BERRY POLYPHENOL ABSORPTION AND THE EFFECT OF NORTHERN BERRIES ON METABOLISM, ECTOPIC FAT ACCUMULATION, AND ASSOCIATED DISEASES

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"Fear less, hope more;
eat less, chew more;
whine less, breathe more;
talk less, say more;
hate less, love more;
and all good things are yours."

To my family

CONTENTS

ABSTRACT

The prevalence of obesity and type 2 diabetes has increased at an alarming rate in developed countries. It seems in the light of current knowledge that metabolic syndrome may not develop at all without NAFLD, and NAFLD is estimated to be as common as metabolic syndrome in western population (23 % occurrence). Fat in the liver is called ectopic fat, which is triacylglycerols within the cells of non-adipose tissue. Serum alanine aminotransferase (ALT) values correlate positively with liver fat proportions, and increased activity of ALT predicts type 2 diabetes independently from obesity. Berries, high in natural bioactive compounds, have indicated the potential to reduce the risk of obesity-related diseases. Ectopic fat induces common endocrine excretion of adipose tissue resulting in the overproduction of inflammatory markers, which further induce insulin resistance by multiple mechanisms. Insulin resistance inducing hyperinsulinemia and lipolysis in adipocytes increases the concentration of free fatty acids and consequently causes further fat accumulation in hepatocytes. Polyphenolic fractions of berries have been shown to reverse inflammatory reaction cascades in *in vitro* and animal studies, and moreover to decrease ectopic fat accumulation.

The aim of this thesis was to explore the role of northern berries in obesity-related diseases. The absorption and metabolism of selected berry polyphenols, flavonol glycosides and anthocyanins, was investigated in humans, and metabolites of the studied compounds were identified in plasma and urine samples (I, II). Further, the effects of berries on the risk factors of metabolic syndrome were studied in clinical intervention trials (III, IV), and the different fractions of sea buckthorn berry were tested for their ability to reduce postprandial glycemia and insulinemia after high-glucose meal in a postprandial study with humans (V).

The marked impact of mixed berries on plasma ALT values (III), as well as indications of the positive effects of sea buckthorn, its fractions and bilberry on omental adiposity and adhesion molecules (IV) were observed. In study V, sea buckthorn and its polyphenol fractions had a promising effect on potprandial metabolism after high-glucose meal. In the literature review, the possible mechanisms behind the observed effects have been discussed with a special emphasis on ectopic fat accumulation. The literature review indicated that especially tannins and flavonoids have shown potential in suppressing diverse reaction cascades related to systemic inflammation, ectopic fat accumulation and insulin resistance development.

LIST OF ABBREVIATIONS

ACC	acetyl-CoA (coenzyme A) carboxylase
ACO	acyl-CoA (coenzyme A) oxidase
ALT	alanine amino transferase
AMPK	adenosine monophosphate activated protein kinases
AST	aspartate amino transferase
CaMKKβ	calcium^{2+}/calmodulin-dependent protein kinase kinase β
CPT-1	carnitine palmityl transferase-1
CRP	c-reactive protein
CVD	cardiovascular diseases
EGCG	epigallocatechin gallate
FA	fatty acid
FAS	fatty acid synthase
GLUT4	glucose transporter 4
ICAM	intercellular adhesion molecule
IKK	IκB kinase
IL	interleukin
(Jak)/STAT	Janus kinase/ signal transducer and activator of transcription
JNK	c-Jun N-terminal kinases
HDL	high density lipoprotein
LDL	low density lipoprotein
LKB1	serine threonine liver kinase B1 (tumor suppressor)
MCP-1	monocyte chemotactic protein-1
meal A	control meal in study V
meal B1	meal in trial V; whole sea buckthorn berry
meal B2	meal in trial V; berry residue extracted by SF-CO_2
meal B3	meal in trial V; berry residue extracted by ethanol after SF-CO_2
meal BB	meal in trial IV; bilberry meal
meal SB	meal in trial IV; sea buckthorn
meal SBe	meal in trial IV; sea buckthorn extract
meal SBo	meal in trial IV; sea buckthorn oil
Mo25	mouse embryo scaffold protein

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatosis
NF-κB	nuclear factor kappa B
NO	nitric oxide
PAI-1	plasminogen activator inhibitor
PAL	phenylalanine ammonia lyase
PGC-1α	prostaglandin 1α
PUFA	polyunsaturated fatty acid
SF-CO_2	supercritical fluid carbon dioxide
sCRP	sensitive C-reactive protein
SIRT1	silent information regulator T1
SREBP	sterol regulatory element binding proteins
STAT-3	signal transducer and activator of transcription-3
STRAD	STE20-related adaptor protein
T2D	type 2 diabetes
TAG	triacylglycerol
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor α
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by roman numerals I-V:

I Lehtonen HM, Rantala M, Suomela JP, Viitanen M, Kallio H. Urinary excretion of the main anthocyanin of lingonberry (Vaccinium vitis-ideae), cyanidin-3-O-galactoside, and its metabolites. *J Agric Food Chem* 2009;57(10):4447–4451

II Lehtonen HM, Lehtinen O, Suomela JP, Viitanen M, Kallio H. Flavonol Glycosides of Sea Buckthorn (Hippophaë rhamnoides ssp. sinensis) and Lingonberry (Vaccinium vitis-idaea) Are Bioavailable in Humans and Monoglucuronidated for Excretion. *J Agric Food Chem* 2010;58(1):620–627

III Lehtonen HM, Suomela JP, Tahvonen R, Vaarno J, Venojärvi M, Viikari J, Kallio H. Berry meals and risk factors associated with metabolic syndrome. *Eur J Clin Nutr* 2010;64:614-621

IV Lehtonen HM, Suomela JP, Tahvonen R, Yang B, Venojärvi M, Viikari J, Kallio H. Different berries and berry fractions have various but slightly positive effects on the variables associated to metabolic diseases in overweight and obese women. *Eur J Clin Nutr* (2010) (in press)

V Lehtonen HM, Järvinen R, Linderborg K, Viitanen M, Venojärvi M, Alanko H, Kallio H. Postprandial hyperglycemia and insulin response are affected by sea buckthorn (Hippophaë rhamnoides) berry and its ethanol-soluble metabolites. *Eur J Clin Nutr* (2010) (Epub ahead of print doi: 10.1038/ejcn.2010.173)

1. INTRODUCTION

About 70-80 % of type 2 diabetics have non-alcoholic fatty liver disease (NAFLD) (Targher et al. 2007), and those obese subjects who tend to accumulate abdominal fat have an increased risk of cardiovascular diseases (Lakka et al. 2002). Normally other tissues than adipose tissue contain only small amounts of fat. Ectopic fat is defined by the deposition of triglycerides within cells of non-adipose tissue that normally contain only small amounts of fat, i.e. liver and other internal organs. Ectopic fat accumulation in the liver and skeletal muscles in humans is a critical determinant of insulin resistance and may predispose to the development of type 2 diabetes. NAFLD can thus be regarded as the hepatic manifestation of metabolic syndrome. This phenomenon can be explained by the impaired insulin sensitivity of a fatty liver, which results in higher glucose (Ryysy et al. 2000, Marchesini et al. 2001, Seppälä-Lindroos et al. 2002), higher very low density lipoprotein (VLDL) production (Adiels et al. 2006, 2007) in the liver, and thus insulin resistance. Serum alanine aminotransferase (ALT) values correlate positively with liver fat proportions, and the increased activity of ALT predicts type 2 diabetes independently from obesity (Vozarova et al. 2002, Hanley et al. 2004, Sattar et al. 2004).

Berries are known to contain high amounts of vitamins, phenolic compounds, trace elements, and fiber, as well as a beneficial fatty acid (FA) composition (Kalt et al. 1999). Some indications of the capability of berries to reduce the risk of certain diseases have lately emerged. Berry polyphenols form a group of compounds with a high potential to affect metabolic diseases and their risk factors. The absorption and metabolism of certain berry polyphenols was investigated in the original research in this thesis from human plasma and urine (I, II).

Northern berries have been implicated in the reduction of risk factors associated with various obesity-related diseases from emerging metabolic syndrome to cardiovascular events (Johansson et al. 2000, Marniemi et al. 2000, Erlund et al. 2008). Mechanisms of action include the modulation of signalling pathways as well as antioxidative properties, although knowledge of the exact metabolic and inflammatory routes involved is only currently emerging. As part of the original research of this thesis three separate human trials were conducted to reveal the effects of berries on ectopic fat accumulation and the risk of diseases related to obesity. First, the effects of the daily consumption of black currant (*Ribes nigrum*), sea buckthorn (*Hippophaë rhamnoides*), bilberry (*Vaccinium myrtillus*), and lingonberry (*Vaccinium vitis-idae*) products on the risk factors associated with obesity-related diseases such as NAFLD were investigated (III). Further, in study IV, sea buckthorn berry, its fractions, and bilberry were studied alone as separate intervention periods to find out if there were differences between berries and different parts of the sea buckthorn berry. Also, sea buckthorn berry and its polyphenol-rich fraction were studied in a postprandial trial where a high-glucose meal was consumed with and without the berry (V). However, clinical trials conducted with whole berry products do not give an insight into which compounds or compound groups might be behind the observed effects. The complicated cascade from increased ectopic fat to hepatic steatosis

intertwined with various inflammatory and lipotoxic mechanisms present various potential action sites for berry polyphenols.

A liver with high fat content also overproduces inflammatory markers such as sensitive C-reactive protein (CRP), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) (Greco et al. 2008). Berries seem to contain some anti-inflammatory compounds, as sea buckthorn (*Hippophae rhamnoides* var. Ljubitelskaja) has been shown to lower the sensitive C-reactive protein (hs-CRP) in a human trial (Larmo et al. 2007). Genes involved in inflammation are upregulated in subjects with a high fat content in the liver, and inflammation in subcutaneous adipose tissue is correlated with liver fat content independently of obesity. On the other hand, the polymorphism of several candidate genes have been associated with NAFLD.

A net retention of lipids within hepatocytes is a prerequisite for the development of NAFLD. Visceral fat induces common endocrine excretion of adipose tissue (Mohamed-Ali et al. 1998), inducing the secretion of TNF-α, IL-6, plasminogen activator inhibitor-1, leptin, and angiotensinogen (Lakka et al. 2002), which further provokes insulin resistance by multiple mechanisms (Cohen et al. 1996). Insulin resistance induces hyperinsulinemia and lipolysis in adipocytes. The increased concentration of free FAs consequently causes further fat accumulation in hepatocytes (Angulo, 2002). Polyphenolic fractions of berries have been shown to reverse inflammatory reaction cascades *in vitro* and in animal studies. Especially tannins have shown potential in suppressing inflammation (Hou et al. 2007, Hwang et al. 2009).

In the literature review of this thesis the key metabolic dysfunctions leading to ectopic fat accumulation in overweight humans and obesity are described. Also, the effects of polyphenolic constituents found in northern berries on the detrimental impacts of a western lifestyle are reviewed.

2. REVIEW OF THE LITERATURE

2.1. STRUCTURES AND OCCURRENCE OF BERRY PHENOLICS

Berries are the small globular or ovate juicy fruits of herbs, shrubs or trees. Generally, berries are high in minerals and vitamins known to be vital for humans. Also, berries contain a wide spectrum of other compounds that have biological functions and affect human health. When the term berry bioactives is defined broadly it contains a very wide spectrum of different compound groups such as lipid compounds found in the seed and pulp oils of berries, polyphenols, sugars and acids, heteropolysaccharides commonly referred as insoluble fiber, cutins from the skin of the berries and soluble fiber like pectins. In this literature review the polyphenols of the berries are presented (chapter 2.1), and their effect on ectopic fat accumulation is discussed (chapter 2.3).

The phenolic compounds act as protecting agents, and often condense in the cuticular surface layers of the plant. Berry polyphenols include flavonoids (anthocyanins, flavonols, and flavan-3-ols), phenolic acids, stilbenoids, and tannins (condensed tannins and hydrolysable tannins). Phenolic compounds are important pigments in berries attracting birds and other animals involved in the pollination and seed dispersion (Faulds and Williamson 1999), and they are also involved in organoleptic properties of edible berries (Strack 1997). As phenolic compounds are part of the stress defence mechanism, their levels respond to environmental stress factors such as UV light (Ryan et al. 2001), and heavy metal toxicity (Kidd et al. 2001). Especially the flavonoid subclass seems to also protect leafs against photo-oxidative damage during senescence in the autumn (Felld et al. 2001).

Polyphenols are a wide range of secondary metabolites synthesized through the shikimate pathway from the same intermediate, phenylalanine, or its precursor, shikimic acid (Harborne 1989). The shikimic acid pathway begins with the condensation of phosphoenolpyruvate from glycolysis and erythrose 4-phosphate from the pentosephosphate pathway, and leads to the formation of the aromatic amino acids phenylalanine and tyrosine. Phenylalanine is the substrate for phenylalanine ammonia-lyase (PAL). Most phenolic compounds, such as phenylpropanoids are synthesized by the phenylpropanoid pathway, the first reaction of which the deamination of phenylalanine to cinnamic acid is catalyzed by PAL. The diversity of phenylpropanoids is produced from different branches of the central pathway converting the cinnamic acid by hydroxylation and esterification to 4-coumaric acid:CoA (Hahlbrock and Scheel 1989) which reacts with malonyl-CoA forming tetrahydroxychalcone, a precursor of diverse flavonoids. In addition, the complexity of phenolic structures arises from different modifications of the basic structures such as hydroxylation, methylation, and glycosylation. Gallic acid, galloylglucoses, gallotannins, and ellagitannins are synthesized from 3-dehydroshikimate (Ossipov et al. 2003). **Figure 1** shows the proposed biosynthetic relationships of the most common phenolic classes.

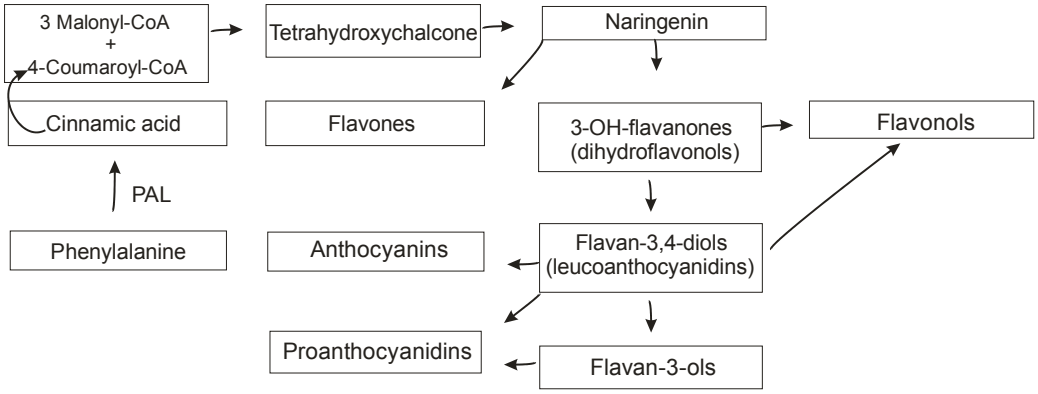

Figure 1: Biosynthesis of common phenolic groups. The phenylpropanoid pathway begins from the conversion of phenylalanine to cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL) and branches to the diverse polyphenol groups. Adapted from Winkel-Shirley et al. 2002.

2.1.1. Flavonoids

The basic structure of flavonoids is phenyl-benzo-γ-pyran, in which two aromatic rings are combined by a three-carbon chain. The synthesis of flavonoids is presented in **Figure 1**, and reviewed by Winkel-Shirley (2002). Flavonoids are derived in a plant from a chalcone structure, which is first converted to flavanones, dihydroflavonols, and flavan-3,4-diols. Further, flavan-3,4-diols are converted to flavonoid subclasses, namely flavonols, flavan-3-ols, proanthocyanidins, and anthocyanidins. Flavonols and flavan-3-ols are abundantly distributed among different branches of the plant kingdom. Anthocyanins are the red and blue pigments of plants, found abundantly in red and blue berries such as chokeberry (*Aronia melanocarpa*), bilberry (*Vaccinium myrtillus*), honeyberry (*Lonicera kamchatka*), black currant (*Ribes nigrum*), and blackberry (*Rubus fruticosus*) (Häkkinen and Törrönen 2000, Buchert et al. 2005). The basic structures of monomeric flavonoids are presented in **Figure 2**. Proanthocyanidins are discussed below in chapter 2.1.3.

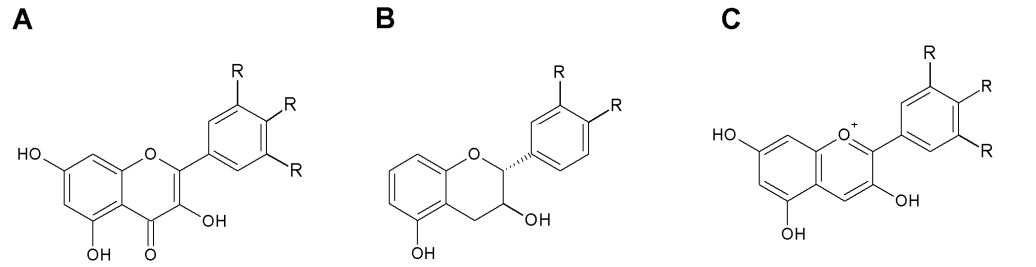

Figure 2: The basic structure of monomeric flavonoids. A=flavonol, B=flavan-3-ol, and C=anthocyanidin. R=OH, CH_3.

2.1.2. Phenolic acids and hydrolysable tannins

Phenolic acids can be divided into two groups, the derivatives of benzoic acid and the derivatives of cinnamic acid. Hydroxybenzoic acids are the constituents of hydrolysable tannins, the polymers of phenolic acids and simple sugars. Hydroxycinnamic acids exist as glycosylated and esterified forms (Manach et al. 2004). A hydroxycinnamic acid ferulic acid **(Fig. 3A)** is the most common phenolic acid in bilberry, chokeberry, and rowan berry (Häkkinen et al. 1999). Gallic acid **(Fig. 3B)** is a trihydroxylated benzoic acid which participates in the formation of hydrolysable tannins. Hydrolysable tannins are complex polymeric structures called gallotannins or ellagitannins **(Fig. 3C)** that hydrolyze into sugars, and gallic acid or ellagic acid (Bate-Smith 1972, Lei et al. 2001). Gallotannins are not universally distributed in higher plants, but occur in certain clearly defined taxonomic groups in both woody and herbaceous dicotyledons. On the other hand, ellagitannins are widely distributed in the lower taxons Hamamelidae, Dilleniidae, and Rosidae (Haslam, 1989). *Rubus* berries are the best known sources of ellagitannins (Serrano et al. 2009), but they are also found in nuts and many fruits such as pomegranate (de Pascual-Teresa et al. 2000).

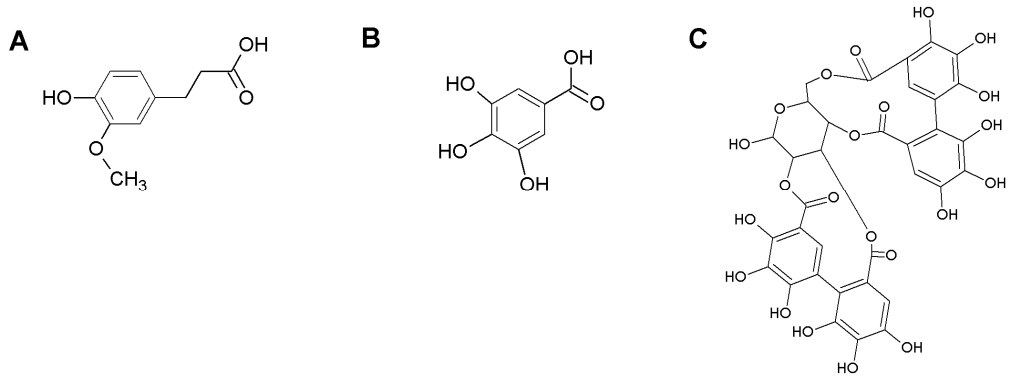

Figure 3: Structures of ferulic acid (A), gallic acid (B), and ellagitannin pedunculagin (C).

2.1.3. Proanthocyanidins (condensed tannins)

Plants synthesize proanthocyanidins from flavan-3-ols (Marles et al. 2003, Xie et al. 2004). Proanthocyanidins are polyhydroxyflavan oligomers or polymers. The structure of a simple trimeric prodelphinidin is presented in **Figure 4**. The monomeric flavanols differ in their hydroxylation pattern in ring A and B as well as in the stereochemistry of C-3. Proanthocyanidin classification is based on chemical structure and hydrolytically obtained anthocyanin monomer. Proanthocyanidins are believed to be ubiquitous, and it has been suggested that they account for a significant fraction of the polyphenols ingested in the western diet (Serrano et al. 2009). Fruits and berries are the best sources, but legumes, nuts and minority cereals also contain quite high amounts of proanthocyanidins. Proanthocyanidins tend to concentrate in the peel of fruits or the bran of grains, and the degree of polymerization being higher in the peel (Serrano et al. 2009).

