Lipids and
Endothelium-Dependent Vasodilation

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ABSTRACT

Impaired endothelium-dependent vasodilation (EDV) is associated with atherosclerotic cardiovascular disease as well as several of its risk factors. The aim of the present thesis was to investigate how lipids influence EDV in the vascular bed of the human forearm.

Apolipoprotein B was inversely associated with both EDV and endothelium-independent vasodilation (EIDV) in healthy subjects aged 20-69 years. HDL cholesterol was associated with the EDV to EIDV ratio (EFI). Small LDL particles and antibodies against oxidized LDL were not associated with endothelial vasodilatory function.

The EFI in young, healthy subjects was positively associated with the alpha-linolenic acid proportion, but inversely associated with myristic acid in men only. Eicosapentaenoic acid was positively associated with EDV, whereas dihomo-gamma-linolenic acid was inversely associated with both EDV and EIDV in men.

Acute elevation of long-chain fatty acids with Intralipid®/heparin infusion in young, healthy subjects impaired EDV after 2 h. This impairment could be prevented by co-infusing vitamin C, diclofenac or L-arginine. Acute elevation of both medium-chain and long-chain fatty acids during Structolipid®/heparin infusion did not impair EDV.

An ordinary meal (34 E% fat) transiently attenuated EDV at 1 hour. No attenuation in EDV was observed after meals containing 20 and 3 E% fat. These findings show that the endothelial vasodilatory function is associated with fatty acid profile in serum in the fasting state and during acute fatty acid elevation, as well as with apolipoprotein B and HDL cholesterol. Furthermore, lowering dietary fat content to 20 E% or less preserves endothelial vasodilatory function and might therefore protect against atherosclerosis.

Key words: Atherosclerosis, endothelium, vasodilation, nitric oxide, fatty acid, meal, diet, lipoprotein, cyclooxygenase, diclophenac, L-arginine, LDL, human

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To my mother
This thesis is based on the following original papers, which will be referred to by their Roman numerals:


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Abbreviations

Ach, acetylcholine  
ADMA, asymmetric dimethylarginine  
Apo, apolipoprotein  
CAD, coronary artery disease  
cGMP, cyclic guanosine monophosphate  
COX, cyclooxygenase  
CVD, cardiovascular disease  
E%, energy percent  
EDV, endothelium-dependent vasodilation  
EFI, endothelial function index  
EIDV, endothelium-independent vasodilation  
FBF, forearm blood flow  
FMD, flow-mediated vasodilation  
HDL, high-density lipoprotein  
HPL, lipoprotein lipase  
MCFA, medium-chain fatty acid  
Mch, methacholine chloride  
MDA, malondialdehyde  
MUFA, monounsaturated fatty acid  
n-3, Ω-3, omega-3  
NEFA, non-esterified fatty acid  
NO, nitric oxide  
NOS, nitric oxide synthase  
oxLDL, oxidized LDL  
oxLDLab, antibodies against oxLDL  
PG, prostaglandin  
PGI2, prostacyclin  
PUFA, polyunsaturated fatty acid  
ROS, reactive oxygen species  
SFA, saturated fatty acid  
SNP, sodium nitroprusside  
TG, triglyceride  
TX, thromboxane  
VLDL, very low-density lipoprotein  
VSMC, vascular smooth muscle cell

Fatty acid nomenclature

10:0, capric acid  
12:0, lauric acid  
14:0, myristic acid  
16:0, palmitic acid  
16:1, palmitoleic acid  
17:0, heptadecanoic acid  
18:0, stearic acid  
18:1, oleic acid  
18:2n-6, LA, linoleic acid  
18:3n-3, ALA, α-linolenic acid  
18:3n-6, GLA, γ-linolenic acid  
20:3n-6, DGLA,dihomo-γ-linolenic acid  
20:4n-6, AA, arachidonic acid  
20:5n-3, EPA, eicosapentaenoic acid  
22:5n-3, docosapentaenoic acid  
22:6n-3, DHA, docosahexanenoic acid
Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the Western World. Coronary artery disease (CAD), peripheral vascular disease and stroke are different manifestations of the same disease – atherosclerosis - first appearing as fatty streaks in the arterial intima as early as in adolescence [1]. Although genetic predisposition is of importance for atherogenesis, a number of environmental risk factors are associated with an enhanced CVD risk. Several of the most important cardiovascular risk factors, e.g. smoking, diabetes mellitus, hypertension, hypercholesterolemia, obesity, a sedentary life style and mental stress, are modifiable by lifestyle changes or medication, whereas age and gender are not. Several of these risk factors are associated with altered endothelial vasodilatory function in arteries [2-7].

Although the existence of an endothelial cell layer, covering the inside of all blood vessels, has been known for several hundred years, full recognition of the endothelium as an important vascular regulator came first in 1980 when Furchgott et al described an endothelium-derived relaxing factor [8]. Moncada et al were the first to show that this factor is nitric oxide (NO) [9]. Since then, additional vasoactive endothelium-derived factors have been identified and the amount of research about endothelial function has exploded. Constituting a monolayer of cells lining the luminal surface of all vessels, the endothelium plays a pivotal role in regulating vascular tone and smooth muscle cell proliferation, the recruitment of monocytes to the intima, as well as for platelet function [10]. These functions are mainly mediated through the release of NO from the endothelium, but also by prostacyclin (PGI2) and other endothelium-derived substances.

Impaired endothelium-dependent vasodilation (EDV) has been reported in a number of disease states, such as CAD, hypercholesterolemia, hypertension, diabetes mellitus, homocysteinemia, smoking and mental stress [2-4, 6, 11-15].

The fact that endothelial dysfunction is observed in atherosclerosis, as well as in subjects with CVD risk factors, implies that disturbances in endothelial function either precede or accompany atherosclerosis. Indeed, impaired EDV has been observed in atherosclerosis-prone humans even before the presence of detectable atherosclerotic lesions on coronary angiograms [16]. Therefore, assessment of EDV is used as a tool to study risk factors for atherosclerosis.
This thesis will address the relationship between lipid metabolism and endothelial vasodilatory function in the human forearm.

Endothelium-derived vasoactive factors

The L-arginine – NO pathway
Several substances, e.g. hormones, neurotransmitters, platelet-derived substances, shear stress, etc., can stimulate EDV. The main mediator of EDV is the free radical NO [9], which is formed from the amino acid L-arginine by the calcium/calmodulin-dependent enzyme nitric oxide synthase (NOS) [17]. NOS exists in three isoforms and in several tissues [18]. The endothelial isoform (eNOS), also expressed in platelets and endocardium, is the major isoform involved in cardiovascular regulation [19]. As eNOS is constitutively expressed, NO is constantly released from the endothelium. Indeed, infusion of the competitive blocker of NOS, L-NO\textsuperscript{O}\textsubscript{1}-monomethyl arginine (L-NMMA), produces vasoconstriction, which reflects the unopposed effects of vasoconstricting forces (i.e. sympathetic nervous system and endothelium-derived constricting factors [20]). Receptor agonists, such as bradykinin, serotonin, thrombin, and adenosine, can increase NO release [10]. Administration of acetylcholine (Ach) and methacholine (Mch), increasing NO-release through muscarinic receptors, is commonly used to investigate EDV during experimental settings [2, 3, 5, 21-27].

Directly after synthesis, NO diffuses out of the endothelial cells and traverses membranes of circulating blood cells and vascular smooth muscle cells (VSMC). NO acts via stimulation of guanylate cyclase activity in the target cell, leading to formation of cyclic guanosine monophosphate (cGMP) [28]. cGMP reduces intracellular calcium levels, which results in relaxation of VSMC. In platelets, an elevated cGMP level reduces adhesion and aggregation [29, 30]. Platelets in turn can activate release of NO and PGI\textsubscript{2} from endothelial cells by producing adenosine diphosphate, adenosine triphosphate, and serotonin [31].

During inflammation, cytokines activate an inducible form of NOS in the endothelium, VSMC and platelets [32]. The increased NO release contributes to enhanced local perfusion.
The cyclooxygenase pathway

Endothelial cells produce eicosanoids, such as PGI$_2$ and thromboxane A$_2$ (TXA$_2$), and PGH$_2$ from arachidonic acid (AA, 20:4n-6) through the cyclooxygenase (COX) pathway [33]. Activation of COX is achieved by hypoxia, shear stress and substances such as Ach and serotonin [10, 34]. Although PGI$_2$ augments EDV, its major effect is probably to inhibit platelet activation [35]. TXA$_2$ and PGH$_2$ counteract the effects of PGI$_2$ and NO by activating the tromboxane receptor on platelets and VSMC [36]. Furthermore, COX is also an important source of superoxide anions, which inactivate NO and cause vasoconstriction [37, 38].

The COX pathway also catalyzes the conversion of dihomo-γ-linolenic acid (DGLA, 20:3n-6) to PGE$_1$ and 15-OH-DGLA. PGE$_1$ causes vasodilation, inhibition of platelet aggregation and inhibition of inflammation, whereas 15-OH-DGLA inhibits the 5- and 12-lipoxygenases, which give rise to proinflammatory agents [33].

Also eicosapentaenoic acid (EPA, 20:5n-3) acts as a substrate for COX-catalyzed conversion to anti-thrombotic and vasodilatory eicosanoids, such as platelet TXA$_3$ and endothelium-derived PGI$_3$ [39].

Other endothelium-derived vasoactive substances

Endothelin-1 is produced in endothelial cells in response to several substances, such as thrombin, interleukin-1, angiotensin II, and epinephrine [40]. Endothelin-1 exerts potent vasoconstrictive as well as proliferative effects on VSMC [41].

Endothelium-derived hyperpolarizing factor is a yet unidentified substance that induces vasorelaxation through hyperpolarization of VSMC [42] by an unknown mechanism. The physiological importance of this substance is currently not known.

Finally, angiotensin II enhances endothelial release of endothelin-1 and superoxide anion, which inactivates NO [43].

In vivo assessment of endothelium-dependent vasodilation

Invasive techniques

The most utilized technique to investigate EDV in peripheral arteries in humans is venous occlusion plethysmography [44] (Described in detail in the Methods section). This method, which is used predominantly in the
forearm, mainly provides information about blood flow in the resistance vessels of skeletal muscle, although other tissues are reflected as well. Ach and Mch are usually used to induce EDV, whereas sodium nitroprusside (SNP) induces EIDV by donating NO to VSMC. One advantage of this method is the opportunity to administer vasoactive drugs locally, without apparent systemic effects, as well as to study arteries in their natural environment under the influence of humoral and neuronal forces.

