=0.005, and r=0.35, P=0.014 respectively) (Figure 5.10B). Expression of mRNA for ATP5G1 was positively correlated with NDUFS8 (r=0.43, P=0.002), UQCRC1 (r=0.65, P<0.001), and COX6B1 (r=0.65, P<0.001). Expression of complex I subunits NDUFS8 and MTND1 was negatively correlated with ROS generation (r=-0.50 P<0.001 and r=-0.49 P<0.001, respectively) (Figure 5.10C). The ER stress markers GRP78, GRP94, GADD34, and CHOP were negatively correlated with mitochondrial content (r=-0.46 P=0.001, r=-0.37 P=0.009, r=-0.4 P=0.006, and r=-0.47 P=0.001, respectively) (Figure 5.10D). Cochaperone ERdj4 was also highly correlated with the other ER chaperones such as GRP94 (r=0.65, P<0.001), GRP78 (r=0.59, P<0.001), ORP150 (r=0.6, P<0.001), and apoptotic CHOP

(r=0.74, P<0.001) respectively. Apoptotic CHOP mRNA expression was highly correlated with the expression of ER stress chaperones and cochaperone such as ORP150 (r=0.73, P<0.001), GRP78 (r=0.70, P<0.001), GRP94 (r=0.57, P<0.001), ERdj4 (r=0.74, P<0.001), as well as other ER stress markers including EDEM1 (r=0.65, P<0.001) (Figure 5.10E). Finally, a fairly high negative correlation was found between ER stress chaperones such as GRP78 (r=-0.52, P<0.001), ORP150 (r=-0.62, P<0.001), cochaperone ERdj4 (r=-0.61, P=0.000) and the apoptotic CHOP (r=-0.57, P<0.001) with the mitochondrial membrane potential (Figure 5.10F).

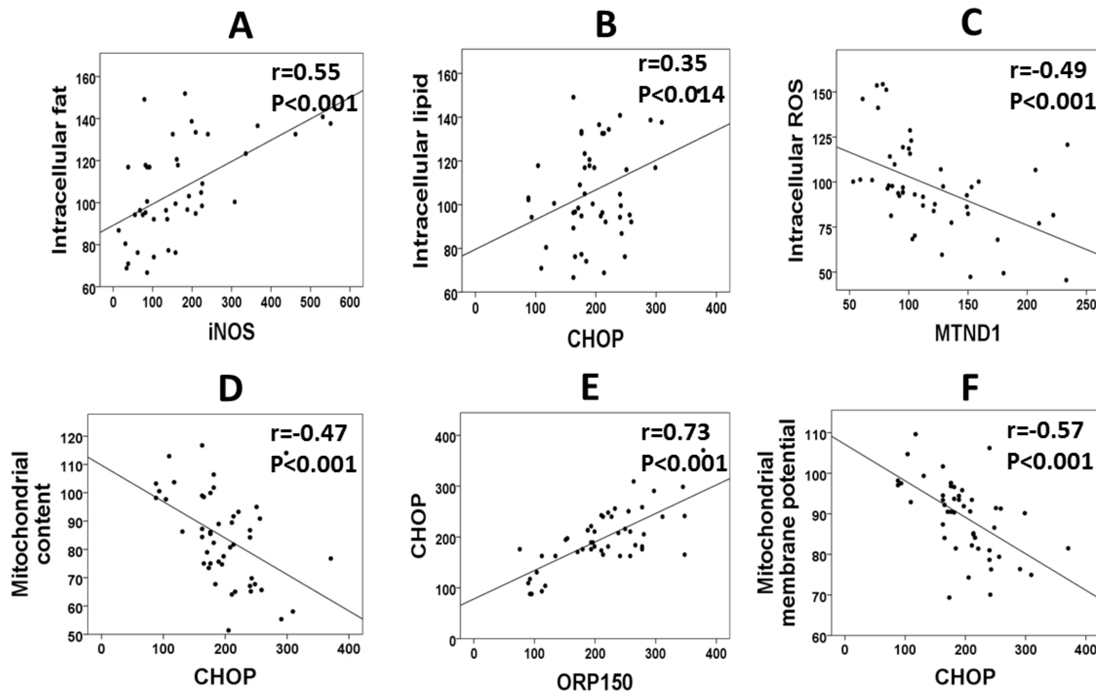

Figure 5. 10. Correlations between different parameters of interest.

These correlations are based on the results previously shown in figure 5.1 to 5.9. (A & B) Positive correlation between *iNOS* and *CHOP* mRNA expression with intracellular triglyceride in HepG2 cells. (C) Inverse correlation between the mitochondrially-encoded complex I subunit and ROS production. (D) Inverse correlation between the apoptotic *CHOP* mRNA expression and mitochondrial content. (E) Positive correlation between the ER chaperone ORP150 and the pro-apoptotic CHOP. (F) Inverse correlation between the apoptotic *CHOP* expression and the mitochondrial membrane potential.

5.4. Discussion

Steatosis and oxidative stress have been suggested to be the “first” and “second hits” of fatty liver progression to NASH [2] and therefore prevention of these outcomes may help prevent steatohepatitis. In the present study treatment with palmitic acid increased intracellular triglyceride and ROS production in HepG2 hepatocytes by 40 and 42%, providing an *in vitro* model of steatosis. Although some studies suggest that simple steatosis is not always progressive in humans provided that it is as the form of neutral lipids, it can be harmful when it is associated with ROS generation or insulin resistance leading to inflammation [316]. Consistent with our results, other studies show similar effects of palmitic acid on steatosis and ROS generation. In one study treatment of HepG2 cells with 400 μM palmitic acid increased intracellular triglyceride and ROS production by 50 and 200% respectively while the polyphenol luteolin protected against the increase [317]. However, some studies report a higher level of steatosis [42] or contrarily no steatosis [318] in HepG2 hepatocytes exposed to palmitic acid. But even when no steatosis was observed with 1 mM palmitic acid, the palmitic acid impaired insulin signaling [318].

Treatment with all polyphenols of interest in our study protected against palmitic acid-induced steatosis and ROS generation. Consistent with these findings, others also show that polyphenols can have strong anti-steatotic [48, 95] and antioxidant effects [47, 238], can inhibit the expression of genes involved in lipogenesis, and can induce genes involved in fatty acid oxidation [44, 73] leading to protection against NAFLD. In the current study, all of the investigated polyphenols similarly and almost completely prevented the increase in ROS induced by palmitic acid.

We also observed that exposure of HepG2 cells to palmitic acid depolarized the mitochondrial membrane potential by about 20%, while most polyphenols (resveratrol, quercetin, catechin, and cyanidin) protected against the depolarization. Interestingly, the same polyphenols protected against the $\Delta\Psi m$ decline with palmitic acid as we observed with oleic acid (Figure 3.6). Collapse of the $\Delta\Psi m$ may be an early sign or requirement for apoptosis [319] and hepatocyte apoptosis is involved in progression of NAFLD to NASH and hepatic fibrosis [320]. Preventing the decline in the mitochondrial membrane potential by polyphenols therefore may have a key role in decreasing hepatocyte apoptosis in NASH. In agreement with our results, polyphenols such as punicalagin (an ellagitannin polyphenol found in pomegranate) [265], curcumin [20], and flavonoids from *Rosa laevigata* Michx fruit [282] have been observed to preserve the mitochondrial membrane

potential in hepatocytes exposed to palmitic acid or in the liver of rodents fed a high-fat diet. Therefore, it appears that one of the mechanisms by which polyphenols protect against mitochondrial dysfunction and lipoapoptosis in NAFLD is via preserving the mitochondrial membrane potential.

One mechanism by which polyphenols may protect against mitochondrial dysfunction caused by palmitic acid is by inducing mitochondrial biogenesis. Mitochondrial biogenesis is defined by both mitochondrial replication and differentiation. Mitochondrial replication involves synthesis of mitochondrial DNA (leading to higher mitochondrial number), while mitochondrial differentiation results in higher expression of mitochondrial proteins (such as respiratory complex subunits), and developing crista of mitochondria leading to improved function of pre-existing mitochondria [141]. In the current study, we evaluated the effect of palmitic acid and polyphenols on both mitochondrial replication and differentiation. We found that palmitic acid decreased mitochondrial content of HepG2 cells by more than 30% and polyphenols could protect against this effect. Mitochondrial content has been shown to be suppressed in HepG2 cells exposed to palmitic acid [255] and in the liver of animals in experimental models of NAFLD and NASH [143, 254, 291]. Consistent with our findings, other studies have also shown polyphenols such as curcumin to protect against free fatty acid-induced inhibition of mitochondrial biogenesis in hepatocytes [20] and such as berberine, resveratrol, and berry anthocyanins to increase mitochondrial biogenesis in the hepatic and skeletal muscle tissue of rodents fed a high-fat diet [291], or in other models of NAFLD [254, 267]. Increased mitochondrial biogenesis leads to increased ability to metabolize excess fatty acids in the liver and this may be an effective and efficient outcome to prevent pathogenesis of NAFLD to progress to NASH. Higher mitochondrial biogenesis is shown by different studies to be negatively associated with ROS production via increased expression of antioxidant enzymes in mitochondria [301]. Mitochondrial biogenesis is also associated with higher fatty acid oxidation [267] and lower inflammation [307] which all can be beneficial for ameliorating NAFLD.

Unlike in our previous study where we found resveratrol at 10 µM to protect against oleic acid-induced inhibition of mitochondrial biogenesis in HepG2 cells (Figure 4.3), resveratrol did not significantly increase mitochondrial content (as measured by MitoTracker Green) in the current study on palmitic acid. In the current study however, resveratrol was the strongest polyphenol in

inducing the expression of mRNA for mitochondrial DNA-encoded subunits of the respiratory chain, suggesting that it increased mitochondrial differentiation and function.

While palmitic acid inhibited mitochondrial content in our study by 33% it had no significant effect on the expression of nuclear and mitochondrial DNA-encoded respiratory complex subunits (except ATP synthase subunit, ATP5G1). In contrast to our results, Garcia-Ruiz et al. [15] found decreased activity and assembly of all mitochondrial complexes by palmitic acid. Although they found a decline in mRNA expression for all mitochondrial DNA-encoded but not nuclear DNA-encoded respiratory complex subunits in HepG2 cells exposed to palmitic acid, we only observed decreased expression of ATP5G1 which is a nuclear DNA-encoded subunit.

Consistent with the results for mitochondrial biogenesis however, polyphenols in our study increased the expression of both nuclear and mitochondrial DNA-encoded subunits, which consequently would bring about improved mitochondrial function. In agreement with these findings, another study [255] showed increased expression of the mitochondrial respiratory complex subunits by polyphenols in HepG2 cells exposed to palmitic acid. Along with inducing mitochondrial biogenesis and increasing the expression of mitochondrial respiratory complex subunits, polyphenols in our study also preserved the mitochondrial membrane potential, which suggests that they can protect against mitochondrial defects in hepatocytes.

ER stress and UPR were other aspects of NAFLD investigated in the current study. Treatment of HepG2 hepatocytes with palmitic acid was found to induce ER stress and UPR evidenced by an adaptive increase in gene expression of several chaperones (GRP58, GRP78, GRP94, ORP150, EDEM1, GADD34, co-chaperone ERdj4, and apoptotic CHOP). Consistent with these results, others have shown that treatment of hepatic cells with saturated fatty acids [26, 321] or of rodents with a high-fat diet [25], induces hepatic ER stress which exacerbates NAFLD. Improved proper folding of unfolded/misfolded proteins by treatment with oral chemical chaperones such as taurine-conjugated ursodeoxycholic acid (TUDCA) and 4-phenyl butyric acid (4-PBA) have shown promise in ameliorating ER stress in diabetic and obese *ob/ob* mice which consequently resulted in alleviation of fatty liver disease, hyperglycemia, and increased insulin sensitivity [192]. Since activation of ER stress markers have been shown to be highly associated with hepatic steatosis, inflammation, impaired insulin signaling, and apoptosis (all implicated in the progression of NAFLD to NASH) [322], we hypothesized that dietary polyphenols may mimic beneficial

effects of chaperones, which by facilitating proper folding of unfolded proteins protect against ER stress in the liver. The polyphenols would thereby inhibit endogenous expression of chaperones that results from ER stress. We observed that treatment of HepG2 cells with different polyphenols prior to treatment with palmitic acid alleviates expression of these chaperones, and therefore ER stress. The effect of polyphenols on hepatic ER stress has been looked at in only a few studies [256-258] . In line with our findings, those studies show that treatment with polyphenols can alleviate ER stress evidenced by decreased ER stress markers such as GRP78, ATF4, CHOP, p-eIF2α, and p-PERK. Resveratrol has also previously been shown to alleviate palmitate-induced ER stress in HepG2 hepatocytes by decreasing gene expression of apoptotic CHOP, and protein expression of p-PERK as well as increasing gene expression of ORP150 in a SIRT1-mediated pathway [257]. In contrast to our results showing berberine had a weak effect on alleviating ER stress markers, berberine was recently shown to suppress ER stress as well as prevent hepatic lipid accumulation, inflammation, and fibrosis in diabetic db/db mice and in methionine choline-deficient mice (MCD), and consequently protected against progression of simple hepatic steatosis to steatohepatitis [258]. These researchers also showed that berberine at 5 μM protected against palmitate/oleate- and tunicamycin-induced ER stress *in vitro*. The study design however, was different from our study (different cell models have been used) and used different stressors which may partly explain the differences between the results.

A potential mechanism by which polyphenols decrease ER stress is by acting as chemical chaperones. Zhang et al. [258] showed a potential chaperone activity of berberine which consequently prevented accumulation of misfolded or unfolded proteins in a hepatocyte cell line and primary hepatocytes. This was apparently the first study revealing that polyphenols such as berberine possess direct chaperone activity whereby the protein-stabilizing function inhibits aggregation of unfolded/misfolded proteins responsible for initiation and progression of ER stress [258]. Since some polyphenols such as catechin, quercetin, and cyanidin in our study decreased the expression of several endoplasmic reticulum chaperones such as ORP150, GRP78, GRP94, and GRP58, these polyphenols may possess chaperone activity similar to that shown by Zhang et al. [258] and consequently decrease expression of ER chaperones by negative feedback. Our results show that treatment of HepG2 hepatocytes with polyphenols can prevent palmitic acid-induced steatosis at least partially due to protection against ER stress. Due to the lack of evidence

in this field, more studies need to be conducted in order to find the chaperonic effect of polyphenols in alleviating ER stress.

Palmitic acid treatment also increased expression of mRNA for the pro-apoptotic protein CHOP, while some polyphenols protected against the increase. Numerous studies show that hepatocyte apoptosis is enhanced in NASH and this is one of the main mechanisms involved in progression of the disease [323]. Failure of the UPR to resolve ER stress can also result in cell death through different mechanisms including CHOP which is one of the main UPR-mediated proapoptotic factors [7]. Therefore, suppression of CHOP may have a beneficial role in prevention of NAFLD. Consistent with our findings in human hepatocytes, others have also shown that polyphenols such as resveratrol attenuated ER stress and consequently decreased protein expression of CHOP in the liver of rats fed a high-fat diet, which led to decreased hepatic steatosis in these rats [256]. However, the role of CHOP in rodents with fatty liver is still controversial since it is reported that while CHOP null ($^{-/-}$) mice have lower steatosis in the liver, they are not protected against ethanol-induced fatty liver disease [324]. Therefore, it has been suggested that CHOP has different roles in apoptosis in humans *vs.* rodents [321]. While increased expression of CHOP by palmitic acid in HepG2 cells and primary human hepatocytes was shown to induce cellular death through activation of a CHOP-NF-κB pathway, this signalling pathway was not conserved in primary rodent hepatocytes and could not induce apoptosis in these cells [321]. The authors suggested that in human cells UPR activation has a major role in the pathogenesis of NASH by inducing expression of CHOP. Therefore, since we used human hepatocytes, we suggest that polyphenols in our study protect against palmitic acid-induced apoptosis in these cells by inhibiting CHOP expression as well as protecting against mitochondrial membrane potential dissipation.

We found a positive correlation between iNOS mRNA and intracellular fat in HepG2 cells. Hepatic expression of iNOS has been shown to be increased in *ob/ob* mice [16], and iNOS knockout (iNOS$^{-/-}$) mice are resistant to a high-fructose diet and accumulate less fat in their livers [312]. By reacting with cytochrome *c* oxidase (complex IV), NO may result in mitochondrial respiratory chain dysfunction, further impairing electron transfer and leading to hepatic lipid accumulation [325].

Our findings also show a negative correlation between the expression of ER stress markers such as GRP78 and CHOP with mitochondrial biogenesis and the mitochondrial membrane potential.

An ER-mitochondrial stress crosstalk has been suggested by some studies [326], so ER stress and mitochondrial dysfunction may work synergistically to exacerbate NAFLD. This ER-mitochondrial stress crosstalk has also been observed in studies regarding apoptosis [327] and hepatic insulin resistance [328]. This crosstalk between mitochondrial dysfunction, ER stress, and depleted ATP stores may promote progression of NAFLD to NASH by inducing apoptotic cell death and necrosis [326], while treatment with polyphenols may protect against these outcomes.

In conclusion, we found that the saturated fat palmitic acid can induce steatosis, increase ROS generation, decrease the mitochondrial membrane potential, inhibit mitochondrial biogenesis and function while inducing ER stress in human hepatocytes. We show that several polyphenols can protect against these changes. The results and correlations show that there might be a crosstalk between mitochondrial dysfunction and endoplasmic reticulum stress which may facilitate progression of simple steatosis to NASH and apoptosis. More studies are needed to prove this crosstalk and to explore molecular mechanisms behind progression of NAFLD to NASH, and the inhibition of these pathways by dietary polyphenols.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interests.

5.5. Transition to CHAPTER 6

So far, CHAPTER 3 and 4 have dealt with the first and second hypotheses (mitochondrial dysfunction and ROS generation) and CHAPTER 5 dealt with the third hypothesis (ER stress). Regarding the fourth hypothesis we could not find any synergism between resveratrol and cyanidin in protection against oleic acid-induced ROS generation (Figure 5.11). Oleic acid significantly induced ROS generation by 123%, and except resveratrol at 1 μM, resveratrol and cyanidin at all

doses (1, 5, 10 μM) alone or in combination gave significant inhibition. However, we could not observe any synergism between resveratrol and cyanidin in inhibition of oleic acid-induced ROS.

CHAPTER 6 deals with breakdown/digestion products of polyphenols. It is still a matter of debate whether breakdown/digestion products of polyphenols contribute to the protection against fatty liver disease. For example, *in vitro* studies (including our results in CHAPTER 4) show that anthocyanins do not induce mitochondrial biogenesis in the cells while studies in rodents show the opposite with capability of inducing mitochondrial biogenesis by anthocyanins. It is believed that the effects observed *in vivo* are due to the breakdown/digestion products of polyphenols formed after ring fission of parent polyphenols by colonic microbiota. CHAPTER 6 will present investigations into the ability of the breakdown/digestion products of polyphenols (quercetin and cyanidin) to contribute to the protective effects against NAFLD.

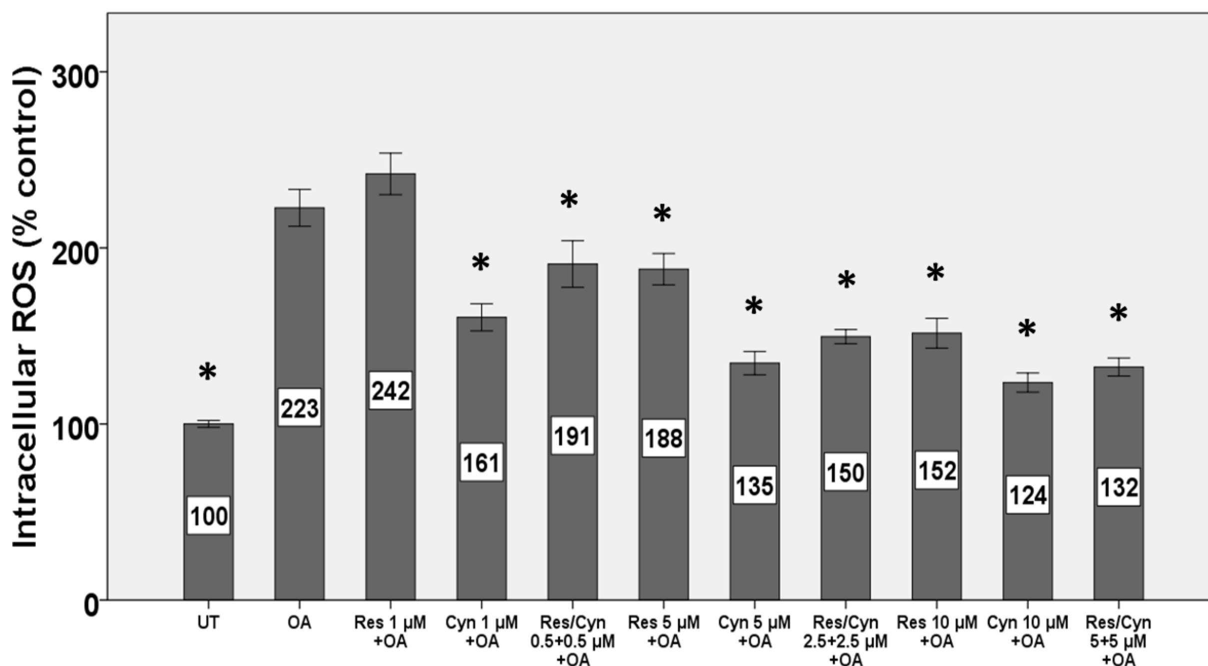


Figure 5. 11. Evaluation of synergism between resveratrol and cyanidin in inhibiting oleic acid-induced ROS generation.