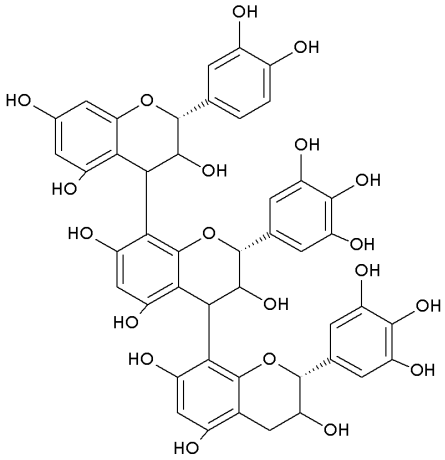

Figure 4: Structure of a simple trimeric prodelphinidin.

2.1.4. Stilbenes

Stilbenes are hydroxylated trans-1,2-diphenylethylenes. The non-flavonoid polyphenolic structure is related to the synthetic estrogen diethylstilbestrol. The most widely investigated stilbene is resveratrol (**Fig. 5**), which is found in red wine, peanuts, and grapes (Alarćon de la Lastra and Villegas 2005), and is also present in small amounts in cranberry and bilberry (Manach et al. 2004). Resveratrol is mainly concentarted in the skin (Jeandet et al. 1991), and thus the content of resveratrol in wine depends on the contact time between the berry skin and the juice.

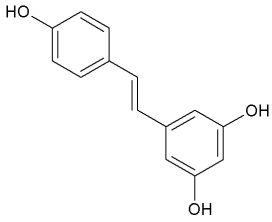

Figure 5: Structure of the most common stilbene, resveratrol.

2.1.5. Lignans

Lignans are one of the two major groups of phytoestrogens, the other being isoflavonoids. Sesamin is the precursor structure for lignans such as matairesinol and secoisolariciresinol occurring in plants. Two enterolignans of mammals, enterodiol and enterolactone, are probably formed from plant lignans in the large bowel as rat studies have indicated (Penalvo et al. 2005a). Plant lignans in foods are mainly glycosides, and found especially in whole-grains, beans, flaxseed, sesame seed, vegetables, berries, and some other fruits (Milder et al.

2005, Penalvo et al. 2005a and b, Thompson et al. 2006). The structure of matairesinol is presented in **Figure 6**.

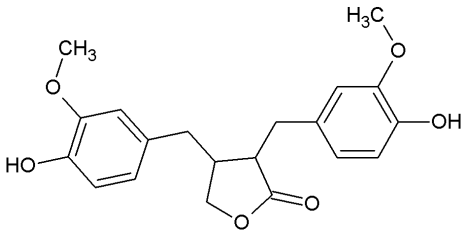

Figure 6: The structure of matairesinol.

2.2. DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE

2.2.1. Diagnostics of nafld

The detection of NAFLD, liver fat content, possible histological changes related to NAFLD and disease activity is somewhat problematic, as the only equivocal means of diagnosis is liver tissue evaluation from biopsy. Laboratory tests such as alanine aminotransferase (ALT) and aspartate amino transferase (AST) are problematic due to the interindividual differences in relation to ALT/AST levels and fibrosis and cirrhosis progression (Neuschwander-Tetri et al 2008). In addition, the upper limit of normal ALT values has been impugned recently (Greenfield et al. 2008). Imaging techniques indicate only the fat content of the liver, and do not detect disease activity. Ultrasound evaluation is accurate when >33 % of the liver is already affected (Saadeh et al. 2002), and computerised tomography and ^{1}H-NMR (proton nuclear magnetic resonance) spectroscopy are not suitable for population screening due to radiation exposure or high cost. Thus, liver markers ALT and AST are currently by far the most commonly used of these methods. ALT values correlate positively with liver fat proportions (Greco et al. 2008), although the development of NAFLD cannot be excluded on the basis of normal ALT values. However, in persons with normal ALT values, even within the reference range, the level of the enzyme is higher in individuals with higher BMI.

2.2.2. Ectopic fat accumulation in obesity

FAs are synthesized mainly in liver from acetyl-CoA, althought most tissues can assemble triacylglycerols from acyl-CoAs and glycerol-3-phosphate. FAs are converted back to acetyl-CoA by β-oxidation and used as fuel, and the synthesis and breakdown is regulated to meet the cellular energy needs.

The traditional perception of adipose tissue as a mere storage place for FAs has been replaced over the past years (Hajer et al. 2008). In addition to its role in insulating and cushioning the body and storing free fatty acids (FFA) after food intake and releasing FFAs during the fasting, the adipose tissue produces a wide range of hormones and cytokines involved in glucose metabolism, lipid metabolism, and feeding behaviour (Frühbeck et al. 2001) as well as in inflammation, coagulation, and blood pressure (Trayhurn and Beattie, 2001).

When caloric intake is equal to caloric expenditure, the liporegulatory system is at rest, and the lean tissues contain little or no unmetabolized lipids. A prolonged positive energy balance promotes an increased mass of adipose tissue. Initially, adipose tissue grows primarily through increases in cell size (adipocyte hypertrophy) and subsequently cell number (adipocyte hyperplasia) (Rosen and MacDougall, 2006). During hyperplasia, adipocytes increase leptin secretion. These are antiobesity hormones that enhance lean tissue oxidation of surplus lipids by activating AMP-activated protein kinase and reducing the activity and expression of lipogenic enzymes (Unger et al. 2003a). These events combine to lower the hepatic concentration of malonyl coenzyme A, a metabolite of the FA synthesis and powerful inhibitor of carnitine palmityl transferase-1 (CPT-1)-mediated fatty acid β-oxidation (McGarry et al. 1977). Leptin also reduces the expression of lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and glycerol-3-phosphate acyl transferase (Zhou et al. 1997). The combination of an increase in FA β-oxidation and a decrease in FA synthesis could account for the reduction in the lipid content of the cell (Unger et al. 2003a). The biochemistry of a normal liporegulation during overnutrition is presented in **Figure 7**.

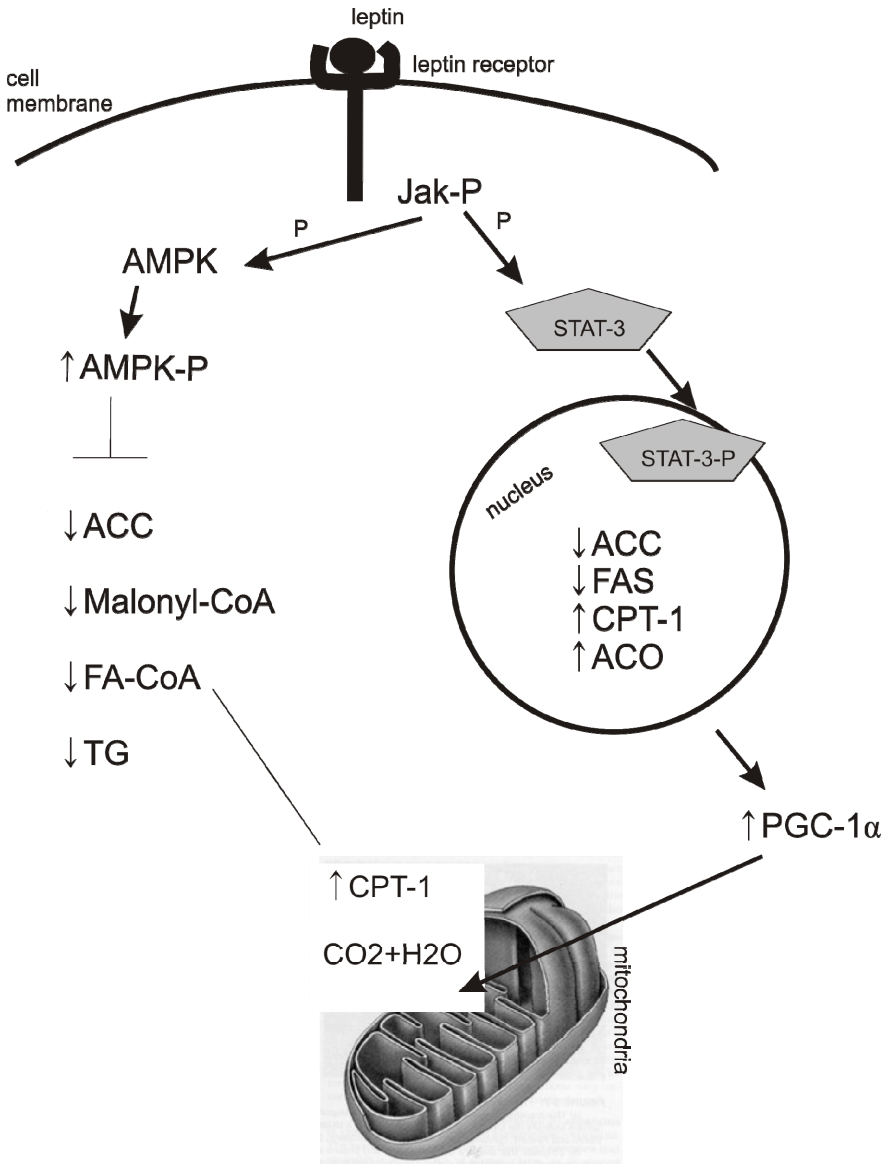

Figure 7: The biochemistry of a normal liporegulation during overnutrition. Normally during overnutrition, increased leptin in the plasma maintains FA oxidation at an appropriate level, thus preventing lipotoxicity. The binding of leptin to its receptor initiates a phosphorylation cascade *via* the Janus kinase (Jak)/STAT-pathway. Phosphorylated STAT-3 enters the nucleus and regulates the transcriptional activity of its target genes. Its effects include the up-regulation of PGC-1α, which is involved in mitochondrial biogenesis, and the enzymes of FA oxidation, CPT-1 and ACO. It also down-regulates lipogenic enzymes, such as ACC and FAS. Another important action of leptin is to phosphorylate AMPK, which activates it. AMPK phosphorylates ACC, which blocks malonyl coenzyme A formation, thus maintaining FA oxidation at a suitable level to prevent lipotoxic effects. (Adapted from Unger 2003a).

ACO = acetyl-CoA oxidase, ACC = acetyl-CoA carboxylase, AMPK = adenosine monophosphate kinases, CPT-1 = carnitine palmityl transferase-1, FAS = fatty acid synthase, (Jak)/STAT-pathway = Janus kinase/ signal transducer and activator of the transcription pathway, STAT-3 = signal transducer and activator of transcription-3, PGC-1α = prostaglandin 1α.

However, ultimately the failure of adipocytes to appropriately buffer the plasma FA concentration can result in disturbances in the otherwise tightly regulated lipid content of non-adipocyte cells (Hill et al. 2009). Undoubtedly, adipocytes have evolved with the potential to store a significant quantity of TAG's, because storage in non-specialist cells was not tenable due to the unavoidable pathogenic consequences (Unger et al. 2003a).

As well as accumulation of lipid in non-adipocytes, obesity can lead to an increase in the volume of non-classical adipose depots, and to adipocyte infiltration into other tissues (Hill et al. 2009). Ectopic fat storage includes both intraabdominal visceral, omental, perivascular, pericardial, perirenal, and retroperitoneal adipose tissue expansions as well as intracellular (liver, skeletal, muscle, heart, pancreas, kidney, and other organs) fat deposition (Blüher, 2009).

Leptin deficiency- or dysfunction-induced abnormal liporegulation leads to ectopic fat accumulation as shown in **Figure 8**. There are remarkable interindividual differences on how fast the deterioration of liporegulation occurs during overnutrition. As leptin has a major regulatory role, defects in leptin signalling such as aleptinemic disorders, relative hypoleptinemia or leptin unresponsiveness give an early push towards lipotoxicity (Unger et al. 2003a). Adiponectin is a relatively lately identified secretory protein belonging to the collectin family and expressed by fat cells (Scherer et al. 1995). The expression of adiponectin is reduced in obese mice and humans. Adiponectin exists in the bloodstream as high-molecular weight-, middle-molecular weight-, and low-molecular weight forms, of which high-molecular weight oligomers have been shown to strongly negatively correlate with ectopic fat, thus possibly representing the form of adiponectin regulating lipid β-oxidation in liver and skeletal muscles (Kantartzis et al. 2009).

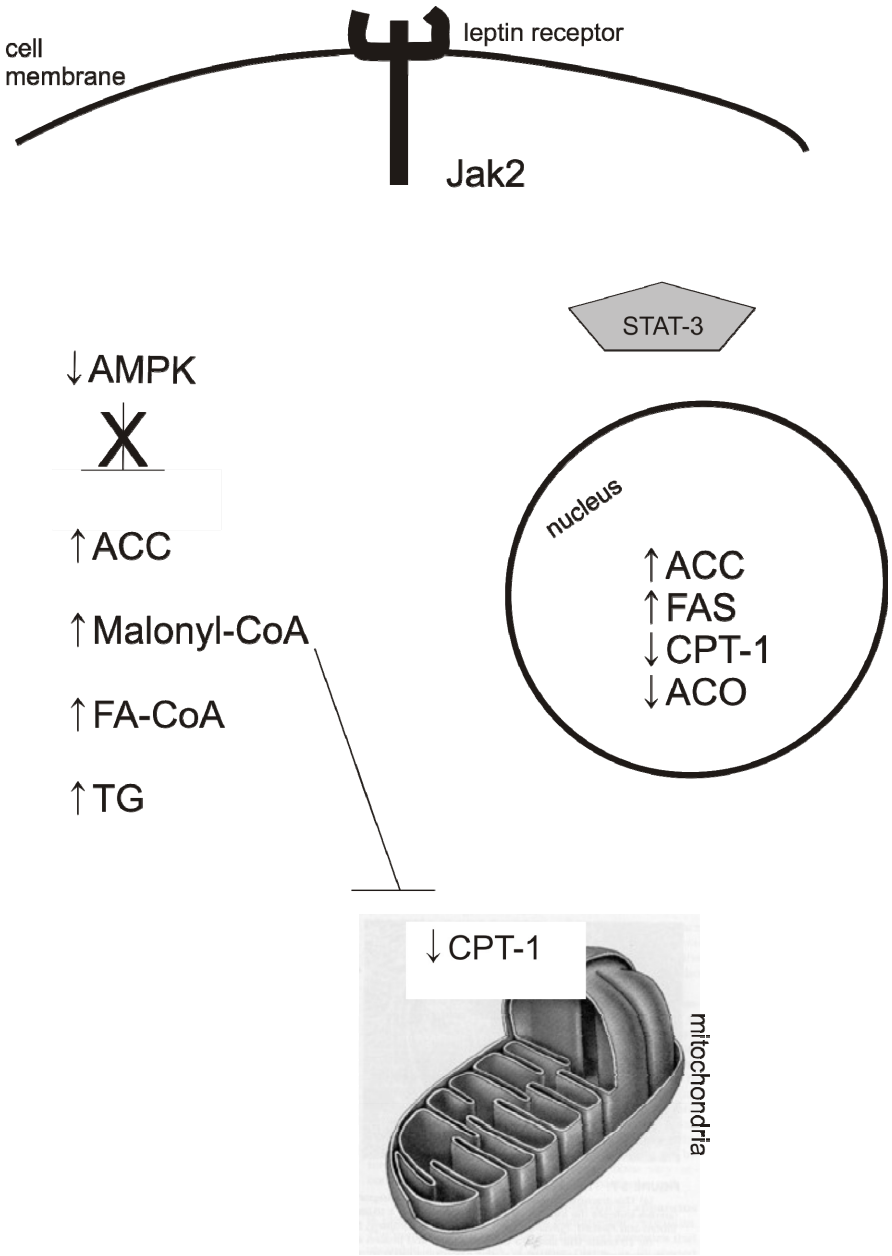

Figure 8: The biochemistry of abnormal liporegulation during overnutrition. When leptin action is lacking, the Jak/STAT pathway is not activated during chronic overnutrition. The high level of ACC expression and activity generates malonyl CoA, the lipogenic precursor and inhibitor of FA oxidation. More fatty acids and triacylglycerols are synthesized and less are oxidized, raising the triacylglycerols fatty acyl CoA content of lean tissues. (Adapted from Unger et al. 2003a).

ACO = acetyl-CoA oxidase, ACC = acetyl-CoA carboxylase, AMPK = adenosine monophosphate kinases, CPT-1 = carnitine palmityl transferase-1, FA = fatty acid, FAS = fatty acid synthase, (Jak)/STAT-pathway = Janus kinase/ signal transducer and activator of the transcription pathway, STAT-3 = signal transducer and activator of transcription-3.

In the normal hepatocyte, energy received from the circulation of glucose, fructose, and lipids is stored as glycogen, and increased lipids are redistributed to peripheral tissues for combustion and storage in adipocytes (Anderson et al. 2008), and the hepatocellular lipid content remains below 5% (Leevy et al. 1962). When the excessive lipid overload in adipocytes leads to metabolic incompetence and macrophage infiltration (Anderson et al. 2008), in hepatocytes the consequences are organelle failure including mitochondrial dysfunction and endoplasmic reticulum (ER) and other organelle stress, as well as hepatic insulin resistance (de Ferranto et al. 2008). In the liver lipotoxicity appears to be induced mainly through ceramide formation, which increases inducible nitric oxide synthase (iNOS) (Shimabukuro et al. 1997). However, it must be noted that the triacylglycerol content of the liver is simply a biomarker of increased hepatic exposure to potentially toxic free FAs, and the interconversion of FFA into more stable TG is in fact the cell's means of protection (Yamaguchi et al. 2007).

2.2.3. Postprandial metabolism and ectopic fat

2.2.3.1. Postprandial carbohydrate metabolism and ectopic fat accumulation

Hormonal regulation of the carbohydrate metabolism is intertwined with the fat and protein metabolism. Insulin, as well as glucagon, somatostatin, and pancreatic polypeptide, are secreted by islets of Langerhans in the pancreas. Insulin is an anabolic and glucagon a catabolic hormone reciprocally regulating the intermediary metabolism of energy nutrients (Ganong, 2003). The binding of insulin to its receptors in skeletal muscle and adipose cells normally triggers the tyrosine kinase activity of β subunit and further phosphorylation cascade which culminates into the translocation of the glucose transporter (GLUT4) from intracytoplasmic vesicles to the plasma membrane facilitating glucose absorption (Quon et al. 1994). Phosphorylation of some cytoplasmic proteins and dephosphorylation of others result also in various other tissue-specific and far-reaching effects of insulin. Insulin induces the expression of the enzymes of FA synthesis through upregulation of the lipogenic transcription factor, SREBP-1 (Foufelle and Ferré, 2002).

The number and the affinity of insulin receptors are affected by exercise, food, and other factors. The number of receptors is decreased in obesity. Defects in the insulin metabolism have extensive and serious consequences. Traditionally, it has been thought that widespread biochemical abnormalities in diabetes can be solely traced to the reduced entry of glucose into various peripheral tissues and the increased liberation of glucose into the circulation by the liver (Ganong, 2003). However, it has also been proposed that glucometabolic insulin resistance is secondary to the overaccumulation of lipids (Boden and Schulman, 2002), and that resistance to insulin-stimulated uptake of surplus glucose might even be a defence against lipid overaccumulation in lean tissues, as glucose is a potent substrate of *de novo* lipogenesis (Unger et al. 2003b). Also, metabolic disorders are related to chronic low-grade inflammation (Shoelson et al. 2006), which is further discussed in chapter 2.2.4.

Postprandial plasma glucose levels depend largely on the rate of hepatic glucose uptake. Thus postprandial glucose metabolism is more strongly intertwined with ectopic fat accumulation than fasting plasma glucose values. NAFLD has been shown to be related to the 2-h or 1-h post-challenge glucose levels but not to fasting plasma glucose (Shiga et al. 2009). Storage of triacylglycerols in the liver following i.e. overeating leads to fat deposition in the hepatocytes and reduces insulin sensitivity of hepatocytes. An insulin resistant liver results in postprandial hyperglycemia (Kawamori et al. 2000). The impairment of the rapid pulsatile secretion of insulin provokes postprandial hyperglycemia further, which then may bring about the delayed hyper-secretion of insulin from β-cells. Delayed hyperinsulinemia increases ectopic fat accumulation thus closing the vicious cycle of NALFD with impaired glucose tolerance.

It is undisputed that the type of food ingested affects postprandial sugar metabolism. It has been shown that diet rich in food items that result in a lower postprandial insulin response modulates the inflammation status (Kallio et al. 2008). Repeated high postprandial insulin response and early postprandial hyperglycemia increase oxidative stress (Ceriello et al. 2008), inflammation (Dandona et al. 2005), and possibly insulin resistance and β-cell dysfunction (Ludwig et al. 2002). High postprandial glycemia and insulinemia are also significant risk factors of cardiovascular diseases in patients with type 2 diabetes (Ginsberg et al. 2001). As Westerners spend most of their time in a postprandial state, it is important to focus on the metabolism of the fed state instead of fasting values only (Lairon et al. 2007).