Quantitative coronary angiography is used to assess coronary EDV during intra-arterial infusion of vasodilators, commonly Ach [45]. EDV in the human forearm and coronary vascular beds are closely related [46]. Intravascular ultrasound is used to assess coronary blood flow by a few investigators [4]. Although coronary angiography and ultrasound enables assessment of EDV and EIDV in a primary target organ, the use of these methods is limited to patients subjected to clinically indicated coronary angiography.

**Figure 1** | A lipoprotein particle

Non-invasive techniques

High resolution ultrasound is a widely spread technique that measures the change in brachial artery diameter in response to increased blood flow, which elicits EDV through increased shear stress on the endothelial surface [20]. Increased blood flow is achieved by the reactive hyperemia, which follows after a short period of arterial occlusion. Sublingual glycercyl trinitrate, on the other hand, produces arterial vasodilation independently of
the endothelium [47]. An advantage with this method is that it can be performed in large-scale studies and does not necessitate arterial perforation, although local intra-arterial infusions with vasodilators and other drugs are not possible. Flow-mediated vasodilation (FMD) in the brachial artery during reactive hyperemia, as assessed with the ultrasound technique, has been shown to be closely related to muscarinic agonist-induced vasodilation, as assessed with strain-gauge plethysmography [48, 49], although this has not been a consistent finding [50].

Lipid metabolism

Upon ingestion, triglycerides (TG) are broken down into monoglycerides and free fatty acids in micellar configuration and are absorbed into intestinal cells together with phospholipids and cholesterol. In the intestinal cells, the monoglycerides are reesterified into TG. TG combine with apolipoprotein (Apo) A and ApoB48 and move into the thoracic duct as chylomicrons, composed of a core of TG and cholesterol esters enveloped by phospholipids, free cholesterol, and apolipoproteins. Subsequently, chylomicrons receive ApoC-II from high-density lipoproteins (HDL). ApoC-II activates lipoprotein lipase (LPL) on endothelial cell membranes, which breaks TG down into glycerol and free fatty acids, also referred to as non-esterified fatty acids (NEFA). NEFA then readily diffuse across cell membranes into cells. However, a substantial proportion of liberated fatty acids escapes, binds to albumin and is transported to the liver [51]. After peripheral cells have digested the TG out of chylomicrons, smaller, cholesterol-rich particles are left, the chylomicron remnants, which are removed from the circulation by hepatocytes through ApoE-receptor mediated endocytosis.

After a meal, most fatty acids from the intestine are passed to the liver, either as albumin-bound fatty acids or as chylomicron remnants. Other sources of fatty acids in the liver include a certain degree of conversion from excess carbohydrate, protein, and alcohol, as well as from receptor-mediated endocytosis of intermediate- and low-density lipoproteins (IDL and LDL, respectively). In the liver, fatty acids are reesterified to TG, which are then utilized to form very low-density lipoproteins (VLDL), composed of a core of TG and cholesterol esters coated by phospholipids, ApoA, ApoB100, as well as free cholesterol (Fig. 1). As with the chylomicrons, VLDL particles acquire ApoC-II and ApoE from HDL shortly after secretion. Again, ApoC-II activates LPL, which hydrolyzes TG and converts VLDL to smaller, cholesterol-rich IDL, and finally to LDL with smaller size, but a greater
proportion of cholesterol. With the conversion of IDL to LDL, most ApoC-II and ApoE dissociate from the particles and reassociate with HDL. Most LDL particles, as well as some IDL, bind to hepatocytes or other cells and are removed from the circulation.

HDL particles are assembled in and secreted from intestinal cells, as well as from hepatocytes. The major function of ApoA-I and ApoA-II-containing HDL, besides constituting a reservoir for apolipoproteins, is to remove cholesterol from cells and to transfer it as cholesterol esters to VLDL or LDL (reverse cholesterol transport). Cholesterol is thereby enabled to reach the liver with VLDL and LDL. The fate of the cholesterol removed from non-hepatic cells is integration into new VLDL particles in the liver or excretion as bile salts into the duodenum.

Most cholesterol in the organism originates from endogenous conversion of saturated fatty acids (SFA) to cholesterol, whereas the remaining one third is of exogenous origin. Accordingly, serum cholesterol is more closely associated with dietary intake of SFA than with dietary cholesterol intake [39]. Monounsaturated fatty acids (MUFA) are neutral, whereas polyunsaturated fatty acids (PUFA) have a mild lowering effect on total and LDL-cholesterol [52].

NEFA is the form in which stored body fat is transported from adipose tissue to its sites of utilization. After an overnight fast, the plasma NEFA concentration is at its highest, reflecting an adaptive response of sparing carbohydrates and mobilizing fat stores. Circulating NEFA can be utilized as fuel by muscles. In addition, NEFA is converted to ketone bodies in the liver, which are utilized as energy by the renal cortex, cardiac myocytes, and the brain during starvation. The enzyme responsible for the lipolysis of stored body fat is hormone-sensitive lipase, which is inhibited by insulin. Under fasting conditions, the insulin level is low and, consequently, lipolysis is increased.

Measurement of plasma TG under fasting conditions reflects circulating VLDL to a large extent. In contrast, the major representatives of plasma TG in the postprandial state are the chylomicrons and chylomicron remnants. Postprandial increase in plasma insulin results in enhanced LPL activity and fatty acid esterification in adipose tissue, resulting in an enhanced clearance of NEFA from the circulation.
Fatty acid structure and function

Fatty acids are compounds containing a hydrocarbon chain, which begins with a methyl group and ends with a carboxyl group (Fig. 2). The carbon atoms can be numbered beginning with the methyl end. The number of the first carbon atom involved in a double bond is used to classify fatty acids for example into omega-3 (n-3) and n-6 fatty acids. SFA lack double bonds, MUFA have one double bond, whereas PUFA have two or more double bonds.

**Fatty acids have several functions:**

- Fatty acids are utilized as fuel. Fatty acids are building blocks for phospholipids and glycolipids, which in turn are constituents of biological membranes. Chain length and number of double bonds influence membrane fluidity.
- Fatty acids modify functions of membrane proteins, i.e. ion channels and receptors. 20-carbon fatty acids are transformed into prostaglandins and thromboxanes through the action of COX, and into leukotrienes through the action of lipoxygenase. Fatty acids are intracellular messengers in the phosphoinositide cascade.

The most prevalent SFA in the diet is palmitic acid (16:0), followed in order of abundance by stearic (18:0), myristic (14:0), and lauric acid (12:0). Major sources of SFA include dairy, beef, pork, and poultry products, whereas the major dietary MUFA, oleic acid (18:1), is abundant in olive oil. Major sources of PUFA in the Western diet are fish, soybean oil, and other vegetable oils. Today, the typical Western diet consists of an n-6/n-3 ratio.
between 10:1 and 25:1 [53]. The World Health Organization recommends an n-6/n-3 ratio of 4-10:1 [54].

Dietary lipids, CVD and the endothelium

The importance of dietary fatty acid composition in the development of CVD has been recognized since decades. The Seven Countries Study by Keys et al showed that SFA intake and serum cholesterol were strongly associated with ischaemic heart disease [55]. Although Cretan men had a high fat intake due to olive oil consumption, their CAD rate was lower than in countries with high SFA consumption. The Ni-Hon-San study compared the dietary habits of Japanese men living in Japan, San Francisco and Hawaii [56]. The increased saturated and total fat intake in Hawaii and San Francisco was accompanied by higher CAD risk. The association between dietary SFA, cholesterol and CVD has later been corroborated by other cross-population and within-population studies [57-63].

Not all SFA affect total cholesterol concentrations in the same manner. Stearic acid (18:0) has little effect on plasma cholesterol concentrations [64], whereas myristic and palmitic acid have been reported to have the greatest cholesterol-raising potential [65].

Fatty acids have been hypothesized to interact with the endothelium through several mechanisms, e.g. disruption of the endothelial barrier function [66], increased oxidative stress [67], or enhancement of α-1-adrenoceptor mediated pressor sensitivity [68]. Being constituents of membrane phospholipids, fatty acids may also play an important role in atherosclerosis by modulating the configuration of membrane-bound proteins, e.g. receptors and ion channels, and thereby influence endothelial cell function [33]. In our group, Sarabi et al investigated if the composition of fatty acids in diet influences endothelial vasodilatory function [69]. In this cross-sectional study, significant inverse associations were found between EDV, as assessed with venous occlusion plethysmography, and the proportion of palmitic acid and palmitoleic acid (16:1) in serum cholesterol esters. However, not only the number of double bonds, but also fatty acid chain length might influence EDV. Indeed, incubation of human umbilical vein endothelial cells with palmitic acid significantly decreased calcium ionophore-stimulated NO production, whereas stearic acid had no effect on EDV [70].
Monounsaturated fatty acids

Although early work suggested that MUFA are neutral with respect to their effects on plasma total cholesterol concentrations, recent studies have shown that, when substituted for dietary SFA, MUFA have a hypocholesterolemic effect [71-76]. The 15-year follow-up of the Seven Countries Study showed an inverse association between oleic acid and coronary deaths [77], a finding that has been confirmed by later studies [78]. Oleic acid has also been shown to reduce the endothelial expression of vascular cell adhesion molecule-1 on endothelial cells [79]. Furthermore, endothelium-dependent flow-mediated vasodilatation was shown to be significantly greater on an oleic acid-rich diet, as compared to a linoleic acid (LA, 18:2n-6) -rich diet in type-2 diabetic patients [80]. These positive associations between EDV and oleic acid intake are in conflict with a study by Ong et al, in which ingestion of a meal rich in oleic acid was associated with an acute impairment of EDV, as assessed with brachial artery ultrasound [81]. Further, oleic acid has been shown to attenuate Ach-induced relaxation in precontracted rabbit artery rings [82, 83]. Thus, the role of oleic acid for vascular function is presently unclear.

Polyunsaturated fatty acids

The major n-6 fatty acid in diet is LA. It is not synthesized by the human body and is therefore an essential fatty acid. LA has a hypocholesterolemic effect when substituted for dietary SFA, and several population studies have shown dietary intake of LA to be inversely associated with CVD [62, 84-87]. However, no relationship was found between dietary intake of LA and cardiovascular death in the Multiple Risk Factor Intervention Trial [88]. Furthermore, in other studies, LA was in fact a predictor of CAD [89, 90].