UT, untreated; OA, oleic acid; Res, resveratrol; Cyn, cyanidin. ROS generation was measured after treatment with different concentrations of resveratrol or cyanidin alone and in combination for 2 h followed by treatment with 1.5 mM oleic acid for 24 h. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The bars represent means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$. The significant difference between groups was tested with one-

way ANOVA followed by Dunnett's post-hoc test. Two-way ANOVA showed no significant interaction between resveratrol and cyanidin.

CHAPTER 6: PHENOLIC BREAKDOWN PRODUCTS OF QUERCETIN AND CYANIDIN CONTRIBUTE TO THE PROTECTION AGAINST STEATOSIS, OXIDATIVE STRESS, AND MITOCHONDRIAL DYSFUNCTION IN AN *IN VITRO* MODEL OF STEATOSIS

Abstract

Scope: Although dietary polyphenols have been shown to induce mitochondrial biogenesis and ameliorate non-alcoholic fatty liver disease (NAFLD) in experimental models, it is largely unknown whether these effects are due to the parent polyphenols or their breakdown/digestion products.

Methods and results: In an *in vitro* model of steatosis using HepG2 hepatocytes, we investigated the effects of quercetin and cyanidin, and their phenolic breakdown/digestion products (caffeic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde) on steatosis, reactive oxygen species (ROS) generation, and mitochondrial biogenesis and function. Treatment with oleic acid for 24 h induced steatosis, and increased ROS generation, while treatment with quercetin and cyanidin and their breakdown/digestion products for 2 h (and thereafter for 24 h) all similarly protected against these inductions. Oleic acid also impaired mitochondrial function evidenced by a decreased mitochondrial membrane potential, while both the parent polyphenols and their breakdown/digestion products prevented the decrease. Oleic acid also decreased the cellular mitochondrial content while 24 h incubation with cyanidin or the breakdown/digestion products protected against this decrease. Interestingly, when HepG2 hepatocytes were incubated with the parent polyphenols or their breakdown/digestion products for only 1 h (to limit spontaneous degradation of quercetin and cyanidin to phenolic products in the medium), only the breakdown/digestion products protected against the oleic acid-induced decrease in mitochondrial content. Moreover, 1 h incubation of HepG2 cells with breakdown/digestion products, but not the parent polyphenols, induced mitochondrial content in the absence of oleic acid.

Conclusion: The results suggest that breakdown/digestion products of polyphenols may contribute to the protective effects of parent polyphenols against NAFLD. This can partly explain the conflicting results found between *in vitro* and *in vivo* studies on the induction of mitochondrial biogenesis by anthocyanins.

Keywords: flavonoids, phenolic degradation products, steatosis, mitochondrial membrane potential, mitochondrial biogenesis

6.1. Introduction

In the last two decades, plant phytochemicals have received growing evidence for their health promoting effects [329]. Recently, the polyphenols found in fruits and vegetables have been extensively studied for their potential health effects against various diseases including obesity, diabetes, cardiovascular diseases, and cancer [330-333]. One disease for which flavonoids may provide a health benefit is non-alcoholic fatty liver disease (NAFLD) [46, 48].

At least part of the health benefits of dietary flavonoids, or of effects observed *in vitro*, may be due to their phenolic digestion/breakdown products. A portion of flavonoids including quercetin and anthocyanins are not absorbed in the small intestine and they reach the colon intact. These flavonoids are digested (broken down) by colonic microbiota resulting in production of phenolic acids including protocatechuic acid (from cyanidin [334] and quercetin [335, 336]), and 2,4,6-trihydroxybenzaldehyde (from cyanidin and other anthocyanins [334]) which are then absorbed. Until recently, little attention was paid to the breakdown/digestion products of flavonoids.

Also *in vitro*, the instability of flavonoids in cell culture media may produce phenolic products that contribute to the observed effects. Although many studies on quercetin have been conducted *in vitro*, instability of the flavonol in the cell culture medium [337, 338] is not often considered, and complicates interpretation of the results. Similarly with anthocyanins under experimental [339] and biological conditions [340, 341], spontaneous degradation to phenolic acids may occur which may contribute to the observed effects. Moreover, doses of flavonoids used in numerous *in vitro* studies are more than are relevant *in vivo* ($\leq$10 µM) and sometimes 100 µM or more has been used [342-344], which is above the physiologically relevant concentrations found in human serum.

In vivo, despite the absorption of some part of ingested flavonoids such as anthocyanins in the small intestine [218, 345], a large proportion may undergo ring fission in the colon and their breakdown/digestion products [340, 346] may be absorbed. These phenolic breakdown products have been suggested to contribute to the beneficial effects *in vivo* [207, 334, 336] and therefore future research on flavonoids bioactivity should take degradation/digestion products into account [329]. While *in vitro* studies for instance show no effect of anthocyanins on sirtuin 1 (SIRT1) activity [279], *in vivo* studies show beneficial effects of anthocyanins on mitochondrial biogenesis

[254]. It is still a question whether the effects of anthocyanins observed *in vivo* is due to the parent compounds or their phenolic digestion/breakdown products.

In the current study, 10 μM quercetin and cyanidin and their phenolic breakdown/digestion products were investigated for their protection against free fatty acid-induced steatosis, ROS generation, and decreases in the mitochondrial membrane potential and mitochondrial content. The results suggest that breakdown products can contribute to the protective effects of the parent polyphenols against NAFLD.

6.2. Materials and Methods

6.2.1. HepG2 cells culture condition

See section 3.2.2.

6.2.2. Treatment with oleic and palmitic acid/BSA and polyphenols

See section 3.2.3.

6.2.3. Measuring intracellular lipid content using Nile Red staining

See section 3.2.4.

6.2.4. Measurement of intracellular ROS

See section 4.2.4.

6.2.5. Measurement of mitochondrial content using MitoTracker Green

To determine mitochondrial content, HepG2 hepatocytes were labelled with a mitochondria-specific dye, MitoTracker Green according to the manufacturer's protocol (Invitrogen, USA). Briefly, 3×10^4 HepG2 cells were seeded per well in 96-well clear-bottom plates. After 24h, the old medium was replaced with fresh medium containing 1% fatty acid-free bovine serum albumin and the cells were pre-treated with 10 μM parent polyphenols, quercetin and cyanidin, or their breakdown/digestion products protocatechuic acid, caffeic acid, and 2,4,6-

trihydroxybenzaldehyde for either 1 h (and removed after 1 h) or 2 h (incubated for 24 h thereafter) followed by treatment with or without oleic acid for 24 h. After 24 h, the old medium was removed and the cells were exposed to 300 nM final concentration of MitoTracker Green dissolved in medium (without fetal bovine serum) and the plate was covered and incubated at 37ºC for 20-30 min. After incubation, the medium containing probe was removed, the cells were carefully washed 3-4 times with phosphate buffered saline (PBS) and the mean fluorescence of 9 different spots of each well was measured using a microplate reader at excitation 485 nm and emission 528 nm. Fluorescence images were captured using a ZOE Fluorescent Cell Imager.

6.2.6. Measuring mitochondrial membrane potential

See section 3.2.11.

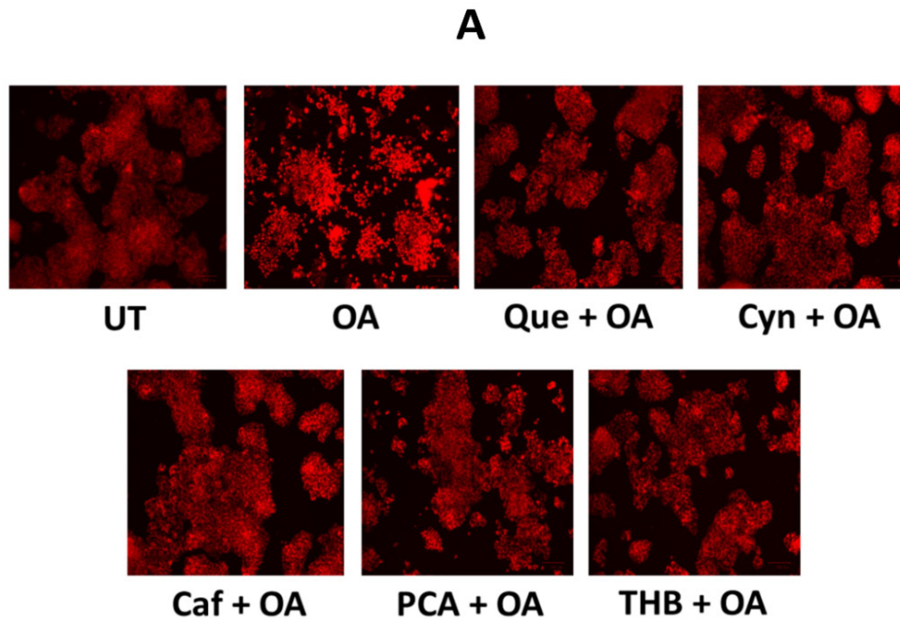
6.2.7. Statistical analysis

Results were expressed as mean values with their standard error of means (SEM) for the number of experiments indicated. Results were analyzed using the Statistical Package for the Social Sciences (SPSS 22) (IBM, USA). One-way ANOVA was used initially, and when any significance was detected, Dunnett's post hoc test was used to compare the mean of each group with the oleic or palmitic acid alone condition. The level of significance was $P<0.05$. Correlation was determined by Pearson's R.

6.3. Results

6.3.1. Intracellular lipid accumulation

Fluorescence images of HepG2 cells stained with Nile Red show that oleic acid increased intracellular lipids while treatment with quercetin, cyanidin, or their phenolic breakdown products inhibited the accumulation (Figure 6.1A). When the fluorescence signals were quantified using a microplate reader, it was revealed that 1.5 mM oleic acid increased intracellular fat in HepG2 cells by 182% while quercetin, cyanidin and all of their breakdown products at 10 µM protected against the increase by 44-52% (Figure 6.1B). The protective effect against steatosis was similar between polyphenols and their breakdown products.



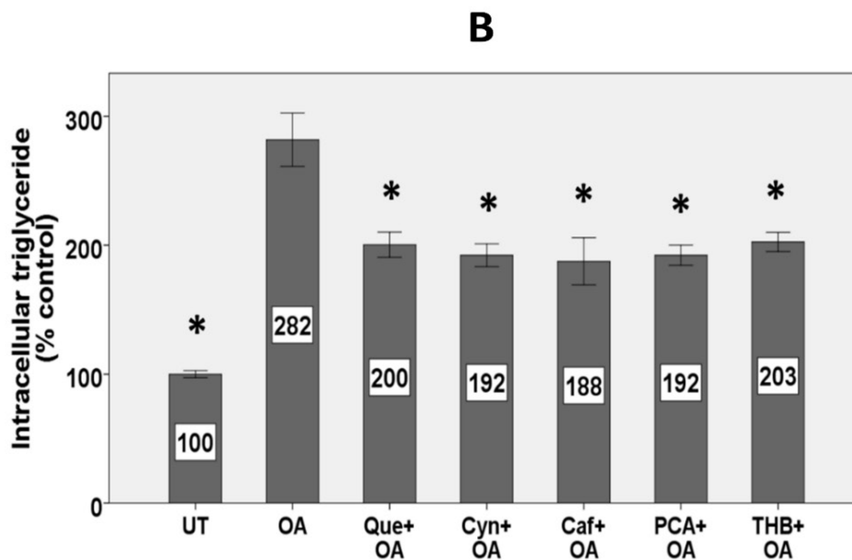


Figure 6. 1. Effects of quercetin, cyanidin and phenolic breakdown products on oleic acid-induced intracellular lipid accumulation.

(UT: untreated, OA: oleic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). HepG2 cells were treated with 10 µM quercetin or cyanidin, or phenolic acids for 2 h (and 24 h thereafter), followed by treatment with 1.5 mM oleic acid for 24 h. Intracellular lipids were stained with the Nile Red fluorescence probe. (A) Representative fluorescence images captured using a ZOE Fluorescent Cell Imager at 20x magnification. (B) Intracellular lipids quantified from the Nile Red fluorescence using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

When the cells were treated with 500 µM palmitic acid, intracellular lipid was increased by 48% while polyphenols and their phenolic products similarly prevented the increase by almost 100% (Figure 6.2). Compared with the oleic acid experiment (Figure 6.1), quercetin, cyanidin, and their phenolic breakdown products showed stronger protection against steatosis from palmitic acid (albeit at a lower concentration than oleic acid).

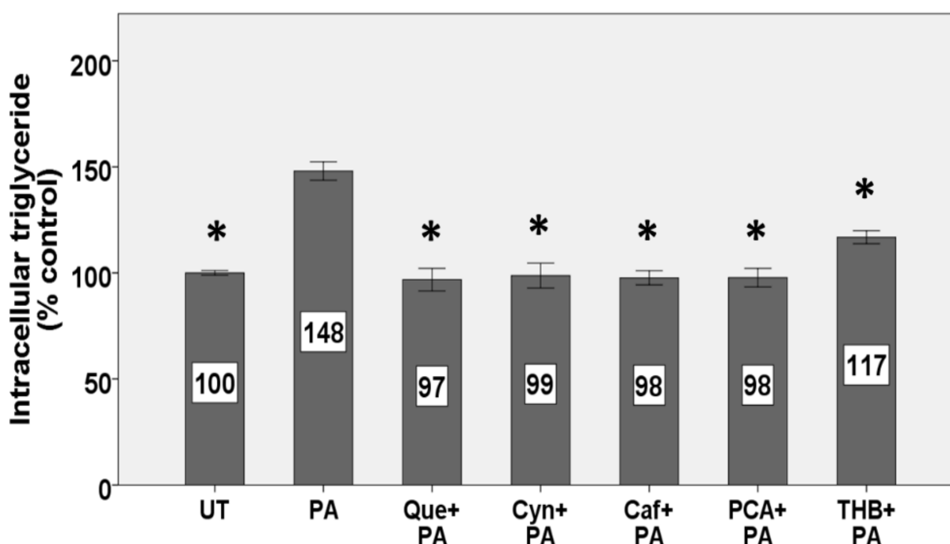


Figure 6. 2. The effect of quercetin, cyanidin and phenolic breakdown products on palmitic acid-induced intracellular lipid accumulation

(UT: untreated, PA: palmitic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). HepG2 cells were pre-treated with 10 µM quercetin or cyanidin, or phenolic acids for 2 h (and 24 h thereafter), followed by treatment with 500 µM palmitic acid for 24 h. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at $P<0.05$.

6.3.2. Intracellular ROS

Fluorescence images of HepG2 cells stained with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) show that oleic acid increased intracellular ROS while treatment with quercetin and cyanidin, and their phenolic breakdown products prevented the increase (Figure 6.3A). Quantification of the fluorescence signals revealed that when the cells are pre-treated with oleic acid, intracellular ROS was increased by 73% and all the phenolic products as well as quercetin prevented the increase by almost 100%. Cyanidin protected by 71% (Figure 6.3B). With palmitic

acid, the same trend was observed in which palmitic acid increased ROS production by 66% while quercetin and the phenolic products gave similar protection (~85%) and cyanidin gave 56% protection (Figure 6.4).

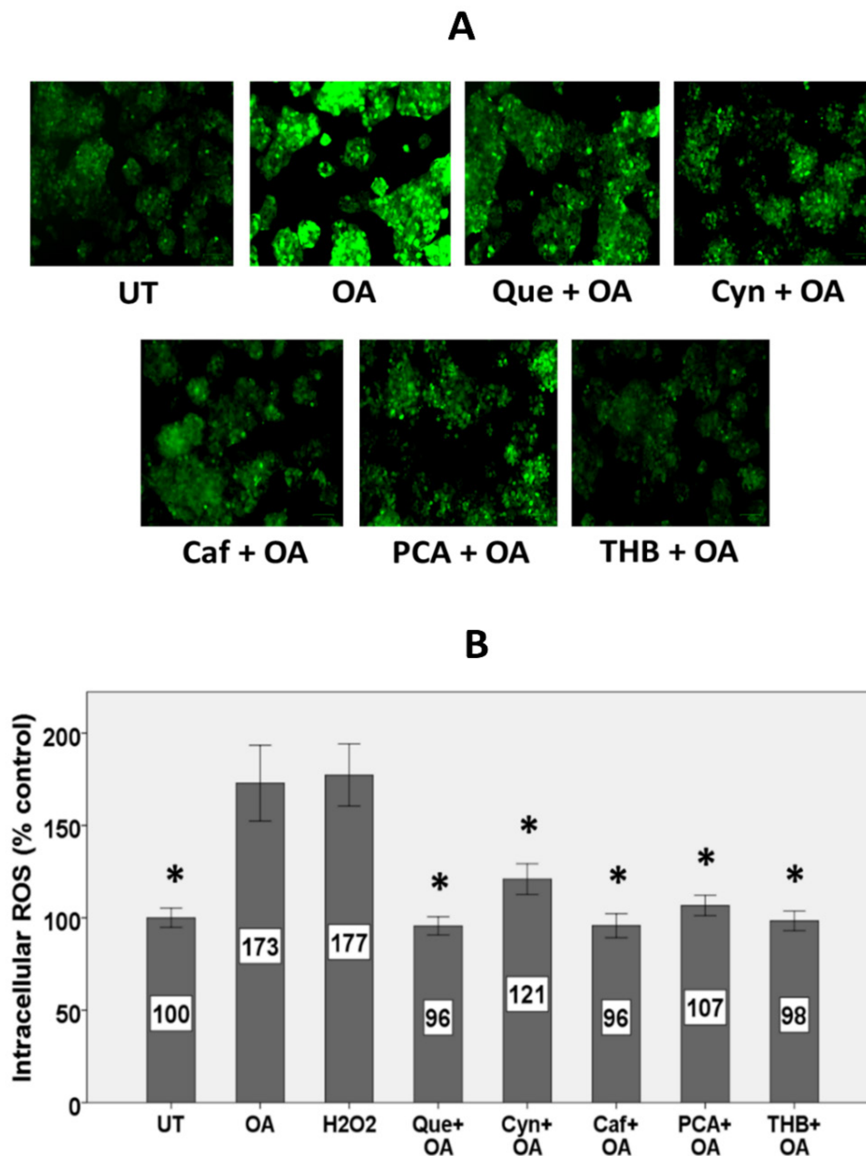


Figure 6. 3. The effect of quercetin and cyanidin and their phenolic breakdown products on intracellular ROS production.

(UT: untreated, OA: oleic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). HepG2 hepatocytes were pre-treated with different polyphenols at 10 µM for 2h (and 24 h thereafter) and then treated with 1.5 mM oleic acid for 24h. Experimental conditions were as in Figure 6.1, and ROS generation was measured using the fluorogenic probe DCFH-DA. (A) Representative fluorescence images at 20x magnification captured by a ZOE Fluorescent Cell Imager after staining HepG2 cells with DCFH-

DA. Higher green fluorescence represents higher intracellular ROS content. (B) Quantification of ROS generation. DCF fluorescence was measured using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at P<0.05.

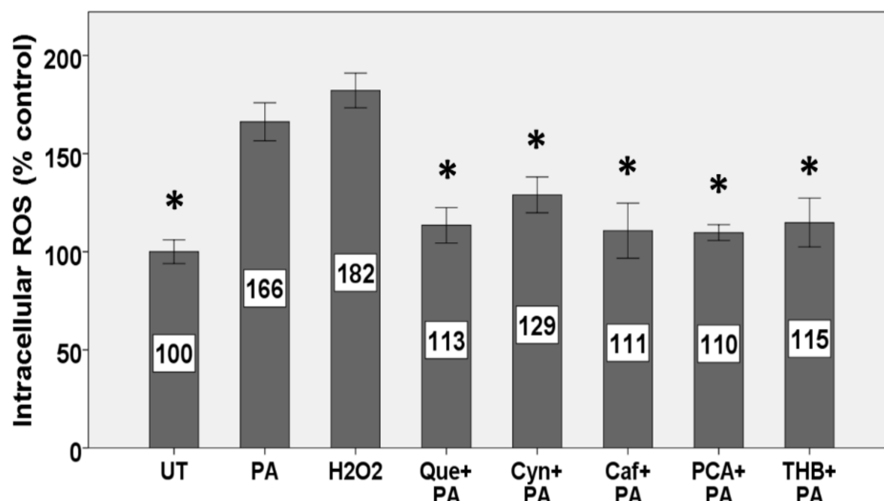


Figure 6. 4. The effects of quercetin, cyanidin and phenolic breakdown products on palmitic acid-induced ROS generation.

Experimental conditions were as in Figure 6.2, and ROS generation was measured using the fluorogenic probe DCFH-DA. DCF fluorescence was measured using a microplate reader. (UT: untreated, PA: palmitic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the palmitic acid alone condition at P<0.05.

6.3.3. Mitochondrial membrane potential

Fluorescence images of HepG2 cells stained with tetramethylrhodamine, ethyl ester (TMRE) show that oleic acid depolarized the mitochondrial membrane potential while treatment with quercetin and cyanidin, and their phenolic breakdown products protected against the depolarization (Figure 6.5A). Quantification of fluorescence signals revealed that when the cells were pre-treated with oleic acid, the mitochondrial membrane potential decreased by almost 40% while quercetin and cyanidin, and their phenolic breakdown products rescued the potential by 46-82% (Figure 6.5B). Protocatechuic acid was the strongest polyphenol with 82% protection while quercetin was the weakest one with 46% protection.