Ectopic fat accumulation requires an excessive input of lipids in one way or another. Increased dietary intake of energy, either as fat or carbohydrate, results in ectopic fat accumulation, as the overall fat mass increases. Despite the undisputed role of energy intake, the types of energy nutrients also have a major effect. A high glycemic index (GI) diet impairs fat β-oxidation resulting in rapid-onset increase in body fat mass and liver fat in mice (Isken et al. 2009). The gene expression profile is also consistent with elevated lipogenesis, and after the long-term exposure of a high-GI diet glucose clearance is impaired (Isken et al. 2009). Other short and long-term animal studies have shown similar results (Kabir et al. 1998, van Schothorst et al. 2009), and some indications of deteriorated fat β-oxidation and enhanced lipogenesis during a high-GI diet in humans exist (Lê and Bortolotti 2008). Even ingestion of different simple sugars results in a different kind of postprandial metabolism and thus shunt lipids differently towards ectopic reserves. For example fructose has been shown to increase postprandial triacylglycerol concentrations, decrease storage efficiency in adipose tissue, and eventually alter regional distribution of triacylglycerols towards ectopic fat accumulation, whereas glucose does not seem to have such impacts (Hoffman and Tschöp, 2009). Stanhope and Havle (2008) have extensively reviewed the effects of fructose consumption on visceral obesity, and they conclude that a diet high in fructose produces an oversupply of lipids within the liver via increased de novo lipogenesis. They also note that while the liver triacylglycerols accumulation is associated with disrupted insulin signaling, the exact downstream steps require further research.

2.2.3.2. Postprandial fat metabolism and ectopic fat accumulation

Adipose tissue buffers the plasma free FA levels by avidly draining them from triacylglycerol-rich lipoproteins in the postprandial state and gently releasing them in the fasting state. Adipose tissue failure is the state when lipids begin to accumulate in non-adipose tissues also. Lacrausta et al. (2007) have proposed that an initial failure of the adipose tissue to buffer postprandial lipids results in the loss of the functional activities of the adipose tissue, i.e. an organ failure. Adipose tissue failure can be either secondary, as is the case in type 1 diabetes when the lack of insulin is responsible for the adipose tissue dysfunction (Goldberg et al. 2001, Schwab et al. 2006) or primary when the excess of demands for the adipose tissue cannot be met due to chronically high food intake (Lacrausta et al. 2007).

The type of fat ingested and other food components consumed, both on a short and long term basis, modify the postprandial lipemia after a particular meal. Lower postprandial lipemia is generally considered less atherogenic (Karpe 1999), and high postprandial lipemia probably stresses adipose tissue leading eventually to adipose tissue failure and ectopic fat accumulation. However, major controversies exist on how different types of dietary fat and carbohydrates affect postprandial lipemia. A low-fat and high-carbohydrate diet seems to deteriorate the blood lipid abnormalities while a high monounsaturated fat diet improves them (Jiménez-Gómez et al. 2010).

In a parallel group intervention study in rats a polyunsaturated fatty acid (PUFA) rich diet protected from the steatosis (Buettner et al. 2006). PUFA enhances lipid β-oxidation and decrease lipid synthesis by modifying the expression of genes related to lipid metabolism (Buettner et al. 2006). On the other side, *trans* fats, common fast foods, and baked goods, cause non-alcoholic steatohepatosis (NASH) in sedentary mice (Tetri et al. 2008). Also, polyunsaturated fat supplementation attenuated the adverse effects of low-fat and high-carbohydrate diet (Jiménez-Gómez et al. 2010).

2.2.4. Ectopic fat and inflammation

There are profound differences between visceral and subcutaneous adipocytes in the metabolism, as the expression of specific receptors, as well as the secretion of specific adipokines and many pro-inflammatory cytokines are predominantly secreted from visceral adipose tissue, whereas adiponectin is mainly expressed in subcutaneous fat (Motoshima et al. 2002, Blühner 2008). The morphology of adipocytes in adipose tissue as well as in visceral adipose tissue defines the adipokine secretion pattern of the cell. Large, lipid-laden adipocytes often present in obesity (Pausova et al. 2006) secrete CRP, IL-6, monocyte chemotactic protein-1 (MCP-1), and plasminogen activator protein-1 (PAI-1) at higher levels compared to fat mass-matched controls with smaller fat cells (Blühner 2008). It can thus be proposed that adipocyte growth is the first push towards adipocyte dysmetabolism. Large adipocytes and increased adipose tissue mass in obesity lead to hypoxia, unless adequate vascularization

supports the vital functions of oxygen and nutrient transport (Trayhurn and Woods, 2004). This may contribute to cell level stress, and further to macrophage infiltration and inflammation (Blühner 2009). Experimental hypoxia has been shown to stimulate secretion of inflammation-related adipokines, including angiopoietin-like protein 4, IL-6, and vascular endothelial growth factor (Trayhurn et al. 2008).

Large lipid-swollen adipocytes also suffer from other inflammation-related stresses in addition to hypoxia. Systemic oxidative stress characterizes the obese state (Rudich et al. 2007), and metabolic stress may result from, for example, incomplete folding of synthesized proteins in the endoplasmic reticulum (Blühner 2009). Cells respond to such intracellular or extracellular stresses by activating numerous stress-responsive signalling pathways (Blühner 2009). Some of these signalling pathways include kinases dedicated to transmit stress response, like p38MAPK and c-Jun N-terminal kinases (JNK) (Blühner 2009). These kinases are more expressed and more phosphorylated in omental adipose tissue than subcutaneous, and the phosphorylation degree correlates with metabolic parameters such as fasting triacylglycerols and glucose (Bashan et al. 2007). However, inflammation in subcutaneous adipose tissue is correlated with liver fat content independently of obesity (Kolak et al. 2007).

Obesity-related hepatic fat accumulation is directly associated with the increased production of inflammatory cytokines (Dielh et al. 2005). A liver with a high fat content overproduces inflammatory markers such as hs-CRP, IL-6 and TNF-α (Kotronen and Yki-Järvinen, 2008). Genes involved in the inflammation are upregulated in subjects with a high fat content in the liver (Greco et al. 2008). Several intervention studies in humans (recently reviewed by Harrison, 2006) have shown that weight loss normalizes the cytokine levels. Also the carbohydrate metabolism is tightly intertwined with cytokine expression and metabolism, as hyperinsulinemia has for example been shown to increase both TNF-α mRNA in adipose tissue (Krogh-Madsen et al. 2004) and TNF-α concentrations in plasma (Ruge et al. 2009).

Human adipose tissue, especially in obese states, is the target of macrophage infiltration (Weisberg et al. 2003). Macrophages may be recruited in response to death of hypertrophied fat cells (Cinti et al. 2005), and to the excessive secretion of pro-inflammatory cytokines (Curat et al. 2004). Also in the liver, cytokines are involved in the recruitment and activation of Kupffer cells, which are resident hepatic macrophages, and cause the transformation and perpetuation of hepatic stellate cells to the myofibroplastic phenotype (Friedman 2008). TNF-α promotes activation of I κB kinase (IKK), which releases active NF-κB (nuclear factor kappa B), a proinflammatory “master switch” that regulates inflammatory mediators. Furthermore, TNF-α antagonices adiponectin, an anti-inflammatory adipocytokine, shift the balance even more into the direction of an inflammation. Macrophage infiltration into omental fat has been associated to the severity of the histological changes in liver biopsies (Cancello et al. 2006).

Eventually, inflammation contributes to the development of insulin resistance, which in turn further induces ectopic fat accumulation. Visceral fat induces common endocrine excretion of adipose tissue (Mohamed-Ali et al. 1998), including secretion of TNF-α, IL-6, plasminogen activator inhibitor-1, leptin, and angiotensinogen (Lakka et al. 2002). These products induce insulin resistance by multiple mechanisms. Leptin induces dephosphorylation of insulin-receptor substrate-1 (Cohen et al. 1996). TNF-α, on the other hand, down-regulates insulin-induced phosphorylation of insulin-receptor substrate-1, and reduces the expression of the insulin-dependent glucose-transport molecule GLUT4 (Hotamisligil et al. 1996). Insulin resistance inducing hyperinsulinemia and lipolysis in adipocytes increases the concentration of FFA and consequently causes further fat accumulation in hepatocytes (Angulo, 2002).

2.3. PROGRESSION OF NON-ALCOHOLIC FATTY LIVER DISEASE, METABOLIC SYNDROME AND TYPE 2 DIABETES

The prevalence of obesity, as well as associated diseases such as type 2 diabetes has increased at an alarming rate in developed countries. Metabolic syndrome and type 2 diabetes have acquired a lot of attention and aroused discussion worldwide, but fatty liver disease (non alcoholic fatty liver disease, NAFLD) has remained a relatively unrecognized manifestation of the syndrome among the general population. However, it seems in light of current knowledge that metabolic syndrome may not develop at all without NAFLD.

Lipid deposition in the liver leads to NAFLD, and this may subsequently develop into nonalcoholic steatohepatitis (NASH). Steatosis, a histologic manifestation of intracytoplasmic lipid in the form of triacylglycerols in hepatocytes, is always present in NAFLD (Puri et al. 2007). Tiniakos et al. (2010) have discussed different aspects of ectopic fat accumulation in their recent review on NAFLD. The intracytoplasmic fat can be either large droplets of macrovesicular fat that fill the cytoplasm of the hepatocyte, or more rarely, foci of hepatocytes with true microvesicular steatosis, i.e. a cluster of several hepatocytes. Mixed large and small droplets often occur in hepatocytes of NAFLD patients (Tiniakos et al. 2010). Dysregulation of lipid content in non-adipocytes can result in lipid-induced apoptosis (lipoapoptosis) and inflammation. Hepatocellular injury and lobular inflammation define NASH (Brunt, 2001). FFA lipotoxicity, oxidative stress, unfolded protein response, adipokine/cytokine effects, mitochondrial injury and inflammation result in histological lesions including megamitochondria (giant mitochondria), glycogenated hepatocyte nuclei in clusters, iron deposition within hepatocytes, ductural reaction (presence of hyperplastic ductural structures accompanied by varying amounts of inflammation and connective tissue at the portal tract interface), and Mallory-Deck bodies (Tiniakos et al. 2010). Hepatocytes may suffer either necrotic or apoptotic death. Lytic necrosis is often preceded by hepatocellular ballooning resulting from microtubular disruption and from alterations to the intermediate filament cytoskeleton (Burt et al. 1998). TAG itself may not be directly involved in lipoapoptosis and has, in fact, been considered to be a protective agent (Listenberger et

al. 2003), but their hydrolysis to fatty acyl-CoA provides an amplified substrate for ceramide synthesis. Ceramide up-regulates iNOS expression, and thus leads to increased intracellular peroxynitrite, a candidate for lipoapoptosis (Bielawska 1997).

Ford (2005) has concluded in his review that the increased prevalence of excessive visceral fat is closely associated with the rising incidence of type 2 diabetes, and the accumulation of lipids in the liver is well correlated with hepatic insulin resistance (Seppälä-Lindroos et al. 2002). About 70-80 % of type 2 diabetics have NAFLD (Targher et al. 2007), and those obese subjects who tend to accumulate abdominal fat have an increased risk of cardiovascular diseases (Lakka et al. 2002). This phenomenon can be explained by the impaired insulin sensitivity of a fatty liver, which results in higher glucose (Ryysy et al. 2000, Marchesini et al. 2001, Seppälä-Lindroos et al. 2002) and higher VLDL (Adiels et al. 2006, Adiels et al. 2007) production in the liver, and thus insulin resistance (Ryysy et al. 2000, Adiels et al. 2006). Lipotoxicity is also believed to cause the failure of pancreatic β-cells (Unger 2001). Initially β-cells undergo hyperplasia compensating for the peripheral insulin resistance. Subsequently, they lose the glucose transporter-2 and glukokinase (Ohneda et al. 1995) and ultimately approximately 50% of the β-cells disappear through lipoapoptosis (Unger 2003a).

When Reaven (1988) originally developed the concept of insulin resistance in 1988, he did not include any marker of obesity in his description. Today, however, central obesity is included in all criteria of metabolic syndrome used and it is clear that in most cases obesity is important for the development of many of the other metabolic syndrome components such as increased triacylglycerol, decreased HDL levels, and hypertension as concluded by Bruce (2009) in a recent review. In particular, increased abdominal adiposity is generally associated with high circulating triacylglycerols, low HDL-cholesterol, and high plasma concentration of apoB-containing lipoproteins (Hill, 2009). Imaging studies with MRI and computed tomography have shown that it is the excess of intra-abdominal or visceral adipose tissue and not the amount of subcutaneous abdominal fat which is the key correlate of the metabolic abnormalities observed in overweight/obese patients (Björnrtorp 1991, Pouliot et al. 1992, Goodpaster et al. 2003, Matsuzawa et al. 1995, Nielsen et al. 1997). Moreover, visceral fat can be regarded as mechanistic link between the metabolic syndrome components as it induces adverse immune and metabolic disturbances (Bays, 2009).

In the light of current knowledge it seems that metabolic syndrome may not develop at all without NAFLD (Kotronen et al. 2007), and NAFLD is estimated to be as common as metabolic syndrome in western populations (23 % occurrence) (Seppälä-Lindroos et al. 2002). NAFLD is commonly associated with obesity, insulin resistance, dyslipidemia, and type 2 diabetes, and can thus be regarded as the hepatic manifestation of metabolic syndrome (Angulo, 2002, Bugianesi and McGullough 2005, Day 2006, McGullough 2006), and the fat content of liver the correlates negatively with hepatic insulin sensitivity, even independently of obesity (Seppälä-Lindroos et al. 2002). It has also been shown, that increased activity of ALT predicts type 2 diabetes independently from obesity (Hanley et al. 2004).

Insulin normally inhibits the production of glucose and VLDL in the liver (Yki-Järvinen 2005). An insulin resistant liver over-produces glucose (Ryysy et al. 2000, Seppälä-Lindroos et al. 2002) and VLDL particles (Adiels et al. 2006), increasing triacylglycerol concentration in the blood and reducing the level of high-density lipoprotein (HDL) levels (Adiels et al. 2007). VLDL production is further provoked by increased the fat content of hepatocytes, as the liver exports surplus fat as VLDL (Hill, 2009). Pancreatic B-cells on the other hand, are not able to export fat as VLDL, and thus lipid overflow results in cell death (Unger, 2003b).

The fat content of the liver in NAFLD patients can be decreased by weight loss (Tiikkainen et al. 2003, Petersen et al. 2005, Larson-Meyer et al. 2006) and rosiglitazone-medication (Carey et al. 2002). A low-fat diet (Westerbacka et al. 2005) and physical activity (Larson-Meyer et al. 2006) probably also have a positive effect, but extensive clinical interventions have not been performed to settle this.

2.4. THE EFFECT OF BERRY POLYPHENOLS ON ECTOPIC FAT ACCUMULATION

The role of nutrition in the progression of obesity-related diseases is nowadays recognized to be important. However, although diet evidently has a major role in the development and progression of NAFLD, it must be noted that genetic predisposition also plays a significant part, as polymorphism of several candidate genes have been associated with NAFLD (Day, 2006). Many reaction cascades intertwine genetic and environmental factors into an outcome of health or disease. For example AMPK (adenosine monophosphate activated protein kinases)-SIRT1 (silent information regulator T1)-cascade serves as an indicator of cellular energy status and is thus important in the regulation of carbohydrate metabolism. The relationships of genetic and environmental factors, pathogenic changes and the onset of diseases are schematized in **Figure 9** together with the regulation on AMPK.

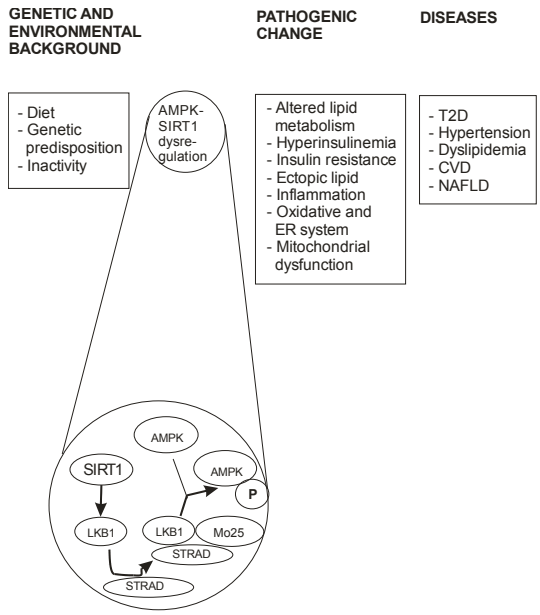

Figure 9: The relationships of genetic and environmental factors, pathogenic changes and the onset of diseases intertwined by the AMPK-SIRT1 cascade. The proposed mechanism for the activation of LKB1 and LKB1 target molecules by SIRT1. SIRT1 activation leads to LKB1 deacetylation and further binding to the STE20-realted adaptor protein (STRAD) and mouse embryo scaffold protein (MO25). The formation of the complex activates LKB's kinase activity and it phosphorylates AMPK. The combination of overnutrition, inactivity, and genetic and other factors interact to produce a state of metabolic susceptibility that is proposed to lead to the dysregulation of AMPK, and thus further to mitochondrial dysfunction, insulin resistance, hyperinsulinemia, and abnormalities in the cellular lipid metabolism, such as modest increases in plasma triglycerols and ectopic lipid deposition in muscles and the liver. The transition from this preclinical stage to the clinically diagnosed metabolic syndrome to overt disease may take many years, and the rate of progression is determined by environmental factors such as diet and exercise. Adapted from Ruderman et al. 2010.

AMPK = adenosine monophosphate kinases, CVD = cardiovascular diseases, LKB1 = serine threonine liver kinase B1, Mo25 = mouse embryo scaffold protein, NAFLD = non-alcoholic fatty liver disease, SIRT1 = silent information regulator T1, STRAD = STE20-related adaptor protein.

2.4.1. The effect of polyphenols on glucose and fat metabolism

The effect of carbohydrate composition on postprandial hyperglycemia and postprandial insulin response is commonly accepted (Ludwig 2000, Kallio et al. 2008). However, even though there is some emerging evidence of the ability of other than macronutrient components of food to alter postprandial sugar metabolism, such evidence is to date scarce and mainly produced by *in vitro* and animal trials. In a recent study, the inclusion of cinnamon in a rice pudding meal lowered the postprandial glucose response (Hlebowicz et al. 2007). Moreover, blueberries were reported to have potential in the attenuation of insulin resistance in an animal trial (DeFuria et al. 2009). Some exotic fruits or their phenolic extracts have also shown positive effects on sugar metabolism in animal models, as Zunino (2009) recently reviewed. Hanamura et al. have shown that crude aeriola polyphenol fraction had a preventive

effect on postprandial hyperglycemia (Hanamura et al. 2006). *Cissus quandrangularis* stem favourably modified plasma glucose and insulin levels in male rats fed a high fat-high fructose diet (Chidambaram et al. 2010). Yet, also controversial results have been reported. Gruendel et al. (2007) found that carob pulp preparation rich in polyphenols deteriorated postprandial glycaemic control. Thus it seems that food matrix and other components present can modulate the effect of polyphenols on glycemic response.

Grape seed polyphenols improve fed state fat metabolism through the regulation of the bile acid pathway gene expression (Del Bas et al. 2005), and black-tea polyphenols suppressed postprandial hypertriacylglycerolemia by suppressing the lymphatic transport of dietary fat (Kobayashi et al 2009) in rats. In an in vitro trial, apple, but not wine, polyphenol extract dose-dependently decreases the esterification of cholesterol and the enterocyte secretion of lipoproteins in Caco2/TC7-enterocytes (Vidal et al. 2005). Consuming a strawberry product 6 weeks in advance and with a high-fat meal mitigated fed-state oxidative stressors such as LDL oxidation and hyperlipemia in a placebo-controlled human trial (Burton-Freeman et al. 2010).

Animal and in vitro studies have investigated which mechanisms might be behind the observed regulatory effects on the carbohydrate and fat metabolism of polyphenols. The ability of polyphenols to inhibit the activity of digestive enzymes probably has some contribution to the smoothing effects on postprandial glycemia and lipemia. Anthocyanins for example inhibit endopeptidases, ellagitannins α-amylases, and flavanols inhibit the activity of pancreatic lipase (McDougall et al. 2005). Glycosylated flavonoids also compete with sugars in absorption as they are able to attach Na+/glucose-transporters (Ruel et al. 2007).

