Comprehensive Analysis of Risk Factors in Offspring of Type 2 Diabetic Patients

Dissertation

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To Whom It May Concern:

I confirm that the following Ph.D. dissertation "Comprehensive Analysis of Risk Factors in Offspring of Type 2 Diabetic Patients" by Michaela Vossen, written in English, is linguistically and grammatically correct.

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Aim and Working Hypothesis

Individuals with at least one type 2 diabetic parent have a substantially higher risk of developing insulin resistance, type 2 diabetes or cardiovascular diseases. Thus, these individuals can be used not only to identify novel risk markers but also to recognize early alterations in metabolism.

Therefore, a study of a cohort of 91 apparently healthy type 2 diabetes offspring was used as a basis for this work which comprised the biostatistical analysis of the given dataset. The participants were invited on two independent test days. Plasma samples were collected before and after an oral glucose tolerance test and after the consumption of a fat-rich breakfast as an oral fat tolerance test. The plasma samples were used for a comprehensive metabolic profiling. The large dataset of fasting and postprandial data was analyzed in this thesis with the help of biostatistical methods. The results were interpreted on the basis of current knowledge and previously published data.

This thesis focused on four aspects. The first aim was to confirm previously reported association between risk factors and fatty acids, measured in total plasma and major phospholipid species, for healthy subjects, type 2 diabetes or cardiovascular diseases patients, in the cohort of healthy offspring from type 2 diabetic patients. Since the role of ω3-fatty acids during the development of type 2 diabetes and cardiovascular diseases remained inconclusive in previous studies, the association of plasma ω3-fatty acids with insulin resistance parameters was the second aspect studied here. The fact that the study focused on the postprandial state and insulin action, allowed, as the third aspect, to analyze plasma triacylglycerol levels during an oral glucose tolerance test as well as an oral fat tolerance test. The fourth aspect which has been analyzed was the association between ω3-fatty acids and active plasminogen activator inhibitor-1 as a marker for fibrinolysis and risk factor for cardiovascular diseases.

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List of Abbreviations

Abbreviation	Name
AA	arachidonic acid
ADMA	asymmetric dimethylarginin
AIR	acute insulin response
ALA	α-linolenic acid
aPAI1	active plasminogen activator inhibitor-1
Apo	apolipoprotein
BMI	body mass index
C-atom	carbon-atom
CVD	cardiovascular disease
d7-HDA	d7-hexadecenoic acid
DBP	diastolic blood pressure
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EDA	eicosadienoic acid
ELOVL	elongation of very long-chain fatty acids
EPA	eicosapentaenoic acid
ETA	eicosatrienoic acid
ETEA	eicosatetraenoic acid
FA	fatty acid
FAS	fatty acid synthase
FFA	free fatty acid
FPLC	fast protein liquid chromatography
GLA	γ-linolenic
HDL	high density lipoprotein
HOMA-%B	homeostasis model assessment of β-cell function
HOMA-IR	homeostasis model assessment of insulin resistance
IDL	intermediate density lipoprotein
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL6	interleukin-6
InsRes	insulin resistance
InsSen	insulin sensitivity
LA	linoleic acid
LDL	low density lipoprotein

Abbreviation	Name
LDL-R	low density lipoprotein receptor
LPC	lysophosphatidylcholine
LPL	lipoprotein lipase
LRP1	low density lipoprotein receptor related protein 1
M	insulin-stimulated glucose disposal
MCP1	monocyte chemotactic protein-1
MetSyn	metabolic syndrome
MUFA	monounsaturated fatty acid
NGT	normal glucose tolerance
OFTT	oral fat tolerance test
OGTT	oral glucose tolerance test
PC	phosphatidylcholine
PCA	principle component analysis
PL	phospholipid
PUFA	polyunsaturated fatty acid
RM	remnant
SBP	systolic blood pressure
SDA	stearidonic acid
SEM	standard error of the mean
SFA	saturated fatty acid
T2D	type 2 diabetes
TAG	triacylglycerol
TC	total cholesterol
TNFα	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
tPAI1	total plasminogen activator inhibitor-1
TRL	triacylglycerol-rich lipoprotein
u-PA	urokinase-type plasminogen activator
VLDL	very low density lipoprotein
Waist	waist circumference
WHR	waist-to-hip ratio