LA serves as the precursor for AA, which has an important function as an eicosanoid precursor. Furthermore, AA has been shown to augment EDV in vitro [91]. A low serum phospholipid proportion of AA has been reported to predict future coronary events [62]. Furthermore, a low AA proportion in serum has also been reported in hypertension, CAD and non-insulin dependent diabetes mellitus [92].

The other major essential fatty acid in the diet is α-linolenic acid (ALA, 18:3n-3). This fatty acid can be rapidly converted in the body to EPA, which in turn can be converted to docosahexaenoic acid (DHA, 22:6n-3) [93]. Dietary or serum cholesterol ester ALA was inversely associated with cardiovascular mortality and stroke in the Multiple Risk Factor Intervention Trial [88, 94] and with the risk of myocardial infarction in health professionals [95]. Furthermore, in a cross-sectional study in a middle-aged population [69], the ALA proportion was an independent positive predictor of EDV.

Fatty acids with 20 or 22 carbon atoms are referred to as highly unsaturated fatty acids (HUFA). HUFA belonging to the n-3 class are commonly referred to as fish oils, as the fat of Arctic fish is the richest
source of these fatty acids. The most important representatives of fish oils are EPA and DHA. Dietary or supplementary intake of fish oil has been shown to lower TG concentrations, to improve arterial compliance and to reduce CAD risk [96]. A high intake of n-3 fatty acids has also been associated with lower platelet aggregation, an attenuated immune response, and a lower blood pressure [97-100]. Furthermore, six weeks of fish oil supplementation in type 2 diabetic patients resulted in a significant improvement in EDV, as measured with strain-gauge plethysmography [101]. n-3 fatty acids have also been shown to improve EDV in porcine atherosclerotic coronary arteries [102], and in bovine coronary artery rings [103]. The protective effect of fish oils is probably to a large extent related to their ability to lower certain lipoprotein levels (LDL, IDL, VLDL, and chylomicrons), and plasma NEFA levels, to increase HDL and to protect myocardial and endothelial function [96]. n-3 HUFA are converted into anti-thrombotic and vasodilatory eicosanoids, such as platelet TXA3 and endothelium-derived PGI3 from EPA [39]. Since a major role of both n-3 and n-6 essential fatty acids is as constituents of membrane phospholipids, they may play an important role in atherosclerosis by modulating the configuration of membrane-bound proteins and thereby influence cell function [33]. Although fish oils are antiatherosclerotic, they, as well as n-6 HUFA, are susceptible to peroxidation. Hence, with increased intake of HUFA, it is important to assure that the dietary source of these fatty acids also provides a sufficient amount of antioxidants such as α-tocopherol [96]. Whether supplementary intake of fish oil is as beneficial as dietary intake is not yet settled.

**Triglycerides**

Serum TG measurements serve as a marker for TG-rich lipoproteins. Several studies have shown that serum TG is positively associated with coronary events. However, the significance of this relationship is frequently weakened when confounders, such as LDL cholesterol, HDL cholesterol, body mass index and plasma glucose, are included in the analyses (Reviewed in [104]). Elevated serum TG is often accompanied by a low HDL cholesterol level and the combination of both can often be observed in type II diabetes. Hence, it is unclear whether hypertriglyceridemia is an independent risk factor for or just a marker of CAD. Cross-section studies on relationships between TG and EDV (as well on NEFA and EDV) face the same problem. In humans with isolated hypertriglyceridemia, both an impaired and a normal EDV have been reported (Reviewed in [105]). Effects of hypertriglyceridemia on EDV have also been investigated during acute elevation of the serum TG level by intravenous infusion of a TG emulsion [106-108]. Hence, in a study by Lundman et al, plasma TG was elevated fourfold during infusion of Intralipid® in healthy young men. The TG elevation was accompanied by a decreased EDV, as assessed with brachial
artery ultrasound [107]. However, Lundman et al also reported a simultaneous 2-fold increase in circulating NEFA, which might have contributed to, or even might have explained the impaired EDV after 1 hour of Intralipid® infusion. Indeed, a previous study by Steinberg et al showed that acute elevation of circulating NEFA during 2-hour infusion of Intralipid® with heparin in young healthy humans impaired EDV, as assessed with the thermodilution technique in the femoral artery [108]. However, in this study there was also a simultaneous elevation of plasma TG. To exclude an effect of elevated TG levels on EDV, Intralipid® was infused without heparin during 2 hours, resulting in a 2-fold significant increase in plasma TG, but only a tendency of increment in plasma NEFA, and no significant change in EDV. Later studies have confirmed the adverse effect of acute hyperlipidemia on EDV [25, 109].

Free radicals and antioxidants

A free radical is defined as a species capable of independent existence that contains one or more unpaired electrons [110]. Important examples are the oxygen-centered radicals superoxide anion, NO, and the fearsome hydroxyl radical. The wider term reactive oxygen species (ROS) includes both oxygen-centered radicals as well as dangerous non-radical oxygen derivates, such as H₂O₂ and ozone. Two free radicals can combine their unpaired electrons in a covalent bond. However, free radicals can also react with non-radical molecules, whereby the free radical either donates one electron to or steals one electron from a non-radical or simply attaches to the non-radical, in all cases transforming the non-radical into a radical. The new radical then attacks a non-radical molecule. Thus, radical attacks lead to chain reactions. The state when ROS generation exceeds the capacity of antioxidant defenses is defined as oxidative stress [111]. Mild oxidative stress leads to increased synthesis of antioxidant defense systems [110], whereas severe oxidative stress deranges cell metabolism by promoting DNA damage (mutagenesis, carcinogenesis), protein oxidation and fragmentation, carbohydrate damage, as well as lipid peroxidation [110, 111]. Particularly lipid peroxidation is believed to play an important role in atherosclerosis (See below).

When fatty acids are attacked by free radicals, they become lipid peroxides. The greater the number of double bonds in a fatty acid, the greater the risk of lipid peroxidation. Thus, PUFA are most vulnerable to free radical attacks. Fortunately, the natural sources of PUFA are also rich in lipid-soluble antioxidants.
Superoxide anion is continuously produced *in vivo*, e.g. by activated phagocytes and endothelial cells [110]. Important sources of superoxide anion include the COX pathway [112], NADH dehydrogenase (a mitochondrial enzyme of the respiratory chain) [113], xanthine oxidase [114], NAD(P)H oxidase [115], and NOS itself [116]. Increased superoxide production has been found in atherosclerosis, hypercholesterolemia, smoking, diabetes and hypertension [117, 118].

Although being less reactive than hydroxyl radical, superoxide rapidly reacts with NO to form peroxynitrite (ONOO-), a compound with vasodilatory actions at low concentrations, and vasoconstrictive actions at high concentrations [119]. The effects of peroxynitrite have been studied during incubation of human LDL and plasma with sydnonimine-1, which liberates both superoxide and NO during autooxidation. Such studies have shown that peroxynitrite, or its derivatives, enhances lipid peroxidation, as assessed by F2-isoprostane measurements or TBARS [120-122]. Furthermore, peroxynitrite has been proposed to be able to undergo protonation and subsequent decomposition to hydroxyl radical and NO2 [123].

Antioxidants, defined as “any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate” [111], are compounds of both non-enzymatic (e.g. tocopherols, vitamin C, glutathione, flavonoids, β-carotene) and enzymatic nature (e.g. superoxide dismutase, glutathione peroxidase, and catalase) [111]. They operate by inhibiting free radical production, intercepting free radicals once formed, and by repairing free radical-induced injury.

The most efficient non-enzymatic antioxidant in the lipid phase is probably the chain-breaking vitamin E (tocopherol) [111], which inactivates free radicals by donating one hydrogen atom. Supplementation of the diet with α-tocopherol has been shown to elevate α-tocopherol levels in LDL by 2- to 3-fold, but after supplementation is abruptly stopped, α-tocopherol levels fall rapidly [124]. Indeed, vitamin E supplementation has been shown to decrease the susceptibility of LDL to oxidation [125]. Vitamin C (ascorbic acid), on the other hand, is capable of recycling α-tocopherol radicals to α-tocopherol on the surface of LDL [124]. Vitamin C is also a scavenger of superoxide anions and inhibits lipid peroxidation in plasma [126].

Oxidized LDL, CVD and the endothelium

Elevated LDL is an important predictor of cardiovascular morbidity and mortality [127]. LDL particles constantly diffuse across the artery wall and
sometimes become trapped in the extracellular matrix of the subendothelial space, the intima [128]. The three most important cell types in the vessel wall – endothelial cells, VSMC and macrophages – constantly release waste products with free radical activity, e.g. superoxide anions, into the extracellular matrix, producing a prooxidative environment in the intima [110]. Upon entrapment, a “lag phase” follows when the oxidative attacks on LDL is eliminated by antioxidants, e.g. tocopherols and β-carotene, that are incorporated in LDL particles [124]. After the antioxidant capacity of the LDL particle has been depleted, the PUFA in LDL become oxidatively modified, a process commonly referred to as lipid peroxidation, and the LDL particle becomes an oxidized LDL (oxLDL) particle [124]. Besides its content of chain-breaking antioxidants, such as tocopherols, the peroxidizability of LDL will depend upon its fatty acid composition, meaning that a high proportion of PUFA will increase the rate of peroxidation [124, 129]. ApoB is also subject to oxidative modification, leading to increased affinity to the “scavenger receptor” on macrophages [130]. Uninhibited endocytosis by macrophages via scavenger receptors leads to development of lipid-laden macrophages (foam cells) [130]. In addition, oxLDL takes part in the development of atheroma through several other mechanisms, such as inhibition of NO release, smooth muscle cell proliferation, platelet activation, and recruitment of monocytes [131, 132]. Once atherosclerotic plaques are established, oxLDL may reduce plaque stability, leading to increased risk of plaque rupture and thrombosis [127]. OxLDL has also been shown to impair EDV [133, 134].

One potentially important consequence of oxLDL formation may be the elicitation of an immune response. Indeed, antibodies against oxLDL (oxLDLab) have been detected in both human and rabbit plasma and in atherosclerotic lesions [135, 136]. However, the role of oxLDLab in atherosclerosis is unclear, as oxLDLab titers have been reported to predict myocardial infarction [137], as well as to be inversely related to the intima-media thickness of the carotid artery in humans [138].