In addition, phenolic compounds seem to exert specific regulatory effects on reaction cascades related to insulin secretion and lipid homeostsatis. Anthocyanins enhance insulin secretion in pancreatic β-cells, and to reduce liver triacylglycerols, and plasma cholesterol in C57BL/6-mice (Jayaprakasam et al. 2006). It seems that all these effects can be traced to common regulatory mechanisms. AMPK serves as a sensor of cellular energy status, being activated by increased AMP/ATP-ratio or by upstream kinase, LKB1 (the tumor suppressor kinase) (Shaw et al. 2004, Shaw et al. 2005), CaMKKβ (Ca2+ /calmodulin-dependent protein kinase kinase β) (Hurley et al 2005), and TAK1 (transforming growth factor-β-activated kinase-1) (Xie et al. 2006). The molecular physiology of a normal and lipotoxic liporegulation is described in more detail in Chapter 2.2.2. Here, the effects of polyphenols on AMPK phosphorylation cascade are described. Hyperglycemia induces dysfunction in hepatic AMPK leading to hepatic lipid accumulation and hyperlipidemia (Zang et al. 2004, Zang et al. 2006). The role of AMPK as the guardians of cellular energy homeostasis has been reviewed extensily by Hardie (2007). Thus, it has been stated that AMPK maintains the balance between ATP production and consumption in all eukaryotic cells (Hardie, 2007). In the liver, AMPK inhibits the production of glucose, cholesterol and triacylglycerols and stimulates FA β-oxidation (Schimmack et al. 2006).

Polyphenols strongly stimulate AMPK (Zang et al. 2006), similarly to metformin (Shaw et al. 2004, Zang et al. 2004,), which results in attenuated hyperlipidemia and atherosclerosis in diabetic mice (Zang et al. 2006). Hwang et al. (2009) have recently reviewed the natural compounds stimulating AMPK, and they pointed out polyphenols as active components of natural products. Evidence was most abundant concerning resveratrol, tannins, curcumin, and quercetin (Hwang et al. 2009). In endothelial cells, AMPK activation induced by EGCG reduced endothelin-1, a known vasoconstrictor (Reiter et al. 2010). The mechanism by which polyphenols exert AMPK activation has been further studied in vitro in human hepatocytes (Hou et al. 2008), and a proposed scheme involving SIRT1 and LKB1 is presented in **Figure 10**.

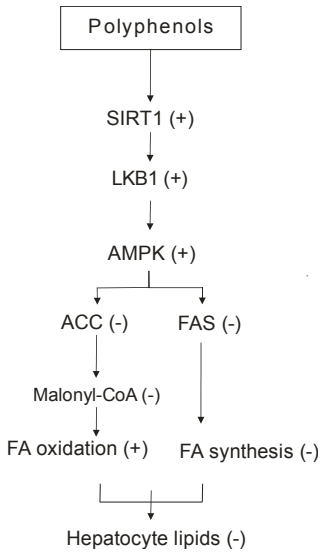

Figure 10: A proposed scheme of the mechanism by which polyphenols exert AMPK activation involving SIRT1 and LKB1. SIRT1-activating polyphenols, stimulate LKB1 phosphorylation as well as AMPK phosphorylation and activation. AMPK activation in turn increases ACC phosphorylation and thus inhibits ACC activity. As a consequence, decreased production of malonyl-CoA results in the up-regulation of FA β-oxidation as well as down-regulation of fatty acid synthesis, thereby leading to hepatocyte lipid reduction. Also, activation of AMPK by polyphenols inhibits glucose-induced expression of FAS, which contributes to the reduction in triacylglycerols through inhibition of fatty acid biosynthesis. Adapted from Hou et al. 2008.

AMPK = AMP-activated protein kinase, FA = fatty acid, FAS = fatty acid synthase, SIRT1 = silent information regulator T1, a mammalian ortholog of Sir2 (silent information regulator 2).

2.4.2. Polyphenols and inflammation

The inflammatory response is a complex self-limiting process coordinated by vasoactive amines, adhesion molecules, lipid-derived eicosanoids, cytokines, and chemokines as reviewed by Lawrence et al. (2002). When the self-limiting nature of this protective mechanism is inappropriately regulated, it is transformed into a detrimental, chronic state of inflammation. The increase in inflammatory tonus is mainly the result of lifestyle and nutritional habits, making the increase controllable as concluded by Bengmark (2006) in his review on nutrition

and chronic diseases. The potential effects of polyphenols on inflammation are extensively investigated, although most studies are of *in vitro* design, mainly cell cultures of human or fat cell lines. Major pathways and other inflammatory mechanisms as well as the effects of polyphenols on their function, are presented below.

2.4.2.1. Polyphenols and the arachidonic acid pathway

Arachidonic acid is released from membrane phospholipids through phospholipase A_2 cleavage, and can be metabolized by the cyclooxygenase pathway into prostaglandins and thromboxan A_2 or by the lipoxygenase pathway to hydroperoxyeicosatetraenoic acids, hydroxyeicosatetraenoic acids, and leucotrienes (Needleman et al. 1986). Dietary polyphenols have inhibited the activity of cyclooxygenases, lipoxygenases and phospholipase A_2 resulting in lower circulating levels of inflammatory eicosanoids *in vitro* (Baumann et al. 1980, Welton et al. 1986, Laughton et al. 1991). In addition to flavonoids (Laughton et al. 1991, Kim et al. 1998, Miles et al. 2005), tannins (Hong et al. 2001, Kundu et al. 2003, Hou et al 2007) have been shown to suppress cyclooxygenase and lipoxygenase activities in *in vitro* and animal studies. In structure-activity studies the galloyl moiety of the tannins rich in green tea appeared important in their inhibitory actions (Hou et al. 2007). It seems that the inhibitory effects of polyphenols is exerted mainly via gene expression modulation (Petroni et al. 1997, Moreno et al. 2003).

2.4.2.2. Polyphenols and nitric oxide synthase

A small amount of nitric oxide (NO) is essential for the maintenance of body functions, but significant increase in NO synthesis by inducible nitric oxide synthase (iNOS) invokes the inflammatory process (Nathan et al. 1992). Versatile polyphenols including apigenin, luteolin, kaempferol, myricetin, quercetin, and genistein, down-regulate iNOS enzyme expression and/or inhibit the iNOS enzyme activity *in vitro* (Kim et al. 1999, Wadsworth et al. 1999, Chen et al. 2001). Also tannins inhibit iNOS, and their ability to interfere with NOS action has been traced to the gallate structure of epigallocatechingallate (EGCG) in structure-activity studies (Chan et al. 1997). Moreover, anthocyanins may induce other forms of NOS, thereby ameliorating endothelial dysfunction and harmonizing blood pressure (Xu et al. 2004). Endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure homeostsasis and vascular integrity, implying that flavonoids may protect against cardiovascular diseases by acting as vasodilatants (Xu et al. 2004). Also, EGCG has been shown to modulate the activity of different NOS isoforms with a net effect of decreased inflammation but enhanced NO synthesis in tissues like endothelium where it is needed (Sutherland et al. 2005).

Inhibition of iNOS expression seems to be mediated by the inhibition of IKB kinase, NF-κB and the STAT1-pathway (Chen et al. 2005), implying that iNOS regulation of polyphenols is

intertwined with the various other metabolic effects of polyphenols related to ectopic fat accumulation.

2.4.2.3. Polyphenols, NF-κB pathway modulation and cytokine system

Cytokines, the major mediators of local, intracellular communication, define the outcome of disease through a balance between pro-inflammatory (*i.e.* IL-6, IL-8, IL-2, TNF-α, IL-1β, IFN-γ) and anti-inflammatory (*i.e.* IL-10, IL-4, TGFβ) cytokines (Santangelo et al 2007). Phenolic compounds have been shown to be able to selectively interfere with cytokine production and/or function. For example, quercetin (Comalada et al. 2006) and catechins (Crouvezier et al. 2001) inhibit TNF-α and IL-6, simultaneously induce IL-10 release, and thus evoke the anti-inflammatory effect. Resveratrol has been shown to suppress cytokine production *in vivo* in rat lungs (Birrell et al. 2005).

The production of cytokines is coordinated by complex reaction pathways, one of the major ones being NF-κB, which regulates the induction of several pro-inflammatory cytokines mainly at the gene expression level, as well as chemokines, adhesion molecules, growth factors, acute-phase proteins, cell proliferation, migration, invasion, iNOS, and immuno-receptors (Nam et al. 2006). The inhibition of NF-κB can thus be considered a potent therapeutic anti-inflammatory target (Karin et al. 2004). Resveratrol especially in its *cis* form is able to attenuate the expression of the NF-κB family of genes (Leiro et al. 2005). Tannins have been widely studied, and have been shown to suppress NF-κB cascade by acting at multiple steps of the activation process. NF-κB stimulators induce IκB proteins to be phosphorylated by IκB kinase, IKK, which releases the regulatory subunit of IκB, NF-κB. The released NF-κB translocates into the nucleus and modifies transcription into inflammatory direction (Hansen et al. 1994). EGCG inactivates IKK and the release of the NF-κB subunit (Wheeler et al. 2004, Aneja et al. 2004, Ichikawa et al. 2004). Also the anti-inflammatory effects of quercetin seem to be due to the inhibition of NF-κB subunit release from IκB as many *in vitro* studies have indicated this (Chen et al. 2005, Min et al. 2007, Garcia-Mediavilla et al. 2007).

2.4.2.4. Polyphenols and the MAPK pathway

The mitogen activated protein kinase (MAPK) pathway is a sequence-specific transcription factor providing assistance to NF-κB (Karin, 1995). The MAPK family consists of Ser/Thr kinases including in mammalian cells at least extracellular signal-regulated kinases ERK1/2, c-Jun amino-terminal kinases JNK 1/2/3, p38-MAP kinase, and ERK5 kinases, of which the JNK and p38 cascades are most involved in inflammation (Mayor et al. 2007). A perspective of the modular activators, intermediate effectors and downstream substrates specifying some of the pato-physiological effects of the p38 cascade are presented in **Figure 11**. Kaempferol, chrysin, apigenin, and luteolin inhibited ERK, JNK, and p38 in respiratory epithelial cells (Chen et al. 2004). Luteolin inhibited more specifically only ERK1/2 and p38 (Xagorari et al. 2002), quercetin had an inhibitory effect on ERK and JNK (Huang et al. 2006),

and catechin inhibited p38, and JNK (Xagorari et al. 2002). Cyanidin-3-O-glucoside inhibited ERK1/2 (Pergola et al. 2006), and delphinidin and cyanidin prevented p38 and JNK activation in human aortic vascular smooth muscle cells (Oak et al. 2006). The effects of resveratrol on the inflammatory MAPK pathway was recently reviewed by Rahman et al. (2006) and they concluded that resveratrol seems to inhibit MAPK in some cells, but induce it in others. Also, the effects are dose dependant as lower concentrations are generally stimulatory and higher inhibitory, although the threshold differed between cell types. On the other hand, widely investigated EGCG elicits extensive anti-MAPK activity in various cell models (Wadsworth et al. 2001, Tokuda et al. 2007, Kundu et al. 2007).

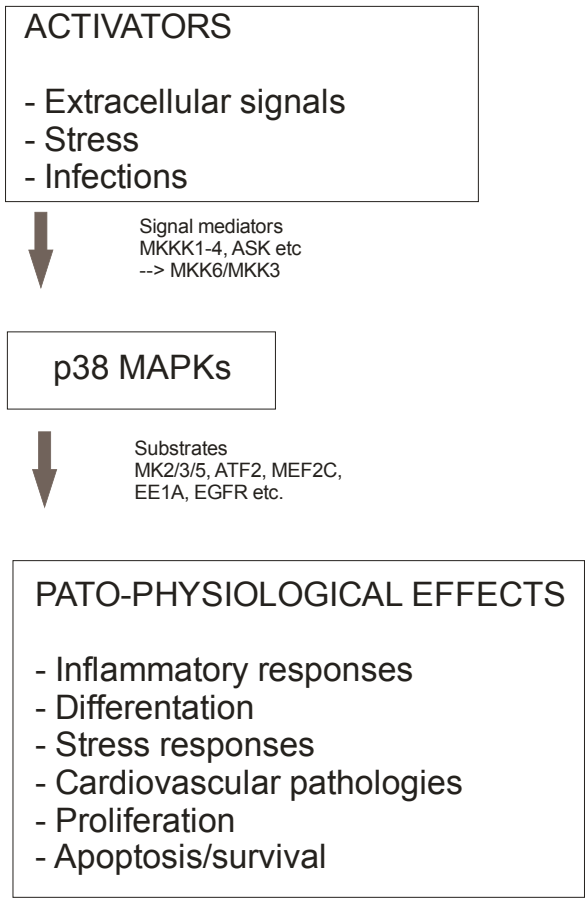

Figure 11: A perspective of the activators, intermediate signal mediators (MAP3K; MKK1-4, ASK and MAP2K;MKK6/MKK3), downstream substrates (MK2/3/5, ATF2, MEF2C, EE1A, EGFR), and patho-physiological effects of the p38 cascade. Adapted from Mayor et al. 2007.

2.4.3. Polyphenols, fat deposition in the liver and pathophysiological consequences

Pomegranate flower ethanol extract is rich in polyphenols, in particular ellagitannins, and has been shown to possess hepatoprotective effects when administered to rats prior to ferric nitrilotriacetate exposure (Kaur et al. 2006). The extract attenuated both the elevation of liver enzymes, and the histopathological effects of ferric nitriloacetate administration such as ballooning degeneration, fatty changes, and necrosis. Also, the polyphenol extract of fenugreek seeds, a common spice in India, alleviated ethanol-induced collagen and lipid accumulation in rat liver similarly to silymarin (Kaviasaran et al. 2007). In both studies, polyphenol extracts concurrently improved plasma lipids by lowering triacylglycerols, cholesterol and FFA (Kaur et al. 2006, Kaviasaran et al. 2007). In high fat-high-fructose fed rats the stem extract of *Cissus quandrangularis* inhibited the activation of hepatocellular enzymes, inhibited lipid deposition in the liver, attenuated the decline in antioxidant status, as well as elevation in lipid peroxidation and protein carbonyl in the liver (Chidambaram et al. 2010). *Hibiscus sabdariffa* extract rich in anthocyanins, flavonoids and protocatechuic acid decreased high-fat diet induced plasma lipid abnormalities such as high LDL cholesterol, as well as attenuated lipid accumulation in the liver (Yang et al. 2010). The effects were probably due to AMPK expression activation also observed in the study, as AMPK activation has previously been indicated to have a pivotal role in lipid metabolism (Hardie, 2007).

Blueberries attenuated hepatic fibrosis measured as serum hyaluronic acid and ALT levels as well as fibrotic changes measured directly from the liver by staining after the rats were killed (Wang et al. 2010). Resveratrol inhibits non-alcoholic fatty liver disease in rats (Bujanda et al. 2008).

Despite the promising results in animal studies, human data is still scarce. However, some human trials exist and sea buckthorn extract has shown hepatic fibrosis alleviation in human cirrhotic patients in a 6 months intervention (Gao et al. 2003).

2.5. SUMMARY

Polyphenols constitute a large group of compounds with relatively versatile structures and ubiqitous prevalence in the plant kingdom. Polyphenols condense in the surface layers of plants, thus existing as high amounts on small berries with high surface-to-volume ratio. Berries are known to be generally healthy and lately more and more indications of their ability to sustain health has emerged. In the literature part of this thesis, the effects of polyphenols on ectopic fat accumulation was discussed by presenting the berry polyphenols and ectopic fat accumulation as biochemical and pathological process as well as reviewing the research performed on polyphenols and ectopic fat accumulation.

The review of the research performed this far on polyphenols and ectopic fat accumulation and related metabolic disturbances revealed indications that various polyphenol subclasses have the potential to affect metabolic diseases and their associated variables. Tannins, resveratrol, and flavonols are polyphenolic compounds and compound groups most investigated in regard to metabolic diseases. Of tannins, mainly epigallocatechins found in green tea have been in the focus of research. It can be concluded, that polyphenols have in many studies shown the potential to have a positive effect on ectopic fat accumulation and related dysfunctions such as whole-body inflammation.

However, studies made with individual polyphenols found in berries are scarce and further investigation is needed to conclude whether or not polyphenols are the most active compound group in berries when the effects on ectopic fat accumulation are considered. Nonetheless, at this point it can be noted that polyphenols and especially the subgroups flavonols and tannins do pose a high potential.

3. AIMS OF THE STUDY

The overall aim of this thesis was to study whether the incorporation of berries into the diet as i) whole berries, ii) industrially produced berry products, and iii) different industrially developed berry fractions could decrease the risk of obesity-related diseases.

The aims of the studies included in this thesis fall into three groups.

The first aim was to develop, optimize, and apply high performance liquid chromatography tandem mass spectrometry analysis of anthocyanins and flavonol glycosides and their metabolites to analyze the absorption and metabolism of these compounds in humans. (I, II)

The second aim was to study the effect of a mixed berry diet on the risk factors associated with obesity-related diseases, and to find out which berries and berry fractions held the most potential. (III, IV)

The third aim was to define how sea buckthorn berry and its extraction residues from food and nutraceutical industry affect the postprandial glucose metabolism. (V)

4. SUBJECTS, MATERIALS, AND METHODS

4.1. STUDY DESIGNS AND ETHICAL CONSIDERATIONS

Studies I and II were conducted as acute postprandial absorption studies, whereas studies III, IV, and V, were conducted as a randomized parallel intervention design (study III) or randomized cross-over design (studies IV, and V). The studies were conducted according to the guidelines laid down in the Declaration of Helsinki (2000), and all procedures involving human subjects were approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all subjects. The study products used in the studies were safe, traditional berry products.

In studies I and II the study subjects consumed berries with yoghurt, and the absorption of polyphenols, anthocyanins in study I and flavonol glycosides in study II, was monitored compared to the baseline in either urine (study I) or in plasma, feces, and urine (study II). Study III was conducted as a randomized 20-week dietary intervention trial with two parallel treatment groups; lifestyle intervention with various berry products (berry group, n=31), or lifestyle intervention (control group, n=30). Physical measurements were made and blood samples were analysed from both groups both before and after the intervention. Study IV was a randomized cross-over intervention study with four berry diets (diets: SB, sea buckthorn; SBe, sea buckthorn extract; SBo, sea buckthorn oil; and BB, bilberry) and five wash-out periods before and after each berry intervention period. Study V was a postprandial randomized cross-over study with three test meals (meals: B1, sea buckthorn berries; B2, berry residue extracted by SF-CO_2; and B3, berry residue extracted by ethanol after SF-CO_2 extraction), and a control meal (meal A).

4.2. STUDY PRODUCTS AND TEST MEALS

The berries used in the studies were lingonberry (studies I, II, and III), sea buckthorn (studies I, II, III, IV, and V), bilberry (studies III, and IV), and black currant (study III). Berries were used as frozen whole berries (studies I, II, III, and IV) as well as various berry products (studies III, IV, and V).

In absorption studies, berries [lingonberry *Vaccinium vitis-ideae*) in studies I and II and sea buckthorn (*Hippophaë rhamnoides* ssp*. mongolica* var. Ljubitelskaja) in study II] were consumed as whole berries with vanilla youghurt.

In study III, the subjects in the berry group consumed 3 portions of berry products daily equalling to 163 g of fresh berries (1138 g/week), and a weekly dose of 3.5 g of berry oils

(equalling to 245 g of berries). Daily berry products were selected from 18 berry products according to a specific product utilisation cycle, and consumption was recorded in a diary. The berry products were designed to replace other snack products in the diet. The berries used as ingredients were lingonberry, sea buckthorn berry (*Hippophaë rhamnoides*, ssp. *mongolica*, var. Ljubitelskaja), bilberry (*Vaccinium myrtillus*), and black currant (*Ribes nigrum*). The berry products were processed by five Finnish food enterprises on their commercial production lines.

In study IV the bilberries were frozen whole berries (BB) and the sea buckthorn berries (*Hippophaë rhamnoides* ssp. *turkistanica*) were air-dried after harvesting (SB). Oils were extracted from berries (berry oil) and seeds (seed oil) with supercritical CO_2. The sea buckthorn oil used in the study was a standardized product containing both berry oil and seed oil (SBo). After CO_2 extraction, the residues were further extracted with 50% aqueous ethanol at 50 ˚C for phenolic compounds. After removal of ethanol, the phenolic extract was spray-dried at 60-70 ˚C into a free-flowing powder with the aid of maltodextrin DE 6. The phenolic extract powder used in the study (SBe) contained native sea buckthorn extract and maltodextrin DE 6 at a ratio of 1:1. All berry diets in study IV were designed to correspond to 100 g of berries daily.

In study V the berries were consumed again in the yoghurt. Sea buckthorn (*Hippophaë rhamnoides* L. ssp. *turkestanica*) berries were used as dried whole berries (B1). The crushed whole berries were extracted by supercritical CO_2 to obtain berry residue (B2). Seeds were broken with a cutting mill to enable the removal of seed oil. The ethanol extraction of the SF-CO_2-extraction residue was carried out with 70% ethanol in water, by a similar batch procedure as previously used by Sandell et al. (2009) to obtain further residue (B3). Glucose (50 g) was served blended into the yoghurt with dried and ground berries or berry extraction residues (meal B1; 40 g of dried and crushed sea buckthorn berries, meal B2; 32.4 g of berry residue extracted by SF-CO_2, and meal B3; 18 g of ethanol extracted CO_2-extraction residue), or without them (meal A, control, 200 g of natural yoghurt with 50 g of glucose). The amounts of fractions correspond to 200 g of fresh sea buckthorn berries. The production of the fraction utilized in studies IV and V are presented as a flow chart in **Figure 12**.