1 Introduction

Diabetes mellitus has reached epidemic proportions and currently (July 2010) affects approximately 285 million people worldwide and the number of cases is estimated to rise to 438 million in 2030 (International Diabetes Federation, 2010). 90%−95% of the affected individuals have type 2 diabetes mellitus (T2D) (former known as non-insulin dependent diabetes mellitus, NIDDM) (Stumvoll M, 2005). In addition to the increasing number of patients suffering from T2D, a steep rise in the clinical complications associated with T2D is expected. Without proper treatment T2D leads to various complications: 1. retinopathy which is the leading cause of visual impairment and blindness in adults in developed countries, 2. neuropathy resulting in a loss of sensation thereby leading to the risk of ulcers and at last foot amputations, 3. nephropathy (kidney failure), and 4. cardiovascular disease (CVD) which may lead to ischemic heart diseases or stroke and accounts for 50% of the deaths caused by T2D (International Diabetes Federation, 2010). The medical and socioeconomic burden of T2D begins at least eight years before the diagnosis is established (Nichols GA, 2000).

1.1 Pathophysiology of Hyperglycemia and Insulin Resistance

In the postprandial state after a carbohydrate-containing meal, pancreatic islet β-cells secrete insulin into the circulation. An estimate of β-cell function can be obtained by homeostasis model assessment for percent β-cell function (HOMA-%B), a factor including fasting plasma insulin (0h-insulin) in the numerator and fasting plasma glucose (0h-glucose) in the denominator (see Analysis of Insulin Resistance Parameters). Then, the secreted insulin stimulates glucose uptake into insulin sensitive tissues and reduces endogenous hepatic glucose production. Since sufficient energy sources are available in the postprandial state, insulin also inhibits lipolysis of triacylglycerol (TAG) and β-oxidation of fatty acids (FA), whereas the biosynthesis of FA is promoted so that energy can be stored as fat. In the fasting state, glucose levels fall and glucagon is released into the circulation to promote hepatic

glucose production (Karlsson HKR, 2007). At the same time, FA biosynthesis is inhibited and TAG are subjected to lipolysis and FA are oxidized to supply energy. In normal glucose tolerant subjects a hyperbolic and balancing relationship exists between insulin secretion and insulin sensitivity (InsSen) which means that a reduction in InsSen can be compensated by an increase in insulin secretion by the pancreatic β-cells (Figure 1) (Weyer C, 1999).

During the transition from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and further to T2D, pancreatic β-cells function decreases and cannot compensate anymore for the reduced InsSen (Figure 1).

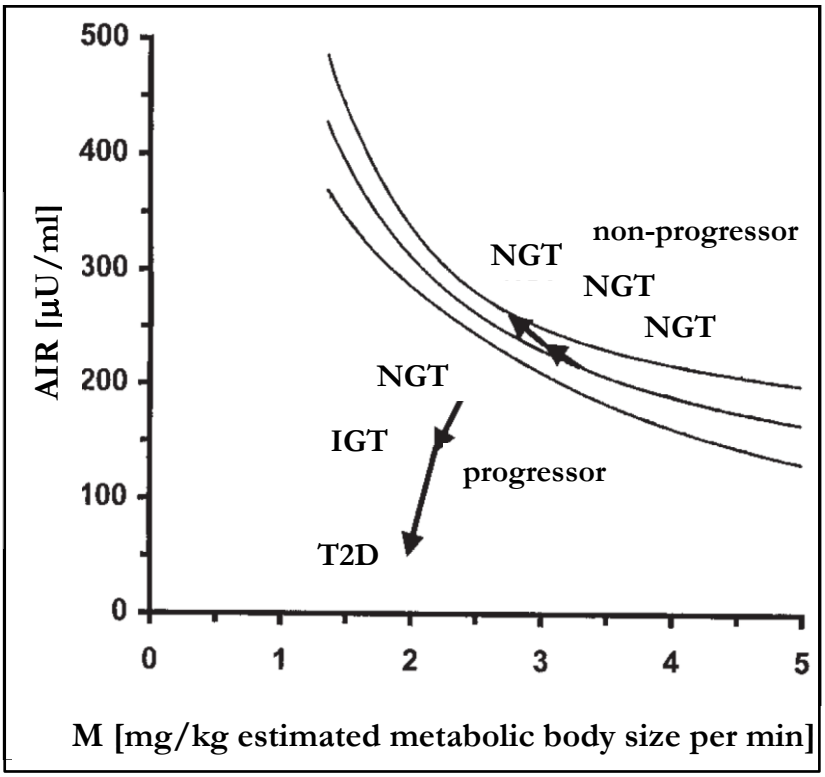

Figure 1. Hyperbolic Relation between β-Cell Function and InsSen
Changes in acute insulin secretory response (AIR) to 25g intravenous glucose relative to changes in rate of total insulin-stimulated glucose disposal at physiological insulin levels during a hyperinsulinemic euglycemic glucose clamp (M), in 11 Pima Indian subjects in whom glucose tolerance changed from NGT to IGT and to T2D (progressor) and in 23 subjects who remained NGT (non-progressor). The lines represent the prediction line and the lower and upper limits of the 95% confidence interval of the regression between AIR and M as derived from a reference population of 277 Pima Indians with NGT (Weyer C, 1999).