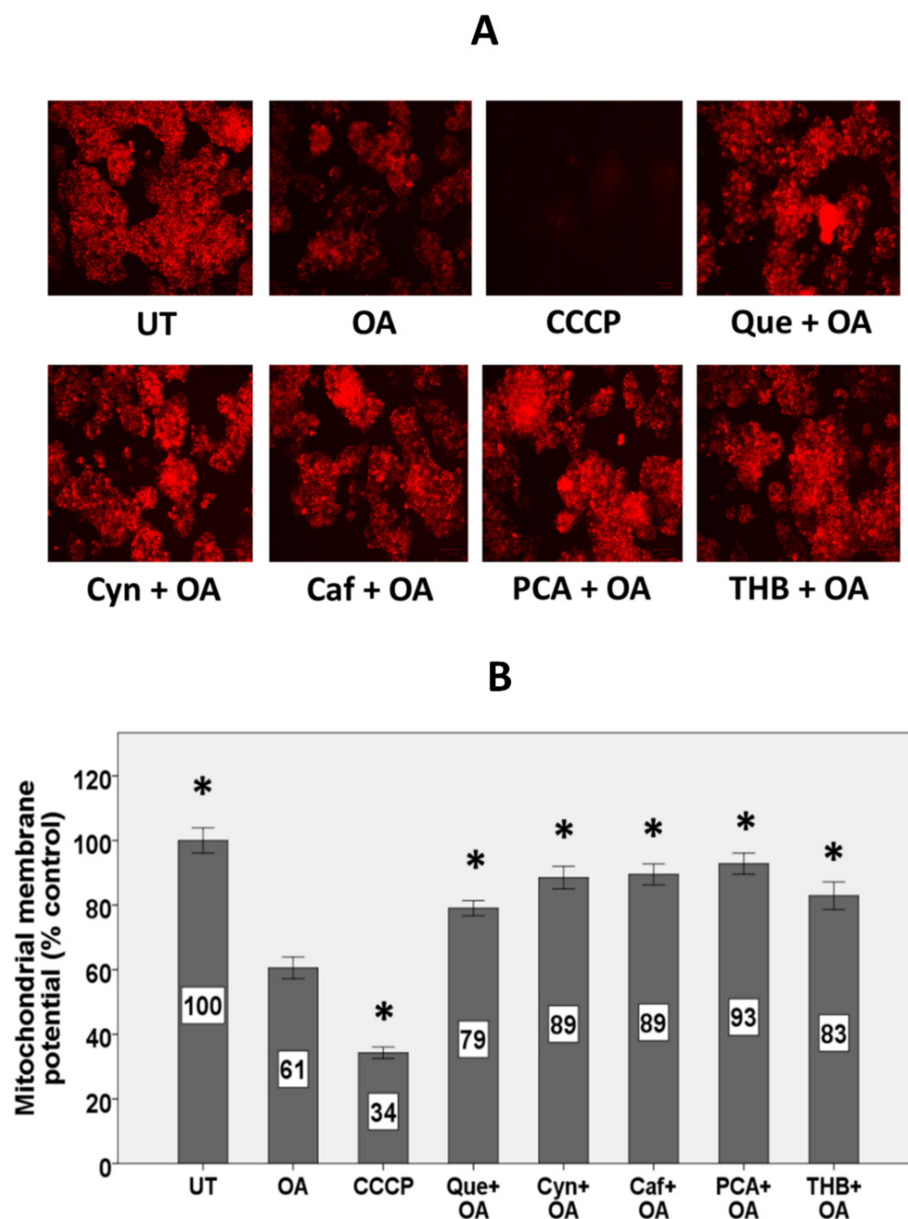


Figure 6. 5. The mitochondrial membrane potential in HepG2 cells.

(UT: untreated, OA: oleic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). The effect of oleic acid and polyphenols and their phenolic breakdown metabolites on the mitochondrial membrane potential ($\Delta\Psi m$) after staining HepG2 cells with TMRE red. (A) Representative fluorescence images captured using a ZOE Fluorescent Cell Imager at 20x magnification (B) Quantification of the mitochondrial membrane potential. Experimental conditions were as in Figure 6.1, and the mitochondrial membrane potential was measured using TMRE. TMRE red fluorescence was quantified using a microplate reader. Untreated cells were set at 100% and the data are presented

as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

6.3.4. Mitochondrial content

Represented in Figure 6.6A, treatment with oleic acid decreased the mitochondrial content evidenced by lower green fluorescence, while treatment with cyanidin and phenolic breakdown products for 2 h (and incubation with them for 24 h thereafter) appeared to inhibit the decrease. When the fluorescence signals were quantified (Figure 6.6B), it was revealed that oleic acid significantly decreased mitochondrial content by 21%, while 2,4,6-trihydroxybenzaldehyde, protocatechuic acid, caffeic acid, and cyanidin protected against this decrease by 157, 100, 95, and 71%, respectively. Quercetin had no significant effect. Interestingly, the effect of phenolic breakdown products was stronger than the parent polyphenols (Figure 6.6B).

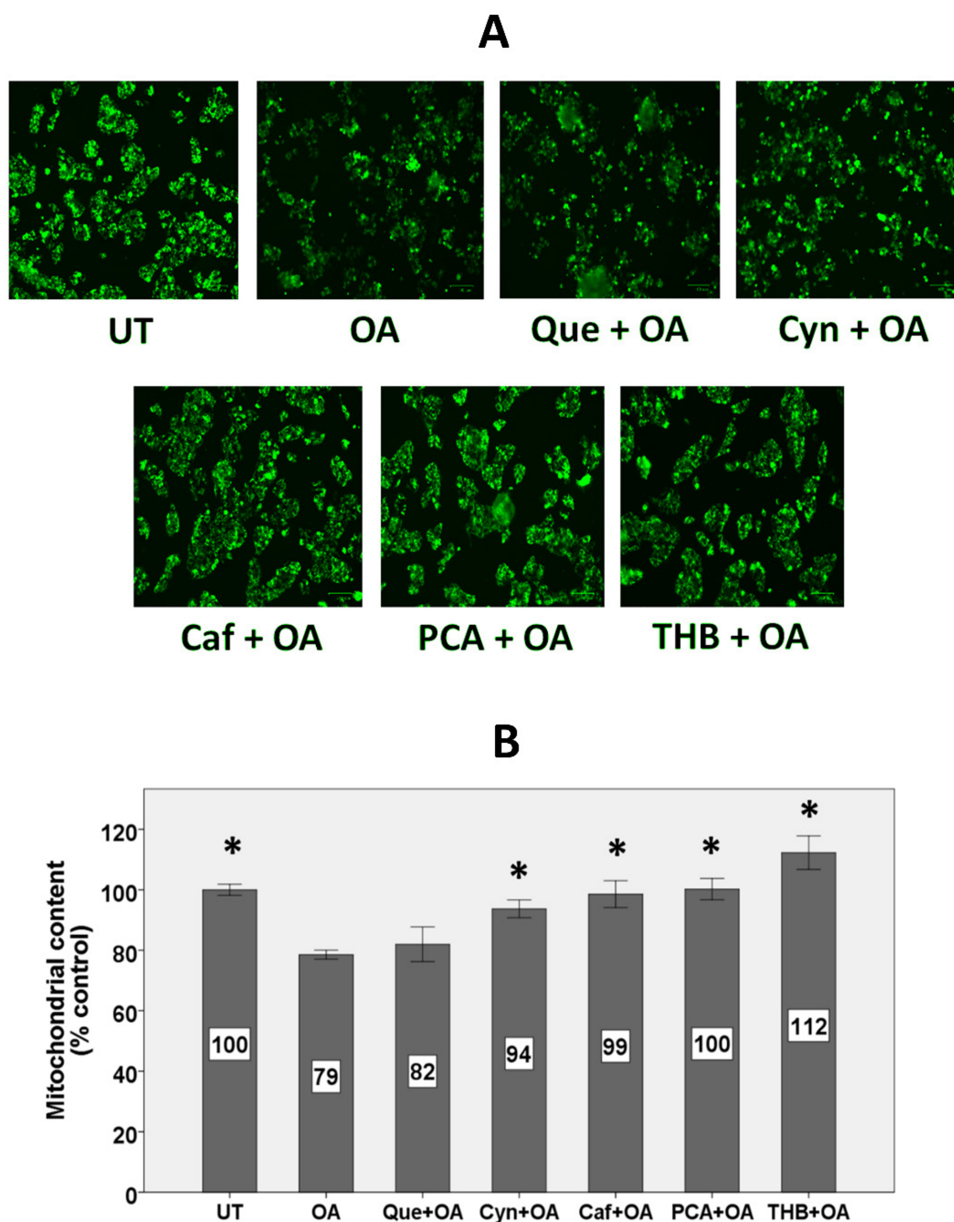


Figure 6. 6. Effects of oleic acid, quercetin, cyanidin and phenolic breakdown products on mitochondrial content.

Experimental conditions were as in Figure 6.1, and mitochondrial content was measured after staining with MitoTracker Green. (A) Fluorescence images were captured using a ZOE Fluorescent Cell Imager at 20x magnification. (B) The MitoTracker Green fluorescence was quantified using a microplate reader. (UT: untreated, OA: oleic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

In another experiment, HepG2 hepatocytes were incubated with quercetin and cyanidin, and their phenolic breakdown products for only 1 h and then removed prior to treatment with oleic acid for 24 h (Figure 6.7). In this experiment, 1 h treatment with the phenolic breakdown products (caffeic acid, protocatechuic acid, 2,4,6-trihydroxybenzaldehyde) prevented the oleic acid-induced decrease in mitochondrial content, while the parent polyphenols quercetin and cyanidin had no significant effect.

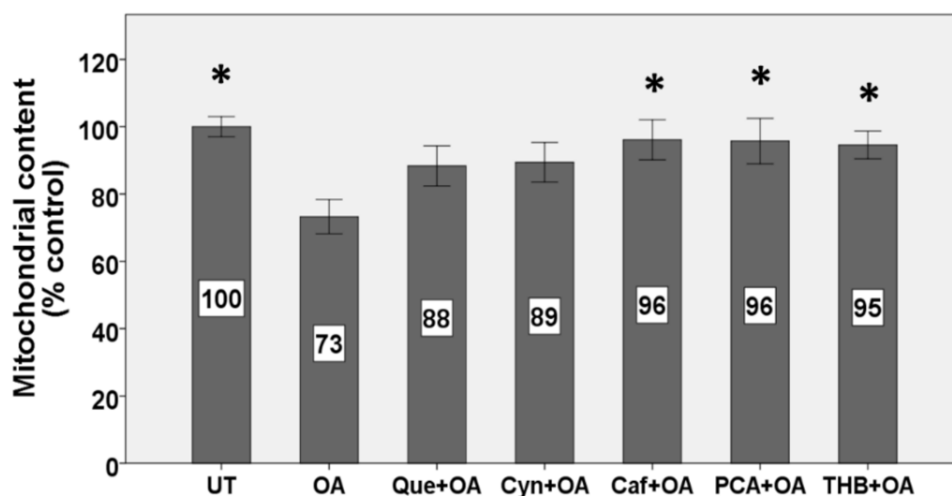


Figure 6. 7. Effects of 1 h treatment with polyphenols on oleic acid-induced suppression of mitochondrial content.

HepG2 cells were incubated with 10 μM quercetin, cyanidin or their phenolic breakdown products for 1 h and then removed prior to treatment with 1.5 mM oleic acid for 24 h. (UT: untreated, OA: oleic acid, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). The MitoTracker Green fluorescence was quantified using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from the oleic acid alone condition at $P<0.05$.

To test whether the polyphenols inhibited an effect of oleic acid or themselves increased mitochondrial content, the cells were incubated with quercetin, cyanidin, or their phenolic breakdown products for 1 h and then removed prior to incubation for 24 h in the absence of oleic acid (Figure 6.8). The results showed that incubation with only protocatechuic and caffeic acid induced mitochondrial content by 37 and 23%, respectively while incubation with the parent

polyphenols quercetin and cyanidin, or the phenolic product 2,4,6-trihydroxybenzaldehyde had no induction of mitochondrial content.

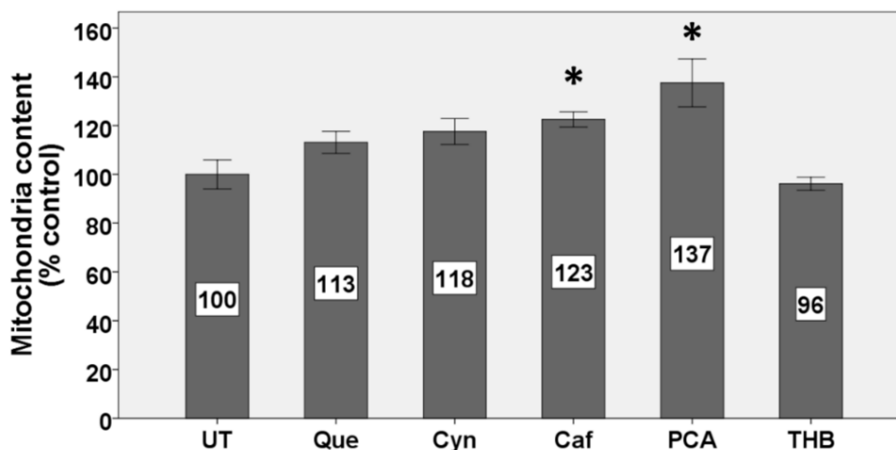


Figure 6. 8. Effects of 1 h treatment with polyphenols on mitochondrial content after 24 h.

HepG2 cells were incubated with 10 µM quercetin, cyanidin or their phenolic breakdown products for 1 h and then removed prior to incubation for 24 h (without oleic acid). (UT: untreated, Que: quercetin, Cyn: cyanidin, Caf: caffeic acid, PCA: protocatechuic acid, THB: 2,4,6-trihydroxybenzaldehyde). The MitoTracker Green fluorescence was quantified using a microplate reader. Untreated cells were set at 100% and the data are presented as % of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 replicate wells in each experiment. (*) Significantly different from UT control at $P<0.05$.

6.4. Discussion

In this study, we compared the effects of the parent flavonoids quercetin and cyanidin, and their degradation products on steatosis, oxidative stress, mitochondrial membrane potential and mitochondrial content in an *in vitro* model of steatosis. We aimed to discover if the phenolic breakdown products have strong enough effects that they might account for some of the protective benefits observed from these flavonoids *in vivo*, or *in vitro*.

The results showed that three of the main breakdown/digestion products of quercetin and cyanidin, (protocatechuic acid, caffeic acid, and 2,4,6-trihydroxybenzaldehyde) had at least equal, and in some measures greater effects than the parent compound. In oleic acid-induced intracellular triglyceride accumulation for example quercetin and cyanidin and their breakdown/digestion products showed similar protection. Consistent with these results, other *in vitro* and *in vivo* studies

have shown that phenolic acids such as protocatechuic acid [347], caffeic acid [348], and 2,4,6-trihydroxybenzaldehyde [349] have anti-steatotic effects. In addition to steatosis, quercetin and cyanidin and their breakdown/digestion products also showed similarity in the prevention of intracellular ROS production. In agreement with these results, other *in vitro* studies [350] have shown that phenolic acids such as protocatechuic acid have potent antioxidant activity and can inhibit oxidative stress.

The results showed that the effectiveness of flavonoids and their breakdown/digestion products on steatosis depended on the type and concentration of free fatty acids used. The protective effect of flavonoids and their breakdown/digestion products against steatosis was stronger when palmitic acid was used to induce steatosis in which polyphenols completely prevented lipid accumulation. This could be due to the lesser steatotic effect of palmitic acid (which increased steatosis by only 48%) compared to the higher steatotic effect of oleic acid (which increased steatosis by 182%).

The results also showed that exposure to oleic acid decreased the mitochondrial membrane potential in HepG2 cells by 40%, while quercetin and cyanidin and their breakdown/digestion products all protected against the decrease. Protection by the phenolic acids protocatechuic acid or caffeic acid against a mitochondrial membrane potential decline has been shown previously in cultured cells exposed to a mitochondrial toxin [351] or ethanol [352]. Since the current study showed that parent flavonoids and their degradation products similarly protected against the mitochondrial membrane potential decline, the breakdown/digestion products of parent polyphenols (quercetin and cyanidin) may contribute to *in vivo* protection against mitochondrial dysfunction and mitochondria-mediated apoptosis in NAFLD. However, studies on the effect or comparison of phenolic degradation products and parent flavonoids on mitochondrial dysfunction and the membrane potential in NAFLD are very limited.

Previous research has shown some protective effects of the investigated flavonoids and phenolic breakdown products against NAFLD. Quercetin [44], cyanidin-3-glucoside [249], and protocatechuic acid [347] have been shown to decrease hepatic steatosis in rodents fed an atherogenic diet. Similar protection of quercetin and cyanidin and their phenolic breakdown products against intracellular lipid accumulation in HepG2 cells, suggest that each of these polyphenols independently possess a lipid lowering effect. In a recent study, 2,4,6-trihydroxybenzaldehyde was also shown to decrease hepatic fat accumulation in mice fed a high-

fat diet [349]. The authors also reported that 2,4,6-trihydroxybenzaldehyde decreased intracellular fat in 3T3-L1 adipocytes although very high doses (50, 100 μM) of this phenolic compound were used [349]. A protective effect against steatosis was also shown with caffeic acid in HepG2 cells treated with oleic acid [348]. Again, very high doses of caffeic acid (100, 150 μM) were used in that study that may not be achievable in human blood. In the current study, we showed that doses more relevant to human serum concentrations can have protective effects.

Decreased content of mitochondria in hepatocytes was another aspect of NAFLD that we investigated for the effects of phenolic breakdown products compared to the parent compounds. Our previous work (Chapter 4) showed that treatment of HepG2 cells with oleic acid decreased mitochondrial content by 20%, and quercetin but not cyanidin could significantly protect against the decrease. In the current study, oleic acid similarly decreased mitochondrial content by 20%, while in this case cyanidin but not quercetin significantly protected against this decrease. The reason for the discrepancy between the two studies is not known. Interestingly however, the phenolic breakdown products had stronger effects than the parent flavonoids in protecting against the decrease in mitochondrial content. Although previous studies have shown protective effects of quercetin and anthocyanins in NAFLD [249, 250], a question is whether the observed effect of quercetin or anthocyanins *in vivo* is due to the parent polyphenol or their degradation products. Previous studies show that anthocyanins for instance, unlike other polyphenols such as quercetin, have no effect on SIRT1 activity *in vitro* [279], but increased liver SIRT1 mRNA and protein expression from dietary anthocyanins has been observed *in vivo* [353]. These results support the possibility that the degradation products of anthocyanins from the colonic microbiota may be responsible for this effect.

The current results also suggest that caution is needed interpreting *in vitro* studies of cells incubated with flavonoids. It is likely that incubation of HepG2 cells with quercetin or cyanidin in culture medium for 24 h produced spontaneous degradation products, as previously shown by other studies [334, 338]. One of those studies [334] reported that after incubation of cyanidin in cell-free medium (DMEM) for 4 h, 96 percent of cyanidin had degraded to primarily protocatechuic acid and 2,4,6-trihydroxybenzaldehyde. Similarly, incubation of quercetin in DMEM resulted in more than 90% degradation to phenolic products after 3 h [338]. Accordingly, the bioactivity of quercetin and cyanidin in the current study may be at least partially attributed to their degradation

products since we observed similarity between parent flavonoids and their breakdown products (after 24 h of incubation) in inhibiting steatosis and ROS generation. Unknown however is the extent to which the parent flavonoid enters the cells in the early part of the incubation, and thereby contributes to some of the effects.

In order to further investigate whether the effects of cyanidin and quercetin is due to the parent polyphenols or their breakdown/digestion products, we incubated the cells with quercetin and cyanidin and their breakdown/digestion products for only 1 h and then replaced the medium with fresh medium containing no polyphenols. This short-term treatment may result in quick uptake of the flavonoids by HepG2 cells and avoid the majority of the spontaneous degradation which occurs following long incubation (24 h) with culture medium. Interestingly, the results show that after 1 h of pre-incubation, only the phenolic degradation products prevented the decrease in mitochondrial content produced by oleic acid, while the flavonoids quercetin and cyanidin had no significant effect. Moreover, in experiments without oleic acid 1 h exposure to the phenolic acids protocatechuic acid and caffeic acid, but not the flavonoids or 2,4,6-trihydroxybenzaldehyde increased the mitochondrial content in HepG2 cells after 24 h. These results suggest that the observed induction of mitochondrial biogenesis in animal models by flavonoids such as anthocyanins [254] may be due to the phenolic degradation products produced by the colonic microbiota.

In conclusion, parent flavonoids quercetin and cyanidin and their breakdown/digestion products similarly protected against steatosis, ROS generation, and the mitochondrial membrane potential decline induced by oleic acid in HepG2 hepatocytes, but only the phenolic degradation products induced mitochondrial biogenesis. The results suggest that phenolic breakdown/digestion products of the flavonoids quercetin and cyanidin are responsible for the observations of protection in cell culture, and for protection by these dietary flavonoids against NAFLD.