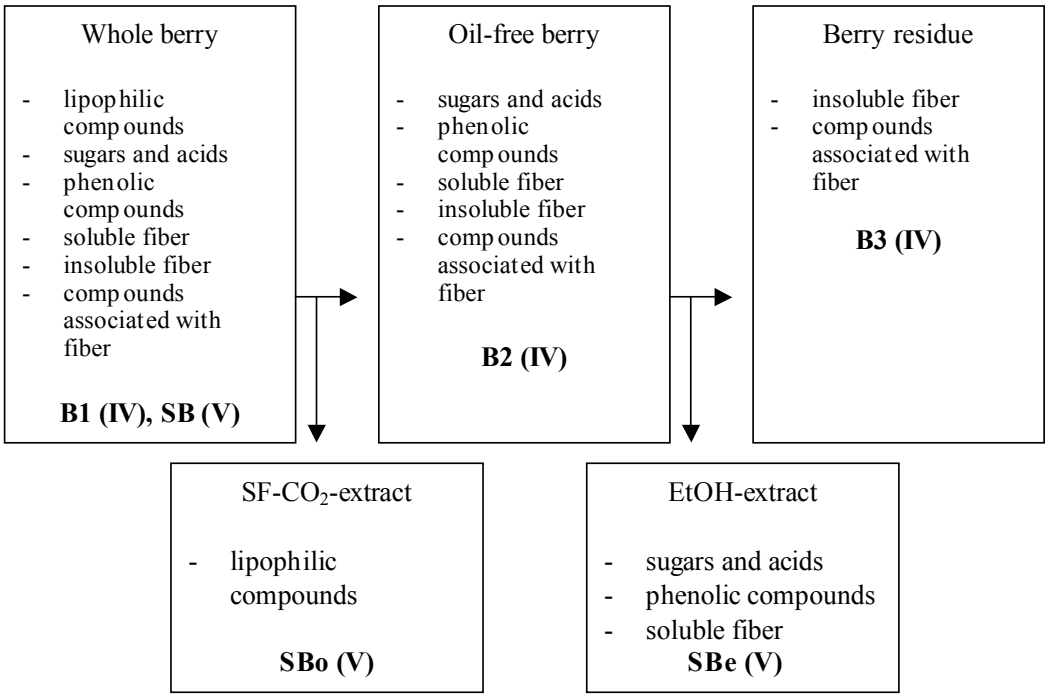

Figure 12: The production of the fraction utilized in studies IV and V.

4.3. SUBJECTS

Study subjects in all the studies were healthy humans with normal liver, kidney and thyroid functions, and with no history of cardiovascular disease or diabetes. In studies I, II, and V the study subjects had a normal weight, but in studies III and V overweight volunteers were recruited. The exact inclusion criteria in studies III and IV were: cholesterol 4.5-8 mmol/L, LDL-cholesterol > 2.5 mmol/L, triacylglycerol < 4 mmol/L, glucose < 6 mmol/L, insulin < 25 mU/L, blood pressure < 160/99 mmHg, hemoglobin > 120 g/L (anemia), TSH 0.3-4.2 mU/L (thyroid function), ALT < 60 U/L (liver function), and creatinine < 115 μmol/L (kidney function). Exclusion criteria were pregnancy, menopause, regular smoking, previously diagnosed diabetes (other than gestational), thyroid, renal, hematological, or hepatic dysfunction, previous myocardial infarction, cardiovascular medication, treatment with lipid-lowering drugs, and ongoing inflammatory disease. In study V the inclusion criteria were: P-ALT < 60 U/L, fP-creatinine < 115 μmol/L, and P-TSH 0.3–4.2 mU/L).

In studies I and II the characteristics of the volunteers (two females and two males) were: age 26 ± 4, and body mass index 20–25 kg/m^2.

For study III, the characteristics of the volunteers (61 females) were: body mass index 29.3 kg/m^2 (berry group), 29.5 kg/m^2 (control group), waist circumference 93.6 cm (berry group) 92.9 cm (control group), and blood pressure 135/89 (berry group), 129/84 (control group).

In study IV, the characteristics of the volunteers (80 females) were: body mass index 29.6 ± 2.1 kg/m^2, waist circumference 95.8 ± 8.5 cm, and blood pressure 133 ± 14.3 / 84.2 ± 7.9 mm Hg.

In study V, the characteristics of the volunteers (10 males) were: body mass index 19.8–26.9 kg/m^2 (excluding one bodybuilder whose BMI was 30.0, AV (n=10) 23.7 ± 3.1), and waist circumference 95.8 ± 8.5 cm.

4.4. LABORATORY METHODS

Before acute meal tests (studies I, II, and V) the subjects ate a standardized evening snack with low flavonoid content (wheat bread, cucumber, water, and a banana) the night before the study visits. After a 10-hour fast, the subjects consumed either the berry meal (studies I, II, and meals B1, B2, and B3 in study V, see **Fig. 12**) or the control meal (meal A in study V). Before the meal, a basal sample of blood (studies II and V), urine (study I and II), and feces (study II) were collected, and after consumption of the breakfast, urine samples were collected for 24 h (0-4h, 4-8h, 8-12h, 12-24h) and feces samples for 48 h (0-12h, 12-36h, 36-48h) (study II) and blood samples were collected at 60, 120, 240, and 480 minutes (study II), or at 30, 60, 90, 120, 180, 270, and 360 minutes (study V). The subjects fasted during the 6-hour study days (study V) or were provided with standardized low-flavonol meals (studies I and II).

In the intervention studies (III and IV) blood samples (two basal samples, as well as samples drawn at 10, 19, and 20 weeks in study III; sampling before and after each berry and wash-out period in study IV) were taken after an overnight fast (12 h) between 07:00 and 11:30 h a.m from each study subject. The study subjects were instructed to avoid alcohol and all medication for 3 days (study III) or 2 days (study IV) before sample collection. In addition, in study III 2 h glucose tolerance tests (50 g of glucose consumed as liquid, capillary blood samples drawn at 0, 30, 60, 90, and 120 min) were performed before and after the intervention period. Plasma, serum, and glucose tubes were centrifuged at 2200 x *g* for 10 min and the supernatant was stored at –80 °C in micro-centrifuge tubes until analyzed further.

4.4.1. Physical measurements and clinical analyses

In studies III and IV, the body weight of bare-foot subjects wearing light indoor clothing was recorded to the nearest 0.1 kg by a calibrated weighing scale (Inbody 3.0, Sunborn Saga Oy, Finland). Body height was recorded to the nearest 0.5 centimeter. Waist circumference was measured midway between the *spina iliaca superior* and the lower rib margin. Blood pressure was measured in duplicate using the automated Omron M4-I device (Omron, UK) in

accordance with standard procedures. ALT, GT, cholesterol, triacylglycerols, and creatinine were analyzed from plasma collected into lithium-heparine tubes, TNF-α, adiponectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and E-selectine from plasma collected into EDTA-tubes, and TSH, insulin, and hs-CRP from serum. Serum total cholesterol, HDL cholesterol, LDL cholesterol, and triacylglycerols (C.V. 2.2%, 3.4%, 3.1%, and 3.2%, respectively) were measured from blood samples by enzymatic photometric methods with commercial kits (Thermo Clinical Labsystems Oy, Espoo, Finland) using the Konelab20i analyser (C.V. 2.2%, 3.4%, 3.1%, and 3.2%, respectively). Plasma glucose (C.V. 2.6%) was analyzed by the enzymatic photometric method using Konelab Glucose HK as a reagent. ALT, γ-glutamyltransferase (GGT), glycated haemoglobin, and serum high-sensitive CRP were analyzed with routine standardized methods (C.V. 4.3%, 3.1%, 4.6 % and 5.3%, respectively), all with a Konelab20i analyser (Thermo Clinical Labsystems Oy, Konelab, Finland) and TSH (C.V. 6.0%) was analyzed using Axsym (Axsym, Abbot Diagnostics, Finland). Serum insulin (C.V. 7.3%) was analyzed by chemiluminescence-immunoassay with a Immulite 1000 analyser (Siemens Medical Solutions, Espoo, Finland). Haematological parameters were measured with a CellDyn analyser. Soluble adhesion molecules sICAM-1, (C.V. 6.6 %), sVCAM-1 (C.V. 6.7%) and adiponectin (C.V. 3.3%) were simultaneously measured from the plasma samples with Millipore's Human CVD1-kit (HCVD1-67AK) and TNF-a (HCYTO-60K) with Millipore's singleplex kit (Millipore, Billerica, MA) using the BioRad Bio-Plex 200 System.

In addition, in study III finger-prick capillary blood samples were collected intoMiniCollect 0.5 mL lithium heparin tubes and glucose, insulin and glucose-dependent insulinotropic peptide (GIP) were analyzed from plasma samples by the enzymatic photometric method. Also, in study III the oxygen radical absorbing capacity (ORAC, C.V. 4.6%) was analyzed from serum using a multi-well plate reader according to the methods previously described (Venojärvi et al. 2008). Hemoglobin A1c (HbA_{1c}) and blood cell count analysis was performed from fresh blood collected into EDTA-tubes by an immunoturbidimetric method.

In study V, blood samples were drawn from the forearms of each subject into glucose tubes (VF-053SFX, for glucose analysis, Oriola, Helsinki, Finland), and serum tubes containing a coagulant activator (VF-054 SPW, Oriola). Plasma glucose, and serum insulin were analyzed with standard biochemical analyses at Turku University Hospital Laboratory. Plasma glucose was determined by a photometric method and serum insulin by an electro-chemiluminescence immunoassay (TYKSLAB, Turku, Finland). Insulin was measured from a single tube with a Roche Modular PPEE analyzer, with commercial reagents provided by Roche Diagnostics GmbH (Mannheim, Germany). TNF-α was analyzed from plasma collected into EDTA-tubes with a Millipore's singleplex kit (VF-054 SPW, Oriola) according to the manufacturer's instructions, using the BioRad Bio-Plex 200 System. TNF-α analyses were performed only for the baseline and for the 60 min samples drawn after meals B1 and A.

4.4.2. Analysis of flavonoids by liquid chromatography and mass spectrometry

In absorption studies the urine (I and II), feces (II) and plasma (II) samples were analyzed for their anthocyanin (I) and flavonol glycoside (II) content.

For urine anthocyanin (I) and flavonol (II) analysis 2 mL of urine was acidified with 200 μL of 0.44 M TFA (trifluoroacetic acid) in water and stored at -80 °C until analyzed (*36*). Acidified urine was purified with solid phase extraction. 96-well plates with 2 mg of Oasis® packing material were pre-treated with 200 μL of methanol and 200 μL of 0.1 % TFA in water. 500 μL of acidified methanol extract of urine (I, II), feces (II) or plasma (II) was added and subsequently washed with 200 μL of 0.1 % TFA in water. After this, the waste container was replaced with a collection plate and anthocyanins (I) and flavonol glycosides (II) were eluted with 25 μL of 0.1 % TFA in methanol. 25 μL of 0.1 % TFA in water was added to enhance the chromatographic behaviour of anthocyanins.

The samples were injected directly from the collection plate wells into the uHPLC system. All MS-analyses were carried out on an uHPLC-MS/MS-equipment consisting of an Acquity UPLC™ system (Waters) with Acquity UPLC™ BEH C_{18} column (1.7 μm, 2.1 x 50 mm, Waters) and of Quattro Premier triple quadrupole mass spectometer (Waters). Elution for anthocyanin analysis (I) was performed using 0.2 % TFA and 1 % HCOOH in water as solvent A and 0.2 % TFA and 1 % HCOOH in acetonitrile as solvent B. For flavonol glycoside analysis (II) elution was performed using 1% acetic acid in water as solvent A and acetonitrile as solvent B. The gradient was optimized to achieve baseline separation of analytes. In study I, initial solvent composition for 0.45 min was 95% A and 5% B. Subsequently, the compounds were eluted with a gradient from 0.45 min to 6.8 min resulting in 88% A and 12% B. After that the proportion of B was increased to 90% for column wash and the initial conditions were stabilized at 7.6-8.0 min. The gradient in study II was the following: initial solvent composition for 0.45 min was 90% A and 10% B. Subsequently, the compounds were eluted with a gradient from 0.45 min to 2 min resulting in 82.5% A and 17.5% B. After that the proportion of B was increased to 70% for column wash and the initial conditions were stabilized at 4.8-5.3 min. The flow rate in both studies was 0.45 mL/min.

Identification of anthocyanins and flavonol glycosides in plasma, urine and feces samples was performed by spiking blank urine with reference compounds and comparing their retention times and parent and product ions in MS/MS. Detection was carried out by using electrospray ionization in a positive ion mode. Anthocyanins were quantified with an external standard. Cyanidin-3-galactoside was chosen as a standard compound and a standard curve was made by spiking anthocyanin-free blank urine with 0, 10, 25, 100 and 500 ng/m/L cyanidin-3-galactoside. Flavonols were quantified with an internal standard, syringetin-3-O-glucoside. To eliminate the error from differences in response, a standard curve was made by spiking flavonol-free blank urine with 0, 40, 200, 1000 and 5000 ng/mL of all reference compounds used (cyanidin, cyanidin 3-O-glucoside, cyanidin 3-O-galactoside, and cyanidin 3-O-rutinoside

in study I; isorhamnetin-3-glucoside, isorhamnetin-3-rutinoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-glucoside, myricetin-3-rhamnoside and syringetin-3-glucoside in study II). In the quantification of compounds for which the reference compounds could not be obtained, a standard curve of the most similar compound was utilized.

In studies I, II, and V, the study products were analyzed for their flavonol glycosides. The berry product (0.2 g) was weighed, homogenized, and extracted once with 4 mL of 0.1% TFA-H_2O and twice with 1 mL of 0.1% TFA-MeOH. Supernatants were combined and diluted with 8 mL of 0.1% TFA-H_2O. Extracts were applied in Supelco (C18 500 mg) solid phase extraction tubes (Bellefonte, USA) preconditioned with 2 mL of methanol and 2 mL of 0.1% TFA-H_2O. The tubes were washed with 2 mL of 0.1% TFA-H_2O and the analytes were eluted with 1 mL of TFA-MeOH-H_2O (40% of 0.1% TFA-H_2O, 60% MeOH). The samples were analyzed as such with the uHPLC-MS/MS method. uHPLC-MS/MS equipment consisted of an Acquity UPLC™ system with a 50 mm x 2.1 mm, 1.7 μm, Acquity UPLC BEH C_{18} column and a Quattro Premier tandem quadrupole mass spectrometer (Waters, Milford, MA). The elution of the samples was performed using 1% acetic acid in water as solvent A and acetonitrile as solvent B. The flow rate was 0.45 mL/min, and the gradient was similar to the one used in the physiological sample analysis in study II (described above). Analysis was carried out by using electrospray ionization in the positive ion mode. The MS/MS data were collected in the MRM mode. In study I quantification was performed with external standard (cyanidin-3-galactoside), whereas in studies I and IV flavonols were quantified with internal standard (syringetin-3-glucoside)

4.5. STATISTICAL ANALYSES

In study III, group comparison analyses were calculated with an unpaired t-test when data was normally distributed and with the Mann-Whitney nonparametric test when data was abnormally distributed. Two-sided tests and significance levels of 0.05 were used throughout. Correlations between relative changes of measured parameters and berry consumption were calculated with the Spearman correlation coefficient. In study V, insulin responses were expressed as the incremental area under the curve (iAUC) with the fasting concentration as a baseline. Also, the peak values of insulin and glucose at 30 min, and the values of insulin at 120 min, and TNF-α at 60 min were used for statistical analyses. A general linear model repeated measurements variance analysis was used for all results as they were normally distributed. In study IV, the changes in the parameters after berry interventions were calculated by a reduction of the average of two wash-out values (one of the samples taken before the berry intervention and the other one taken after another 35 d wash-out following the berry intervention) from the value obtained at the end of the berry intervention. A general linear model repeated measurements variance analysis was used for all results to compare the changes in different berry interventions to the average change of all different

wash-out periods. All analyses were performed with SPSS software (SPSS Inc., Chicago, IL), version 14.0.

5. RESULTS AND DISCUSSION

5.1. ABSORPTION AND METABOLISM OF FLAVONOL GLYCOSIDES IN NORTHERN BERRIES

Method development and optimization was successful althought pH-labile anthocyanins possessed a tricky target. The uHPLC-MS/MS run of nine minutes utilizing small solvent volumes together with small 96-well plate solid phase extraction columns made the method economical and environmentally responsible. Utilization of small solid phase extraction columns also enabled the simultaneous extraction, purification and concentration of anthocyanins from urine samples without evaporation steps.

In study I, cyanidin-3-galactoside was the major anthocyanin in lingonberries consumed by the subjects (92.3% (w/w)), cyanidin-3-glucoside (7.7% (w/w)) being the minor one. Of the total anthocyanin derivatives obtained from urine after lingonberry ingestion, 46.7% was intact cyanidin-3-galactoside, 30.7% was peonidin galactoside and 22.6% was in the glucuronidated form. The total excreted amount of cyaniding-3-galactoside in the urine was less (AUC 1019 ng) than the amount of its two metabolites combined (AUC 1274 ng).

The main metabolites detected were glucuronidated and methylated derivatives of cyanidin, which is in accordance with previous animal (Ichiyanagi et al. 2005, Wu et al. 2004) and human (Talavéra et al. 2005, Wu et al. 2002, Kay et al. 2005, Kay et al. 2004) studies carried out with pure cyanidin compounds or extracts rich in cyanidins. Thus, the results suggest that cyanidin-3-galactoside is absorbed and metabolized by similar routes to other cyanidin glycosides investigated to date, althought relative amounts of intact and metabolized forms were somewhat different than previously reported. The maximum concentration of cyanidin derivatives in the urine after the ingestion of lingonberries with yoghurt appeared slightly later than reported in earlier cyanidin absorption studies (Bitsch et al. 2004, Frank et al. 2007, Frank et al. 2003, McGhie et al. 2003). In these studies, cyanidin glycosides were ingested in the form of an extract or concentrated juice. It is interesting to note that anthocyanins in our study design also remained in the urine longer, even up to 12 hours. We also detected lower maximum concentrations of individual anthocyanins in the urine compared with many previous studies. Lower peak concentration is evidently a consequence of the slower absorption.

Also, the relative amounts of intact and metabolized forms were somewhat different than previously reported. In study I, of the total anthocyanin derivatives obtained from the urine 46.7 % was intact cyanidin-3-galactoside, 30.7 % was peonidin galactoside and 22.6 % was in a glucuronidated form (**Fig. 13**).

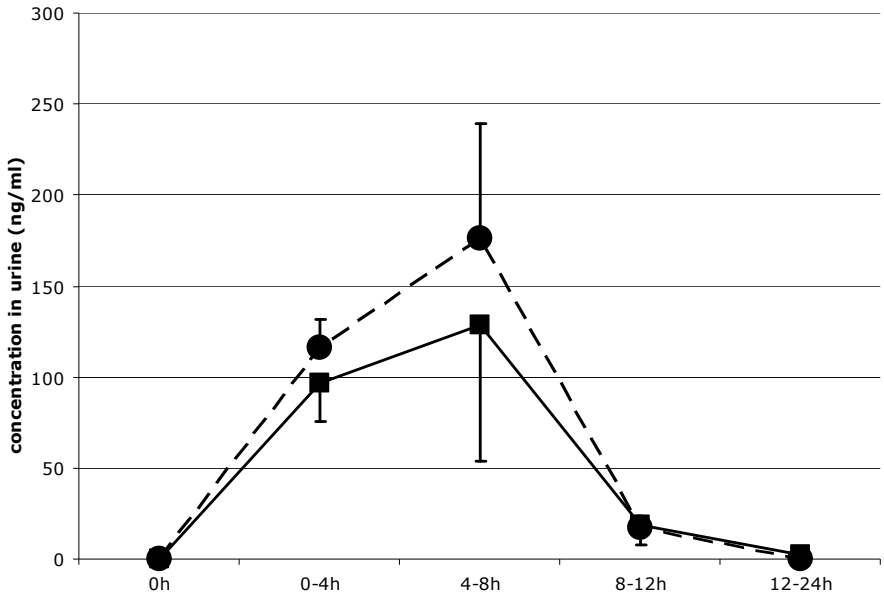

Figure 13: The amounts of total anthocyanin derivatives obtained from urine. 46.7 % was intact cyanidin-3-galactoside (squares □), and 53.3 % was metabolized (dots •).

In study II, the HPLC-MS/MS method was further optimized for flavonol glycoside analysis. Run time of 5.3 min made the method ecological as solvent usage was minimal. Detection and quantification limits for the reference compounds were 0.75 ng/mL and 1.5–3.1 ng/mL in standard dilutions, respectively. Isorhamnetin-3-rutinoside was the major flavonol in sea buckthorn berries [54% (wt-%)], isorhamnetin-3-glucoside, isorhamnetin-3-galactoside and quercetin-3-glucoside being the minor ones, accounting for 22, 8, and 13% (wt-%, respectively). In lingonberries quercetin-3-rhamnoside was the main flavonol [33% (wt-%)], and quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-xyloside/-arabinoside were present as lower levels of 29, 4, 6, 5, and 19% (wt-%, respectively) of the total flavonols.