The cellular and molecular mechanisms responsible for T2D result from a dysregulation of glucose metabolism as it occurs in insulin resistant states (Figure 2). Insu-

lin resistance (InsRes) is characterized by impaired insulin signaling in liver, skeletal muscle, and/or adipose tissue (Björnholm M, 2005). InsRes may affect only particular signaling pathways in particular tissues. In contrast to InsRes which impairs insulin signaling pathways specifically, a diminished insulin secretion has consequences on all insulin signaling pathways.

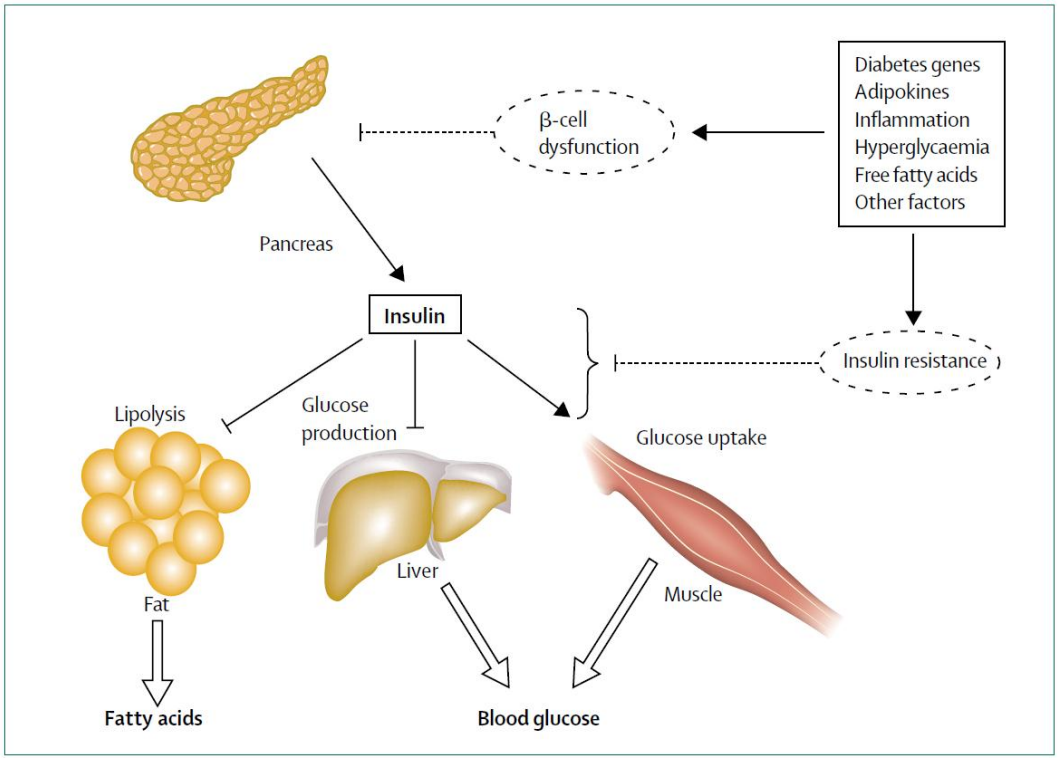

Figure 2. Pathophysiology of Hyperglycemia and InsRes in T2D

Normally, insulin suppresses hepatic glucose production, stimulates glucose uptake by skeletal muscle, and reduces release of FA from fat tissue. Various factors influence insulin secretion and action thereby contributing to the pathogenesis of T2D. Reduced insulin secretion due to β-cell dysfunction will lower insulin effect in its target tissues. In InsRes states, insulin action will be impaired in one or several of the major target tissues resulting in elevated plasma FA and glucose levels which in turn will worsen both insulin secretion and InsRes (Stumvoll M, 2005).