The inflow rate of LDL particles into the arterial wall has been suggested to be increased for small LDL particles [139]. Indeed, small LDL particle size has been shown to be associated with atherosclerosis, as assessed by coronary angiography [140, 141] and carotid ultrasonography [142, 143], although this has not been a consistent finding [144-146].

Antioxidants and CVD

Many, but not all cohort and case-control studies have shown consumption of antioxidants, such as vitamin C and E and β-carotene, as assessed by food
questionnaires or serum levels, to be associated with reduced rates of CVD (See [147, 148] for review). Some studies have also proposed a cardioprotective role for flavonoids.

In primary prevention studies, low dose α-tocopherol does not reduce the incidence of coronary events (Alpha-Tocopherol, Beta-Carotene [ATBC] study), and β-carotene either has no effect or increases the incidence of coronary events and cancer death (ATBC study, Beta-Carotene and Retinol [CARET] study, Physician's Health study). Secondary preventions with small populations and shorter duration of follow-up have shown some benefit from α-tocopherol (Cambridge Heart Antioxidant Study [CHAOS] and Secondary Prevention with Antioxidants of CVD in Endstage Renal Disease [SPACE] study), but larger randomized studies indicate no benefit from treatment with α-tocopherol (Heart Outcomes Prevention Evaluation [HOPE] study, GISSI-Prevenzione study, Primary Prevention Project [PPP]). Neither do recent studies with antioxidant combinations show any benefit (HDL-Atherosclerosis Treatment Study [HATS], Heart Protection Study [HPS]).

One possible explanation for the discrepancy between observational studies and randomized trials might be that antioxidant-rich foods are also rich in other beneficial dietary factors. Indeed, food rich in antioxidants generally contain less cholesterol and SFA and more unsaturated fatty acids as well as fiber. Furthermore, results of observational studies are more likely to reflect the dietary intake not only during the study time frame, but also during the total life span of the studied individuals, which is important as atherogenesis starts early in life [1]. Furthermore, the mode of industrial production of food, as well as how dishes are prepared, is likely to affect the levels of antioxidants and lipid peroxides in what we eat. Hence, the antioxidant/oxidant status of ingested food is likely to be as important as in vivo ROS generation.
AIMS OF THE THESIS

1. To investigate whether LDL particle size, oxLDLab, apolipoproteins or lipoproteins are related to EDV in healthy humans.

2. To test the hypothesis that serum fatty acid profile, reflecting dietary intake, influences EDV.

3. To investigate whether the negative effect of acute NEFA elevation on EDV is due to increased oxidative stress, COX activation or a relative intracellular L-arginine deficiency.

4. To investigate whether an acute elevation of circulating NEFA with a high proportion of saturated medium-chain fatty acids (MCFA) influences EDV differently as compared to an acute elevation of long-chain fatty acids (LCFA).

5. To test the hypothesis that the acute postprandial attenuation in EDV, observed after ingestion of an ordinary Western meal, can be abolished by lowering dietary fat content.
Methods

Subjects

**Subjects in study I**
The study sample was randomly recruited from the population registry of Uppsala, Sweden and consisted of 58 healthy subjects, aged 20 to 69 years. None of the subjects were on regular medication or had a history of any disease known to affect the cardiovascular system or a history of any metabolic or other serious disease. None of the women were on contraceptive or estrogen replacement therapy. Subjects with blood pressure higher than 160/95 mmHg, fasting hyperglycemia (>6.0 mmol/l) or pronounced hypercholesterolemia (>7.0 mmol/l) at the investigation were not included.

**Subjects in study II-V**
The characteristics of the studied samples are shown in Table 1.

Young subjects aged 20-30 years without any known cardiovascular, metabolic or gastrointestinal disorders were recruited from the general population of Uppsala. Subjects on regular medication, vitamin supplements at doses exceeding U.S. Food and Drug Administration recommendations, and habitual smokers and snuff users were not allowed to enter the studies. Recruitment was arranged through advertisements in university and community buildings. An initial telephone interview served to exclude subjects not fulfilling the inclusion criteria and to inform them about the protocol and potential discomfort. Upon their arrival at our laboratory, history was repeated, and, when indicated, a physical examination was conducted. The subjects also received detailed written information about the study, to which they gave their written consent. The subjects in study II originated from studies III-V.

The Ethics Committee of Uppsala University approved the studies and each participant gave informed consent.
Protocols

Protocols - General
Subjects were instructed to fast overnight and were not allowed to drink anything but water in the morning. Occasional users of tobacco, snuff or alcohol were instructed to refrain from usage from 6 p.m. the preceding evening. All studies began at 8 a.m. with the subjects supine. Room temperature was maintained at 20-22°C. An arterial catheter (1.0 mm, Ohmeda, Swindon, UK) was inserted into the brachial artery for infusion of vasodilators (Fig. 3). An intravenous catheter was placed in an antecubital vein for collection of blood samples. In order to establish stable conditions after cannulation of the artery, baseline registrations were performed only when basal FBF had reached a steady state after 40 to 60 minutes.

Protocols - Study I & II
In these cross-sectional studies, hemodynamical and biochemical data were collected after an overnight fast as described above.

Protocol - Study III
Acute elevation of circulating NEFA was achieved by intravenous infusion

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Basic characteristics of the samples in studies II-V.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study II</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>74</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24±3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22±2</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/l)</td>
<td>4.1±0.8</td>
</tr>
<tr>
<td>Serum triglyceride (mmol/l)</td>
<td>0.7±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD or n
of the lipid emulsion Intralipid® (200 mg/ml, Fresenius Kabi, Uppsala, Sweden) together with heparin (Heparin Pharmacia, Pharmacia & Upjohn, Uppsala, Sweden) during 2 hours. The role of heparin was to increase LPL activity and thereby to elevate circulating NEFA levels. Intralipid® was administered to 34 subjects during 2 hours at an initial rate of 0.5 ml/kg during 10 min and at 90 ml/h thereafter. Heparin was given at a total dose of 340 U during the 2-hour period.

In addition, 24 of these subjects received one of three intervention drugs as a continuous infusion into the brachial artery; vitamin C 1000 mg/h (n=8) (C-vitamin, Apoteksbolaget, Umeå, Sweden), diclophenac 30 mg/h (n=8) (Voltaren®, Novartis Healthcare A/S, Copenhagen, Denmark) or L-arginine 750 mg/h (n=8) (L-argininhydroklorid, Apoteksbolaget, Umeå, Sweden). The intervention drug infusions started 20 min prior to the start of the Intralipid® infusion and continued until the end of Intralipid® infusion at a rate of 0.5 ml/min. The remaining 10 subjects received only Intralipid® and heparin and constituted the non-intervention group.

Mch and SNP were administered in one arm, whereas the other arm served as a control for basal FBF. Arterial blood samples were drawn at baseline and after 2 hours of lipid infusion.

To exclude any effects of the intra-arterial infusion procedure itself on basal FBF, Mch-FBF, and SNP-FBF, six subjects received an intra-arterial infusion of saline at a rate of 0.5 ml/min (Intra-arterial control study). Vasodilations were performed at baseline and after 1 hour of intra-arterial saline infusion.

Another 8 subjects were given a slow intravenous saline infusion instead of Intralipid® to exclude any effects of time or the procedure of intravenous administration itself on basal FBF, Mch-FBF and SNP-FBF (Time-control study). Vasodilations were performed at baseline and after 2 hours of intravenous saline infusion.

**Protocol- Study IV**

The vasodilatory and biochemical outcomes of the 10 subjects of the non-intervention group of study III were compared with those of 10 other subjects, in whom an acute elevation of circulating NEFA was achieved by intravenous infusion of Structolipid® (200 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) and heparin (Heparin, Fresenius Kabi AB, Uppsala, Sweden) during 2 hours. The protocol was identical to that of study III and the amount of infused lipid was equal in both groups.

**Protocol- Study V**
Meals were designed by associate Professor B. Karlström and prepared by a professional cook. Each subject consumed a meal with either an ordinary fat content in which 34 energy % (E%) was derived from fat (n=10), a low-fat meal (20 E% fat, n=8), or a minimum-fat meal (3 E% fat, n=8, Table 2). Subjects weighing less than 70 kg received a 700 kCal-meal, otherwise a 900 kCal-meal was given. The 34 E% and 20 E% fat meals consisted of minced meat (beef) in tomato sauce, rice, a vegetable mix (peas, corn and paprika), bread, butter and water. The 3 E% fat meal consisted of cooked cod in tomato sauce with rice and beans, vegetables, and bread. A carbohydrate beverage (250 ml, Nutrical®, Nutricia Nordica AB, Stockholm, Sweden) accompanied the 3 E% fat meal, whereas the 34 E% and 20 E% fat meals were served with a glass of water (250 ml).

After fasting blood and urine samples had been obtained and a baseline endothelial function test had been performed, each subject consumed a meal during 15 minutes. The male: female ratio was 1:1 in each meal group. Measurements of endothelial function and blood samplings were repeated at 1 and 2 hours after the beginning of each meal. A second urine sample was obtained after 2 hours.

Due to the complexity of the protocol, vasodilation to Mch and SNP was only evaluated at the highest infusion rates (4 and 10 µg/min, respectively) used in the other studies.

### Table 2 | Composition of the meals

<table>
<thead>
<tr>
<th></th>
<th>Ordinary meal</th>
<th>Low-fat meal</th>
<th>Minimum-fat meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Fat (E%)</td>
<td>34</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>saturated (E%)</td>
<td>26</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>monounsaturated (E%)</td>
<td>38</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>polyunsaturated (E%)</td>
<td>36</td>
<td>33</td>
<td>57</td>
</tr>
<tr>
<td>Carbohydrate (E%)</td>
<td>51</td>
<td>66</td>
<td>84</td>
</tr>
<tr>
<td>Protein (E%)</td>
<td>15</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>147</td>
<td>78</td>
<td>114</td>
</tr>
</tbody>
</table>

Values are energy percent (E%) of the total energy in meals.