After the consumption of sea buckthorn, 5.1% of flavonols excreted in the urine were detected intact, and 94.9% as the glucuronidated form. Isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, quercetin-3-glucoside and kaempherol-3-rutinoside were detected in feces, isorhamnetin-3-glucoside, quercetin-3-glucoside, isorhamnetin glucuronide and quercetin glucuronide in the urine and isorhamnetin glucuronide and kaempherol glucuronide in the plasma. Lingonberry meal resulted in somewhat different proportions of intact and glucuronidated forms as 13.8% of flavonols analyzed from the urine were glycosides, and 86.2% glucuronidated forms. After the consumption of lingonberries, quercetin-3-galactoside, quercetin-3-rhamnoside, and quercetin-3-x-sugar were detected in the feces, quercetin-3-x-sugar, quercetin glucuronide, quercetin-3-rhamnoside in the urine and quercetin-3-rhamnoside, quercetin glucuronide and kaempherol glucuronide in the plasma. Compounds

detected after the consumption of lingonberry and sea buckthorn were not detectable before the consumption of the berries, and the concentrations decreased in plasma and urine almost to the baseline values during the collection period. For feces the 48 h collection period was too short as the analytes were detectable at the 48 h timepoint. Amounts of glucuronides and glycosides in urine are presented in **Figure 14**. Berries thus seem to serve as foods or food components providing a relatively slow and steady flavonol supply, when compared to previous studies of flavonol absorption (Wang et al. 2003, Mullen et al. 2006, Bonetti et al. 2007)

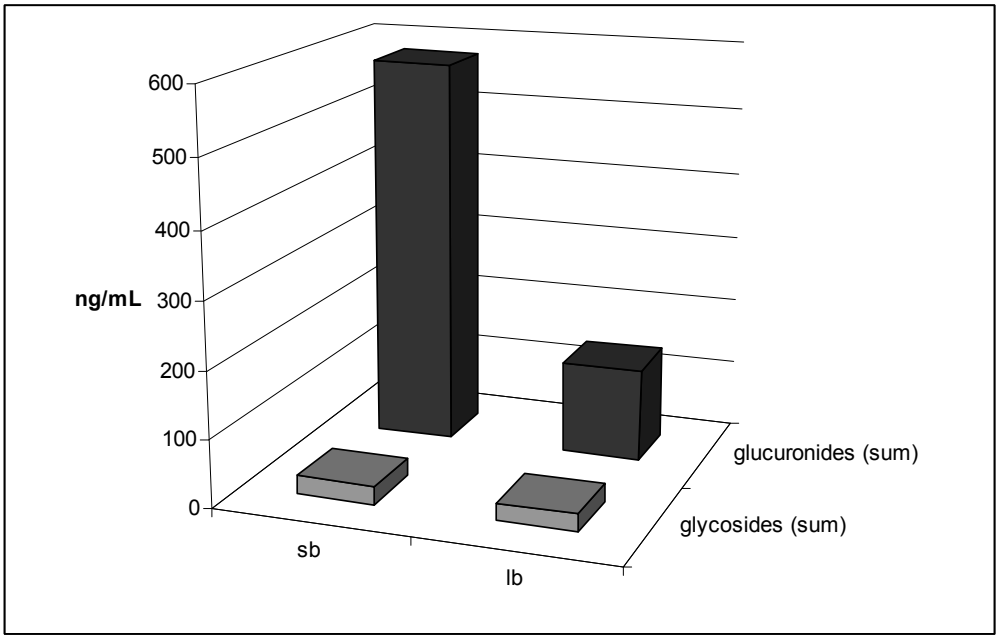

Figure 14: Concentrations of flavonol glucuronides and glycosides in urine analyzed in study II after the consumption of a berry breakfast. sb= sea buckthorn, lb=lingonberry

However, Interindividual differences both at the absorption extent and time as well as relative proportions of different metabolites were large. The concentrations of intact compounds and metabolites in the urine of individual study subjects is presented in **Figure 15**. As a summary, it can be stated that both anthocyanins and flavonol glycosides ingested in the studied berries are absorbed and the absorbed compounds are vastly metabolized. Metabolites tend to be in the more easily soluble and excreted form, i.e. in glucuronides.

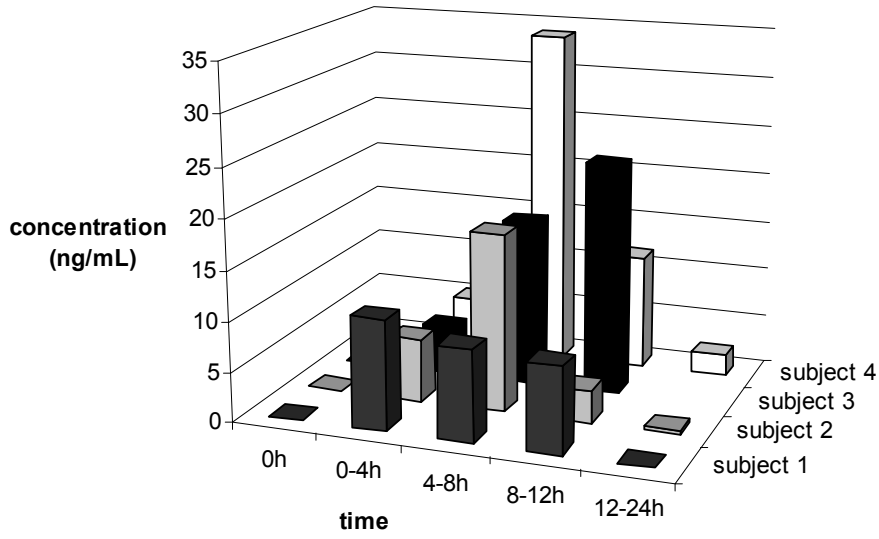

Figure 15: Concentrations of isorhamnetin-3-glucoside in the urine samples of the four study subjects after the ingestion of sea buckthorn.

5.2. EFFECT OF NORTHERN BERRIES ON ECTOPIC FAT ACCUMULATION AND THE RISK OF OBESITY-RELATED DISEASES

In study III, plasma ALT decreased and plasma adiponectin increased in the berry group ($P<0.001$ and $P=0.002$, respectively), but there was no statistically significant change in the lifestyle group. The changes in plasma ALT differed statistically significantly ($P=0.003$) between the groups (**Fig. 16A**). As there was a slight increasing trend in the adiponectin in the control group (Δ 0.56 μg/mL in control group, Δ 1.61 μg/mL in the berry group), the difference between the groups did not reach a level of significance. No statistically significant changes in either of groups were recorded in BMI, waist circumference, fasting plasma insulin levels, fasting plasma cholesterol or triacylglycerol levels, hs-CRP, TNF-α or ORAC.

Changes in the ALT activities of the individual study subjects in the berry group in study III displayed a decreasing trend in 24 subjects out of 28 (**Fig. 16B**), whereas changes in the lifestyle group were random (data not shown). This supports the conclusions drawn from the statistical results. Because ALT is affected by alcohol consumption, the average intake of alcohol of the study subjects was estimated from food diaries and compared between the groups. There was no difference in the average alcohol consumption ($P=0.973$), and neither in the compliance to alcohol restriction before the sample collection, between the groups.

Changes of adiponectin and ALT correlated negatively and statistically significantly ($P=0.049$, $R=0.08$) when all the study subjects were taken into account. Within the berry group there was a

significant correlation between berry consumption and both an adiponectin increase (P=0.021, R=0.204) and an ALT decrease (P=0.039, R=0.159). Slight variation in berry consumption within the berry group explained 20.4 % of the adiponectin increase and 15.9 % of ALT decrease during intervention, although berry intake in this group was already very high. Within the lifestyle group there was no correlation between the reported berry consumption and ALT and adiponectin changes. Thus it can be stated that berries probably caused the decrease of ALT values and the increase of the adiponectin values in the berry group. The current study showed that the daily consumption of over 150 g of northern berries in various forms as part of the normal diet had a positive effect on ALT and adiponectin levels, but the small amount of berries consumed as part of the normal diet in the lifestyle group was not enough to evoke such an impact.

The decrease in ALT activity in the berry group was 23%. Ratziu et al. (2010) recently reported in their study on rosiglitazone, a decrease of 24% both after 4-6 months and even a 2 year extension phase. As rosiglitazone is a medication with many side effects, it is remarkable that the same effect could be obtained by eating safe and traditional berries.

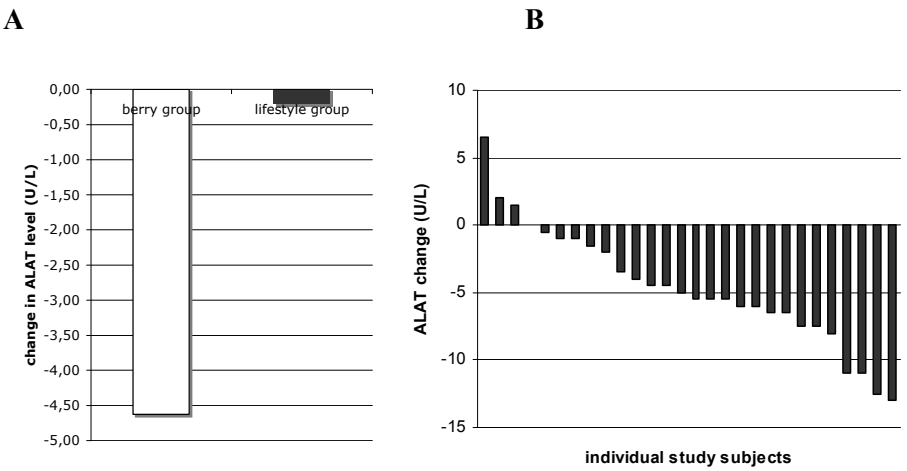

Figure 16: The changes in plasma ALT on average in both groups (**A**), and the changes of individual study subjects in the berry group (**B**).

In study IV, VCAM concentrations decreased after BB and SBo diets, and ICAM after a SBe diet. Also, the values not reaching statistical significance tended to show a decreasing trend after other berry intervention periods in VCAM and ICAM values. ICAM and VCAM belong to the immunoglobulin super-family, and they are involved in the process of tethering leukocyte to and transmigration across the endothelium (Konstantopuolos et al. 1996). Firm adhesion and transmigration of leucocytes across the endothelium requires ICAM and VCAM, and this step is the earliest stage in the development of the atherosclerotic lesion. Thus, these endothelial markers are implicated in the pathogenesis of atherosclerosis and CVD (Hope et al. 2003), and they are also elevated in obesity and have been shown to decrease with weight loss (Keogh et al. 2007). Major change observed in study IV, both statistically significant and more profound

in subjects with greater baseline BMI, was the decrease in VCAM levels during sea buckthorn berry oil intervention. Also, statistically significant changes in waist circumference after BB (Δ -1.2 cm, $p = 0.041$) and SB (Δ -1.1 cm, $p = 0.008$) periods were observed. A decreasing but non-significant trend was observed also after the other berry interventions, [SBo (Δ -1.2 cm ± 3.8, NS, $p = 0.077$) and SBe (Δ -0.1 cm ± 4.1, NS)]. Accordingly, there was a small decrease in weight after the BB diet (Δ -0.2 kg, $p = 0.028$) but after the SBe and SBo interventions the decrease was statistically non-significant.

It must be noted that energy intake during the SB diet was higher than in wash-out periods, mainly due to increased carbohydrates, mostly sugar, and fat intake from other sources than the berry itself. These changes were due to other modifications of the diet that occurred during the SB period, possibly because of the strong taste of the berry. It seems that study subjects increased the use of sugar- and fat-rich foods, although the energy intake was intended to be similar. This might have hindered some effects of the SB diet and explains why SBo and SBe diets had more impact on VCAM and ICAM values than the whole berry (SB) diet. It is also interesting to notice that BB seemed to be more effective than SB, although previously isorhamnetin-3-glucuronide, a major metabolite of isorhamnetin glucosides of sea buckthorn, has been shown to modulate the expression of VCAM (Tribolo et al. 2008). Also aronia (*Aronia melanocarpa* E.) extract has been shown to lower the levels of adhesion molecules concurrently with IL-6 and CRP, and the elevation of adiponectin (Naruszewicz et al. 2007).

Adiponectin is an adipose-secreted cytokine, which is present at decreased plasma levels in subjects with obesity (Arita et al. 1999) or type 2 diabetes (Hotta et al. 2000). According to a recent review, experimental and epidemiological studies have provided abundant evidence that adiponectin improves insulin sensitivity, and has potent antiatherosclerotic effects (Stefan et al. 2002). In study III, adiponectin increased during a mixed berry diet, although the differences between the berry and lifestyle groups was not statistically significant. In study IV, adiponectin was decreased more or less during all berry interventions. This contradiction seems less confusing in the light of the knowledge that adiponectin values (Arita et al. 1999), similarly to GHbA1c (American Diabetes Association 2003), change more slowly than, for example, ICAM and VCAM values. The rate of formation of $GHbA_{1c}$ is directly proportional to the ambient glucose concentration. Since erythrocytes are freely permeable to glucose, the level of $GHbA_{1c}$ in a blood sample reflects the glycemic control of the previous 120 days, the average erythrocyte life span (American Diabetes Association 2003). As the duration of intervention periods was only 33-35 days, the changes observed in GHbA1c and adiponectin values may reflect more the effect of wash-out periods than the berry intervention and vice versa. In any case, when considering the nature of the formation of $GHbA_{1c}$, intervention periods in this study were quite short (5 weeks) to make strong conclusions.

There was no decrease in ALT activity after any intervention period in trial IV. Thus it seems that the effects observed in study III cannot be due to bilberry or sea buckthorn. It also

seems that either lingonberry and/or black currant modulate the fat metabolism of the liver into a positive direction or the positive effect requires simultaneous incorporation of different berries into the diet. However, it must be noted that there were also other differences between trials III and IV as intervention periods in the latter (IV) were shorter (5 weeks instead of 20), and the daily amount of berries less (100 g instead of 160 g). However, sampling was performed also at the 10-week time point in trial III, and the change in ALT values was already at the same level as after 20 weeks. This implies that some trend could be expected already after 5 weeks in this particular value. Another notion should be made about sea buckthorn varieties, as in the first trial berries were consumed frozen and were the organically cultivated Ljubitelskaja variety (*Hippophaë rhamnoides* ssp *mongolica*) grown in Finland, whereas in the second trial, sea buckthorn berries were dried *Hippophaë rhamnoides* ssp *turkestanica* berries grown in China. Despite the lack of decline in ALT values, trial IV showed the other positive effects of berries on the risk factors of obesity-related diseases. Based on the results, it can be stated that different berries and berry fractions have quite different effects on metabolic syndrome and type 2 diabetes risk factors.

5.3. EFFECT OF SEA BUCKTHORN AND ITS FRACTIONS ON THE POSTPRANDIAL GLUCOSE METABOLISM

The main flavonol glycosides in the products were isorhamnetin-3-rutinoside, -glucoside, and –galactoside. One portion contained 22.8 mg (B1), 68.0 mg (B2), and 1.8 mg (B3) of flavonol glycosides. Whole berry meal (meal B1) in study V had a balancing effect on postprandial hyperglycemia when compared to the control meal (meal A) (Δ glucose concentration of 30 min peak value – 120 min value -1.0 mmol/L, p=0.042 versus the values in control meal). Also the insulin peak concentrations were lower after meal B1 than after meal A (Δ insulin concentration of 30 min peak value between the meals -22 mU/L, p = 0.039). The differences between meals B1 and A were even greater when the 120 min value was also considered, because the berries suppressed the subsequent drop in insulin values after the peak concentration (Δ insulin concentration of 30 min peak value – 120 min value between the meals -30.4 mU/L, p = 0.036). The phenomenon could also be observed as a trend of lower iAUC values ($\Delta iAUC^{30\ min}$ of insulin between the meals -297.2, p=0.062, $\Delta iAUC^{120\ min}$ of insulin between the meals -363.1, p=0.507). Similarly, the oil-free berry meal (meal B2, see **Fig 12** for the production flow of berry fractions) had a positive effect on insulin metabolism (Δ concentration of 30 min peak value – 120 min value -25.9 mU/L, p=0.037). In contrast, the berry residue (meal B3) had no effect on the peak insulin concentration, and the iAUC of 120 minutes was even slightly higher than of the control meal. Postprandial insulin responses after each berry meal and control meal are presented in **Figure 17**.

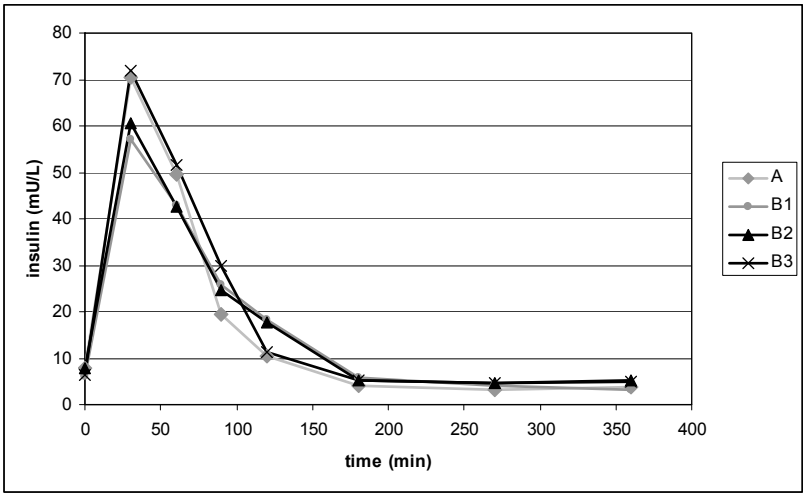

Figure 17: Postprandial insulin responses after each berry meal and control meal. A=control meal, B1=whole sea buckthorn meal, B2= oil-free berry meal and B3= berry residue. For production flow, see **Fig 12**.

Removal of the lipophilic compounds (SBo in **Fig. 12**) did not affect the advantageous effects of the berries on insulin metabolism (p=0.608) but the removal of the fraction soluble in 70 % EtOH (SBe in **Fig. 12**) eliminated the observed effects. The effective compounds seem thus to be in the ethanol soluble fraction rather than in the lipophilic fractions. However, it must be noted that meal B2 had higher content of flavonol glycosides than meal B1 when analyzed by HPLC-MS/MS. Amounts of different products in one meal had to be estimated before flavonol analysis results were obtained, and thus the erraneous approximation of extraction efficiency resulted in higher content of flavonol glycosides in the extract than whole berry.

Elevated plasma glucose concentrations are thought to alter metabolism, create oxidative stress, and induce apoptosis in many cell types (Evans et al. 2002). Oscillating glucose has been shown to be an even more important risk factor of metabolic diseases and their further complications than the mean glucose (Ceriello et al. 2008). The rapid absorption of glucose challenges the homeostasis mechanisms of the body, complicating in effect the transition from the postprandial state to the postabsorptive state (Wolever et al. 1995). The effect of carbohydrate composition on postprandial hyperglycemia and postprandial insulin response is commonly accepted (Kallio et al. 2008, Ludwig, 2000). Yet, even though there is some emerging evidence of the ability of other than macronutrient components of food to alter postprandial sugar metabolism, such evidence is to date scarce and mainly produced by *in vitro* and animal trials. Some exotic fruits or their phenolic extracts have also shown positive effects on sugar metabolism in animal models, as Zunino (2009) recently reviewed. However, prior to study V of this thesis, no investigations were available concerning the potential of northern berries to alter postprandial metabolism. In study V, sea buckthorn berries had a stabilizing effect on postprandial hyperglycemia. Furthermore, these berries suppressed the

peak postprandial insulin response after a high-glucose meal. Thus, sea buckthorn berries consumed as part of a high-glucose meal containing simple carbohydrates seemed to be able to modify the postprandial glucose metabolism towards a metabolic response typical after ingestion of complex carbohydrates.

The berries as part of a high-glucose meal containing simple carbohydrates seemed to be able to modify the postprandial glucose metabolism towards a metabolic response typical after ingestion of complex carbohydrates. In addition, the insulin response was milder compared to the control, and this was also observed with the ethanol extract of sea buckthorn (V). According to accumulating evidence, diets designed to lower the insulin response to ingested carbohydrates may improve access to stored metabolic fuels, decrease hunger, and promote weight loss (Brand-Miller et al. 2002).

As a summary, it can be concluded that polyphenols possess the potential for decreasing the risk of metabolic dysfunctions, and alleviating ectopic fat accumulation. In the future, the potential of lingonberry and black currant on ectopic fat accumulation should be studied in clinical trials. It is also highly recommendable to perform another larger clinical trial with mixed berries. Berries are ecologic northern resources, the potential of which should be noted and utilized. Safe, natural alternatives for medication are especially important in the fight against childhood obesity and the occurrence of obesity-related diseases among children and adolescents.