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Conflicts of interest

The authors declare no conflicts of interests.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUDING REMARKS

7.1. General discussion

The main goal of this thesis was to compare the effects of different classes of dietary polyphenols on molecular mechanisms underlying NAFLD. We were interested to know which polyphenols are the most effective in protecting against the disease and by what molecular mechanisms. In the first manuscript (CHAPTER 3), we investigated the effects of polyphenols on steatosis as the "first hit" of NAFLD, and mitochondrial dysfunction. Treatment of HepG2 cells with oleic acid increased intracellular lipid accumulation and distorted morphology of the cells. However, treatment with different polyphenols inhibited steatosis and preserved the eye shape morphology of HepG2 cells. Although protective effects of individual polyphenols against steatosis have been previously shown in studies *in vitro* [35-37] and *in vivo* [48, 73], we found different classes of polyphenols show similar effectiveness in the context of protection against lipid accumulation. To further elucidate the mechanism of protection, we did a gene expression study and we found that different polyphenols inhibit steatosis by inducing the expression of genes involved in fatty acid oxidation and inhibiting those of lipogenesis. Polyphenols also modulated the expression of PPARs favoring amelioration of NAFLD. In the second manuscript (CHAPTER 4), we found that oleic acid induces oxidative stress and inflammation ("second hits" of NAFLD), while polyphenols prevent these outcomes by inducing the expression of the antioxidant enzyme Mn-SOD, inducing uncoupling protein 2, and suppressing TNFα mRNA. Again, different classes of polyphenols similarly protected against ROS production while they differed in inhibiting TNFα expression in which anthocyanins showed no protection. We further found that inducing mRNA and activity of SIRT1, and producing phosphorylation of $AMPK\alpha^{Thr172}$ and deacetylation of PGC1α are some of the mechanisms of protection by dietary polyphenols against steatosis, oxidative stress, and inflammation. Interestingly, all these factors (SIRT1, p-$AMPK\alpha^{Thr172}$, PGC1α) have key roles in induction of mitochondrial biogenesis. As discussed earlier, decreased mitochondrial biogenesis and fewer healthy and functional mitochondria have been reported in different models of NAFLD/NASH [20, 143, 354] as well as in our study. In line with our previous results, we found

different polyphenols concomitantly induce the expression of other genes involved in mitochondrial biogenesis such as PGC1α, NRF1, and TFAM. Increased mitochondrial biogenesis correlates with reduced ROS generation by inducing the expression of mitochondrial antioxidant enzymes via PGC1α [145, 301]. Mitochondrial biogenesis can also lead to increased fatty acid β-oxidation [267] and decreased inflammation [307] which contribute to the alleviation of NAFLD/NASH. Although most polyphenols in this study similarly induced mitochondrial biogenesis, anthocyanins were unique in that they ameliorated steatosis and oxidative stress without inducing mitochondrial biogenesis. This may be partly explained by the fact that anthocyanins have no effect on SIRT1 activity *in vitro* [279]. Anthocyanins however, strongly protected against the oleic acid-induced decrease of UCP2 mRNA which may partly explain their protective effects against steatosis and ROS generation. Higher mitochondrial content (volume) was further observed by polyphenols when we stained mitochondria with the mitochondria-specific dye MitoTracker Green. Due to the pivotal roles of mitochondria in lipid metabolism, ROS generation, lipid peroxidation, cytokine production and apoptosis [13], induction of mitochondrial content may have a critical role in prevention of progression of fatty liver to NASH.

In addition to mitochondrial content, mitochondrial function is also affected in NAFLD. An accumulating body of evidence suggests NAFLD as a mitochondrial disease due to the abnormality in function and morphology of hepatic mitochondria [4, 10, 17]. Therefore, it is not surprising that progression of the disease is correlated with defective mitochondrial function. Treatment of HepG2 hepatocytes with oleic acid also induced mitochondrial dysfunction evidenced by the lower expression of mitochondrial respiratory complex I and V subunits, depleted intracellular ATP stores (when bypassing glycolysis using galactose medium), and decreased mitochondrial membrane potential. However, different polyphenols protected against these effects and induced the expression of mitochondrial respiratory complex I-IV subunits, replenished intracellular ATP stores, and prevented depolarization of the mitochondrial membrane potential. We also found inverse correlations between mitochondrial content and function with ROS production and TNFα expression.

Although oleic acid induced steatosis, increased ROS generation, decreased mitochondrial content, and induced mitochondrial dysfunction, it was unable to induced ER stress. In the third manuscript (CHAPTER 5), palmitate was shown to induce endoplasmic reticulum stress

concomitant with the induction of mitochondrial dysfunction (evidenced by lower mitochondrial content, and decreased mitochondrial membrane potential). As discussed earlier, endoplasmic reticulum stress has been suggested to be one of the "multiple hits" involved in the progression of NAFLD to NASH [22]. Several studies have reported increased ER stress in hepatocytes treated with palmitic acid [26], or in animals [27] and humans [29] with hepatic steatosis. We found that palmitic acid increases the expression of several endoplasmic reticulum chaperones and apoptotic CHOP. Polyphenols differed in their effect on alleviating ER stress and only quercetin, catechin, and cyanidin were able to inhibit the expression of several chaperones concomitant with decreasing pro-apoptotic CHOP. We hypothesize that dietary polyphenols mimic beneficial effects of the chemical chaperones which by facilitating proper folding of unfolded proteins protect against ER stress in the liver, and therefore inhibit endogenous expression of chaperones that results from ER stress. A potential chemical chaperone activity of a polyphenol has been shown previously by Zhang et al. [258], who showed a potential chaperone activity of berberine which consequently prevented accumulation of misfolded or unfolded proteins in a hepatocyte cell line and primary hepatocytes. It appears that polyphenols such as berberine possess direct chaperone activity which by its protein-stabilizing function ameliorates ER stress and therefore inhibits aggregation of unfolded/misfolded proteins responsible for initiation and progression of ER stress [258]. Since some polyphenols such as catechin, quercetin, and cyanidin in this study decreased the expression of several endoplasmic reticulum chaperones simultaneously, it is most likely that these polyphenols possess a chaperone activity similar to what was shown by Zhang et al. and consequently decrease expression of most ER chaperones by negative feedback.

In addition, we found that besides inducing the expression of nuclear DNA-encoded respiratory complex subunits, polyphenols can induce the expression of mitochondrial DNA-encoded subunits (CHAPTER 5). This shows that by improving the assembly of mitochondrial complexes and therefore improving mitochondrial function, polyphenols may prevent progression of simple fatty liver to advanced stages like NASH.

We also found a correlation between ER stress and mitochondrial dysfunction. Our findings show inverse correlations between the expression of ER stress markers such as GRP78 and CHOP and mitochondrial content and the mitochondrial membrane potential. These correlations are consistent with findings that a synergistic steatohepatitis induced by moderate obesity and alcohol

results in increased level of GRP78 and CHOP while inhibiting genes involved in mitochondrial biogenesis such as PGC1α, NRF1, and TFAM in the liver of mice [326]. Therefore, it is likely that ER stress and mitochondrial dysfunction work synergistically to facilitate progression of NAFLD to NASH and fibrosis.

In this study, both oleic and palmitic acids have been used as the stressors. The effects of oleic and palmitic acid were different in some aspects. Although oleic and palmitic acid both increased ROS generation, and decreased mitochondrial content, oleic acid was stronger in inducing steatosis and mitochondrial dysfunction (evidenced by lower mitochondrial membrane potential and mRNA expression of mitochondrial complex subunits). Palmitic acid was however stronger in inducing ER stress and less potent in inducing mitochondrial dysfunction. The difference in fatty acid saturation and even doses (1.5 mM *vs.* 500 μM) may partly explain the difference in the observed effects. It has been shown by many studies that treatment of the cells or animals with palmitic acid activates ER stress and induces lipotoxicity [355-357]. Along with tunicamycin, palmitic acid has been used as a common model for inducing ER stress in cells. This is likely because palmitic acid does not effectively induce triglyceride synthesis [358] and may not effectively be incorporated into neutral triglycerides [359]. Another reason is that palmitic acid may induce ER stress by modification of calcium concentration in the ER lumen [360] likely due to increasing the stiffness of the ER membrane. Palmitic acid is the most prevalent saturated fatty acid found in triglycerides of meats and dairy products, as well as in palm oils that are widely used by the food industry. Upon consumption of saturated fats, the liver is exposed to high levels of palmitic acid that can contribute to NAFLD. Oleic and palmitic acid are the most abundant fatty acids in hepatic triglycerides of healthy subjects and patients with fatty liver disease [201]. Since the results show that polyphenols can prevent ER stress while inducing mitochondrial biogenesis and function, and protect against the deleterious effects induced by oleic or palmitic acid, they may be effective in protection against NAFLD. Well-designed randomized clinical trials however, should be conducted to prove the efficacy of polyphenols in protection against NAFLD/NASH in humans.

Since using either oleic or palmitic acid in the cell models of steatosis has its own advantages and limitations, some studies suggest using the mixture of these fatty acids (oleate/palmitate 2:1 ratio). This has been suggested by some studies as an effective *in vitro* model of steatosis due to the higher steatotic effect of oleic acid and lipotoxicity of palmitic acid [361].

In the last manuscript (CHAPTER 6), we aimed to answer the question of whether the effects of polyphenols in NAFLD are due to the parent polyphenols or due to their breakdown/digestion products. For example, *in vitro* studies (including our results in CHAPTER 4) show that anthocyanins such as cyanidin and cyanidin-3-glucoside do not increase SIRT1 activity [279] in the cells while studies on rodents show the opposite with the capability of inducing mitochondrial biogenesis by purified anthocyanins from black currant and bilberry [254]. It is still largely unknown whether the effects observed *in vivo* by anthocyanins are due to their breakdown/digestion products after ring fission of the parent flavonoids by colonic microbiota. Interestingly, we observed that 24 h treatment of HepG2 cells with polyphenols such as quercetin and cyanidin and their breakdown/digestion products (2,4,6-trihydroxybenzaldehyde, protocatechuic acid, and caffeic acid) similarly decreased oleic acid-induced steatosis, ROS generation, and induced mitochondrial function. Moreover, when the cells were pre-treated with these polyphenols and their breakdown products for only 1 h (to prevent spontaneous degradation of the parent flavonoids by medium), only breakdown/digestion products but not the parent flavonoids induced mitochondrial biogenesis. This further demonstrates that the breakdown/digestion products of polyphenols may contribute to the preventive effects of the parent polyphenols against NAFLD.

7.2. Concluding remarks

In conclusion, it seems that mitochondrial biogenesis and dysfunction and endoplasmic reticulum stress contribute to the progression of NAFLD while different polyphenols, with similar effectiveness but somewhat different mechanisms, can protect against these changes. Different polyphenols similarly protected against the increase in intracellular lipid accumulation and ROS generation but differed in their effects on mitochondrial content, mitochondrial dysfunction, and ER stress. There was no clear trend to the effects of different polyphenols, but they may use different mechanisms to protect against NAFLD. Due to the differences in structure and properties of different classes of polyphenols, such differences may be expected. More studies on animals and humans are needed to elucidate the contributing mechanisms.

7.3. Limitations of the study

There are some limitations for this study:

1. **The model that we used here was an *in vitro* model of steatosis:** Although *in vitro* models are good models for identifying and testing the molecular mechanisms, they do not show all the features of NAFLD and NASH in humans. NAFLD and NASH are complex diseases in which many factors and even other tissues can affect the disease initiation and progression. For example, inflammatory cytokines and adipokines (such as adiponectin, resistin, and leptin) produced by adipose tissue can have negative effects on the steatotic liver. Infiltrating macrophages in the liver (Kupffer cells) which are a critical source of ROS and inflammatory cytokines are also missing in our model. In the liver, hepatic stellate cells are also available which produce collagen and result in fibrosis which is also missing in an *in vitro* model of steatosis. Investigating all of these contributors and their intertwined mechanisms is not possible in an *in vitro* model of steatosis.
2. **The activity of the mitochondrial respiratory complexes has not been measured:** In order to investigate the effects of free fatty acids and polyphenols on mitochondrial dysfunction, we measured the mitochondrial respiratory complex subunits mRNA expression, the mitochondrial membrane potential, and intracellular ATP stores. Another limitation was that for exploring the effects on mitochondrial function however, the activity of mitochondrial respiratory complexes was not measured that could give us a better idea about the effectiveness of polyphenols on mitochondrial function.
3. **Lack of direct measurement of mitochondrial function:** due to the lack of a Seahorse XF analyzer, we could not measure the real-time oxygen consumption and activity of mitochondria in the cells. The Seahorse XF analyzer is a very useful instrument for studying mitochondria that would give real-time data about mitochondrial function, ATP stores, and a shift of metabolism from glycolysis to oxidative phosphorylation.

7.4. Future directions

The effects of different classes of polyphenols on mitochondrial dysfunction and endoplasmic reticulum stress should be measured in *in vivo* experimental models. Investigating protective effects of polyphenols and their breakdown/digestion products of polyphenols on genetically obese

mice on a methionine- and choline-deficient diet will also be interesting as this model will develop the main features of human NASH and fibrosis. A Seahorse XF analyzer can also be used to evaluate the mitochondrial activity of isolated hepatic, adipose tissue and muscle cells of rodents fed polyphenols and a high-fat high-sucrose diet. Doing microarrays or investigating genomics and proteomics of the liver, adipose or muscle tissue of rodents treated with different classes of polyphenols and an atherogenic diet can be excellent future goals to elucidate the contributing genes or proteins affected in NAFLD/NASH. Animal models overexpressing or null for SIRT1 ($^{-/-}$) can also be a great future direction to further elucidate the role of mitochondrial biogenesis in prevention of NAFLD.

Moreover, translating the results to humans with NAFLD/NASH is difficult due to invasiveness of a liver biopsy. In order to translate the results, some of the experiments however can be investigated on the liver, or adipose tissue biopsy of obese subjects who undergo bariatric surgery.

7.5. Appendix

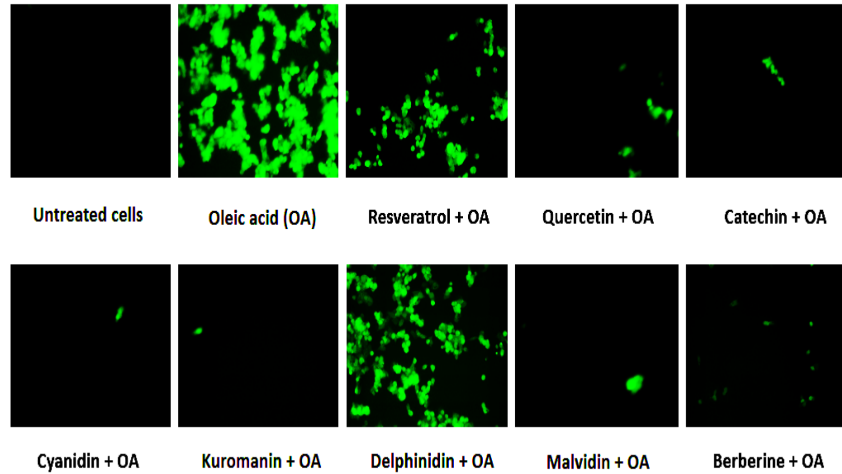

Figure 7.1. Effect of polyphenols on intracellular ROS production. Fluorescence images (at 40x magnification) captured by a fluorescence microscope showing effect of polyphenols on oleic acid-induced ROS generation after staining HepG2 cells with DCFH-DA. Higher green fluorescence represents higher intracellular ROS content

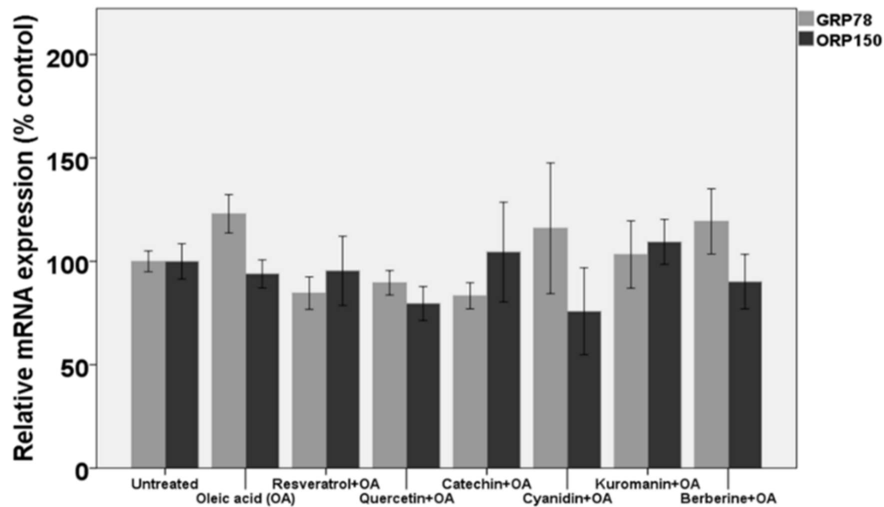

Figure 7.2. mRNA expression of some of the ER stress markers after treatment with 10 µM polyphenols for 2 h and 1.5 mM oleic acid for 24 h. *Oleic acid did not induce ER stress in HepG2 cells.*

Gene	Species	Primer sequence (5'-3')	Cycles (Ct values)
SIRT1	Human	Forward: AGT GGC AAA GGA GCA GAT TAG Reverse: CTG CCA CAA GAA CTA GAG GAT AAG	24
SIRT3	Human	Forward: GAA AGC CTA GTG GAG CTT CTG Reverse: GGG CAG CCATCA TCC TAT TT	24
PPARα	Human	Forward: TCC TCG GTG ACT TAT CCT GT Reverse: GCGTGG ACT CCGTAATGA TAG	27
PPARγ	Human	Forward: GCCTGC ATC TCC ACCTTATTA Reverse: ATC TCC ACA GAC ACG ACATTC	23
CPT1A1	Human	Forward: AGC GTT CTT CGT GAC GTT AG Reverse: CGG CCGTGT AGT AGA GAT TTG	24
FAS	Human	Forward: TGT CCT GGG AGG AGT GTA AA Reverse: CTG CTC CAC GAA CTC AAA CA	21
GAPDH	Human	Forward: ATG GGT GTG AAC CAT GAG AAG Reverse: GAGTCC TTC CAC GAT ACC AAA G	17

Table 7.1. The sequences of primers used to measure the expression of genes of interest in CHAPTER 3

Gene	Species	Primer sequence (5'-3')	Cycles (Ct values)
UCP2	Human	Forward: GAA CGG GAC ACCTTT AGA GAA G Reverse: CAG CAA CAA GAC GAG ATA GAG G	26
Mn-SOD	Human	Forward: GGA GAT GTT ACA GCC CAG ATA G Reverse: CGT TAG GGCTGA GGTTTG T	19
PGC1α	Human	Forward: TGA ACT GAG GGA CAGTGA TTT C Reverse: CCC AAG GGT AGCTCA GTT TAT C	23
NRF1	Human	Forward: GTATCT CAC CCT CCA AAC CTA AC Reverse: CCA GGATCA TGC TCT TGT ACT T	24
TFAM	Human	Forward: ATA GGC ACA GGA AAC CAGTTA G Reverse: GCA GAA GTC CAT GAG CTG AAT A	24
TNFα	Human	Forward: GGA TGG ATG GAG GTG AAA GTA G Reverse: TGA TCC TGA AGA GGA GAG AGA A	33
NDUFS8	Human	Forward: GAT CCC GAG ATG GAC ATG AAG Reverse: TTC TCG AAC GGGTAGTTG ATG	23
SDHb	Human	Forward: CAT TCT CTG TGC CTG CTG TA Reverse: GGA GTC AAT CAT CCA GCG ATA G	23
UQCRC1	Human	Forward: GAG CAC CAG CAA CTG TTA GA Reverse: TGA AGC GGC ATG GAGTAA G	21
COX6B1	Human	Forward: CCA ACC AGA ACC AGA CTA GAA A Reverse: GTA CCATTC GCA CAC AGA GAT A	21
ATP5G1	Human	Forward: TGT CTG CCT CCT TCT TGA ATA G Reverse: ACA ACA CTG GTCTGG AACTC	21
ATP5F1	Human	Forward: GTC CCT TTG TTG CAG ACT TTG Reverse: CCT GTT GTG ACT TCT CCGTAT C	21
GAPDH	Human	Forward: ATG GGT GTG AAC CAT GAG AAG Reverse: GAGTCC TTC CAC GAT ACC AAA G	17

Table 7.2. The sequences of primers used to measure the expression of genes of interest in CHAPTER 4

Gene	Species	Primer sequence (5'-3')	Cycles (Ct values)
iNOS	Human	Forward: TGTTTGAACACATCTGCAGACACG Reverse: CAAGGTCAGGTGGGATTTCGAAGA	36
NDUFS8	Human	Forward: GAT CCC GAG ATG GAC ATG AAG Reverse: TTC TCG AAC GGGTAGTTG ATG	23
UQCRC1	Human	Forward: GAG CAC CAG CAA CTG TTA GA Reverse: TGA AGC GGC ATG GAGTAA G	20
COX6B1	Human	Forward: CCAACCAGAACCAGACTAGAAA Reverse: GTACCATTCGCACACAGAGATA	21
ATP5G1	Human	Forward: TGTCTGCCTCCTTCTTGAATAG Reverse: ACAACACTGGTCTGGAACTC	21
MTND1	Human	Forward: CTT AGCTCT CAC CAT CGCTCT T Reverse: AGATTG TTT GGG CTA CTG CTC G	19
MTCYB	Human	Forward: GAT CCT CCA AAT CAC CAC AGG AC Reverse: GGA GGATAATGC CGATGT TTC AG	17
MTCO1	Human	Forward: GAG CTG GGC CAG CCA GGC AA Reverse: GGA AAC GCC ATATCG GGG GCA	15
MTATP6	Human	Forward: GCC GCA GTA CTG ATC ATT CTA TTT C Reverse: TCG GTT GTT GAT GAG ATA TTT GGA	14
GRP58	Human	Forward: CCT ATC CCA GAG AGC AAT GAT G Reverse: TAC AAT GAC CAC ACC AAG GG	19
GRP78	Human	Forward: AACCATCCCGTGGCATAAA Reverse: GGACATACATCAAGCAGTACCA	17
GRP94	Human	Forward: CGC CTT CCT TGT AGC AGATAA Reverse: CGT CCT AGA GTGTTT CCT CTT G	18
ORP150	Human	Forward: TAA CAC CACTGC CCA GAATAT C Reverse: CAT CCC AGCTTC CTT AGT CTT C	21
ERdj4	Human	Forward: ATGTGTTTGGTTCCAGCCTA Reverse: GACCTAAGAGTGCCAACAACTA	24
ATF4	Human	Forward: GGA GAT AGG AAG CCA GAC TAC A Reverse: GGC TCA TAC AGA TGC CAC TAT C	19
EDEM1	Human	Forward: GTGCCCTCCCTGAGAGATATAA Reverse: GATTCTTGGTTGCCTGGTAGAG	23
GADD34	Human	Forward: GAA CCT CTA CTT CTG CCT TGT C Reverse: GTC TTC CTG GCT CCT TTA CTT C	23
CHOP	Human	Forward: CTCACTCTCCAGATTCCAGTCA Reverse: GACCACTCTGTTTCCGTTTCC	21
GAPDH	Human	Forward: ATG GGT GTG AAC CAT GAG AAG Reverse: GAGTCC TTC CAC GAT ACC AAA G	16

Table 7.3. The sequences of primers used to measure the expression of genes of interest in CHAPTER 5

References

[1] Bellentani, S., Marino, M., Epidemiology and natural history of non-alcoholic fatty liver disease (NAFLD). *Annals of Hepatology* 2009, *8*, S4-S8.