Venous occlusion plethysmography

Forearm blood flow (FBF) was measured by venous occlusion plethysmography with mercury-in-silastic strain gauges (Elektromedicin,
Kullavik, Sweden, Fig. 3). The strain gauges were placed at the upper third of the forearm, which rested comfortably slightly above heart level. The strain gauge was connected to a calibrated plethysmograph. Venous occlusion pressure of 40 mm Hg was achieved by rapid inflation of a blood pressure cuff on the upper arm. FBF was calculated as the mean value of five consecutive readings. EDV was assessed as FBF during intra-arterial infusion of Mch chloride (Mch-FBF, Metakolinklorid, Apoteksbolaget, Umeå, Sweden) at the rates of 2 and 4 µg/min in the brachial artery. Endothelium-independent vasodilation (EIDV) was assessed as FBF during infusion of sodium nitroprusside (SNP-FBF, Nitropress®, Abbott Labs, Chicago, TL, USA) at the rates of 5 and 10 µg/min. Registrations were performed during the 5th minute of vasodilation. Mch-FBF and SNP-FBF were registered in random order and washout periods of 20 minutes were allowed between drugs. Basal FBF, as well as Mch-FBF and SNP-FBF, were expressed as ml/min per 100 ml of forearm tissue. The endothelial function index (EFI), reflecting the endothelial contribution to vasodilation, was calculated as the Mch-FBF to SNP-FBF ratio at the highest rates of Mch and SNP (except in study I, in which the relative increase from baseline FBF [%] during vasodilations was used). This latter approach is less appropriate during interventional studies such as studies III-IV, as basal FBF might be affected by the interventions.
Figure 3 | Venous occlusion plethysmography
The short-term (2 hours) and long-term (3 weeks) reproducibility of Mch-FBF and SNP-FBF with venous occlusion plethysmography has a coefficient of variation of 5-7% in our laboratory [149]. We have recently found that Mch infusion (4 µg/min) results in a significant increase in forearm venous plasma nitrite and nitrate concentrations in healthy volunteers [150]. The forearm release of plasma nitrite and nitrate showed a more than ten-fold increase, indicating that Mch mediates vasodilation through increased NO production.

Blood pressure measurements

Blood pressure was registered by an automatic device (Omron® Hem 705c, Omron Corporation, Tokyo, Japan).

Biochemical analysis

Triglyceride, cholesterol and NEFA
Enzymatic kits were used to determine serum concentrations of TG and cholesterol (IL Test Triglyceride 181610-60 and IL Test Cholesterol 181618-10, Instrumentation Laboratory Company, Lexington, MA, USA), and NEFA (NEFA C 994-75409, Wako Chemicals GmbH, Neuss, Germany) in a Monarch 2000 multicentrifugal analyzer (Instrumentation Laboratory Company, Lexington, MA, USA). Serum TG and NEFA levels were adjusted for in vitro lipolysis.

Fatty acid composition
Whole serum fatty acid composition was analyzed with gas-liquid chromatography as described elsewhere [151].

8-iso-PGF$_{2\alpha}$
Urine or plasma 8-iso-PGF$_{2\alpha}$ was analyzed by a newly developed radioimmunoassay [152].
Glucose and insulin
Plasma glucose was measured in a Beckman glucose analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Insulin was assayed in EDTA plasma with an enzymatic-immunological assay (Enzymmun®, Boehringer Mannheim, Germany) in an ES300 automatic analyzer (Boehringer, Mannheim, Germany).

Serum tocopherols
Two tocopherols, α- and γ-tocopherol were analyzed with high-performance liquid chromatography [153]. Serum tocopherol concentrations were divided by the sum of the plasma cholesterol and TG concentrations [154].

Lipoprotein levels and LDL particle size
LDL, VLDL and HDL were isolated by a combination of preparative ultracentrifugation [155] and precipitation with a sodium phosphotungstate and magnesium chloride solution [156].

LDL particle size was assessed on commercially available non-denaturating 2% to 16% polyacrylamide gradient gels as previously described [143, 157]. The coefficient of variation (same serum run on different gels on different days) for LDL peak particle size was 0.3%, with a correlation coefficient of r=0.99. In order to minimize the reading error from the gels, each lane was scanned twice. Mean values from the 2 readings were used in study I.

Antibody titers against oxLDL
Antibody titers were determined with a solid phase ELISA, as earlier described [158, 159]. Malondialdehyde-treated LDL (MDA-LDL) was prepared as described by Palinski et al [160]. As a routine procedure, modifications were checked by controlling the electrophoretic mobility in agarose gel of the modified lipoproteins. Antibody titer was defined as titer=absorbance (patient serum- postcoat)/(internal antibody titer standard serum-postcoat). For IgG the post-coated wells gave no absorbance. Therefore this correction was made only for IgM. We chose to determine MDA-LDL antibodies as MDA is a prominent epitope of oxLDL and seems to be more sensitive than other ligands for antibody detection [161, 162].
Apolipoproteins
Concentrations of serum ApoA-I and ApoB were determined by immunoturbidimetry in a Monarch apparatus (Orion Diagnostica, Espoo, Finland).

Statistical analysis

Differences within each subject were calculated by ANOVA for repeated measurements. Relationships between continuous variables were evaluated by linear regression analysis. Interactions between several independent variables were evaluated by stepwise multiple regression analysis. Two-tailed p-values were used. P<0.05 was regarded as significant.
Results and Discussion

Study I.
Endothelial vasodilatory function is predicted by circulating apolipoprotein B and HDL in healthy humans

Results

Univariate regression analysis was used to study whether Mch-FBF, SNP-FBF or EFI were associated with LDL particle size, oxLDLab, apolipoproteins, and lipoproteins. In this study, the relative increase from basal FBF was used to evaluate EDV and EIDV. EFI was calculated as the EDV/EIDV ratio. When indices of cholesterol metabolism, that were significantly associated with EDV, EIDV or EFI in univariate regression analysis, were introduced into a multiple stepwise regression model together with apolipoprotein B and apolipoprotein B.

Figure 4 | Univariate regression analysis between serum apolipoprotein B and endothelium-dependent (EDV) and endothelium-independent vasodilation (EIDV, r = -0.43, p < 0.01 and r = -0.34, p < 0.05, respectively). EDV and EIDV are expressed as the relative increase (%) in forearm blood flow during methacholine (4 µg/min) and sodium nitroprusside (10 µg/min), respectively.

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with age and gender, ApoB was inversely related to both EDV and EIDV ($r=-0.43$, $p<0.01$ for EDV and $r=-0.34$, $p<0.05$ for EIDV, Fig. 4), whereas HDL cholesterol was positively related to the EFI ($r=0.35$, $p<0.01$, Fig. 5). Neither LDL peak particle size, nor IgM and IgG titers against oxLDL were significantly related to EDV, EIDV or EFI.

### Discussion

As oxLDL participates in atherogenesis [131-134, 163], several investigators have hypothesized that there is a relationship between the immune response against oxLDL and atherosclerosis. Indeed, low titers of oxLDLab have been found to predict a thicker carotid intima-media thickness and previous myocardial infarction [138, 158], although other studies have reported a positive relationship between oxLDLab and atherosclerosis or myocardial infarction [137, 162] or no relationship at all [125]. In the present study, neither EDV, EIDV nor the EFI were significantly related to oxLDLab. This is in accordance with a recent study in patients with type 1 diabetes, in which no correlation was found between the vasodilatory response to Ach in forearm resistance vessels and oxLDLab [164] despite elevated titers of oxLDLab. Taken together, these data do not support a relationship between the immune response to oxLDL and endothelial vasodilatory function.

According to several studies, individuals with small, dense LDL particles run a higher risk of developing CAD [165-167]. Although Vakkilainen et al found a significant relationship between small LDL particles and impaired EDV in healthy middle-aged men [168], we found no relationship between LDL particle size and endothelial vasodilatory function in the present study. This could be explained by differences in metabolic profiles of the samples. Thus, O’Brien et al found a significant positive relationship between LDL...
particle size and EDV in a sample of middle-aged men with type 2 diabetes, whereas no relationship was found between LDL particle size and EDV in healthy controls [27]. However, in the study by Vakkilainen et al, mean values of body mass index, serum TG and cholesterol, and LDL particle size showed closer resemblance to the type 2 diabetic group in the study by O’Brien et al than to the healthy subjects in our present study. Furthermore, in the Quebec Cardiovascular Study, small LDL particles were significant predictors of CAD risk only at plasma ApoB levels above 1.2 g/l [169]. Hence, the low ApoB levels in our sample might therefore contribute to the lack of correlation between EDV and LDL particle size. Thus, the lack of correlation between LDL particle size and EDV in the present study might be due to that the subjects were healthy and had a favorable metabolic risk factor profile.

Our finding that EFI, reflecting the endothelial contribution to vasodilation, was positively associated with HDL cholesterol is corroborated by other studies in healthy young men and in type 2 diabetic patients [27, 163]. Thus, the independent relationship between HDL cholesterol and EFI in the present study further supports a protective role for HDL cholesterol on endothelial vasodilatory function.

The independent inverse associations between ApoB and both EDV and EIDV in the present study would suggest that ApoB predicts structural vascular changes rather than endothelial dysfunction per se. Indeed, an elevated ApoB level has been found to be independently related to increased carotid intima-media thickness and arterial stiffness in healthy subjects [170-172].

In conclusion, the inverse relationships between ApoB and both EDV and EIDV indicate that ApoB might be an early marker of vascular changes in healthy subjects, whereas HDL seems to be more specifically related to endothelial vasodilatory function.
Study II.

Endothelial vasodilatory function is related to the proportions of saturated fatty acids and alpha-linolenic acid in young men, but not in women

Results

Univariate regression analysis was used to investigate relationships between individual fatty acids and Mch-FBF, SNP-FBF and EFI. Regression analysis was performed in the whole sample, as well as gender-specifically.