6. CONCLUSIONS

An alarming trend in developed countries is that obesity, metabolic syndrome and type 2 diabetes are developing at increasingly younger ages (Franks et al. 2010). The potential of berries to decrease the risk of these ailments could be of primary importance for children and adolescents as berries are safe and readily accepted by them. In the original research of this thesis, the aim was to study whether berries could have positive effects on the risk factors of obesity-related diseases.

In the first part of the research (studies I, II) a high performance liquid chromatography tandem mass spectrometry analysis of anthocyanins and flavonol glycosides was developed, optimized and applied in a small postprandial trial. Both anthocyanins and flavonl glycosides of berries were shown to be bioavailable and metabolized in humans.

In the second part, a mixed berry diet was shown to decrease ALT values in a 5-month intervention study in humans, suggesting a eduction in liver fat content (III). This effect was not there when sea buckthorn and bilberry were consumed for 5 weeks, although other positive changes in waist circumference and adhesion molecules were seen (IV). It might be that a synergistic effect of various berries is needed to affect the ALT values related to ectopic fat accumulation, or the intervention time needs to be longer than 5 weeks. An explanation can also be that one of the berries not used in study IV, lingonberry or black currant, is the most active. However, it can be concluded that berries have various but positive effects on the risk factors of obesity-related diseases.

The third aim was to study the effects of sea buckthorn berry on the postprandial glucose metabolism. Study V showed that sea buckthorn berries decreases the postprandial hyperglycemia and insulinemia induced by a high-glucose meal. The effects could be mainly traced down to the polyphenol-rich fraction of the berry.

Although the compounds behind the the observed effects could not be revealed, some suggestions could be made. As polyphenolic fraction seemed to be the most active one in the modulation of postprandial sugar metabolism (V), it could be postulated to be the active fraction also in study III. In the literature review, the complex mechanisms behind ectopic fat accumulation were introduced with an emphasis on the possible control points where bioactive compounds could exert their regulatory effects. Based on the literature review, tannins seem to be one of the compound groups with the most potential. This supports the conclusion made based on the results from studies III and IV, that of the studied berries the one berry with most potential in alleviating ectopic fat accumulation is probably lingonberry or black currant, both berries being rich in tannins. However, it must be noted that the most investigated tannins are hydrolyzable tannins found in green tea, and the most abundant tannins in berries are often condensed tannins.

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REFERENCES

Adiels M, Westerbacka J, Soro-Paavonen A, Häkkinen AM, Vehkavaara S, Caslake MJ, Packard C, Olofsson SO, Yki-Järvinen H, Taskinen MR, Borén J. Acute suppression of VLDL1 secretion rate by insulin is associated with hepatic fat content and insulin resistance. *Diabetologia* 2007;50:2356-2365.

Adiels M, Taskinen MR, Packard C, Caslake MJ, Soro-Paavonen A, Westerbacka J, Vehkavaara S, Häkkinen A, Olofsson SO, Yki-Järvinen H, Borén J. Overproduction of large VLDL particles is driven by increased liver fat content in man. *Diabetologia* 2006;49:755-765.

Alarćon de la Lastra C, Villegas I. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. *Mol Nutr Food Res* 2005;49:405-430.

Aneja R, Hake PW, Burroughs TJ, Denenberg AG, Wong HR, Zingarelli B. Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats. *Mol Med* 2004;10:55-62.

Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346:1221-1231.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999;257:79-83.

Bashan N, Dorfman K, Tarnovscki T, Harman-Boehm I, Liberty IF, Blüher M, Ovadia S, Maymon-Zilberstein T, Potashnik R, Stumvoll M, Avinoach E, Rudich A. Mitogen-activated protein kinases, inhibitory kappaB kinase, and insulin signalling in human omental versus subcutaneous adipose tissue in obesity. *Endocrinology* 2007;148:2955-2962.

Bate-Smith EC. Detection and determination of ellagitannins. *Phytochem* 1972;11:1153-1156.

Baumann J, von Bruchhausen F, Wurm G. Flavonoids and related compounds as inhibitors of arachidonic acid peroxidation. *Prostaglandins* 1980;20:627-639.

Bays E. "Sick fat", metabolic disease, and atherosclerosis. *Am J Med* 2009;122:S26-S37.

Bengmark S. Impact of nutrition on ageing and disease. *Curr Opin Clin Nutr Metab Care* 2006;9:2-7.

Bielawska A, Shapiro J, Jiang L, Melkonyan HS, Piot C, Wolfe CL, Tomei LD, Hannun YA, Umansky SR. Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. *Am J Pathol* 1997;151:1257-1263.

Bitsch R, Netzel M, Sonntag S, Strass G, Frank T, Bitsch I. Urinary Excretion of Cyanidin Glucosides and Glucuronides in Healthy Humans After Elderberry Juice Ingestion. *J Biomed Biotech* 2004;2004:343–345.

Björntorp P. Metabolic implications of body fat distribution. *Diabetes Care* 1991;14:1132-1143.

Blûher M. The inflammatory process of adipose tissue. *Pediatr Endocrinol Rev* 2008;6:24-31.

Blûher M. Adipose tissue dysfunction in obesity. *Exp Clin Endocrinol Diabetes* 2009;117:241-250.

Boden G and Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and β-cell dysfunction. *J Clin Invest* 2002;32(Suppl. 3):14-23.

Bonetti, A.; Marotti, I.; Dinelli, G. Urinary excretion of kaempferol from common beans (Phaseolus vulgaris L.) in humans. *Int J Food Sci Nutr* 2007;58:261-269.

Brand-Miller J, Holt S, Pawlak D, McMillan J. Glycemic index and obesity. *J Am J Clin Nutr* 2002;76:S281-S285.

Bruce K, Byrne C. The metabolic syndrome: common origins of a multifactorial disorder. *Postgrad Med J* 2009;85:614-621.

Brunt E. Nonalcoholic steatohepatitis: definition and pathology. *Sem Liv Dis* 2001;21:3-16.

Buchert J, Koponen JM, Suutarinen M, Mustaranta A, Lille M, Törrönen R, Poutanen K. Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and blackcurrant juices. *J Sci Food Agric* 2005;85:2548-2556.

Bugianesi E, McCullough A, Marchesini G. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatol* 2005;42:987-1000.

Bujanda L, Hijona E, Larzabal M, Beraza M, Aldazabal P, García-Urkia N, Sarasqueta C, Cosme A, Irastorza B, González A, Arenas JI Jr. Resveratrol inhibits nonalcoholic fatty liver disease in rats. *BMC Gastroenterol* 2008;8:40-48.

Burt AD, Mutton A, Day CP. Diagnosis and interpretation of steatosis and steatohepatitis. *Semin Diagn Pathol* 1998;15:246-258.

Burton-Freeman B, Linares A, Hyson D, Kappagoda T. Strawberry modulates LDL oxidation and postprandial lipemia in response to high-fat meal in overweight hyperlipidemic men and women. *J Am Coll Nutr* 2010;29:46-54.

Carey D, Cowin G, Galloway G, Galloway G, Jones N, Richards J, Biswas N, Doddrell D. Effect of rosiglitazone on insulin sensitivity and body composition in type 2 diabetic patients. *Obes Res* 2002;10:1008-1015.

Ceriello A, Esposito K, Piconi L, Ihnat M, Thorpe J, Testa R, Boemi M, Giugliano D. Glucose "peak" and glucose "spike": Impact on endothelial function and oxidative stress. *Diabetes Res Clin Pract* 2008;82:262–267.

Chan MM, Fong D, Ho CT, Huang HI. Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem Pharmacol* 1997;54:1281-1286.

Chen YC, Shen SC, Lee WR, Hou WC, Yang LL, Lee TJ. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J Cell Biochem* 2001;82:537-548.

Chen CC, Chow MP, Huang WC, Lin YC, Chang YJ. Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappaB: structure-activity relationships. *Mol Pharmacol* 2004;66:683-693.

Chen JC, Ho FM, Pei-Dawn Lee Chao, Chen CP, Jeng KC, Hsu HB, Lee ST, Wen Tung Wu, Lin WW. Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IkappaB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. *Eur J Pharmacol* 2005;521:9-20.

Chidambaram J, Venkatraman. Cissus quandrangularis stem alleviates insulin resistance, oxidative injury and fatty liver disease in rats fed high fat plus fructose diet. *Food Chem Toxicol* 2010 (in press).

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005;46:2347-2355.

Cohen B, Novick D, Rubinstein M. Modulation of insulin activities by leptin. *Science* 1996;274:1185-1188.

Comalada M, Ballester I, Bailón E, Sierra S, Xaus J, Gálvez J, de Medina FS, Zarzuelo A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. *Biochem Pharmacol* 2006;72:1010-1021.

Crouvezier S, Powell B, Keir D, Yaqoob P. The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro. *Cytokine* 2001;13:280-286.

Curat C, Miranville A, Sengenés C Diehl M, Tonus C, Busse R, Bouloumié A. From blood monocytes to adipose tissue-resident macrophages:induction of diabedesis by human mature adipocytes. *Diabetes* 2004;53:1285-1292.

Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: A comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circul* 2005;111:1448–1454.

Day CP. Non-alcoholic fatty liver disease: current concepts and management strategies. *Clin Med* 2006;6:19-25.

Day C. Genes or environment to determine alcoholic liver disease and non-alcoholic fatty liver disease. *Liver Int* 2006;26:1021-1028.

DeFuria J, Bennett G, Strissel KJ, Perfield JW 2nd, Milbury PE, Greenberg AS, Obin MS. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *J Nutr* 2009;139:1510-6.

Del Bas J, Fernández-Larrea J, Blay M, Ardévol A, Salvadó M, Arola L, Bladé C. Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *FASEB J* 2005;19:479-481.

Erlund I, Koli G, Alfthan J, Marniemi J, Puukka P, Mustonen P, Mattila P, Jula A. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *Am J Clin Nutr* 2008;87:323-331.

Evans J, Goldfine I, Maddux B, Grodsky G. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* 2002;23:599–622.

Faulds CB, Williamson G. The role of hydroxycinnamates in the plant cell wall. *J Sci Food Agric* 1999;79:393-395.

Felld TS, Lee DW, Holbrook NM. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiol* 2001;127:566-574.

Ford ES. Prevalence of the metabolic syndrome defoned by the International Diabetes Federation among adults in the U.S. *Diab Care* 2005;28:2745-2749.

Foufelle F, Ferré P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor SREBP-1c. *Biochem J* 2002;366:377-391.

Frank T, Janssen M, Netzel G, Christian B, Bitsch I, Netzel M. Absorption and excretion of elderberry (*Sambucus nigra L.*) anthocyanins in healthy humans. *Meth Find Exp Clin Pharm* 2007;29:525–533.

Frank T, Netzel M, Strass G, Bitsch R, Bitsch, I. Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. *Can J Physiol Pharm* 2003;81:423–436.

Frühbeck G, Gómez-Ambrosi J, Muruzabai FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signalling in energy

metabolism regulation. *Am J Physiol* 2001;280:E827-E847.

Ganong W. Review of medical physiology. United States of America: McGraw-Hill Companies, 2003, p. 322-329. ISBN 0-07-140236-5

Gao Z-L, Gu X-H, Cheng F-T, Jiang F-H. Effect of sea buckthorn on human fibrosis: A clinical study. *World J Gastroenterol* 2003;9:1615-1617.

García-Mediavilla V, Crespo I, Collado PS, Esteller A, Sánchez-Campos S, Tuñón MJ, González-Gallego J. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *Eur J Pharmacol* 2007;557:221-229.

Ginsberg H, Illingworth R. Postprandial dyslipidemia: an atherogenic disorder common in patients with diabetes mellitus. *Am J Cardiol* 2001;88:9–15.

Goldberg RB, Capuzzi D. Lipid disorders in type 1 and type 2 diabetes. *Clin Lab Med* 2001;21:147-172.

Goodpaster BH, Krishnaswami S, Resnick H, Kelley DE, Haggerty C, Harris TB, Schwartz AV, Kritchevsky S, Newman AB. Association between regional adipose tissue distribution and both type 2 diabetes and impaired glucose tolerance in elderly man and women. *Diabetes Care* 2003;26:372-379.

Greco D, Kotronen A, Westerbacka J, Puig O, Arkkila P, Kiviluoto T, Laitinen S, Kolak M, Fisher RM, Hamsten A, Auvinen P, Yki-Järvinen H. Gene expression in human NAFLD. *Am J Physiol* 2008;294:G1281-G1287.

Greenfield V, Cheung O, Sanyal AJ. Recent advances in nonalcholic fatty liver disease. *Curr Opin Gastroenterol* 2008;24:320-327.

Gruendel S, Otto B, Garcia AL, Wagner K, Mueller C, Weickert M.O, Heldwein W, Koednick, C. Carob pulp preparation rich in insoluble dietary fibre and polyphenols increases plasma glucose and serum insulin responses in combination with a glucose load in humans. *British J Nutr* 2007;98:101-105.

Hahlbrock K, Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. *Annual Rev Plant Phys Plant Mol Biol* 1989;347-369.

Hanamura T, Mayama C, Aoki H, Hirayama Y, Shimizu M. Antihyperglycemic effect of polyphenols from Acerola (*Malpighia emarginata* DC.) fruit. *Biosci Biotechnol Biochem* 2006;70:1813-1820.

Hanley A, Williams K, Festa A, Wagenknecht LE, D'Agostino RB, Kempf J, zinman B, Haffner S. Elevations in markers of liver injury and risk of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* 2004;53:2623-2632.

Hansen SK, Baeuerle PA, Blasi F. Purification, reconstitution, and I kappa B association of the c-Rel-p65 (RelA) complex, a strong activator of transcription. *Mol Cell Biol* 1994;14:2593-2603.

Hajer G, Haeften T, Visseren F. Adipose tissue dysfunction in obesity, diabetes, and vascular disease. *Eur Heart J* 2008;29:2959-2971.

Harborne JB. General procedures. In: Dey PM, Harborne JB, eds. Methods in Plant Biochemistry. London, UK: Academic Press, 1989, p.1-27. In: Riihinen K: Phenolic compounds in berries. Kuopio Univ Publ C Nat Environ Sci 2005;187:1-97.

Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 2007;8:774-785.

Haslam E. Plant polyphenols – vegetable tannins revisited, Cambridge University Press, Cambridge 1989. In: Serrano J, Puupponen-Pimiä R, Dauer A, Aura AM, Saura-Calixto F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Mol Nutr Food Res* 2009:53:S310-S329.

Hlebowicz J, Darwiche G, Bjrgell O, Almér L. Effect of cinnamon on postprandial blood glucose, gastric emptying, and satiety in healthy subjects. *Am J Clin Nutr* 2007;85:1552–1556.

Hoffmann SM, Tschöp MH. Dietary sugars: A fat difference. *J Clin Invest* 2009; 119:1089–1092.

Hong J, Smith TJ, Ho CT, August DA, Yang CS. Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. *Biochem Pharmacol* 2001;62:1175-1183.

Hope SA, Meredith IT. Cellular adhesion molecules and cardiovascular disease. Part I. Their expression and role in atherogenesis. *Intern Med J* 2003;33:380-386.

(a) Hou DX, Masuzaki S, Hashimoto F, Uto T, Tanigawa S, Fujii M, Sakata Y. Green tea proanthocyanidins inhibit cyclooxygenase-2 expression in LPS-activated mouse macrophages: molecular mechanisms and structure-activity relationship. *Arch Biochem Biophys* 2007;460:67-74.

(b) Hou DX, Luo D, Tanigawa S, Hashimoto F, Uto T, Masuzaki S, Fujii M, Sakata Y. Prodelphinidin B-4 3'-O-gallate, a tea polyphenol, is involved in the inhibition of COX-2 and iNOS via the downregulation of TAK1-NF-kappaB pathway. Biochem Pharmacol 2007;74:742-751.

Hou X, Xu S, Maitland-Toolan, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierbicki M, Verbeuren TJ, Cohen RA, Zang M. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008;283:20015-20026.

Hotamisligil G, Peraldi P, Budavari A. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996;271:665-668.

Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA. The Ca2+/calmodulin-dependent protein kinase kinases are AMP-

activated protein kinase kinases. *J Biol Chem* 2005;280:29060-29066.

Huang SM, Wu CH, Yen GC. Effects of flavonoids on the expression of the pro-inflammatory response in human monocytes induced by ligation of the receptor for AGEs. *Mol Nutr Food Res* 2006;50:1129-1139.

Hwang JT, Kwon DY, Yoon SH. AMP-activated protein kinase: a potential target for the diseases prevention by natural occurring polyphenols. *N Biotechnol* 2009;26:17-22.

Häkkinen S, Törrönen R. Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique. *Food Res Int* 2000:33:517-524.

Häkkinen S, Hienonen M, Kärenlampi S, Mykkänen H, Ruuskanen J, Törrönen R. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res Int* 1999;32:345-353.

Ichikawa D, Matsui A, Imai M, Sonoda Y, Kasahara T. Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line, J774.1. *Biol Pharm Bull* 2004;27:1353-1358.

Ichiyanagi T, Shida Y, Rahman M, Hatano Y, Matsumoto H, Hirayama M, Konishi T. Metabolic pathway of cyanidin 3-O-beta-D-glucopyranoside in rats. *J Agric Food Chem* 2005;53:145–150.

Isken F, Klaus S, Petzke KJ, Loddenkemper C, Pfeoffer AFH, Weickert MO. Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am J Physiol Endocrinol Metab* 2010;298:E287-E295.

Jayaprakasam B, Olson LK, Schutzki RE, Tai MH, Nair MG. Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (Cornus mas). *J Agric Food Chem* 2006;54:243-8.

Jeandet P, Bessis R, Gautheron B. The production of resveratrol (3,5,4′-trihydroxystilbene) by grape berries in different developmental stages. *Am J Enol Vitic* 1991;42:41-46.

Jiménez-Gómez Y, Marín C, Peérez-Martínez P, Hartwich J, Malczewska-Malec M, Golabek I, Kiec-Wilk B, Cruz-Teno C, Rodríguez F, Gómez P, Gómez-Luna MJ, Defoort C, Gibney MJ, Pérez-Jiménez F, Roche HM, López-Miranda J. A low-fat, high-complex carbohydrate diet supplemented with long-chain (n-3) fatty acids alters the postprandial lipoprotein profile in patients with metabolic syndrome. *J Nutr* 2010;140:1595-1601.

Johansson A, Korte H, Yang B, Stanley J, Kallio H. Sea buckthorn berry oil inhibits platelet aggregation. *J Nutr Biochem* 2008;11:491-495.

Kabir M, Rizkalla SW, Champ M, Luo J, Boillot J, Bruzzo F, Slama G. Dietary amylose-amylopectin starch content affects glucose and lipid metabolism in adipocytes of normal and diabetic rats. *J Nutr* 1998;128:35-43.

Kallio P, Kolehmainen M, Laaksonen DE, Pulkkinen L, Atalay M, Mykkänen H, Uusitupa M, Poutanen K, Niskanen L. Inflammation markers are modulated by responses to diets differing in postprandial insulin responses in individuals with the metabolic syndrome. *Am J Clin Nutr* 2008;87:1497–1503.

Kantartzis K, Staiger H, Machann J, Schick F, Claussen CD, Machicao F, Fritsche A, Häring HU, Stefan N. Adiponectin oligomers and ectopic fat in liver and skeletal muscle in humans. *Obesity* (Silver Spring) 2009;17:390-392.

Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 1995;270:16483-16486.

Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17-26.

Karpe F. Postprandial lipoprotein metabolism and atherosclerosis. *J Intern Med* 1999;246:341-355.

Kaur C, Jabbar Z, Athar M, Alam M. Punica granatum (pomegranate) flower extract possesse potent antioxidant activity and abrogates fe-NTA induced hepatotoxicity in mice. *Food Chem Toxicol* 2006;44:984-993.

Kaviarasan S, Viswanathan P, Anuradha C. Fenugreek seed (Trigonella foecum graecum) polyphenols inhibit ethanol-induced collagen and lipid accumulation in rat liver. *Cell Biol Toxicol* 2007;23:373-383.

Kawamori R. The role of the liver on postprandial glycaemia-emphasis on hepatic glucose uptake. *Int J Clin Prat Suppl* 2000;112:19-22.

Kay C. D, Mazza G, Holub B, Wang J. Anthocyanin metabolites in human urine and serum. *Br J Nutr* 2004;9: 933-942.

Kay C. D, Mazza G, Holub B. J. Anthocyanins exist in the circulation primarily as metabolites in adult men. *J Nutr* 2005**;**13:2582-2588.

Keogh JB, Luscombe-Marsh ND, Noakes M, Wittert GA, Clifton PM. Long-term weight maintenance and cardiovascular risk factors are not different following weight loss on carbohydrate-restricted diets high in either monounsaturated fat or protein in obese hyperinsulinaemic men and women. *Br J Nutr* 2007;97:405-410.