[2] Day, C. P., James, O. F., Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998, *114*, 842-845.

[3] Herruzo, J. S., Ruiz, I. G., Carreras, M. P., Yague, M. M., Non-alcoholic fatty liver disease. From insulin resistance to mitochondrial dysfunction. *Revista Española de Enfermedades Digestivas* 2006, *98*, 844.

[4] Begriche, K., Massart, J., Robin, M. A., Bonnet, F., Fromenty, B., Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. *Hepatology* 2013, *58*, 1497-1507.

[5] Nassir, F., Ibdah, J. A., Role of mitochondria in nonalcoholic fatty liver disease. *International Journal of Molecular Sciences* 2014, *15*, 8713-8742.

[6] Mantena, S. K., King, A. L., Andringa, K. K., Eccleston, H. B., Bailey, S. M., Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol-and obesity-induced fatty liver diseases. *Free Radical Biology and Medicine* 2008, *44*, 1259-1272.

[7] Pagliassotti, M. J., Endoplasmic reticulum stress in nonalcoholic fatty liver disease. *Annual Review of Nutrition* 2012, *32*, 17-33.

[8] Tilg, H., Moschen, A. R., Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010, *52*, 1836-1846.

[9] Wei, Y., Rector, R. S., Thyfault, J. P., Ibdah, J. A., Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World Journal of Gastroenterology* 2008, *14*, 193.

[10] Pessayre, D., Fromenty, B., NASH: a mitochondrial disease. *Journal of Hepatology* 2005, *42*, 928-940.

[11] Ibdah, J. A., Perlegas, P., Zhao, Y., Angdisen, J., Borgerink, H., Shadoan, M. K., Wagner, J. D., Matern, D., Rinaldo, P., Cline, J. M., Mice heterozygous for a defect in mitochondrial trifunctional protein develop hepatic steatosis and insulin resistance. *Gastroenterology* 2005, *128*, 1381-1390.

[12] Petrosillo, G., Portincasa, P., Grattagliano, I., Casanova, G., Matera, M., Ruggiero, F. M., Ferri, D., Paradies, G., Mitochondrial dysfunction in rat with nonalcoholic fatty liver: involvement of complex I, reactive oxygen species and cardiolipin. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 2007, *1767*, 1260-1267.

[13] Begriche, K., Igoudjil, A., Pessayre, D., Fromenty, B., Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 2006, *6*, 1-28.

[14] Pérez-Carreras, M., Del Hoyo, P., Martín, M. A., Rubio, J. C., Martín, A., Castellano, G., Colina, F., Arenas, J., Solis-Herruzo, J. A., Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 2003, *38*, 999-1007.

[15] García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Muñoz-Yagüe, T., Solís-Herruzo, J. A., In vitro treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis. *Disease Models and Mechanisms* 2015, *8*, 183-191.

[16] García-Ruiz, I., Rodríguez-Juan, C., Díaz-Sanjuan, T., del Hoyo, P., Colina, F., Muñoz-Yagüe, T., Solís-Herruzo, J. A., Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* 2006, *44*, 581-591.

[17] García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Grau, M., Colina, F., Muñoz-Yagüe, T., Solís-Herruzo, J. A., High-fat diet decreases activity of the oxidative phosphorylation

complexes and causes nonalcoholic steatohepatitis in mice. *Disease Models and Mechanisms* 2014, *7*, 1287-1296.
[18] Kuo, J.-J., Chang, H.-H., Tsai, T.-H., Lee, T.-Y., Positive effect of curcumin on inflammation and mitochondrial dysfunction in obese mice with liver steatosis. *International Journal of Molecular Medicine* 2012, *30*, 673-679.
[19] Liu, J., Shen, W., Zhao, B., Wang, Y., Wertz, K., Weber, P., Zhang, P., Targeting mitochondrial biogenesis for preventing and treating insulin resistance in diabetes and obesity: Hope from natural mitochondrial nutrients. *Advanced drug delivery reviews* 2009, *61*, 1343-1352.
[20] Kuo, J.-J., Chang, H.-H., Tsai, T.-H., Lee, T.-Y., Curcumin ameliorates mitochondrial dysfunction associated with inhibition of gluconeogenesis in free fatty acid-mediated hepatic lipoapoptosis. *International Journal of Molecular Medicine* 2012, *30*, 643-649.
[21] Marra, F., Gastaldelli, A., Baroni, G. S., Tell, G., Tiribelli, C., Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis. *Trends in Molecular Medicine* 2008, *14*, 72-81.
[22] Takaki, A., Kawai, D., Yamamoto, K., Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). *International Journal of Molecular Sciences* 2013, *14*, 20704-20728.
[23] Malhi, H., Gores, G. J., *Seminars in Liver Disease*, © Thieme Medical Publishers 2008, pp. 360-369.
[24] Flamment, M., Kammoun, H. L., Hainault, I., Ferre, P., Foufelle, F., Endoplasmic reticulum stress: a new actor in the development of hepatic steatosis. *Current Opinion in Lipidology* 2010, *21*, 239-246.
[25] Özcan, U., Cao, Q., Yilmaz, E., Lee, A.-H., Iwakoshi, N. N., Özdelen, E., Tuncman, G., Görgün, C., Glimcher, L. H., Hotamisligil, G. S., Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004, *306*, 457-461.
[26] Cao, J., Dai, D.-L., Yao, L., Yu, H.-H., Ning, B., Zhang, Q., Chen, J., Cheng, W.-H., Shen, W., Yang, Z.-X., Saturated fatty acid induction of endoplasmic reticulum stress and apoptosis in human liver cells via the PERK/ATF4/CHOP signaling pathway. *Molecular and Cellular Biochemistry* 2012, *364*, 115-129.
[27] Kammoun, H. L., Chabanon, H., Hainault, I., Luquet, S., Magnan, C., Koike, T., Ferré, P., Foufelle, F., GRP78 expression inhibits insulin and ER stress–induced SREBP-1c activation and reduces hepatic steatosis in mice. *The Journal of Clinical Investigation* 2009, *119*, 1201-1215.
[28] Mu, Y.-p., Ogawa, T., Kawada, N., Reversibility of fibrosis, inflammation, and endoplasmic reticulum stress in the liver of rats fed a methionine–choline-deficient diet. *Laboratory Investigation* 2010, *90*, 245-256.
[29] Gregor, M. F., Yang, L., Fabbrini, E., Mohammed, B. S., Eagon, J. C., Hotamisligil, G. S., Klein, S., Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* 2009, *58*, 693-700.
[30] Puri, P., Mirshahi, F., Cheung, O., Natarajan, R., Maher, J. W., Kellum, J. M., Sanyal, A. J., Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology* 2008, *134*, 568-576.
[31] Koteish, A., Diehl, A. M., *Seminars in Liver Disease*, Copyright© 2001 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel.:+ 1 (212) 584-4662 2001, pp. 089-104.

[32] Lieber, C. S., Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol* 2004, *34*, 9-19.
[33] Feldstein, A. E., Canbay, A., Guicciardi, M. E., Higuchi, H., Bronk, S. F., Gores, G. J., Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. *Journal of Hepatology* 2003, *39*, 978-983.
[34] Donato, M. T., Lahoz, A., Jimenez, N., Perez, G., Serralta, A., Mir, J., Castell, J. V., Gómez-Lechón, M. J., Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metabolism and Disposition* 2006, *34*, 1556-1562.
[35] Vidyashankar, S., Varma, R. S., Patki, P. S., Quercetin ameliorate insulin resistance and up-regulates cellular antioxidants during oleic acid induced hepatic steatosis in HepG2 cells. *Toxicology in Vitro* 2013, *27*, 945-953.
[36] Kang, O., Kim, S., Seo, Y., Joung, D., Mun, S., Choi, J., Lee, Y., Kang, D., Lee, H., Kwon, D., Curcumin decreases oleic acid-induced lipid accumulation via AMPK phosphorylation in hepatocarcinoma cells. *European Review for Medical and Pharmacological Sciences* 2013, *17*, 2578-2586.
[37] Ou, T. T., Hsu, M. J., Chan, K. C., Huang, C. N., Ho, H. H., Wang, C. J., Mulberry extract inhibits oleic acid-induced lipid accumulation via reduction of lipogenesis and promotion of hepatic lipid clearance. *Journal of the Science of Food and Agriculture* 2011, *91*, 2740-2748.
[38] Malhi, H., Bronk, S. F., Werneburg, N. W., Gores, G. J., Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *Journal of Biological Chemistry* 2006, *281*, 12093-12101.
[39] Gomez-Lechon, M., Donato, M., Castell, J., Jover, R., Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Current drug metabolism* 2004, *5*, 443-462.
[40] Gomez-Lechon, M. J., Donato, M. T., Martínez-Romero, A., Jiménez, N., Castell, J. V., O'Connor, J.-E., A human hepatocellular in vitro model to investigate steatosis. *Chemico-Biological Interactions* 2007, *165*, 106-116.
[41] Yin, J., Luo, Y., Deng, H., Qin, S., Tang, W., Zeng, L., Zhou, B., Hugan Qingzhi medication ameliorates hepatic steatosis by activating AMPK and PPARα pathways in L02 cells and HepG2 cells. *Journal of Ethnopharmacology* 2014, *154*, 229-239.
[42] Park, J.-Y., Kim, Y., Im, J. A., Lee, H., Oligonol suppresses lipid accumulation and improves insulin resistance in a palmitate-induced in HepG2 hepatocytes as a cellular steatosis model. *BMC Complementary and Alternative Medicine* 2015, *15*, 1.
[43] Visioli, F., Lastra, C. A. D. L., Andres-Lacueva, C., Aviram, M., Calhau, C., Cassano, A., D'Archivio, M., Faria, A., Favé, G., Fogliano, V., Polyphenols and human health: a prospectus. *Critical Reviews in Food Science and Nutrition* 2011, *51*, 524-546.
[44] Jung, C. H., Cho, I., Ahn, J., Jeon, T. I., Ha, T. Y., Quercetin reduces high-fat diet-induced fat accumulation in the liver by regulating lipid metabolism genes. *Phytotherapy Research* 2013, *27*, 139-143.
[45] Alberdi, G., Rodríguez, V. M., Macarulla, M. T., Miranda, J., Churruca, I., Portillo, M. P., Hepatic lipid metabolic pathways modified by resveratrol in rats fed an obesogenic diet. *Nutrition* 2013, *29*, 562-567.
[46] Hwang, Y. P., Choi, J. H., Han, E. H., Kim, H. G., Wee, J.-H., Jung, K. O., Jung, K. H., Kwon, K.-i., Jeong, T. C., Chung, Y. C., Purple sweet potato anthocyanins attenuate hepatic lipid accumulation through activating adenosine monophosphate–activated protein kinase in human HepG2 cells and obese mice. *Nutrition Research* 2011, *31*, 896-906.
[47] Bagul, P. K., Middela, H., Matapally, S., Padiya, R., Bastia, T., Madhusudana, K., Reddy, B. R., Chakravarty, S., Banerjee, S. K., Attenuation of insulin resistance, metabolic syndrome and

hepatic oxidative stress by resveratrol in fructose-fed rats. *Pharmacological Research* 2012, *66*, 260-268.
[48] Kobori, M., Masumoto, S., Akimoto, Y., Oike, H., Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. *Molecular Nutrition & Food Research* 2011, *55*, 530-540.
[49] Jhun, J. Y., Moon, S.-J., Yoon, B. Y., Byun, J. K., Kim, E. K., Yang, E. J., Ju, J. H., Hong, Y. S., Min, J. K., Park, S. H., Grape seed proanthocyanidin extract–mediated regulation of STAT3 proteins contributes to treg differentiation and attenuates inflammation in a murine model of obesity-associated arthritis. *PloS One* 2013, *8*, e78843.
[50] Xiao, J., Ho, C. T., Liong, E. C., Nanji, A. A., Leung, T. M., Lau, T. Y. H., Fung, M. L., Tipoe, G. L., Epigallocatechin gallate attenuates fibrosis, oxidative stress, and inflammation in non-alcoholic fatty liver disease rat model through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-kappa B pathways. *European Journal of Nutrition* 2014, *53*, 187-199.
[51] Aoun, M., Michel, F., Fouret, G., Casas, F., Jullien, M., Wrutniak-Cabello, C., Ramos, J., Cristol, J.-P., Coudray, C., Carbonneau, M.-A., A polyphenol extract modifies quantity but not quality of liver fatty acid content in high-fat–high-sucrose diet-fed rats: possible implication of the sirtuin pathway. *British Journal of Nutrition* 2010, *104*, 1760-1770.
[52] Masterjohn, C., Bruno, R. S., Therapeutic potential of green tea in nonalcoholic fatty liver disease. *Nutrition Reviews* 2012, *70*, 41-56.
[53] Onyekwere, C., Ogbera, A., Samaila, A., Balogun, B., Abdulkareem, F., Nonalcoholic fatty liver disease: Synopsis of current developments. *Nigerian Journal of Clinical Practice* 2015, *18*, 703-712.
[54] Mcavoy, N. C., Ferguson, J. W., Campbell, I. W., Hayes, P. C., Non-alcoholic fatty liver disease: natural history, pathogenesis and treatment: review. *South African Journal of Diabetes and Vascular Disease* 2007, *4*, 9-16.
[55] Ludwig, J., Viggiano, T. R., Mcgill, D. B., Oh, B., *Mayo Clinic Proceedings* 1980, pp. 434-438.
[56] Sheth, S. G., Gordon, F. D., Chopra, S., Nonalcoholic steatohepatitis. *Annals of Internal Medicine* 1997, *126*, 137-145.
[57] Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Liu, Y. C., McCullough, A. J., Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999, *116*, 1413-1419.
[58] Bellentani, S., Scaglioni, F., Marino, M., Bedogni, G., Epidemiology of non-alcoholic fatty liver disease. *Digestive Diseases* 2010, *28*, 155-161.
[59] Leite, N. C., Villela-Nogueira, C. A., Pannain, V. L., Bottino, A. C., Rezende, G. F., Cardoso, C. R., Salles, G. F., Histopathological stages of nonalcoholic fatty liver disease in type 2 diabetes: prevalences and correlated factors. *Liver International* 2011, *31*, 700-706.
[60] Hannah Jr, W. N., Harrison, S. A., Lifestyle and dietary interventions in the management of nonalcoholic fatty liver disease. *Digestive Diseases and Sciences* 2016, *61*, 1365-1374.
[61] Saviano, M. C., Brunetti, F., Rubino, A., Franzese, A., Vajro, P., Argenziano, A., Puzziello, A., Iannucci, M. P., Liver involvement in obese children (ultrasonography and liver enzyme levels at diagnosis and during follow-up in an Italian population). *Digestive Diseases and Sciences* 1997, *42*, 1428-1432.
[62] Tominaga, K., Kurata, P. J. H., Chen, Y. K., Fujimoto, E., Miyagawa, S., Abe, I., Kusano, Y., Prevalence of fatty liver in Japanese children and relationship to obesity. *Digestive Diseases and Sciences* 1995, *40*, 2002-2009.

[63] Marchesini, G., Natale, S., Manini, R., Agostini, F., Review article: the treatment of fatty liver disease associated with the metabolic syndrome. *Alimentary Pharmacology and Therapeutics* 2005, *22*, 37-39.

[64] Marchesini, G., Petta, S., Dalle Grave, R., Diet, weight loss, and liver health in nonalcoholic fatty liver disease: pathophysiology, evidence, and practice. *Hepatology* 2016.

[65] Misra, U. K., Bradford, B. U., Handier, J. A., Thurman, R. G., Chronic ethanol treatment induces H2O2 production selectively in pericentral regions of the liver lobule. *Alcoholism: Clinical and Experimental Research* 1992, *16*, 839-842.

[66] Basaranoglu, M., Basaranoglu, G., Sentürk, H., From fatty liver to fibrosis: a tale of “second hit”. *World Journal of Gastroenterology* 2013, *19*, 1158-1165.

[67] Yilmaz, Y., Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Alimentary Pharmacology and Therapeutics* 2012, *36*, 815-823.

[68] Brumbaugh, D. E., Friedman, J. E., Developmental origins of nonalcoholic fatty liver disease. *Pediatric Research* 2013, *75*, 140-147.

[69] Mouralidarane, A., Soeda, J., Visconti-Pugmire, C., Samuelsson, A. M., Pombo, J., Maragkoudaki, X., Butt, A., Saraswati, R., Novelli, M., Fusai, G., Maternal obesity programs offspring nonalcoholic fatty liver disease by innate immune dysfunction in mice. *Hepatology* 2013, *58*, 128-138.

[70] Musso, G., Gambino, R., Cassader, M., Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Progress in Lipid Research* 2009, *48*, 1-26.

[71] Bechmann, L. P., Hannivoort, R. A., Gerken, G., Hotamisligil, G. S., Trauner, M., Canbay, A., The interaction of hepatic lipid and glucose metabolism in liver diseases. *Journal of Hepatology* 2012, *56*, 952-964.

[72] Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., Parks, E. J., Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *The Journal of Clinical Investigation* 2005, *115*, 1343-1351.

[73] Ahn, J., Cho, I., Kim, S., Kwon, D., Ha, T., Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet. *Journal of Hepatology* 2008, *49*, 1019-1028.

[74] Choi, S. S., Diehl, A. M., Hepatic triglyceride synthesis and nonalcoholic fatty liver disease. *Current Opinion in Lipidology* 2008, *19*, 295-300.

[75] Hui, J. M., Hodge, A., Farrell, G. C., Kench, J. G., Kriketos, A., George, J., Beyond insulin resistance in NASH: TNF-α or adiponectin? *Hepatology* 2004, *40*, 46-54.

[76] Arkan, M. C., Hevener, A. L., Greten, F. R., Maeda, S., Li, Z.-W., Long, J. M., Wynshaw-Boris, A., Poli, G., Olefsky, J., Karin, M., IKK-β links inflammation to obesity-induced insulin resistance. *Nature Medicine* 2005, *11*, 191-198.

[77] Ahmed, M. H., Byrne, C. D., Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD). *Drug Discovery Today* 2007, *12*, 740-747.

[78] Byrne, C. D., Olufadi, R., Bruce, K. D., Cagampang, F. R., Ahmed, M. H., Metabolic disturbances in non-alcoholic fatty liver disease. *Clinical Science* 2009, *116*, 539-564.

[79] Tessari, P., Coracina, A., Cosma, A., Tiengo, A., Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutrition, Metabolism and Cardiovascular Diseases* 2009, *19*, 291-302.

[80] Gutiérrez-Juárez, R., Pocai, A., Mulas, C., Ono, H., Bhanot, S., Monia, B. P., Rossetti, L., Critical role of stearoyl-CoA desaturase–1 (SCD1) in the onset of diet-induced hepatic insulin resistance. *The Journal of Clinical Investigation* 2006, *116*, 1686-1695.