When variables associated with vasodilatory parameters in univariate analysis were introduced into multiple stepwise regression models in men together with age, systolic and diastolic blood pressure, body mass index, serum levels of NEFA, TG and cholesterol as confounders, ALA (r=0.47, p<0.01) was positively associated with the EFI (Fig. 6), whereas myristic acid was inversely related to this index (r=-0.39, p<0.01, Fig. 7). DGLA was inversely associated with both Mch-FBF and SNP-FBF (r = -0.34, p<0.05, r = -0.40, p<0.01, respectively), whereas EPA was positively associated with SNP-FBF (r=0.57, p<0.001), but not with Mch-FBF. No significant associations between fatty acids and EFI or Mch-FBF were found in women.
Discussion

ALA was independently and positively associated with the EFI in men. Dietary or serum cholesterol ester ALA has been shown to be inversely associated with cardiovascular mortality and stroke in the Multiple Risk Factor Intervention Trial [88, 94] and with the risk of myocardial infarction in health professionals [95]. Furthermore, in a cross-sectional study in a middle-aged population [69], the ALA proportion was positively associated with EDV. ALA can be converted to HUFA, which have been shown to improve EDV in pigs and type 2 diabetic patients [101, 102]. n-3 fatty acids could exert their anti-atherosclerotic and EDV-modulating properties through conversion of 20-c and 22-c n-3 fatty acids into anti-thrombotic and vasodilatory eicosanoids, such as platelet TXA3 and endothelium-derived PGI1 from EPA [39]. n-3 fatty acids may also modulate the configuration of membrane-bound proteins, i.e. receptors and ion channels, and thereby influence cell function [33]. Furthermore, n-3 and n-6 fatty acids compete for the same elongating and desaturating enzyme systems, e.g. delta-6-desaturase [173]. Indeed, supplementation with EPA has been shown to inhibit the delta-6-desaturase-catalyzed conversion of DGLA to AA [174], from which both thrombotic and vasoconstrictive eicosanoids (e.g. TXA2) are formed as well as PGI2.

As could be expected from previous epidemiological studies showing a relationship between SFA and subsequent CAD [55-57, 59, 61-63, 175], the present study showed an inverse independent relationship between myristic acid and EFI.

The main source of DGLA is from conversion of γ-linolenic acid (GLA, 18:3n-6) to DGLA, since DGLA only exists in trace amounts in the human diet (mother's milk and organ meats such as liver). GLA is found in evening primrose, borage and black currant oils. In the present study, DGLA was

![Figure 7](image)

Univariate regression analysis between the endothelial function index (EFI) and the proportion myristic acid (14:0) in men ($r = -0.36$, $p<0.05$). EFI was calculated as the forearm blood flow during infusion of methacholine 4 µg/min divided by forearm blood flow during infusion of nitroprusside 10 µg/min.

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inversely associated with both Mch-FBF and SNP-FBF in men. A high serum proportion of DGLA can either reflect a high dietary intake of GLA or its n-6 precursors, or a decreased rate of desaturation of DGLA to AA by delta-6-desaturase. However, the AA/DGLA ratio, an often-used index to evaluate delta-6-desaturase activity, was not related to vasodilatory function in the present study (data not presented in the paper). A negative role for DGLA in human health was suggested by a prospective study, in which the proportion of DGLA was significantly higher among Swedes who suffered myocardial infarction during the subsequent 19 years [175]. Furthermore, in the same population, DGLA in serum cholesterol esters was higher among patients with left ventricular systolic dysfunction as compared to controls [176]. On the other hand, DGLA has been shown to counteract hypertension in spontaneously hypertensive rats and a low serum DGLA level has been shown to predict myocardial infarction in middle-aged men [62]. The fact that DGLA is a precursor of anti-thrombotic and vasodilatory eicosanoids [177] corroborates the latter studies.

EPA was independently related to EIDV in the present study. This fatty acid has been associated with a reduced number of coronary events [62]. Altered EIDV indicates altered conditions downstream to the endothelium, as SNP donates NO at or inside the VSMC. Since a major role of both n-3 and n-6 fatty acids is as constituents of membrane phospholipids, the positive relationship between EIDV and EPA might be due to an impact on VSMC membrane properties, for example by modulating membrane fluidity or the configuration of membrane proteins [33]. We failed to detect any effect of EPA on EDV, although this fatty acid has been shown to relax arteries in an endothelium-dependent manner [178]. Our findings suggest that EPA, which is associated with a reduced number of coronary events [62], rather is related to vasodilation in general.

The relationships between arterial vasodilation and fatty acid composition were more pronounced or only present in men. A possible explanation could be that the protective role of estrogens against CVD and impaired EDV before menopause may override possible relationships between fatty acids and arterial vasodilation [179, 180].

In conclusion, the present study shows that an elevated serum proportion of myristic acid and a decreased ALA proportion are independently related to a reduced endothelial vasodilatory function in healthy individuals in the third decade of life. The relationships were only present or more pronounced in males. As the fatty acid composition reflects dietary fat quality, these findings might be of major importance for public health.
Study III.
Vitamin C, diclophenac, and L-arginine protect endothelium-dependent vasodilation against elevated circulating fatty acid levels in humans

Results
Acute elevation of circulating fatty acids was achieved by intravenous infusion of the long-chain TG emulsion Intralipid® together with heparin. Serum NEFA and TG increased in all groups after 2 hours (from 0.2±0.1 to 1.5±0.7 mmol/l, p<0.001, and from 0.7±0.3 to 2.9±0.8 mmol/l, p<0.001, respectively). Serum 8-iso-PGF$_2\alpha$ was significantly elevated by 60±69% at 2 hours in the total sample (p<0.001). The increments of this isoprostane did not reach significance in the individual groups, except in the L-arginine group (p<0.05). In the non-intervention group, Mch-FBF was attenuated by

![Figure 8](image-url)
19±13 % (p<0.01 during Mch 4 µg/min, Fig. 8) after 2 hours. However, no significant reduction in Mch-FBF was seen when diclofenac or L-arginine was co-infused with Intralipid®/heparin. Mch-FBF increased significantly in the vitamin C co-infusion group (from 15.3±4.2 to 18.9±6.5 ml/min per 100 ml tissue, p<0.05 during Mch 4 µg/min). SNP-FBF increased slightly, but significantly, in the non-intervention and vitamin C groups (from 16.6±4.2 to 18.4±5.2 ml/min per 100 ml tissue, p<0.01, and from 16.0±5.4 to 19.3±6.8 ml/min per 100 ml, p<0.05, respectively, during SNP 10 µg/min). SNP was unchanged in the other groups. The EFI was significantly reduced in the Intralipid® non-intervention group (from 1.10 ± 0.25 to 0.79 ± 0.08, p<0.01), but not in the other groups.

No changes in Mch-FBF or SNP-FBF were seen during the intra-arterial control protocol or the time-control protocol.

Discussion
The endothelium-protecting effect of the COX-inhibitor diclofenac in the present study indicates that the COX pathway is a mediator of fatty acid action on EDV. In the present study, increased availability of AA might have stimulated COX-dependent production of vasoconstricting prostaglandins and thromboxanes. Although Intralipid® lacks 20-carbon fatty acids, its most abundant fatty acid is LA, which can be converted to AA. However, the COX pathway is also an important source of superoxide anion in endothelial cells [181]; thus the benefit of COX-inhibition might also be a decreased generation of this oxidant, which reacts with NO to produce the vasoconstrictor peroxynitrite. Hence, both diclofenac and vitamin C might protect NO from degradation by superoxide anion - in the case of diclofenac by blocking the generation of superoxide ion by the COX pathway and in the case of vitamin C by scavenging superoxide anion. Indeed, studies comparing the effects of vitamin C or a COX inhibitor have shown both kinds of substances to augment EDV in hypertension, obesity, and after acute endogenous estrogen deprivation due to bilateral ovariectomy, all of these being conditions in which COX-derived superoxide anions have been proposed as responsible for the impairment in EDV through inactivation of NO [7, 21, 182]. Furthermore, Taddei et al showed that separate infusions of vitamin C and the COX inhibitor indomethacin augmented EDV, whereas co-infusion of these substances provided no additional effect in hypertensive patients [21]. Hence, this latter study further supports the hypothesis that vitamin C and COX inhibition interferes with EDV at different levels of a common pathway. Although vitamin C is scavenger of superoxide ions, its ability to elevate EDV was accompanied by a tendency of increased plasma 8-iso-PGF_2α in the present study. Similarly, vitamin C did not affect plasma F_2-isoprostane levels when taken as a 500
mg daily supplement by Finnish men during 1 year [183]. Hence, in the present study, plasma 8-iso-PGF$_{2\alpha}$ should only be used to indicate increased lipid peroxidation, but not as an assessment of the antioxidative effect of vitamin C.

Acute administration of L-arginine preserved EDV in the presence of an elevated NEFA level in the present study. Indeed, acute administration of L-arginine has also been shown to improve EDV in hypercholesterolemic subjects with or without CAD and in hypercholesterolemic rabbit aortic rings [184-188]. As asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, is elevated in atherosclerosis and hypercholesterolemia [189], exogenous L-arginine might compensate for an increased ADMA level, and thereby restore NO formation in these conditions, and perhaps also in the present study. Alternatively, in the present study, exogenous L-arginine might have compensated a relative intracellular L-arginine deficiency due to enhanced NO synthase activity in order to compensate for increased NO breakdown by e.g. superoxide anions [190]. On the other hand, a study by Mitchell et al has shown that there is an abundance of L-arginine in cultured endothelial cells [191]. Another possibility is that L-arginine infusion might have reduced oxidative stress in the present study, since treatment with L-arginine improved EDV and reduced superoxide release by aortic rings from cholesterol-fed rabbits [192]. However, the present study cannot differentiate whether the maintained EDV during L-arginine infusion reflected a protecting effect against NEFA-induced endothelial dysfunction or if L-arginine infusion simply provided supranormal L-arginine availability for NO synthase, resulting in increased NO production.

In conclusion, EDV was protected by vitamin C, diclophenac and L-arginine during acute NEFA elevation, suggesting that these drugs operate through a common pathway.
Study IV.
Acute elevations of medium-chain and long-chain fatty acids have different impact on endothelium-dependent vasodilation in humans

Results
Acute NEFA elevation was achieved by intravenous infusion of either Structolipid® or Intralipid® together with heparin (NEFA rose from 0.2±0.1 to 1.4±0.5 mmol/l, p<0.001, and from 0.4±0.1 to 1.8±0.4 mmol/l, p<0.001, respectively). Serum TG increased significantly in both groups (from 0.8±0.2 to 3.1±1.0 mmol/l, p<0.001 in the Intralipid® group, and from 0.7±0.3 to 4.3±1.2 mmol/l, p<0.001 in the Structolipid® group). In the Intralipid® group, the PUFA proportion increased from 39% to 52% (p<0.001), and SFA decreased from 35% to 25% (p<0.001) after 2 hours. In the Structolipid® group, there were only minor changes in the proportions of SFA (from 34% to 32%, p<0.001), and PUFA (from 40% to 43%, p<0.05) after 2 hours. Urine 8-iso-PGF$_{2\alpha}$ was significantly elevated after 2 hours in the Structolipid® group (70±65%, p<0.05), but not in the Intralipid® group.