Kidd PS, Llugany M, poschenrieder C, Gunsé B, Barceló J. The role of root exudaces in aluminium resistance ans silicon-induced amelioration of aluminium-toxicity in three variety of maize (Zea mays L.). *Exp Bot* 2001;52:1339-1352.

Kim HP, Mani I, Iversen L, Ziboh VA. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostagl Leuk Ess Fat Ac* 1998;58:17-24.

Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem Pharmacol* 1999;58:759-765.

Kobayashi M, Ichitani M, Suzuki Y, Unno T, Sugawara T, Yamahira T, Kato M, Takihara T, Sagesaka Y, Kakuda T, Ikeda I. Black-tea polyphenols suppress postprandial hypertriacylglycerolemia by suppressing lymphatic transport of dietary fat in rats. *J Agric Food Chem* 2009;57:7131-7136.

Kolak M, Westerbacka J, Velagapudi V, Wågsäter D, Yetukuri L, Makkonen J, Rissanen A, Häkkinen AM, Lindell M, Bergholm R, Hamsten A, Eriksson P, Fisher RM, Oresic M, Yki-Järvinen H. Adipose tissue inflammation and increased ceramide content characterize subjects with high liver fat content independent of obesity. *Diabetes* 2007;56:1960-1968.

Konstantopoulos K, McIntire LV. Effects of fluid dynamic forces on vascular cell adhesion. *J Clin Invest* 1996;98:2661-2665.

Kotronen A, Westerbacka J, Bergholm R, Pietiläinen K, Yki-Järvinen H. Liver fat in the metabolic syndrome. *J Clin Endocr Metab* 2007;92:3490-3497.

Kotronen A, Yki-Järvinen H. Fatty liver: a novel component of the metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008;28:27-38.

Krogh-Madsen R, Møller K, Dela F, Kronborg G, Jauffred S, Pedersen B. Effect of hyperglycemia and hyperinsulinemia on the response of IL-6, TNF-alpha, and FFAs to low-dose endotoxemia in humans. *Am J Physiol Endocrinol Metab* 2004;286:E766-E772.

Kundu JK, Surh YJ. Epigallocatechin gallate inhibits phorbol ester-induced activation of NF-kappa B and CREB in mouse skin: role of p38 MAPK. *Ann N Y Acad Sci* 2007;1095:504-512.

Kundu JK, Na HK, Chun KS, Kim YK, Lee SJ, Lee SS, Lee OS, Sim YC, Surh YJ. Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells. *J Nutr* 2003;133:S3805-S3810.

Kähkönen M, Hopia A, Heinonen M. Berry phenolics and their antioxidant activity. *J Agric Food Chem* 2001;49:4076-4082.

Laclaustra M, Corella D, Ordovas JM. Metabolic syndrome pathophysiology: The role of adipose tissue. *Nutr Metabol Cardiov Dis* 2007;17:125-139.

Lopez-Miranda J, Williams C, Lairon D. Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. *Br J Nutr* 2007;98:458–473.

Lakka HM, Lakka TH, Tuomilehto J, Salonen JT. Abdominal obesity is associated with increased risk of acute coronary events in men. *Eur Heart J* 2002;23:706-713.

Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S, Smith SR, Alfonso A, Ravussin E. Effect of calorie restriction with or without exercise on insulin sensitivity, beta-cell function, fat cell size, and ectopic lipid in overweight subjects. *Diabetes Care* 2006;29:1337-1344.

Laughton MJ, Evans PJ, Moroney MA, Hoult JR, Halliwell B. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. *Biochem Pharmacol* 1991;42:1673-1681.

Lawrence T, Willoughby D, Gilroy D. Anti-inflammatory lipid medaitors and insights into the resolution of inflammation. *Nat Rev Immunol* 2002;2:787-795.

Lê KA, Bortolotti M. Role of dietary carbohydrates and macronutrients in the pathogenesis of nonalcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* 2008;11:477-482.

Lei Z, Jervis J, Helm RF. Use of methanolysis for the determination of total ellagic and gallic acid contents of woods and wood products. *J Agric Food Chem* 2001;49:1165-1168.

Listenberger L, Han X, Lewis S, Cases S, Farese RV Jr, Ory DS, Schaffer JE. Triacylglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci USA* 2003;100:3077-3082.

Ludwig D. Dietary glycemic index and obesity. *J Nutr* 2000;130:S280-S283.

Ludwig D. The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. *JAMA* 2002;287:2414–2423.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: Food sources and availability. *Am J Clin Nutr* 2004;79:727-747.

Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McGullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001;50:1844-1850.

Marles M, Ray H, Gruber MY. New perspectives on proanthocyanidin biochemistry and molecular regulation. *Phytochem* 2003;64:367-383.

Marniemi J, Hakala P, Mäki J, Ahotupa M. Partial resistance of low density lipoprotein to oxidation in vivo after increased intake of berries. *Nutr Metab Cardiovasc Dis* 2000;10:331-337.

Matsuzawa Y, Shimomura I, Nakamura T, Keno Y, Kotani K, Tokunaga K. Pathophysiology and pahogenesis of visceral fat obesity. *Obes Res* 1995;3:S187-S194.

Mayor F Jr, Jurado-Pueyo M, Campos PM, Murga C. Interfering with MAP kinase docking interactions: implications and perspective for the p38 route. *Cell Cycle* 2007;6:528-533.

McCullough A. Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenter* 2006;40:S17-29.

McDougall G, Stewarr D. The inhibitory effects of berry polyphenols on digestive enzymes. *Biofact* 2005;23:189-195.

McGarry JD, Mannaerts GP, Foster DW. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest* 1977;60:265-270.

McGhie T. K, Ainge G. D, Barnett L. E, Cooney J. M, Jensen D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J Agric Food Chem* 2003**;**51:4539–4548.

Milder I, Arts I, vandePutte B, Venema D, Hollman P. Lignan contents of Dutch plant foods: a database including lariciresinol, pinorecinol, secoisolariciresinol ant´´d matairesinol. *Br J Nutr* 2005;93:393-402.

Miles EA, Zoubouli P, Calder PC. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. *Nutr* 2005;21:389-394.

Min YD, Choi CH, Bark H, Son HY, Park HH, Lee S, Park JW, Park EK, Shin HI, Kim SH. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. *Inflamm Res* 2007;56:210-215.

Mohamed-Ali V, Pinkney J, Coppack S. Adipose tissue as an endocrine and paracrine organ. *Int J Obes* 1998;22:1145-1158.

Moreno JJ. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radic Biol Med* 2003;35:1073-81.

Nam NH. Naturally occurring NF-kappaB inhibitors. *Mini Rev Med Chem* 2006;6:945-951.

Motoshima H, Wu X, Sinha M, Rosato EL, Barbot DJ, Rosato FE, Goldstein BJ. Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosigliatzone. *J Clin Endocrinol Metab* 2002;87:5662-5667.

Mullen, W.; Edwards, C.; Crozier, A. Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. *Br J Nutr* 2006;96:107-116.

Naruszewicz M, Laniewskab I, Millo B, Dluzniewski M. Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients with myocardial infarction (MI). *Atherosclerosis* 2007;194:e179-e184.

Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J*. 1992;6:3051-3064.

Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. *Annu Rev Biochem* 1986;55:69-102.

Nielsen S, Jensen MD. Obesity and cardiovascular disease: is body structure a factor? *Curr Opin Lipidol* 1997;8:200-204.

Oak MH, Bedoui JE, Madeira SV, Chalupsky K, Schini-Kerth VB. Delphinidin and cyanidin inhibit PDGF(AB)-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. *Br J Pharmacol* 2006;149:283-290.

Ossipov V, Salminen JP, Ossipova S, Haukioja E, Pihlaja K. Gallic acid and hydrolysable tannins are formed in birch leaves from an intermediate compound of the shikimate pathway. *Bioch System Ecol* 2003;31:3-16.

Pausova Z. From big fat cells to hig blood pressure: apathway to obesity-associated hypertension. *Curr Opin Nephrol Hypertens* 2006;15:173-178.

de Pascual-Teresa S, Santos-Buelga C, Rivas-Gonzalo J,. Quntitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J Agric Food Chem* 2000;48:5331-5337.

Penalvo J, Heinonen S, Aura A, Adlercreutz H. Dietary sesamin is converted to enterolactone in humans. *J Nutr* 2005a;135:1056-1062.

Penalvo J, Haajanen K, Botting N, Adlercreutz H. Quantification of lignan in food using isotope dilution gas chromatography-mass spectrometry. *J Agric Food Chem* 2005;53:9242-9347.

Pergola C, Rossi A, Dugo P, Cuzzocrea S, Sautebin L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide* 2006;15:30-39.

Petersen K, Dufour S, Befroy D, Lehrke M, Hendler R, Shulman G. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* 2005;54:603-608.

Petroni A, Blasevich M, Papini N, Salami M, Sala A, Galli C. Inhibition of leukocyte leukotriene B4 production by an olive oil-derived phenol identified by mass-spectrometry. *Thromb Res* 1997;87:315-322.

Pouliot MC, Després JP, Nadeau A, Moorjani S, Prud'homme D, Lupien PJ, Tremblay A, Bouchard C. Visceral obesity in men. Association in glucose tolerance, plasma insulin, and lipoprotein levels. *Diabetes* 1992;41:826-834.

Puri P, Baillie RA, Wiest M, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007;46:1081-1090.

Quon MJ, Butte AJ, Zarnowski MJ, Sesti G, Cushman SW, Taylor SI. Insulin receptor substrate 1 mediates the stimulatory effect of insulin on GLUT4

translocation in transfected rat adipose cells. *J Biol Chem* 1994;269:27920-27924.

Ratziu V, Charlotte F, Bernhardt C, Giral P, Halbron M, Lenaour G, Hartmann-Heurtier A, Bruckert E, Poynard T; LIDO Study Group. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. *Hepatology* 2010;51:445-453.

Reaven G. Role of insulin resistance in human disease. *Diabetes* 1988;37:1595-1607.

Reiter CE, Kim JA, Quon MJ. Green tea polyphenol epigallocatechin gallate reduces endothelin-1 expression and secretion in vascular endothelial cells: roles for AMP-activated protein kinase, Akt, and FOXO1. *Endocrinol* 2010;151:103-114.

Rosen ED, MacDougall OA. Adipocyte differentation from the inside out. *Nat Rev Mol Cell Biol* 2006;7:885-896.

Ruderman N, Xu XJ, Nelson L, Cacicedo J, Saha A, Lan F, Ido Y. AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* 2009;298:751-760.

Rudich A, Kanety H, Bashan N. Adipose stress-sensing kinases:linking obesity to malfunction. *Trends Endocrinol Metab* 2007;18:291-299.

Ruel G, Couillard C. Evidences of the cardioprotective potential of fruits: The case of cranberries. *Mol Nutr Food Res* 2007:51;692-701.

Ruge T, Lockton J, Renstrom F, Lystig T, Sukonina V, Svensson M, Eriksson J. Acute hyperinsulinemia raises plasma interleukin-6 in both nondiabetic and type 2 diabetes mellitus subjects, and this effect is inversely associated with body mass index. *Metabol Clin Experim* 2009;58:860–866.

Ryan KG, Swinny Ee, Winefield C, Marlham KR. Flavonoid and UV photoprotection in *Arabidopsis* mutants. *Z Naturforsch* 2001;56c:745-754.

Ryysy L, Häkkinen AM, Goto T, Vehkavaara s, Westerbacka J, Halavaara J, Yki-Järvinen H. Hepatic fat content and insulin action on free fatty acids and glucose metabolism rather than insulin absorption are associated with insulin requirements during insulin therapy in type 2 diabetic patients. *Diabetes* 2000;49:749-758.

Sandell M, Laaksonen O, Järvinen R, Rostiala N, Pohjanheimo T, Tiitinen K, Kallio H. Orosensory profiles and chemical composition of black currant (*Ribes nigrum*) juice and fractions of press residue. *J Agric Food Chem* 2009;57:3718–3728.

Santangelo c, Vari R, Scazzochio B, Benedetto R, Filesi C, Masella R. Polyphenols, intracellular signalling and inflammation. *Ann Ist Super Sanita* 2007;43:394-405.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995;270:26746-26749.

Schimmack G, Defronzo RA, Musi N. AMP-activated protein kinase: Role in metabolism and therapeutic implications. *Diabetes Obes Metab* 2006;8:591-602.

van Schotthorst EM, Bunschoten A, Schrauwen P, Mensink RP, Keijer J. Effetcs of a high-fat, low- versus high-glycemic index diet: retardation of insulin resistance involves adipose tissue modulation. *FASEB J* 2009;23:1092-1101.

Schwab KO, Doerfer J, Hecker W, Grulich-Henn J, Wiemann D, Kordonouri O, Beyer P, Holl RW; DPV Initiative of the German Working Group for Pediatric Diabetology. Spectrum and prevalence of atherogenic risk factors in 27,358 children, adolescents, and young adults with type 1 diabetes: crosssectional data from the German diabetes documentation and quality management system (DPV). *Diabetes Care* 2006;29:218-225.

Seppälä-Lindroos A, Vehkavaara S, Häkkinen AM, Goto T, Westerbacka J, Sovijärvi A, Halavaara J, Yki-Järvinen H. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocr Metab* 2002;87:3023-3028.

Serrano J, Puupponen-Pimiä R, Dauer A, Aura AM, Saura-Calixto F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Mol Nutr Food Res* 2009:53:S310-S329.

Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A* 2004;101:3329-3335.

Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 2005;310:1642-1646.

Shiga T, Moriyoshi Y, Nagahara H, Shiratori K. Nonalcoholic fatty liver is a risk factor for postprandial hyperglycemia, but not for impaired fasting glucose. *J Gastroenterol* 2009;44:757-764.

Shimabukuro M, Ohneda M, Lee Y, Unger RH. Role of nitric oxide in obesity-induced β cell disease. *J Clin Invest* 1997;100:290-295.

Shoelson S, Lee J, Goldfine A. Inflammation and insulin resistance. *J Clin Invest* 2006;116:1793–1801.

Stanhope KL, Havel PJ. Fructose consumption: potential mechanisms for its effects to increase visceral adiposity and induce dyslipidemia and insulin resistance. *Curr Opin Lipidol* 2008;19:16-24.

Strack D. Phenolic metabolism. In: Dey PM, Harborne JB, eds. Plant Biochemistry. London, UK: Academic Press, 1997, p.387-416. In: Riihinen K: Phenolic

compounds in berries. Kuopio Univ Publ C Nat and Environ Sci 2005;187:1-97.

Sutherland BA, Shaw OM, Clarkson AN, Jackson DN, Sammut IA, Appleton I. Neuroprotective effects of (-)-epigallocatechin gallate following hypoxia-ischemia-induced brain damage: novel mechanisms of action. *FASEB J* 2005;19:258-260.

Talavéra S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison J.-L, Rémésy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* 2005;53:3902–3908.

Targher G, Berolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007;30:1212-1218.

Thompson L, Boucher B, Liu Z, Cotterchio M, Kreiger N. Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. *Nutr Cancer* 2006;54:184-201.

Tiikkainen M, Bergholm R, Vehkavaara S. Effects of identical weight loss on body composition and features of insulin resistance in obese women with high and low liver fat content. *Diabetes* 2003;52:701-707.

Tiniakos D, Vos M, Brunt E. Nonalcoholic fatty liver disease: Pathology and pathogenesis. *Annu. Rev Pathol Mech Dis* 2010;5:145-171.

Tokuda H, Takai S, Matsushima-Nishiwaki R, Akamatsu S, Hanai Y, Hosoi T, Harada A, Ohta T, Kozawa O. (−)-epigallocatechin gallate enhances prostaglandin F2alpha-induced VEGF synthesis via upregulating SAPK/JNK activation in osteoblasts. *J Cell Biochem* 2007;100:1146-1153.

Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 2001;60:329-339.

Trayhurn P, Woods IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue *Br J Nutr* 2004;92:347-355.

Trayhurn P, Wang B, Wood IS. Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *Br J Nutr* 2008;100:227-235.

Tribolo, S.; Lodi, F.; Connor, C.; Suri, S.; Wilson, V.; Taylor, M.; Needs, P., Kroon, P.; Hughes, D. Comparative effects of quercetin and its predominant human metabolites on adhesion molecule expression in activated human vascular endothelial cells. *Atherosclerosis* 2008;197:50-56.

Unger R, Orci L. Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J* 2001;15:312-321.

Unger R (a). Minireview: Weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology* 2003;144:5159-5165.

Unger R (b). Lipid overload and overflow: metabolic trauma and the metabolic syndrome. Trends *Endocrinol Metab* 2003;14:398-403.

Venojärvi M, Aunola S, Puhke R, Marniemi J, Hämäläinen H, Halonen JP, Lindström J, Rastas M, Hällsten K, Nuutila P, Hänninen O, Atalay M. Exercise training with dietary counselling increases mitochondrial chaperone expression in middle-aged subjects with impaired glucose tolerance. *BMC Endocr Disord* 2008;8:3-14.

Vidal R, Hernandez-Vallejo S, Pauquai T, Texier O, Rousset M, Chambaz J, Demignot S, Lacorte JM. Apple procyanidins decrease cholesterol esterification and lipoprotein secretion in Caco-2/TC7 enterocytes. *J Lipid Res* 2005;46:258-68.

Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem Pharmacol* 1999;57:941-949.

Wadsworth TL, Koop DR. Effects of Ginkgo biloba extract (EGb 761) and quercetin on lipopolysaccharide-induced release of nitric oxide. *Chem Biol Interact* 2001;137:43-58.

Wang, F.; Yao, T.; Zeng, S. Determination of quercetin and kaempferol in human urine after orally administrated tablet of ginkgo biloba extract by HPLC. *J Pharm Biomed Anal* 2003;33:317-321.

Wang Y-P, Cheng M-L, Zhang B-F, Mu M, Wu J. Effects of blueberry on hepatic fibrosis and transcription factor Nrf2 in rats. *World J Gastroenetrol* 2010;16:2657-2663.

Weisberg SP, MacCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796-1808.

Welton AF, Tobias LD, Fiedler-Nagy C, Anderson W, Hope W, Meyers K, Coffey JW. Effect of flavonoids on arachidonic acid metabolism. *Prog Clin Biol Res* 1986;213:231-242.

Westerbacka J, Lammi K, Häkkinen A, Rissanen A, Salminen I, Aro A, Yki-Järvinen H. Dietary fat content modifies liver fat in overweight nondiabetic subjects. *J Clin Endocrin Metab* 2005;90:2804-2809.

Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V, Wong HR. Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. *J Nutr* 2004;134:1039-1044.

Winkel-Shirley B. Biosynthesis of flavonoids and effects of stress. *Curr Op Plant Biol* 2002;5:218-223.

Wolever T, Bentum-Williams A, Jenkins D. Physiological modulation of plasma free fatty acids concentrations by diet: metabolic implications in nondiabetic subjects. *Diabetes Care* 1995;18:962–970.

Wu X, Pittman H. E, McKay S, Prior R. L. Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *J Nutr* 2005;13: 2417–2424.

Wu X, Pittman H. E, Prior R. L. Pelargonidin is absorbed and metabolized differently than cyanidin after marionberry consumption in pigs. *J Nutr* 2004;134:2603–2610.

Wu X, Cao G, Prior R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J Nutr* 2002;132:1865–1871.

Xagorari A, Roussos C, Papapetropoulos A. Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. *Br J Pharmacol* 2002;136:1058-1064.

Xie DY, Sharma SB, Dixon RA. Anthocyanidin reductases from Medicago truncatula and Arabidopsis thaliana. *Arch Biochem Biophys* 2004;422:91-102.

Xie M, Zhang D, Dyck JR, Li Y, Zhang H, Morishima M, Mann DL, Taffet GE, Baldini A, Khoury DS, Schneider MD. A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci U S A* 2006;103:17378-17383.

Xu JW, Ikeda K, Yamori Y. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. *Hypertension* 2004;44:217-222.

Yang M-Y, Peng C-H, Chan K-C, Yang Y-S, Huang C-N, Wang C-J. The hypolipidemic effect of Hibiscus sabdariffa polyphenols via inhibiting lipogenesis and promoting hepatic lipid clearance. *J Agric Food Chem* ***2010***;58:850-859.

Yki-Järvinen H. Fat in the liver and insulin resistance. *Annals of medicine* 2005;37:347-356.

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA. AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem* 2004;279:47898-47905.

Zang M, Xu S, Maitland-Toolan KA, Zuccollo A, Hou X, Jiang B, Wierzbicki M, Verbeuren TJ, Cohen RA. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. *Diabetes* 2006;55:2180-2191.

Zhou YT, Shimabukuro M, Koyama K, Lee Y, Wang MY, Trieu F, Newgard CB, Unger RH. Induction by leptin uncoupling protein-2 and enzymes of fatty acid oxidation. *Proc Natl Acad Sci USA* 1997;94:6386-6390.

Zunino S. Type 2 diabetes and glycemic response to grapes or grape products. *J Nutr* 2009;139:1794S–1800S.

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