[81] Kotronen, A., Seppänen-Laakso, T., Westerbacka, J., Kiviluoto, T., Arola, J., Ruskeepää, A.-L., Orešič, M., Yki-Järvinen, H., Hepatic stearoyl-CoA desaturase (SCD)-1 activity and diacylglycerol but not ceramide concentrations are increased in the nonalcoholic human fatty liver. *Diabetes* 2009, *58*, 203-208.
[82] Cohen, P., Miyazaki, M., Socci, N. D., Hagge-Greenberg, A., Liedtke, W., Soukas, A. A., Sharma, R., Hudgins, L. C., Ntambi, J. M., Friedman, J. M., Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. *Science* 2002, *297*, 240-243.
[83] Miyazaki, M., Dobrzyn, A., Sampath, H., Lee, S.-H., Man, W. C., Chu, K., Peters, J. M., Gonzalez, F. J., Ntambi, J. M., Reduced adiposity and liver steatosis by stearoyl-CoA desaturase deficiency are independent of peroxisome proliferator-activated receptor-α. *Journal of Biological Chemistry* 2004, *279*, 35017-35024.
[84] Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendziorski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M., Attie, A. D., Loss of stearoyl–CoA desaturase-1 function protects mice against adiposity. *Proceedings of the National Academy of Sciences* 2002, *99*, 11482-11486.
[85] Klaus, S., Pültz, S., Thöne-Reineke, C., Wolfram, S., Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *International Journal of Obesity* 2005, *29*, 615-623.
[86] Yamaguchi, K., Yang, L., McCall, S., Huang, J., Yu, X. X., Pandey, S. K., Bhanot, S., Monia, B. P., Li, Y. X., Diehl, A. M., Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 2007, *45*, 1366-1374.
[87] Fabbrini, E., Mohammed, B. S., Magkos, F., Korenblat, K. M., Patterson, B. W., Klein, S., Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology* 2008, *134*, 424-431.
[88] Liu, Y., Wang, D., Zhang, D., Lv, Y., Wei, Y., Wu, W., Zhou, F., Tang, M., Mao, T., Li, M., Inhibitory effect of blueberry polyphenolic compounds on oleic acid-induced hepatic steatosis in vitro. *Journal of Agricultural and Food Chemistry* 2011, *59*, 12254-12263.
[89] Frühbeck, G., Méndez-Giménez, L., Fernández-Formoso, J.-A., Fernández, S., Rodríguez, A., Regulation of adipocyte lipolysis. *Nutrition Research Reviews* 2014, *27*, 63-93.
[90] Koek, G., Liedorp, P., Bast, A., The role of oxidative stress in non-alcoholic steatohepatitis. *Clinica Chimica Acta* 2011, *412*, 1297-1305.
[91] Commerford, S. R., Peng, L., Dubé, J. J., O'Doherty, R. M., In vivo regulation of SREBP-1c in skeletal muscle: effects of nutritional status, glucose, insulin, and leptin. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 2004, *287*, R218-R227.
[92] Hardie, D. G., AMP-activated protein kinase—an energy sensor that regulates all aspects of cell function. *Genes and Development* 2011, *25*, 1895-1908.
[93] Tomita, K., Tamiya, G., Ando, S., Kitamura, N., Koizumi, H., Kato, S., Horie, Y., Kaneko, T., Azuma, T., Nagata, H., AICAR, an AMPK activator, has protective effects on alcohol-induced fatty liver in rats. *Alcoholism: Clinical and Experimental Research* 2005, *29*, 240S-245S.
[94] Perfield, J. W., Ortinau, L. C., Pickering, R. T., Ruebel, M. L., Meers, G. M., Rector, R. S., Altered hepatic lipid metabolism contributes to nonalcoholic fatty liver disease in leptin-deficient Ob/Ob mice. *Journal of Obesity* 2013, *2013*.

[95] Pu, P., Gao, D.-M., Mohamed, S., Chen, J., Zhang, J., Zhou, X.-Y., Zhou, N.-J., Xie, J., Jiang, H., Naringin ameliorates metabolic syndrome by activating AMP-activated protein kinase in mice fed a high-fat diet. *Archives of Biochemistry and Biophysics* 2012, *518*, 61-70.
[96] Li, X., Zhang, S., Blander, G., Jeanette, G. T., Krieger, M., Guarente, L., SIRT1 deacetylates and positively regulates the nuclear receptor LXR. *Molecular Cell* 2007, *28*, 91-106.
[97] Feige, J. N., Auwerx, J., DisSIRTing on LXR and cholesterol metabolism. *Cell Metabolism* 2007, *6*, 343-345.
[98] Liang, G., Yang, J., Horton, J. D., Hammer, R. E., Goldstein, J. L., Brown, M. S., Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *Journal of Biological Chemistry* 2002, *277*, 9520-9528.
[99] Baraille, F., Planchais, J., Dentin, R., Guilmeau, S., Postic, C., Integration of ChREBP-mediated glucose sensing into whole body metabolism. *Physiology* 2015, *30*, 428-437.
[100] Iizuka, K., Bruick, R. K., Liang, G., Horton, J. D., Uyeda, K., Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proceedings of the National Academy of Sciences of the United States of America* 2004, *101*, 7281-7286.
[101] Dentin, R., Girard, J., Postic, C., Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* 2005, *87*, 81-86.
[102] Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical Investigation* 2001, *108*, 1167-1174.
[103] Sanyal, A. J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W. B., Contos, M. J., Sterling, R. K., Luketic, V. A., Shiffman, M. L., Clore, J. N., Angulo, P., Nonalcoholic steatohepatitis: Association of insulin resistance and mitochondrial abnormalities. Editorial. *Gastroenterology* 2001, *120*, 1281-1285.
[104] Miele, L., Grieco, A., Armuzzi, A., Candelli, M., Forgione, A., Gasbarrini, A., Gasbarrini, G., Hepatic mitochondrial beta-oxidation in patients with nonalcoholic steatohepatitis assessed by 13C-octanoate breath test. *The American Journal of Gastroenterology* 2003, *98*, 2335.
[105] Rolo, A. P., Teodoro, J. S., Palmeira, C. M., Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radical Biology and Medicine* 2012, *52*, 59-69.
[106] Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., Van Breusegem, F., ROS signaling: the new wave? *Trends in Plant Science* 2011, *16*, 300-309.
[107] D'Autréaux, B., Toledano, M. B., ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature Reviews Molecular Cell Biology* 2007, *8*, 813-824.
[108] Orr, A. L., Quinlan, C. L., Perevoshchikova, I. V., Brand, M. D., A refined analysis of superoxide production by mitochondrial sn-glycerol 3-phosphate dehydrogenase. *Journal of Biological Chemistry* 2012, *287*, 42921-42935.
[109] Venditti, P., Di Stefano, L., Di Meo, S., Mitochondrial metabolism of reactive oxygen species. *Mitochondrion* 2013, *13*, 71-82.
[110] Sumida, Y., Yoshikawa, T., Okanoue, T., Role of hepatic iron in non-alcoholic steatohepatitis. *Hepatology Research* 2009, *39*, 213-222.
[111] Dongiovanni, P., Fracanzani, A. L., Fargion, S., Valenti, L., Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. *Journal of Hepatology* 2011, *55*, 920-932.

[112] VIDELA, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., Varela, N., Contreras, J., Lazarte, R., Csendes, A., Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clinical Science* 2004, *106*, 261-268.
[113] Sumida, Y., Nakashima, T., Yoh, T., Furutani, M., Hirohama, A., Kakisaka, Y., Nakajima, Y., Ishikawa, H., Mitsuyoshi, H., Okanoue, T., Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. *Journal of Hepatology* 2003, *38*, 32-38.
[114] Malaguarnera, L., Madeddu, R., Palio, E., Arena, N., Malaguarnera, M., Heme oxygenase-1 levels and oxidative stress-related parameters in non-alcoholic fatty liver disease patients. *Journal of Hepatology* 2005, *42*, 585-591.
[115] Zein, C. O., Yerian, L. M., Gogate, P., Lopez, R., Kirwan, J. P., Feldstein, A. E., McCullough, A. J., Pentoxifylline improves nonalcoholic steatohepatitis: A randomized placebo-controlled trial. *Hepatology* 2011, *54*, 1610-1619.
[116] Ucar, F., Sezer, S., Erdogan, S., Akyol, S., Armutcu, F., Akyol, O., The relationship between oxidative stress and nonalcoholic fatty liver disease: Its effects on the development of nonalcoholic steatohepatitis. *Redox Report* 2013, *18*, 127-133.
[117] Niki, E., Lipid peroxidation: physiological levels and dual biological effects. *Free Radical Biology and Medicine* 2009, *47*, 469-484.
[118] Andreyev, A. Y., Kushnareva, Y. E., Starkov, A., Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Moscow)* 2005, *70*, 200-214.
[119] Ježek, P., Hlavatá, L., Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *The International Journal of Biochemistry & Cell Biology* 2005, *37*, 2478-2503.
[120] Kowaltowski, A. J., de Souza-Pinto, N. C., Castilho, R. F., Vercesi, A. E., Mitochondria and reactive oxygen species. *Free Radical Biology and Medicine* 2009, *47*, 333-343.
[121] Schrader, M., Fahimi, H. D., Peroxisomes and oxidative stress. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 2006, *1763*, 1755-1766.
[122] Morris, E. M., Rector, R. S., Thyfault, J. P., Ibdah, J. A., Mitochondria and redox signaling in steatohepatitis. *Antioxidants & Redox Signaling* 2011, *15*, 485-504.
[123] Melov, S., Coskun, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A. S., Zastawny, T. H., Dizdaroglu, M., Goodman, S. I., Huang, T.-T., Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proceedings of the National Academy of Sciences* 1999, *96*, 846-851.
[124] Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nature Genetics* 1995, *11*, 376-381.
[125] Gardner, P. R., Aconitase: sensitive target and measure of superoxide. *Methods in Enzymology* 2002, *349*, 9-23.
[126] Takami, Y., Uto, H., Tamai, T., Sato, Y., Ishida, Y. i., Morinaga, H., Sakakibara, Y., Moriuchi, A., Oketani, M., Ido, A., Identification of a novel biomarker for oxidative stress induced by hydrogen peroxide in primary human hepatocytes using the 2-nitrobenzenesulfenyl chloride isotope labeling method. *Hepatology Research* 2010, *40*, 438-445.
[127] Baskol, G., Baskol, M., Kocer, D., Oxidative stress and antioxidant defenses in serum of patients with non-alcoholic steatohepatitis. *Clinical Biochemistry* 2007, *40*, 776-780.
[128] Nobili, V., Pastore, A., Gaeta, L. M., Tozzi, G., Comparcola, D., Sartorelli, M. R., Marcellini, M., Bertini, E., Piemonte, F., Glutathione metabolism and antioxidant enzymes in patients affected by nonalcoholic steatohepatitis. *Clinica Chimica Acta* 2005, *355*, 105-111.

[129] Seki, S., Kitada, T., Yamada, T., Sakaguchi, H., Nakatani, K., Wakasa, K., In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *Journal of Hepatology* 2002, *37*, 56-62.

[130] Hardwick, R. N., Fisher, C. D., Canet, M. J., Lake, A. D., Cherrington, N. J., Diversity in antioxidant response enzymes in progressive stages of human nonalcoholic fatty liver disease. *Drug Metabolism and Disposition* 2010, *38*, 2293-2301.

[131] Caballero, F., Fernández, A., Matías, N., Martínez, L., Fucho, R., Elena, M., Caballeria, J., Morales, A., Fernández-Checa, J. C., García-Ruiz, C., Specific contribution of methionine and choline in nutritional nonalcoholic steatohepatitis impact on mitochondrial s-adenosyl-l-methionine and glutathione. *Journal of Biological Chemistry* 2010, *285*, 18528-18536.

[132] Braunersreuther, V., Viviani, G. L., Mach, F., Montecucco, F., Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World Journal of Gastroenterology* 2012, *18*, 727-735.

[133] Pessayre, D., Role of mitochondria in non-alcoholic fatty liver disease. *Journal of Gastroenterology and Hepatology* 2007, *22*, S20-S27.

[134] Montecucco, F., Mach, F., Does non-alcoholic fatty liver disease (NAFLD) increase cardiovascular risk? *Endocrine, Metabolic and Immune Disorders-Drug Targets* 2008, *8*, 301-307.

[135] Crespo, J., Cayón, A., Fernández-Gil, P., Hernández-Guerra, M., Mayorga, M., Domínguez-Díez, A., Fernández-Escalante, J. C., Pons-Romero, F., Gene expression of tumor necrosis factor α and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001, *34*, 1158-1163.

[136] Asrih, M., Jornayvaz, F. R., Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *Journal of Endocrinology* 2013, *218*, R25-R36.

[137] Duarte, N., Coelho, I. C., Patarrão, R. S., Almeida, J. I., Penha-Gonçalves, C., Macedo, M. P., How inflammation impinges on NAFLD: a role for Kupffer cells. *BioMed research international* 2015, *2015*.

[138] Navarro, A., Boveris, A., The mitochondrial energy transduction system and the aging process. *American Journal of Physiology-Cell Physiology* 2007, *292*, C670-C686.

[139] Serviddio, G., Sastre, J., Bellanti, F., Viña, J., Vendemiale, G., Altomare, E., Mitochondrial involvement in non-alcoholic steatohepatitis. *Molecular Aspects of Medicine* 2008, *29*, 22-35.

[140] Mandelker, L., Introduction to oxidative stress and mitochondrial dysfunction. *Veterinary Clinics of North America: Small Animal Practice* 2008, *38*, 1-30.

[141] Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Roca, P., Oliver, J., Mitochondrial dynamics is affected by 17β-estradiol in the MCF-7 breast cancer cell line. Effects on fusion and fission related genes. *The International Journal of Biochemistry & Cell Biology* 2012, *44*, 1901-1905.

[142] Aoun, M., Michel, F., Fouret, G., Schlernitzauer, A., Ollendorff, V., Wrutniak-Cabello, C., Cristol, J.-P., Carbonneau, M.-A., Coudray, C., Feillet-Coudray, C., A grape polyphenol extract modulates muscle membrane fatty acid composition and lipid metabolism in high-fat–high-sucrose diet-fed rats. *British Journal of Nutrition* 2011, *106*, 491-501.

[143] Aharoni-Simon, M., Hann-Obercyger, M., Pen, S., Madar, Z., Tirosh, O., Fatty liver is associated with impaired activity of PPARγ-coactivator 1α (PGC1α) and mitochondrial biogenesis in mice. *Laboratory Investigation* 2011, *91*, 1018-1028.

[144] Finck, B. N., Kelly, D. P., PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *The Journal of Clinical Investigation* 2006, *116*, 615-622.

[145] St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J. M., Rhee, J., Jäger, S., Handschin, C., Zheng, K., Lin, J., Yang, W., Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006, *127*, 397-408.
[146] Lee, W. J., Kim, M., Park, H.-S., Kim, H. S., Jeon, M. J., Oh, K. S., Koh, E. H., Won, J. C., Kim, M.-S., Oh, G. T., AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1. *Biochemical and Biophysical Research Communications* 2006, *340*, 291-295.
[147] Puigserver, P., Tissue-specific regulation of metabolic pathways through the transcriptional coactivator PGC1-α. *International Journal of Obesity* 2005, *29*, S5-S9.
[148] Farrell, G. C., Animal models of steatohepatitis. *Fatty liver disease: NASH and related disorders. Massachusetts: Blackwell* 2005, 91-108.
[149] Sakaida, I., Okita, K., The role of oxidative stress in NASH and fatty liver model. *Hepatology Research* 2005, *33*, 128-131.
[150] Cortez-Pinto, H., Chatham, J., Chacko, V., Arnold, C., Rashid, A., Diehl, A. M., Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* 1999, *282*, 1659-1664.
[151] Tarantino, G., Saldalamacchia, G., Conca, P., Arena, A., Non-alcoholic fatty liver disease: Further expression of the metabolic syndrome. *Journal of Gastroenterology and Hepatology* 2007, *22*, 293-303.
[152] Caldwell, S. H., Swerdlow, R. H., Khan, E. M., Iezzoni, J. C., Hespenheide, E. E., Parks, J. K., Parker, W. D., Mitochondrial abnormalities in non-alcoholic steatohepatitis. *Journal of Hepatology* 1999, *31*, 430-434.
[153] Vial, G., Dubouchaud, H., Couturier, K., Cottet-Rousselle, C., Taleux, N., Athias, A., Galinier, A., Casteilla, L., Leverve, X. M., Effects of a high-fat diet on energy metabolism and ROS production in rat liver. *Journal of Hepatology* 2011, *54*, 348-356.
[154] Fromenty, B., Pessayre, D., Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. *Pharmacology and Therapeutics* 1995, *67*, 101-154.
[155] Chavin, K. D., Yang, S., Lin, H. Z., Chatham, J., Chacko, V. P., Hoek, J. B., Walajtys-Rode, E., Rashid, A., Chen, C.-H., Huang, C.-C., Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *Journal of Biological Chemistry* 1999, *274*, 5692-5700.
[156] Tailleux, A., Wouters, K., Staels, B., Roles of PPARs in NAFLD: potential therapeutic targets. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* 2012, *1821*, 809-818.
[157] Wang, Y.-X., PPARs: diverse regulators in energy metabolism and metabolic diseases. *Cell Research* 2010, *20*, 124-137.
[158] Méndez-Sánchez, N., Arrese, M., Zamora-Valdés, D., Uribe, M., Current concepts in the pathogenesis of nonalcoholic fatty liver disease. *Liver International* 2007, *27*, 423-433.
[159] Semple, R. K., Chatterjee, V. K. K., O'Rahilly, S., PPARγ and human metabolic disease. *The Journal of Clinical Investigation* 2006, *116*, 581-589.
[160] Nan, Y.-M., Han, F., Kong, L.-B., Zhao, S.-X., Wang, R.-Q., Wu, W.-J., Yu, J., Adenovirus-mediated peroxisome proliferator activated receptor gamma overexpression prevents nutritional fibrotic steatohepatitis in mice. *Scandinavian Journal of Gastroenterology* 2011, *46*, 358-369.
[161] Wu, C., Chu, E., Lam, C., Cheng, A., Lee, C., Wong, V., Sung, J., Yu, J., PPARγ is essential for protection against nonalcoholic steatohepatitis. *Gene Therapy* 2010, *17*, 790-798.

[162] Yu, J., Zhang, S., Chu, E. S., Go, M. Y., Lau, R. H., Zhao, J., Wu, C.-W., Tong, L., Zhao, J., Poon, T. C., Peroxisome proliferator-activated receptors gamma reverses hepatic nutritional fibrosis in mice and suppresses activation of hepatic stellate cells in vitro. *The International Journal of Biochemistry & Cell Biology* 2010, *42*, 948-957.

[163] Lalloyer, F., Staels, B., Fibrates, glitazones, and peroxisome proliferator–activated receptors. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010, *30*, 894-899.

[164] Fiévet, C., Staels, B., Efficacy of peroxisome proliferator-activated receptor agonists in diabetes and coronary artery disease. *Current Atherosclerosis Reports* 2009, *11*, 281-288.

[165] Wilson-Fritch, L., Burkart, A., Bell, G., Mendelson, K., Leszyk, J., Nicoloro, S., Czech, M., Corvera, S., Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Molecular and Cellular Biology* 2003, *23*, 1085-1094.

[166] Cortez-Pinto, H., Machado, M. V., Uncoupling proteins and non-alcoholic fatty liver disease. *Journal of Hepatology* 2009, *50*, 857-860.

[167] Sluse, F. E., Jarmuszkiewicz, W., Navet, R., Douette, P., Mathy, G., Sluse-Goffart, C. M., Mitochondrial UCPs: new insights into regulation and impact. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 2006, *1757*, 480-485.

[168] Souza, B. M. d., Assmann, T. S., Kliemann, L. M., Gross, J. L., Canani, L. H., Crispim, D., The role of uncoupling protein 2 (UCP2) on the development of type 2 diabetes mellitus and its chronic complications. *Arquivos Brasileiros de Endocrinologia & Metabologia* 2011, *55*, 239-248.

[169] Fisler, J. S., Warden, C. H., Uncoupling proteins, dietary fat and the metabolic syndrome. *Nutrition & Metabolism* 2006, *3*, 1.

[170] Fülöp, P., Derdák, Z., Sheets, A., Sabo, E., Berthiaume, E. P., Resnick, M. B., Wands, J. R., Paragh, G., Baffy, G., Lack of UCP2 reduces fas-mediated liver injury in ob/ob mice and reveals importance of cell-specific UCP2 expression. *Hepatology* 2006, *44*, 592-601.

[171] Poulsen, M. M., Larsen, J. Ø., Hamilton-Dutoit, S., Clasen, B. F., Jessen, N., Paulsen, S. K., Kjær, T. N., Richelsen, B., Pedersen, S. B., Resveratrol up-regulates hepatic uncoupling protein 2 and prevents development of nonalcoholic fatty liver disease in rats fed a high-fat diet. *Nutrition Research* 2012, *32*, 701-708.

[172] Collins, P., Jones, C., Choudhury, S., Damelin, L., Hodgson, H., Increased expression of uncoupling protein 2 in HepG2 cells attenuates oxidative damage and apoptosis. *Liver International* 2005, *25*, 880-887.

[173] Vincent, A. M., Olzmann, J. A., Brownlee, M., Sivitz, W., Russell, J. W., Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. *Diabetes* 2004, *53*, 726-734.

[174] Franco, R., Sánchez-Olea, R., Reyes-Reyes, E. M., Panayiotidis, M. I., Environmental toxicity, oxidative stress and apoptosis: menage a trois. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 2009, *674*, 3-22.

[175] Silva, J. P., Wahlestedt, C., Role of Sirtuin 1 in metabolic regulation. *Drug Discovery Today* 2010, *15*, 781-791.

[176] Rodgers, J. T., Puigserver, P., Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1. *Proceedings of the National Academy of Sciences* 2007, *104*, 12861-12866.

[177] Cao, Y., Jiang, X., Ma, H., Wang, Y., Xue, P., Liu, Y., SIRT1 and insulin resistance. *Journal of Diabetes and Its Complications* 2016, *30*, 178-183.