Figure 9  Forearm blood flow during infusion of methacholine chloride at 4 µg/min (Mch-FBF) and sodium nitroprusside at 10 µg/min (SNP-FBF) at baseline and after 2 hours of infusion with Intralipid® plus heparin or Structolipid® plus heparin. Means ± SEM are given. * p<0.05, ** p<0.01 vs baseline.
The 2-hour reduction in Mch-FBF in the Intralipid group (from 18.2±6.2 to 14.5±4.9 ml/min per 100 ml, p<0.01 during Mch 4 µg/min) was not seen in the Structolipid group (Fig. 9). Both lipid emulsions increased SNP-FBF significantly after 2 hours (from 16.6±4.2 to 18.4±5.2 ml/min per 100 ml, p<0.01 in the Intralipid group, and from 19.1±8.1 to 21.1±8.8 ml/min per 100 ml, p<0.05 in the Structolipid group during SNP 10 µg/min). The EFI decreased significantly in the Intralipid group (from 1.10±0.25 to 0.79±0.08, p<0.01), but was unchanged in the Structolipid group.

Discussion
Acute elevation of circulating NEFA, induced by infusion of Intralipid and heparin, has previously been shown to impair EDV in humans [25, 107-109] and to produce elevated levels of malondialdehyde, a widely used marker of lipid peroxidation [109, 193]. However, urine 8-iso-PGF$_{2α}$ increased significantly in the Structolipid group, whereas no significant elevation of this lipid peroxidation marker was detected in the Intralipid group. Owing to the small sample size of the 8-iso-PGF$_{2α}$ analysis in the Intralipid group, no conclusions can be made about differences in lipid peroxidation between the groups in the present study. However, the fact that EDV was unaffected in the Structolipid group, despite significantly elevated 8-iso-PGF$_{2α}$ levels, suggests that lipid peroxidation is not the mechanism whereby EDV was impaired in the Intralipid group.

A quite different plasma fatty acid composition was seen after 2 hours in the Intralipid group with a significantly lower proportion of plasma MCFA and a higher proportion of PUFA as compared to the Structolipid group. Individual fatty acids have been shown to either enhance or attenuate EDV. Hence, supplementation with fish oil improved EDV in pigs after 4 weeks [102]. We have recently found that EDV is positively related to ALA in serum phospholipids in humans [69], whereas in vitro studies have shown that AA produces EDV [91], whereas oleic acid inhibits EDV in rabbit femoral artery rings [82].

Although Intralipid lacks 20-carbon fatty acids, its most abundant fatty acid is LA (52% in Intralipid, 33% in Structolipid), which is converted to AA in vivo. In the present study, increased availability of AA might have stimulated COX-dependent production of vasoconstricting prostaglandins and thromboxanes, such as TXA$_2$ and PGH$_2$. On the other hand, the COX pathway also is an important source of free radical superoxide anions in endothelial cells [112]. Superoxide anion rapidly reacts with NO to form the powerful oxidant peroxynitrite [194]. Indeed, inhibition of the COX pathway has previously been shown to restore EDV in essential hypertension and to protect EDV during experimental ischemia [195, 196]. We demonstrated that the attenuated EDV observed during Intralipid and heparin infusion in
humans could be counteracted by concomitant infusion of the COX inhibitor diclofenac [197]. Selective blockers of TXA₂ and PGH₂ and assessment of endothelium-derived superoxide anion production are needed to further clarify the role of these NO antagonists in NEFA-induced endothelial dysfunction.

In conclusion, an acute elevation of LCFA attenuated EDV, whereas EDV was maintained during simultaneous elevation of both MCFA and LCFA. These findings emphasize that fatty acid composition influences EDV. Hence, in order to understand the effects of fatty acids on endothelial vasodilatory function, it is necessary to investigate fatty acids individually.
Study V.
The Effect of a Mixed Meal on Endothelium-Dependent Vasodilation in Healthy Humans is Dependent on Fat Content

Results
In the 34 E% fat group, Mch-FBF was significantly decreased by 14±0.9%
(from 15.8±3.9 to 13.8±4.1, p<0.01) at 1 hour, but returned to fasting levels at 2 hours (Fig. 10). No significant change was observed for Mch-FBF in the low-fat (20 E% fat) group. In the minimum-fat (3 E% fat) group, Mch-FBF actually increased significantly between 1 and 2 hours (from 21.2±5.0 to 23.4±5.8, p<0.01). No significant changes were observed for SNP-FBF in any of the groups. Also EFI was significantly reduced in the 34 E% fat group after 1 hour (p<0.05), but not after 2 hours compared to baseline. No such reduction in EFI was seen during meals with lower fat content. The EFI increased significantly after 2 hours in the minimum-fat (3 E% fat) group (p<0.05).

The lipid peroxidation markers plasma malondialdehyde and urine 8-iso-PGF$_{2\alpha}$ were not significantly changed in any of the groups (Table 3.).

### Table 3 | Circulating levels of lipids, glucose, and insulin before and at 1 and 2 h after ingestion of meals with different fat content.

<table>
<thead>
<tr>
<th></th>
<th>Serum cholesterol (mmol/l)</th>
<th>Serum TG (mmol/l)</th>
<th>Serum NEFA (mmol/l)</th>
<th>Plasma glucose (mmol/l)</th>
<th>Plasma insulin (mU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ordinary meal</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(34 E% fat, n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.6 ± 0.5</td>
<td>1.0 ± 0.8</td>
<td>0.40 ± 0.21</td>
<td>5.1 ± 0.9</td>
<td>6.4 ± 3.9</td>
</tr>
<tr>
<td>1 hour</td>
<td>3.6 ± 0.5</td>
<td>1.1 ± 0.7</td>
<td>0.40 ± 0.30</td>
<td>6.0 ± 1.1**</td>
<td>27.8 ± 14.4**</td>
</tr>
<tr>
<td>2 hours</td>
<td>3.6 ± 0.5</td>
<td>1.2 ± 0.7**</td>
<td>0.33 ± 0.16</td>
<td>5.9 ± 0.8***</td>
<td>21.9 ± 10.2***</td>
</tr>
</tbody>
</table>

| **Low-fat meal**         |                             |                   |                     |                         |                       |
| (20 E% fat, n=8)         |                             |                   |                     |                         |                       |
| Baseline                 | 4.5 ± 0.5                   | 1.1 ± 0.6         | 0.36 ± 0.08         | 4.8 ± 0.8               | 3.7 ± 0.8             |
| 1 hour                   | 4.6 ± 0.5                   | 1.4 ± 0.8*        | 0.16 ± 0.05***      | 6.4 ± 1.1*              | 28.6 ± 10.4***        |
| 2 hours                  | 4.6 ± 0.5                   | 1.6 ± 1.0*        | 0.20 ± 0.09**       | 5.8 ± 0.8*              | 22.9 ± 5.1***         |

| **Minimum-fat meal**     |                             |                   |                     |                         |                       |
| (3 E% fat, n=8)          |                             |                   |                     |                         |                       |
| Baseline                 | 4.0 ± 0.7                   | 0.8 ± 0.2         | 0.48 ± 0.13         | 5.3 ± 0.5               | 4.2 ± 1.9             |
| 1 hour                   | 3.9 ± 0.6*                  | 0.7 ± 0.2         | 0.10 ± 0.05***      | 7.9 ± 2.0*              | 63.9 ± 34.5**         |
| 2 hours                  | 3.9 ± 0.6*                  | 0.6 ± 0.2**       | 0.08 ± 0.04***      | 6.5 ± 1.7               | 41.2 ± 20.2***        |

Means ± SD are given. * p<0.05, ** <0.01 and *** p<0.001 compared to baseline.
By univariate regression analysis including all groups, the increase in plasma insulin was significantly associated with the changes in Mch-FBF after 2 hours in the total sample ($r=0.45$, $p<0.05$ at 2 hours, $r=0.29$ at 1 hour). There were, however, no significant relationships between changes in Mch-FBF, SNP-FBF and EFI and changes in serum NEFA and TG in the total sample.

Discussion

Our findings are corroborated by several previous studies showing that EDV decreases after meals containing an average to high fat percentage [81, 198-201], although this has not been a consistent finding [202-204]. Furthermore, almost fat-free meals have been shown to preserve EDV [198-200]. Taken together, the above cited studies have shown that virtually fat-free meals preserve EDV, whereas meals containing 34 to 60 E% fat decrease EDV in most studies. To our knowledge, the present study is the first to show that a moderate lowering of fat content from 34 to 20 E% fat or less preserves EDV.

The present study also investigated several potential mechanisms whereby a 34 E% fat meal might attenuate EDV, e.g. changes in NEFA, TG and insulin levels, as well as biomarkers of lipid peroxidation. Substantial differences in 2-hour insulin levels were observed between the groups in the present study, with the lowest increase in the ordinary (34 E% fat) meal group. Furthermore, changes in insulin levels correlated significantly with Mch-FBF at 2 hours in the total sample. Indeed, insulin has been shown to be an endothelium-dependent vasodilator [205, 206]. Furthermore, acute hyperinsulinemia has been shown to enhance Mch-FBF and to maintain Mch-FBF during acutely elevated circulating NEFA levels in healthy humans [109]. As insulin was elevated in proportion to the postprandial increase in glucose concentration, we observed greater increases in insulin levels the greater the carbohydrate content and the lower the fat content in the meals. It has been demonstrated that insulin inhibits hormone-sensitive lipase and stimulates lipoprotein lipase, which will result in a decreased circulating NEFA level and incorporation of NEFA into adipose tissue TG [207]. Thus, in the present study the postprandial NEFA levels were reduced in proportion to the elevation of insulin levels. As the 2-hour change in Mch-FBF was related to the change in insulin levels at 2 hours, the combination of only a modest rise in insulin together with the blunted reduction of circulating NEFA in the 34 E% fat group, might contribute to the reduced Mch-FBF seen in this group [201].

Interestingly, Mch-FBF and the EFI increased significantly after the 3 E% fat meal, although no increment in endothelial vasodilatory function was observed after the 20 E% fat meal. This difference may be attributed to the significantly higher insulin level seen after the 3 E% fat meal as compared to
the 20 E% fat meal. However, a special characteristic of the 3 E% fat meal was that the fat was mainly derived from fish (cod) in contrast to the other meals. Although a high dietary or supplementary intake of fish oils is associated with enhanced EDV and reduced CVD risk [24, 208, 209], ingestion of two different high-fat meals, containing 50 E% fat with different SFA proportion, decreased FMD similarly after 4 hours, whereas a fat-free meal preserved FMD [200].

There were no significant increments in plasma MDA and urine 8-iso-PGF$_{2\alpha}$ in any of the groups. This could indicate that lipid peroxidation is of minor importance in the settings of the present study. Alternatively, since these lipid peroxidation biomarkers are non-specific indices of oxidative stress, they may have failed to respond to those factors that are responsible for the attenuated EDV in the present study. Indeed, ingestion of a high-fat meal rich in used cooking fat failed to elevate the levels of lipid peroxidation biomarkers (thiobarbituric acid reacting substances and fluorescent lipid oxidation products) [203], despite the high levels of lipid peroxidation products in used cooking fat. Thus, the present study cannot provide evidence that the reduction in EDV in the 34 E% fat group is due to increased lipid peroxidation.

In conclusion, our findings indicate that a dietary fat content of 20 E% or less might preserve endothelial vasodilatory function and thereby protect against atherosclerosis.
General discussion

A high LDL cholesterol level may be attributed either to large LDL particles or an increased number of these particles. The latter seems to be more important, as indicated by the inverse relationship between ApoB and vasodilatory function in study I. A high number of LDL particles is likely to increase the rate at which these are trapped in the intima. Thus, lowering LDL particle number should be one of the aims for cholesterol-lowering therapy.

Lipoprotein particles are also carriers of fatty acids, and once trapped, the fatty acid profile will influence the degree of lipid peroxidation in the intima. A high PUFA proportion might lead to a higher degree of lipid peroxidation, unless antioxidant defense is adequate [124, 129]. However, increased lipid peroxidation may not be the only mechanism whereby fatty acids impair EDV. Indeed, in study IV, increased lipid peroxidation during acute elevation of both MCFA and LCFA (with Structolipid®/heparin) was not accompanied by an impaired EDV, indicating that lipid peroxidation is not the sole mechanism behind EDV suppression during acute LCFA elevation (with Intralipid®/heparin). However, the fact that both the COX inhibitor diclophenac and the superoxide scavenger vitamin C prevented the impairment in EDV seen during LCFA elevation suggests that these interventions might have reduced the bioavailability of COX-derived superoxide anion, which rapidly inactivates NO [194]. As an elevated NEFA level, as seen in insulin resistance, may be adverse to the endothelium due to increased superoxide-mediated inactivation of NO, a reasonable endothelium-protecting strategy would be to assure an adequate vitamin C intake.

The positive relationship between ALA and EFI and EIDV in study II, suggests a positive role for n-3 PUFA on the endothelium, as well as on vasodilation in general, as long as adequate antioxidative protection is assured. Indeed, a protective role for n-3 PUFA against CVD has been recognized for decades [39, 88, 94-96]. PUFA, and especially HUFA, may be important for endothelial function by lowering LDL cholesterol [96], by being precursors for anti-thrombotic and vasodilatory eicosanoids [39], and by modulating the configuration of membrane-bound proteins and thereby influencing cell function [33]. SFA on the other hand are known to increase CVD risk [55, 56, 61-63]. Hence, the association between myristic acid and the EFI in study II is not surprising.
However, dietary fatty acid profile was not a factor that differed significantly between the 34 and 20 E% fat meals in study V. Instead, lowering the fat content to 20 E% or less, in favor of an increased carbohydrate percentage, abolished the negative effect on EDV, seen during the 34 E% fat meal. These results suggest that dietary fat intake in the Western diet should be less than 34 E% fat.

Taken together, the studies of this thesis highlight several aspects of lipid metabolism with regard to endothelial vasodilatory function. Thus, the number of LDL particles, as assessed as ApoB, the cholesterol-removing capacity of HDL, the plasma fatty acid profile, reflecting dietary intake, as well as total fat content in meals all seem to influence endothelial vasodilatory function.

Study limitations

A number of correlations were performed in studies I and II and, with the chosen significance level of 5%, one correlation in twenty might be significant merely by chance. This risk increased when the genders were analyzed separately in study II. Therefore, these results must be interpreted with caution until reproduced by other investigators. However, as the major results in studies I and II agree with findings in prospective and case-control studies relating fatty acid composition and lipoprotein profile to CAD [62, 175, 210-213], it is unlikely that the major results of the present studies occurred merely by chance. The fact that several of the correlations were independent of other factors of importance for arterial vasodilation, such as blood pressure, age and serum cholesterol level, further emphasizes the validity of the findings.

The statement that lipid peroxidation increased during acute NEFA elevation after 2 hours in study III is based on significant elevations of 8-iso-PGF$_{2\alpha}$ in the total sample. A disadvantage with this approach is that the degree of lipid peroxidation may have been modified by the interventions. Unfortunately, a between-group comparison of plasma 8-iso-PGF$_{2\alpha}$ could not be performed in a proper way owing to insufficient number of 8-iso-PGF$_{2\alpha}$ data in the non-intervention group. Neither could such a between-group comparison be performed in study IV due to the same reason. However, increments in 8-iso-PGF$_{2\alpha}$ were observed in all of the intervention groups, and these increments were significant in the L-arginine and Structolipid® groups without any decrease in EDV in any of these groups. This fact, together with the finding by Kaikkonen et al that vitamin C did not decrease F$_2$-isoprostanes significantly in Finnish men [183], suggests that lipid peroxidation was not the main mechanism responsible for the decreased EDV after Intralipid®/heparin infusion.
Another limitation in studies III and IV is that *in vitro* lipolysis was not prevented by addition of an inhibitor, such as paraoxon, tetrahydrolipstatin or hypertonic saline in the original study protocols [214-216]. The magnitude of *in vitro* lipolysis was corrected afterwards by adjusting the TG and NEFA values with factors obtained in a later performed control study, in which hypertonic saline was added [216]. Thus, the pronounced elevation of NEFA and TG reported in studies III and IV must be interpreted with caution. However, the measurements of NEFA and TG were not a major aim of these studies and merely serve to show that these lipids were elevated to a similar extent in the subgroups.

**Future perspectives**

The results of study I should be validated in clinical trials aiming at reducing ApoB levels and increasing HDL cholesterol levels.

The findings of studies II-V, which are based on young, healthy subjects, should be confirmed in older populations with and without overt atherosclerotic CVD.

The mechanism behind endothelial dysfunction during acute NEFA elevation needs further clarification. To solve this matter in future studies, it is essential to measure the effects of elevated NEFA concentration on levels of L-arginine, ADMA, superoxide anion, as well as on different markers of inflammation.

Study V suggests an acute beneficial effect on endothelial function by lowering dietary fat content to 20 E% or less. However, apart from confirming these findings, future studies need to further characterize the breakeven for endothelial function somewhere between 34 and 20 E%. Furthermore, the effects of long-term dietary interventions with different fatty acid and antioxidant profiles need to be further clarified. Such studies are essential for future recommendations regarding dietary fat percentage. The current recommended fat intake of 30 E% or less e.g. by the Swedish National Food Administration and the U.S. Food and Drug Administration might be too high.

**Methodological considerations**

Assessment of FBF by venous occlusion plethysmography is a widely used method measuring blood flow predominantly through skeletal muscle resistance arteries. However, skin and subcutaneous tissue has been shown to substantially contribute to FBF during resting conditions [217, 218].

Both Mch and Ach are muscarinic agonists, whose vasodilator effects have been shown to be attenuated to a similar extent by the NOS inhibitor L-
NMMA [219]. Mch was used in the present investigations, as Mch has been shown to be more resistant to degradation by cholinesterase than Ach [219].

Hand circulation has traditionally been excluded by applying a wrist cuff. However, as it has been shown that this procedure has no significant influence on blood flow measurements [149], wrist cuffs were not applied in the present studies.

In study I, EDV and EIDV are expressed as the relative increase (%) in FBF during vasodilation with Mch or SNP, respectively. However, in study II, EDV and EIDV are instead expressed as the absolute value of FBF (Mch-FBF and SNP-FBF in ml/min per 100 ml of forearm tissue). One of the reasons for choosing this latter approach is that several fatty acids were related to basal FBF in study II. Hence, incorporating basal FBF into the assessment of vasodilatory function might have biased the correlations between fatty acids and vasodilatory function.

Mch-FBF and SNP-FBF were also used in studies III-V. With this approach EDV and EIDV will not be underestimated due to increasing basal FBF as seen in study V and in previous studies of our group. The EFI is not influenced if basal FBF increases by an intervention, as this index expresses the ratio between Mch-FBF and SNP-FBF independently of basal FBF. Furthermore, the EFI provides an index that minimizes the influences of drug distribution and the differences in forearm volume. Furthermore, we have found this index to be more closely related than Mch-FBF to several risk factors, such as blood pressure, smoking, and mental stress [13, 220, 221]. However, the EFI should be interpreted with caution when SNP-FBF changes over time, as a depressed EFI over time may reflect either an attenuated response to Mch or an increased response to SNP. Hence, EDV and EIDV data should always be presented together with this index.
General summary

► In healthy subjects aged 20-69 years, ApoB was inversely associated with EDV and EIDV, whereas HDL cholesterol was positively associated with the EFI, independently of confounders. Neither oxLDLab nor small LDL particles were related to endothelial vasodilatory function.

► In a population sample of young, healthy subjects, the ALA proportion was positively associated with the EFI, whereas myristic acid was inversely associated with the EFI in men only, independently of confounders. EPA was positively related to EIDV, whereas DGLA was inversely related to both EDV and EIDV in men only.

► Acute LCFA elevation during Intralipid®/heparin infusion in young, healthy subjects impaired EDV after 2 hours. This impairment could be prevented by infusion of vitamin C, diclophenac or L-arginine.

► Acute elevation of both MCFA and LCFA during Structolipid®/heparin infusion did not impair EDV after 2 hours in young, healthy subjects.

► An ordinary meal containing 34 E% fat transiently attenuated EDV in young, healthy subjects. No such attenuation in EDV was observed after meals containing 20 or 3 E% fat.
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