[178] Feige, J. N., Lagouge, M., Canto, C., Strehle, A., Houten, S. M., Milne, J. C., Lambert, P. D., Mataki, C., Elliott, P. J., Auwerx, J., Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. *Cell Metabolism* 2008, *8*, 347-358.
[179] Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., Puigserver, P., Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature* 2005, *434*, 113-118.
[180] Dominy, J. E., Lee, Y., Gerhart-Hines, Z., Puigserver, P., Nutrient-dependent regulation of PGC-1α's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* 2010, *1804*, 1676-1683.
[181] Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F., Ido, Y., AMPK and SIRT1: a long-standing partnership? *American Journal of Physiology-Endocrinology And Metabolism* 2010, *298*, E751-E760.
[182] Hou, X., Xu, S., Maitland-Toolan, K. A., Sato, K., Jiang, B., Ido, Y., Lan, F., Walsh, K., Wierzbicki, M., Verbeuren, T. J., SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *Journal of Biological Chemistry* 2008, *283*, 20015-20026.
[183] Walker, A. K., Yang, F., Jiang, K., Ji, J.-Y., Watts, J. L., Purushotham, A., Boss, O., Hirsch, M. L., Ribich, S., Smith, J. J., Conserved role of SIRT1 orthologs in fasting-dependent inhibition of the lipid/cholesterol regulator SREBP. *Genes and Development* 2010, *24*, 1403-1417.
[184] Schug, T. T., Li, X., Sirtuin 1 in lipid metabolism and obesity. *Annals of Medicine* 2011, *43*, 198-211.
[185] Giralt, A., Villarroya, F., SIRT3, a pivotal actor in mitochondrial functions: metabolism, cell death and aging. *Biochemical Journal* 2012, *444*, 1-10.
[186] Hirschey, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* 2010, *464*, 121-125.
[187] Yu, J., Auwerx, J., The role of sirtuins in the control of metabolic homeostasis. *Annals of the New York Academy of Sciences* 2009, *1173*, E10-E19.
[188] Ahn, B.-H., Kim, H.-S., Song, S., Lee, I. H., Liu, J., Vassilopoulos, A., Deng, C.-X., Finkel, T., A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proceedings of the National Academy of Sciences* 2008, *105*, 14447-14452.
[189] Schröder, M., Kaufman, R. J., ER stress and the unfolded protein response. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 2005, *569*, 29-63.
[190] Basseri, S., Austin, R. C., Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential. *Biochemistry research international* 2011, *2012*.
[191] Salvadó, L., Palomer, X., Barroso, E., Vázquez-Carrera, M., Targeting endoplasmic reticulum stress in insulin resistance. *Trends in Endocrinology and Metabolism* 2015, *26*, 438-448.
[192] Özcan, U., Yilmaz, E., Özcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., Görgün, C. Z., Hotamisligil, G. S., Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006, *313*, 1137-1140.

[193] Chen, Y., Wu, Z., Zhao, S., Xiang, R., Chemical chaperones reduce ER stress and adipose tissue inflammation in high fat diet-induced mouse model of obesity. *Scientific Reports* 2016, *6*, 27486.

[194] Takahashi, Y., Soejima, Y., Fukusato, T., Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World Journal of Gastroenterology* 2012, *18*, 2300-2308.

[195] Aubert, J., Begriche, K., Delannoy, M., Morel, I., Pajaud, J., Ribault, C., Lepage, S., McGill, M. R., Lucas-Clerc, C., Turlin, B., Differences in early acetaminophen hepatotoxicity between obese ob/ob and db/db mice. *Journal of Pharmacology and Experimental Therapeutics* 2012, *342*, 676-687.

[196] Anstee, Q. M., Goldin, R. D., Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *International Journal of Experimental Pathology* 2006, *87*, 1-16.

[197] Brix, A. E., Elgavish, A., Nagy, T. R., Gower, B. A., Rhead, W. J., Wood, P. A., Evaluation of liver fatty acid oxidation in the leptin-deficient obese mouse. *Molecular Genetics and Metabolism* 2002, *75*, 219-226.

[198] Wortham, M., He, L., Gyamfi, M., Copple, B. L., Wan, Y.-J. Y., The transition from fatty liver to NASH associates with SAMe depletion in db/db mice fed a methionine choline-deficient diet. *Digestive Diseases and Sciences* 2008, *53*, 2761-2774.

[199] Larter, C. Z., Yeh, M. M., Animal models of NASH: getting both pathology and metabolic context right. *Journal of Gastroenterology and Hepatology* 2008, *23*, 1635-1648.

[200] Romestaing, C., Piquet, M.-A., Bedu, E., Rouleau, V., Dautresme, M., Hourmand-Ollivier, I., Filippi, C., Duchamp, C., Sibille, B., Long term highly saturated fat diet does not induce NASH in Wistar rats. *Nutrition & Metabolism* 2007, *4*, 1.

[201] Araya, J., Rodrigo, R., Videla, L. A., Thielemann, L., Orellana, M., Pettinelli, P., Poniachik, J., Increase in long-chain polyunsaturated fatty acid n− 6/n− 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clinical Science* 2004, *106*, 635-643.

[202] Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* 2004, *79*, 727-747.

[203] D Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., Polyphenols, dietary sources and bioavailability. *Annali-Istituto Superiore di Sanita* 2007, *43*, 348.

[204] Graf, B. A., Milbury, P. E., Blumberg, J. B., Flavonols, flavones, flavanones, and human health: epidemiological evidence. *Journal of Medicinal Food* 2005, *8*, 281-290.

[205] Virgili, F., Marino, M., Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. *Free Radical Biology and Medicine* 2008, *45*, 1205-1216.

[206] S Panickar, K., A Anderson, R., Role of dietary polyphenols in attenuating brain edema and cell swelling in cerebral ischemia. *Recent Patents on CNS Drug Discovery* 2010, *5*, 99-108.

[207] Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P., Tognolini, M., Borges, G., Crozier, A., Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling* 2013, *18*, 1818-1892.

[208] de Pascual-Teresa, S., Moreno, D. A., García-Viguera, C., Flavanols and anthocyanins in cardiovascular health: a review of current evidence. *International Journal of Molecular Sciences* 2010, *11*, 1679-1703.

[209] Thilakarathna, S. H., Rupasinghe, H., Flavonoid bioavailability and attempts for bioavailability enhancement. *Nutrients* 2013, *5*, 3367-3387.

[210] Shih, P.-H., Yeh, C.-T., Yen, G.-C., Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *Journal of Agricultural and Food Chemistry* 2007, *55*, 9427-9435.
[211] Macheix, J.-J., Fleuriet, A., *Fruit phenolics*, CRC press 1990.
[212] Langcake, P., Pryce, R., A new class of phytoalexins from grapevines. *Cellular and Molecular Life Sciences* 1977, *33*, 151-152.
[213] Crozier, A., Borges, G., Ryan, D., The glass that cheers: Phenolic and polyphenolic constituents and the beneficial effects of moderate red wine consumption. *The Biochemist* 2010, *32*, 4.
[214] Lewandowska, U., Szewczyk, K., Hrabec, E. b., Janecka, A., Gorlach, S., Overview of metabolism and bioavailability enhancement of polyphenols. *Journal of Agricultural and Food Chemistry* 2013, *61*, 12183-12199.
[215] Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., Queipo-Ortuño, M. I., Benefits of polyphenols on gut microbiota and implications in human health. *The Journal of Nutritional Biochemistry* 2013, *24*, 1415-1422.
[216] Matuschek, M. C., Hendriks, W. H., McGhie, T. K., Reynolds, G. W., The jejunum is the main site of absorption for anthocyanins in mice. *The Journal of Nutritional Biochemistry* 2006, *17*, 31-36.
[217] Nurmi, T., Mursu, J., Heinonen, M., Nurmi, A., Hiltunen, R., Voutilainen, S., Metabolism of berry anthocyanins to phenolic acids in humans. *Journal of Agricultural and Food Chemistry* 2009, *57*, 2274-2281.
[218] Gee, J. M., DuPont, M. S., Day, A. J., Plumb, G. W., Williamson, G., Johnson, I. T., Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *The Journal of Nutrition* 2000, *130*, 2765-2771.
[219] Perez-Vizcaino, F., Duarte, J., Santos-Buelga, C., The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids. *Journal of the Science of Food and Agriculture* 2012, *92*, 1822-1825.
[220] Dangles, O., Dufour, C., Manach, C., Morand, C., Remesy, C., Binding of flavonoids to plasma proteins. *Methods in Enzymology* 2000, *335*, 319-333.
[221] Van Het Hof, K. H., Wiseman, S. A., Yang, C. S., Tijburg, L. B., Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Experimental Biology and Medicine* 1999, *220*, 203-209.
[222] Aziz, A. A., Edwards, C. A., Lean, M. E., Crozier, A., Absorption and excretion of conjugated flavonols, including quercetin-4′-O-β-glucoside and isorhamnetin-4′-O-β-glucoside by human volunteers after the consumption of onions. *Free Radical Research* 1998, *29*, 257-269.
[223] Pietta, P.-G., Flavonoids as antioxidants. *Journal of Natural Products* 2000, *63*, 1035-1042.
[224] Guo, J.-J., Hsieh, H.-Y., Hu, C.-H., Chain-breaking activity of carotenes in lipid peroxidation: a theoretical study. *The Journal of Physical Chemistry B* 2009, *113*, 15699-15708.
[225] Bandy, B., Bechara, E. J., Bioflavonoid rescue of ascorbate at a membrane interface. *Journal of Bioenergetics and Biomembranes* 2001, *33*, 269-277.
[226] Gómez-Zorita, S., Fernández-Quintela, A., Macarulla, M., Aguirre, L., Hijona, E., Bujanda, L., Milagro, F., Martínez, J., Portillo, M., Resveratrol attenuates steatosis in obese Zucker rats by decreasing fatty acid availability and reducing oxidative stress. *British Journal of Nutrition* 2012, *107*, 202-210.

[227] Obrenovich, M. E., Nair, N. G., Beyaz, A., Aliev, G., Reddy, V. P., The role of polyphenolic antioxidants in health, disease, and aging. *Rejuvenation Research* 2010, *13*, 631-643.

[228] Nakamoto, K., Takayama, F., Mankura, M., Hidaka, Y., Egashira, T., Ogino, T., Kawasaki, H., Mori, A., Beneficial effects of fermented green tea extract in a rat model of non-alcoholic steatohepatitis. *Journal of Clinical Biochemistry and Nutrition* 2009, *44*, 239-246.

[229] Marcolin, E., San-Miguel, B., Vallejo, D., Tieppo, J., Marroni, N., González-Gallego, J., Tuñón, M. J., Quercetin treatment ameliorates inflammation and fibrosis in mice with nonalcoholic steatohepatitis. *The Journal of Nutrition* 2012, *142*, 1821-1828.

[230] Marcolin, É., Forgiarini, L. F., Rodrigues, G., Tieppo, J., Borghetti, G. S., Bassani, V. L., Picada, J. N., Marroni, N. P., Quercetin decreases liver damage in mice with non-alcoholic steatohepatitis. *Basic & Clinical Pharmacology & Toxicology* 2013, *112*, 385-391.

[231] Crespo, I., García-Mediavilla, M. V., Almar, M., González, P., Tuñón, M. J., Sánchez-Campos, S., González-Gallego, J., Differential effects of dietary flavonoids on reactive oxygen and nitrogen species generation and changes in antioxidant enzyme expression induced by proinflammatory cytokines in Chang Liver cells. *Food and Chemical Toxicology* 2008, *46*, 1555-1569.

[232] Kuzu, N., Bahcecioglu, I. H., Dagli, A. F., Ozercan, I. H., Ustündag, B., Sahin, K., Epigallocatechin gallate attenuates experimental non-alcoholic steatohepatitis induced by high fat diet. *Journal of Gastroenterology and Hepatology* 2008, *23*, e465-e470.

[233] Weltman, M. D., Farrell, G. C., Hall, P., Ingelman-Sundberg, M., Liddle, C., Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology* 1998, *27*, 128-133.

[234] Gupte, A. A., Lyon, C. J., Hsueh, W. A., Nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a key regulator of the antioxidant response to protect against atherosclerosis and nonalcoholic steatohepatitis. *Current Diabetes Reports* 2013, *13*, 362-371.

[235] Yu, Z.-W., Li, D., Ling, W.-H., Jin, T.-R., Role of nuclear factor (erythroid-derived 2)-like 2 in metabolic homeostasis and insulin action: A novel opportunity for diabetes treatment. *World Journal of Diabetes* 2012, *3*, 19-28.

[236] Meakin, P. J., Chowdhry, S., Sharma, R. S., Ashford, F. B., Walsh, S. V., McCrimmon, R. J., Dinkova-Kostova, A. T., Dillon, J. F., Hayes, J. D., Ashford, M. L., Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Molecular and Cellular Biology* 2014, *34*, 3305-3320.

[237] Cui, Y., Wang, Q., Li, X., Zhang, X., Experimental nonalcoholic fatty liver disease in mice leads to cytochrome p450 2a5 upregulation through nuclear factor erythroid 2-like 2 translocation. *Redox Biology* 2013, *1*, 433-440.

[238] Vitaglione, P., Morisco, F., Mazzone, G., Amoruso, D. C., Ribecco, M. T., Romano, A., Fogliano, V., Caporaso, N., D'Argenio, G., Coffee reduces liver damage in a rat model of steatohepatitis: the underlying mechanisms and the role of polyphenols and melanoidins. *Hepatology* 2010, *52*, 1652-1661.

[239] Zhu, W., Jia, Q., Wang, Y., Zhang, Y., Xia, M., The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP–PKA-dependent signaling pathway. *Free Radical Biology and Medicine* 2012, *52*, 314-327.

[240] Guo, M., Perez, C., Wei, Y., Rapoza, E., Su, G., Bou-Abdallah, F., Chasteen, N., Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton Transactions* 2007, 4951-4961.

[241] Wang, G.-L., Fu, Y.-C., Xu, W.-C., Feng, Y.-Q., Fang, S.-R., Zhou, X.-H., Resveratrol inhibits the expression of SREBP1 in cell model of steatosis via Sirt1–FOXO1 signaling pathway. *Biochemical and Biophysical Research Communications* 2009, *380*, 644-649.

[242] Park, K. G., Min, A. K., Koh, E. H., Kim, H. S., Kim, M. O., Park, H. S., Kim, Y. D., Yoon, T. S., Jang, B. K., Hwang, J. S., Alpha-lipoic acid decreases hepatic lipogenesis through adenosine monophosphate-activated protein kinase (AMPK)-dependent and AMPK-independent pathways. *Hepatology* 2008, *48*, 1477-1486.

[243] da Costa Guerra, J. F., Maciel, P. S., de Abreu, I. C. M. E., Pereira, R. R., Silva, M., de Morais Cardoso, L., Pinheiro-Sant'Ana, H. M., de Lima, W. G., Silva, M. E., Pedrosa, M. L., Dietary açai attenuates hepatic steatosis via adiponectin-mediated effects on lipid metabolism in high-fat diet mice. *Journal of Functional Foods* 2015, *14*, 192-202.

[244] Seymour, E. M., Singer, A. A., Kirakosyan, A., Urcuyo-Llanes, D. E., Kaufman, P. B., Bolling, S. F., Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. *Journal of Medicinal Food* 2008, *11*, 252-259.

[245] Jia, Y., Kim, J.-Y., Jun, H.-j., Kim, S.-J., Lee, J.-H., Hoang, M. H., Kim, H. S., Chang, H. I., Hwang, K.-Y., Um, S.-J., Cyanidin is an agonistic ligand for peroxisome proliferator-activated receptor-alpha reducing hepatic lipid. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* 2013, *1831*, 698-708.

[246] Ono, M., Fujimori, K., Antiadipogenic effect of dietary apigenin through activation of AMPK in 3T3-L1 cells. *Journal of Agricultural and Food Chemistry* 2011, *59*, 13346-13352.

[247] Kang, W., Hong, H. J., Guan, J., Kim, D. G., Yang, E.-J., Koh, G., Park, D., Han, C. H., Lee, Y.-J., Lee, D.-H., Resveratrol improves insulin signaling in a tissue-specific manner under insulin-resistant conditions only: in vitro and in vivo experiments in rodents. *Metabolism: Clinical and Experimental* 2012, *61*, 424-433.

[248] Chen, S., Li, J., Zhang, Z., Li, W., Sun, Y., Zhang, Q., Feng, X., Zhu, W., Effects of resveratrol on the amelioration of insulin resistance in KKAy mice. *Canadian Journal of Physiology and Pharmacology* 2012, *90*, 237-242.

[249] Guo, H., Xia, M., Zou, T., Ling, W., Zhong, R., Zhang, W., Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1. *The Journal of Nutritional Biochemistry* 2012, *23*, 349-360.

[250] Ying, H.-Z., Liu, Y.-H., Yu, B., Wang, Z.-Y., Zang, J.-N., Yu, C.-H., Dietary quercetin ameliorates nonalcoholic steatohepatitis induced by a high-fat diet in gerbils. *Food and Chemical Toxicology* 2013, *52*, 53-60.

[251] Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. *Cell* 2006, *127*, 1109-1122.

[252] Gomes, A. P., Duarte, F. V., Nunes, P., Hubbard, B. P., Teodoro, J. S., Varela, A. T., Jones, J. G., Sinclair, D. A., Palmeira, C. M., Rolo, A. P., Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 2012, *1822*, 185-195.

[253] Peng, F., Mitochondrial uptake of anthocyanidins and protection from oxidative stress. 2012.
[254] Tang, X., Shen, T., Jiang, X., Xia, M., Sun, X., Guo, H., Ling, W., Purified anthocyanins from bilberry and black currant attenuate hepatic mitochondrial dysfunction and steatohepatitis in mice with methionine and choline deficiency. *Journal of Agricultural and Food Chemistry* 2015, *63*, 552-561.
[255] Hiramitsu, M., Shimada, Y., Kuroyanagi, J., Inoue, T., Katagiri, T., Zang, L., Nishimura, Y., Nishimura, N., Tanaka, T., Eriocitrin ameliorates diet-induced hepatic steatosis with activation of mitochondrial biogenesis. *Scientific Reports* 2014, *4*, 3708.
[256] Pan, Q.-R., Ren, Y.-L., Liu, W.-X., Hu, Y.-J., Zheng, J.-S., Xu, Y., Wang, G., Resveratrol prevents hepatic steatosis and endoplasmic reticulum stress and regulates the expression of genes involved in lipid metabolism, insulin resistance, and inflammation in rats. *Nutrition Research* 2015, *35*, 576-584.
[257] Jung, T. W., Lee, K.-T., Lee, M. W., Ka, K.-H., SIRT1 attenuates palmitate-induced endoplasmic reticulum stress and insulin resistance in HepG2 cells via induction of oxygen-regulated protein 150. *Biochemical and Biophysical Research Communications* 2012, *422*, 229-232.
[258] Zhang, Z., Li, B., Meng, X., Yao, S., Jin, L., Yang, J., Wang, J., Zhang, H., Zhang, Z., Cai, D., Berberine prevents progression from hepatic steatosis to steatohepatitis and fibrosis by reducing endoplasmic reticulum stress. *Scientific Reports* 2016, *6*.
[259] Wang, Z.-s., Lu, F.-e., Xu, L.-j., Dong, H., Berberine reduces endoplasmic reticulum stress and improves insulin signal transduction in Hep G2 cells. *Acta Pharmacologica Sinica* 2010, *31*, 578-584.
[260] Wu, Z., Zhao, J., Xu, H., Lyv, Y., Feng, X., Fang, Y., Xu, Y., Maternal quercetin administration during gestation and lactation decrease endoplasmic reticulum stress and related inflammation in the adult offspring of obese female rats. *European Journal of Nutrition* 2014, *53*, 1669-1683.
[261] Rodriguez, J., Gilson, H., Jamart, C., Naslain, D., Pierre, N., Deldicque, L., Francaux, M., Pomegranate and green tea extracts protect against ER stress induced by a high-fat diet in skeletal muscle of mice. *European Journal of Nutrition* 2015, *54*, 377-389.
[262] Yatsuji, S., Hashimoto, E., Tobari, M., Taniai, M., Tokushige, K., Shiratori, K., Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *Journal of Gastroenterology and Hepatology* 2009, *24*, 248-254.
[263] Raffaella, C., Francesca, B., Italia, F., Marina, P., Giovanna, L., Susanna, I., Alterations in hepatic mitochondrial compartment in a model of obesity and insulin resistance. *Obesity* 2008, *16*, 958-964.
[264] Satapati, S., Sunny, N. E., Kucejova, B., Fu, X., He, T. T., Méndez-Lucas, A., Shelton, J. M., Perales, J. C., Browning, J. D., Burgess, S. C., Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *Journal of Lipid Research* 2012, *53*, 1080-1092.
[265] Zou, X., Yan, C., Shi, Y., Cao, K., Xu, J., Wang, X., Chen, C., Luo, C., Li, Y., Gao, J., Mitochondrial dysfunction in obesity-associated nonalcoholic fatty liver disease: the protective effects of pomegranate with its active component punicalagin. *Antioxidants & Redox Signaling* 2014, *21*, 1557-1570.
[266] Schneider, A. R., Kraut, C., Lindenthal, B., Braden, B., Caspary, W. F., Stein, J., Total body metabolism of 13C-octanoic acid is preserved in patients with non-alcoholic steatohepatitis, but

differs between women and men. *European Journal of Gastroenterology and Hepatology* 2005, *17*, 1181-1184.
[267] Kim, W. S., Lee, Y. S., Cha, S. H., Jeong, H. W., Choe, S. S., Lee, M.-R., Oh, G. T., Park, H.-S., Lee, K.-U., Lane, M. D., Berberine improves lipid dysregulation in obesity by controlling central and peripheral AMPK activity. *American Journal of Physiology-Endocrinology And Metabolism* 2009, *296*, E812-E819.
[268] Sitepu, I., Ignatia, L., Franz, A., Wong, D., Faulina, S., Tsui, M., Kanti, A., Boundy-Mills, K., An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of Microbiological Methods* 2012, *91*, 321-328.
[269] Cottet-Rousselle, C., Ronot, X., Leverve, X., Mayol, J. F., Cytometric assessment of mitochondria using fluorescent probes. *Cytometry Part A* 2011, *79*, 405-425.
[270] Guo, H., Liu, G., Zhong, R., Wang, Y., Wang, D., Xia, M., Cyanidin-3-O-β-glucoside regulates fatty acid metabolism via an AMP-activated protein kinase-dependent signaling pathway in human HepG2 cells. *Lipids in Health and Disease* 2012, *11*, 1.
[271] Pajuelo, D., Fernández-Iglesias, A., Díaz, S., Quesada, H., Arola-Arnal, A., Bladé, C., Salvadó, J., Arola, L. s., Improvement of mitochondrial function in muscle of genetically obese rats after chronic supplementation with proanthocyanidins. *Journal of Agricultural and Food Chemistry* 2011, *59*, 8491-8498.
[272] Yu, S., Matsusue, K., Kashireddy, P., Cao, W.-Q., Yeldandi, V., Yeldandi, A. V., Rao, M. S., Gonzalez, F. J., Reddy, J. K., Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor γ1 (PPARγ1) overexpression. *Journal of Biological Chemistry* 2003, *278*, 498-505.
[273] Gavrilova, O., Haluzik, M., Matsusue, K., Cutson, J. J., Johnson, L., Dietz, K. R., Nicol, C. J., Vinson, C., Gonzalez, F. J., Reitman, M. L., Liver peroxisome proliferator-activated receptor γ contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *Journal of Biological Chemistry* 2003, *278*, 34268-34276.
[274] Sharma, A. K., Bharti, S., Ojha, S., Bhatia, J., Kumar, N., Ray, R., Kumari, S., Arya, D. S., Up-regulation of PPARγ, heat shock protein-27 and-72 by naringin attenuates insulin resistance, β-cell dysfunction, hepatic steatosis and kidney damage in a rat model of type 2 diabetes. *British Journal of Nutrition* 2011, *106*, 1713-1723.
[275] Berlanga, A., Guiu-Jurado, E., Porras, J. A., Auguet, T., Molecular pathways in non-alcoholic fatty liver disease. *Clinical and Experimental Gastroenterology* 2014, *7*, 221-239.
[276] Morgan, K., Uyuni, A., Nandgiri, G., Mao, L., Castaneda, L., Kathirvel, E., French, S. W., Morgan, T. R., Altered expression of transcription factors and genes regulating lipogenesis in liver and adipose tissue of mice with high fat diet-induced obesity and nonalcoholic fatty liver disease. *European Journal of Gastroenterology and Hepatology* 2008, *20*, 843-854.
[277] Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *International Journal of Molecular Medicine* 2007, *20*, 351-358.
[278] Deng, X. Q., Chen, L. L., Li, N. X., The expression of SIRT1 in nonalcoholic fatty liver disease induced by high-fat diet in rats. *Liver International* 2007, *27*, 708-715.
[279] Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L.-L., Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* 2003, *425*, 191-196.

[280] Lage, R., Diéguez, C., Vidal-Puig, A., López, M., AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in Molecular Medicine* 2008, *14*, 539-549.
[281] Zou, Y.-X., Shen, W.-Z., Liao, S.-T., Liu, F., Zheng, S.-Q., Blumberg, J. B., Chen, C.-Y. O., Mulberry leaf phenolics ameliorate hyperglycemia-induced oxidative stress and stabilize mitochondrial membrane potential in HepG2 cells. *International Journal of Food Sciences and Nutrition* 2014, *65*, 960-966.
[282] Zhang, S., Zheng, L., Dong, D., Xu, L., Yin, L., Qi, Y., Han, X., Lin, Y., Liu, K., Peng, J., Effects of flavonoids from Rosa laevigata Michx fruit against high-fat diet-induced non-alcoholic fatty liver disease in rats. *Food Chemistry* 2013, *141*, 2108-2116.
[283] Dott, W., Mistry, P., Wright, J., Cain, K., Herbert, K. E., Modulation of mitochondrial bioenergetics in a skeletal muscle cell line model of mitochondrial toxicity. *Redox Biology* 2014, *2*, 224-233.
[284] Marroquin, L. D., Hynes, J., Dykens, J. A., Jamieson, J. D., Will, Y., Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. *Toxicological Sciences* 2007, *97*, 539-547.
[285] Yin, J., Gao, Z., Liu, D., Liu, Z., Ye, J., Berberine improves glucose metabolism through induction of glycolysis. *American Journal of Physiology-Endocrinology And Metabolism* 2008, *294*, E148-E156.
[286] Marchesini, G., Brizi, M., Morselli-Labate, A. M., Bianchi, G., Bugianesi, E., McCullough, A. J., Forlani, G., Melchionda, N., Association of nonalcoholic fatty liver disease with insulin resistance. *The American Journal of Medicine* 1999, *107*, 450-455.
[287] Silverman, J. F., O'Brien, K. F., Long, S., Leggett, N., Khazanie, P. G., Pories, W. J., Norris, H. T., Caro, J. F., Liver pathology in morbidly obese patients with and without diabetes. *American Journal of Gastroenterology* 1990, *85*.
[288] Akhlaghi, M., Non-alcoholic fatty liver disease: beneficial effects of flavonoids. *Phytotherapy Research* 2016, *30*, 1559-1571.
[289] Rodriguez-Ramiro, I., Vauzour, D., Minihane, A., Polyphenols and non-alcoholic fatty liver disease: impact and mechanisms. *Proceedings of the Nutrition Society* 2016, *75*, 47-60.
[290] Cave, M., Deaciuc, I., Mendez, C., Song, Z., Joshi-Barve, S., Barve, S., McClain, C., Nonalcoholic fatty liver disease: predisposing factors and the role of nutrition. *The Journal of Nutritional Biochemistry* 2007, *18*, 184-195.
[291] Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006, *444*, 337-342.
[292] Zhang, D., Xie, L., Jia, G., Cai, S., Ji, B., Liu, Y., Wu, W., Zhou, F., Wang, A., Chu, L., Comparative study on antioxidant capacity of flavonoids and their inhibitory effects on oleic acid-induced hepatic steatosis in vitro. *European Journal of Medicinal Chemistry* 2011, *46*, 4548-4558.
[293] Agnello, M., Morici, G., Rinaldi, A. M., A method for measuring mitochondrial mass and activity. *Cytotechnology* 2008, *56*, 145-149.
[294] Te Sligte, K., Bourass, I., Sels, J., Driessen, A., Stockbrűgger, R., Koek, G., Non-alcoholic steatohepatitis: review of a growing medical problem. *European Journal of Internal Medicine* 2004, *15*, 10-21.
[295] Eghbaliferiz, S., Iranshahi, M., Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins and Carotenoids: Updated Review of Mechanisms and Catalyzing Metals. *Phytotherapy Research* 2016.

[296] Boots, A. W., Haenen, G. R., Bast, A., Health effects of quercetin: from antioxidant to nutraceutical. *European Journal of Pharmacology* 2008, *585*, 325-337.
[297] Ritov, V. B., Menshikova, E. V., He, J., Ferrell, R. E., Goodpaster, B. H., Kelley, D. E., Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* 2005, *54*, 8-14.
[298] Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metabolism* 2015, *21*, 739-746.
[299] Sparks, L. M., Xie, H., Koza, R. A., Mynatt, R., Hulver, M. W., Bray, G. A., Smith, S. R., A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* 2005, *54*, 1926-1933.
[300] Ploumi, C., Daskalaki, I., Tavernarakis, N., Mitochondrial biogenesis and clearance: a balancing act. *The FEBS Journal* 2016.
[301] Austin, S., St-Pierre, J., PGC1α and mitochondrial metabolism–emerging concepts and relevance in ageing and neurodegenerative disorders. *Journal of Cell Science* 2012, *125*, 4963-4971.
[302] Baffy, G., Uncoupling protein-2 and non-alcoholic fatty liver disease. *Frontiers in Bioscience* 2005, *10*, 2082-2096.
[303] Epstein, F. H., Tilg, H., Diehl, A. M., Cytokines in alcoholic and nonalcoholic steatohepatitis. *New England Journal of Medicine* 2000, *343*, 1467-1476.
[304] Miwa, S., Brand, M., Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochemical Society Transactions* 2003, *31*, 1300-1301.
[305] Cho, K. W., Kim, Y. O., Andrade, J. E., Burgess, J. R., Kim, Y.-C., Dietary naringenin increases hepatic peroxisome proliferators–activated receptor α protein expression and decreases plasma triglyceride and adiposity in rats. *European Journal of Nutrition* 2011, *50*, 81-88.
[306] Jabůrek, M., Miyamoto, S., Di Mascio, P., Garlid, K. D., Ježek, P., Hydroperoxy fatty acid cycling mediated by mitochondrial uncoupling protein UCP2. *Journal of Biological Chemistry* 2004, *279*, 53097-53102.
[307] Handschin, C., Choi, C. S., Chin, S., Kim, S., Kawamori, D., Kurpad, A. J., Neubauer, N., Hu, J., Mootha, V. K., Kim, Y.-B., Abnormal glucose homeostasis in skeletal muscle–specific PGC-1α knockout mice reveals skeletal muscle–pancreatic β cell crosstalk. *The Journal of Clinical Investigation* 2007, *117*, 3463-3474.
[308] Handschin, C., Spiegelman, B. M., The role of exercise and PGC1α in inflammation and chronic disease. *Nature* 2008, *454*, 463-469.
[309] Wang, D., Wei, Y., Pagliassotti, M. J., Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006, *147*, 943-951.
[310] Enos, R. T., Velázquez, K. T., Murphy, E. A., Insight into the impact of dietary saturated fat on tissue-specific cellular processes underlying obesity-related diseases. *The Journal of Nutritional Biochemistry* 2014, *25*, 600-612.
[311] Gentile, C. L., Pagliassotti, M. J., The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *The Journal of Nutritional Biochemistry* 2008, *19*, 567-576.
[312] Spruss, A., Kanuri, G., Uebel, K., Bischoff, S. C., Bergheim, I., Role of the inducible nitric oxide synthase in the onset of fructose-induced steatosis in mice. *Antioxidants & Redox Signaling* 2011, *14*, 2121-2135.

[313] Mantena, S. K., Vaughn, D. P., Andringa, K. K., Eccleston, H. B., King, A. L., Abrams, G. A., Doeller, J. E., Kraus, D. W., Darley-Usmar, V. M., Bailey, S. M., High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function in vivo. *Biochemical Journal* 2009, *417*, 183-193.
[314] Spruss, A., Kanuri, G., Wagnerberger, S., Haub, S., Bischoff, S. C., Bergheim, I., Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology* 2009, *50*, 1094-1104.
[315] Arunkumar, E., Karthik, D., Anuradha, C. V., Genistein sensitizes hepatic insulin signaling and modulates lipid regulatory genes through p70 ribosomal S6 kinase-1 inhibition in high-fat–high-fructose diet-fed mice. *Pharmaceutical Biology* 2013, *51*, 815-824.
[316] Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., Fantoni, L. I., Marra, F., Bertolotti, M., Banni, S., Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of Gastroenterology and Hepatology* 2009, *24*, 830-840.
[317] Liu, J. F., Ma, Y., Wang, Y., Du, Z. Y., Shen, J. K., Peng, H. L., Reduction of lipid accumulation in HepG2 cells by luteolin is associated with activation of AMPK and mitigation of oxidative stress. *Phytotherapy Research* 2011, *25*, 588-596.
[318] Lee, J.-y., Cho, H.-K., Kwon, Y. H., Palmitate induces insulin resistance without significant intracellular triglyceride accumulation in HepG2 cells. *Metabolism: Clinical and Experimental* 2010, *59*, 927-934.
[319] Ly, J. D., Grubb, D., Lawen, A., The mitochondrial membrane potential ($\Delta\psi m$) in apoptosis; an update. *Apoptosis* 2003, *8*, 115-128.
[320] Ribeiro, P. S., Cortez-Pinto, H., Solá, S., Castro, R. E., Ramalho, R. M., Baptista, A., Moura, M. C., Camilo, M. E., Rodrigues, C. M., Hepatocyte apoptosis, expression of death receptors, and activation of NF-κB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *The American Journal of Gastroenterology* 2004, *99*, 1708-1717.
[321] Willy, J. A., Young, S. K., Stevens, J. L., Masuoka, H. C., Wek, R. C., CHOP links endoplasmic reticulum stress to NF-κB activation in the pathogenesis of nonalcoholic steatohepatitis. *Molecular Biology of the Cell* 2015, *26*, 2190-2204.
[322] Zhang, X.-Q., Xu, C.-F., Yu, C.-H., Chen, W.-X., Li, Y.-M., Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease. *World Journal of Gastroenterology* 2014, *20*, 1768-1776.
[323] Wieckowska, A., Zein, N. N., Yerian, L. M., Lopez, A. R., McCullough, A. J., Feldstein, A. E., In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006, *44*, 27-33.
[324] Ji, C., Mehrian-Shai, R., Chan, C., Hsu, Y. H., Kaplowitz, N., Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcoholism: Clinical and Experimental Research* 2005, *29*, 1496-1503.
[325] Brown, G. C., Cooper, C., Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Letters* 1994, *356*, 295-298.
[326] Xu, J., Lai, K. K., Verlinsky, A., Lugea, A., French, S. W., Cooper, M. P., Ji, C., Tsukamoto, H., Synergistic steatohepatitis by moderate obesity and alcohol in mice despite increased adiponectin and p-AMPK. *Journal of Hepatology* 2011, *55*, 673-682.

[327] Chami, M., Oulès, B., Szabadkai, G., Tacine, R., Rizzuto, R., Paterlini-Bréchot, P., Role of SERCA1 truncated isoform in the proapoptotic calcium transfer from ER to mitochondria during ER stress. *Molecular Cell* 2008, *32*, 641-651.
[328] Lim, J. H., Lee, H. J., Jung, M. H., Song, J., Coupling mitochondrial dysfunction to endoplasmic reticulum stress response: a molecular mechanism leading to hepatic insulin resistance. *Cellular Signalling* 2009, *21*, 169-177.
[329] Watson, R. R., Preedy, V. R., Zibadi, S., *Polyphenols in human health and disease*, Academic Press 2013.
[330] Calle, E. E., Rodriguez, C., Walker-Thurmond, K., Thun, M. J., Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. *New England Journal of Medicine* 2003, *348*, 1625-1638.
[331] Field, A. E., Coakley, E. H., Must, A., Spadano, J. L., Laird, N., Dietz, W. H., Rimm, E., Colditz, G. A., Impact of overweight on the risk of developing common chronic diseases during a 10-year period. *Archives of Internal Medicine* 2001, *161*, 1581-1586.
[332] Katzmarzyk, P. T., Reeder, B. A., Elliott, S., Joffres, M. R., Pahwa, P., Raine, K. D., Kirkland, S. A., Paradis, G., Body mass index and risk of cardiovascular disease, cancer and all-cause mortality. *Canadian Journal of Public Health/Revue Canadienne de Sante'e Publique* 2012, 147-151.
[333] Larsson, S. C., Wolk, A., Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. *The American Journal of Clinical Nutrition* 2007, *86*, 556-565.
[334] Kay, C. D., Kroon, P. A., Cassidy, A., The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. *Molecular Nutrition & Food Research* 2009, *53*, S92-S101.
[335] Duda-Chodak, A., Tarko, T., Satora, P., Sroka, P., Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. *European Journal of Nutrition* 2015, *54*, 325-341.
[336] Stevens, J. F., Maier, C. S., The chemistry of gut microbial metabolism of polyphenols. *Phytochemistry Reviews* 2016, *15*, 425-444.
[337] Xiao, J., Högger, P., Stability of dietary polyphenols under the cell culture conditions: avoiding erroneous conclusions. *Journal of Agricultural and Food Chemistry* 2015, *63*, 1547-1557.
[338] Maini, S., Hodgson, H. L., Krol, E. S., The UVA and aqueous stability of flavonoids is dependent on B-ring substitution. *Journal of Agricultural and Food Chemistry* 2012, *60*, 6966-6976.
[339] Sadilova, E., Stintzing, F., Carle, R., Thermal degradation of acylated and nonacylated anthocyanins. *Journal of Food Science* 2006, *71*, C504-C512.
[340] Aura, A.-M., Martin-Lopez, P., O'Leary, K. A., Williamson, G., Oksman-Caldentey, K.-M., Poutanen, K., Santos-Buelga, C., In vitro metabolism of anthocyanins by human gut microflora. *European Journal of Nutrition* 2005, *44*, 133-142.
[341] Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F., Gallo, A., Scalfi, L., Fogliano, V., Protocatechuic acid is the major human metabolite of cyanidin-glucosides. *The Journal of Nutrition* 2007, *137*, 2043-2048.
[342] Mendes, A., Desgranges, C., Chèze, C., Vercauteren, J., Freslon, J. L., Vasorelaxant effects of grape polyphenols in rat isolated aorta. Possible involvement of a purinergic pathway. *Fundamental and Clinical Pharmacology* 2003, *17*, 673-681.

[343] Kamata, K., Makino, A., Kanie, N., Oda, S.-i., Matsumoto, T., Kobayashi, T., Kikuchi, T., Nishimura, M., Honda, T., Effects of anthocyanidin derivative (HK-008) on relaxation in rat perfused mesenterial bed. *Journal of Smooth Muscle Research* 2006, *42*, 75-88.
[344] Andriambeloson, E., Magnier, C., Haan-Archipoff, G., Lobstein, A., Anton, R., Beretz, A., Stoclet, J. C., Andriantsitohaina, R., Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *Journal of Nutrition* 1998, *128*, 2324-2333.
[345] Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., Plumb, G. W., Morgan, M. R., Williamson, G., Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters* 2000, *468*, 166-170.
[346] Kay, C. D., Mazza, G., Holub, B. J., Wang, J., Anthocyanin metabolites in human urine and serum. *British Journal of Nutrition* 2004, *91*, 933-942.
[347] Liu, W.-H., Lin, C.-C., Wang, Z.-H., Mong, M.-C., Yin, M.-C., Effects of protocatechuic acid on trans fat induced hepatic steatosis in mice. *Journal of Agricultural and Food Chemistry* 2010, *58*, 10247-10252.
[348] Liao, C. C., Ou, T. T., Huang, H. P., Wang, C. J., The inhibition of oleic acid induced hepatic lipogenesis and the promotion of lipolysis by caffeic acid via up-regulation of AMP-activated kinase. *Journal of the Science of Food and Agriculture* 2014, *94*, 1154-1162.
[349] Kim, K.-N., Kang, M.-C., Kang, N., Kim, S.-Y., Hyun, C.-G., Roh, S. W., Ko, E.-Y., Cho, K., Jung, W.-K., Ahn, G., 2, 4, 6-Trihydroxybenzaldehyde, a potential anti-obesity treatment, suppressed adipocyte differentiation in 3T3-L1 cells and fat accumulation induced by high-fat diet in C57BL/6 mice. *Environmental Toxicology and Pharmacology* 2015, *39*, 962-968.
[350] Sroka, Z., Cisowski, W., Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology* 2003, *41*, 753-758.
[351] Guan, S., Jiang, B., Bao, Y., An, L., Protocatechuic acid suppresses MPP+-induced mitochondrial dysfunction and apoptotic cell death in PC12 cells. *Food and Chemical Toxicology* 2006, *44*, 1659-1666.
[352] Lee, K.-M., Kang, H.-S., Yun, C.-H., Kwak, H.-S., Potential in vitro protective effect of quercetin, catechin, caffeic acid and phytic acid against ethanol-induced oxidative stress in SK-Hep-1 cells. *Biomol Ther* 2012, *20*, 492-498.
[353] Ren, T., Huang, C., Cheng, M., Dietary blueberry and bifidobacteria attenuate nonalcoholic fatty liver disease in rats by affecting SIRT1-mediated signaling pathway. *Oxidative Medicine and Cellular Longevity* 2014, *2014*.
[354] Benn, T., Kim, B., Park, Y.-K., Wegner, C. J., Harness, E., Nam, T.-G., Kim, D.-O., Lee, J. S., Lee, J.-Y., Polyphenol-rich blackcurrant extract prevents inflammation in diet-induced obese mice. *The Journal of Nutritional Biochemistry* 2014, *25*, 1019-1025.
[355] Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., Ory, D. S., Schaffer, J. E., Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *Journal of Lipid Research* 2006, *47*, 2726-2737.
[356] Wei, Y., Wang, D., Topczewski, F., Pagliassotti, M. J., Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *American Journal of Physiology-Endocrinology And Metabolism* 2006, *291*, E275-E281.
[357] Erbay, E., Babaev, V. R., Mayers, J. R., Makowski, L., Charles, K. N., Snitow, M. E., Fazio, S., Wiest, M. M., Watkins, S. M., Linton, M. F., Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. *Nature Medicine* 2009, *15*, 1383-1391.

[358] Lee, J. N., Kim, H., Yao, H., Chen, Y., Weng, K., Ye, J., Identification of Ubxd8 protein as a sensor for unsaturated fatty acids and regulator of triglyceride synthesis. *Proceedings of the National Academy of Sciences* 2010, *107*, 21424-21429.
[359] Fu, S., Watkins, S. M., Hotamisligil, G. S., The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metabolism* 2012, *15*, 623-634.
[360] Wei, Y., Wang, D., Gentile, C. L., Pagliassotti, M. J., Reduced endoplasmic reticulum luminal calcium links saturated fatty acid-mediated endoplasmic reticulum stress and cell death in liver cells. *Molecular and Cellular Biochemistry* 2009, *331*, 31.
[361] Gómez-Lechón, M. J., Donato, M. T., Martínez-Romero, A., Jiménez, N., Castell, J. V., O'Connor, J.-E., A human hepatocellular in vitro model to investigate steatosis. *Chemico-Biological Interactions* 2007, *165*, 106-116.