InsRes can be estimated by homeostasis model assessment for InsRes (HOMA-IR), a calculated factor containing the product of 0h-insulin and 0h-glucose (see Analysis of Insulin Resistance Parameters). Low HOMA-IR values indicate high InsSen, whereas high HOMA-IR values indicate lower InsSen (thus InsRes) (Matthews DR, 1985). Since HOMA-IR is a continuous parameter and not used in routine clinical practice, a concrete cut-off value defining InsRes does not exist. Reported cut-off values are 1.77 (Esteghamati A, 2010), 2.26 (Radikova Z, 2006),

2.77 (Bonora E, 1998), and 4.65 (Stern SE, 2005) depending on the study population and the applied definition criteria. In the present study, 1.77 was chosen as a cut-off value since it was shown to predict metabolic syndrome (MetSyn) in a population-based cohort (Esteghamati A, 2010).

In insulin resistant states, the effect of insulin on glucose uptake by skeletal muscle and/or endogenous glucose production by the liver is lower than expected. Despite elevated insulin levels, the diminished response of insulin target tissues to insulin leads to increased biosynthesis of FA and more release of free FA (FFA) from adipose tissue and decreased FA β-oxidation resulting in an accumulation of TAG in the liver (Stumvoll M, 2005). Elevated TAG levels in combination with diminished insulin action leads to a reduction in insulin-stimulated glucose uptake in muscle and an increased hepatic glucose production causing hyperglycemia (Figure 2) (Karlsson HKR, 2007).

InsRes is a major risk factor for the development of T2D (Reaven GM, 2008) and CVD (Grundy SM, 2007). InsRes is strongly associated with obesity mainly caused by physical inactivity and consumption of high-energy diets. InsRes and obesity are both features of the MetSyn which also includes atherogenic dyslipidemia (Howard BV, 1999), altered adipokine secretion (Aguilera CM, 2008), and subclinical chronic inflammation (Hotamisligil GS, 2006).

Adipose tissue of obese subjects, storing excessive amounts of fat, secretes more glycerol and FFA, which further induce InsRes and impair β-cell function (Kahn SE, 2006). Adipose tissue also affects metabolism by releasing hormones, like leptin and adiponectin, and pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin 6 (IL6) and monocyte chemoattractant protein-1 (MCP1). In contrast to FFA and other adipokines, concentrations of adiponectin decrease in obesity and InsRes reducing its insulin-sensitizing effects. Adiponectin improves InsSen in liver and muscle by increasing FA oxidation and reducing plasma FA levels (Aguilera CM, 2008). TNFα, IL6 and MCP1 trigger and attract further potential

mediators of inflammation and inhibit insulin signaling thereby further promoting InsRes (Khan S, 2002).

Previous studies have established that individuals with at least one parent suffering from T2D (T2D offspring) have a substantially higher risk of developing T2D or pre-diabetic impairments in glucose and lipid metabolism (Meigs JB, 2000; Srinvasan SR, 2003; Haffner SM, 1989). Cohorts consisting of T2D offspring are well-suited to study InsRes mechanisms, as they include a disproportionally high number of insulin resistant subjects (Vaag A, 2001). This demonstrates a genetic predisposition for T2D in T2D offsprings although only a small number of potential responsible genes have been identified (O'Rahilly S, 2009).

1.2 Lipids and their Role in Insulin Resistance

Fatty Acids

FA play an important role in InsRes and CVD. FA are needed as energy sources, for membrane synthesis and maintenance as well as regulatory processes. They can either be taken up with the diet or synthesized by the body itself. FA are composed of a carboxy-group and a long aliphatic tail, which is either saturated or unsaturated containing one or more double-bonds. The number of carbon atoms and double-bonds are characteristics for FA. Saturated FA (SFA) usually consist of 4 to 24 carbon-atoms (C-atom) and have no double-bonds. In the present study, only SFA with 14 to 24 C-atoms were measured. Monounsaturated FA (MUFA) contain one double-bond and polyunsaturated FA (PUFA), like ω3- and ω6-FA, have two to six double-bonds. All these double-bonds occur in a cis-confirmation. A multi-enzyme called FA synthase (FAS) can synthesize SFA up to palmitic acid from acetyl-CoA and malonyl-CoA precursors. Palmitic acid is composed of 16 C-atoms and no double bonds which is denoted as C16:0.

The pathway comprising SFA and MUFA with 14 to 24 C-atoms and the chemical structures of myristic and myristoleic acid are displayed in Figure 3.

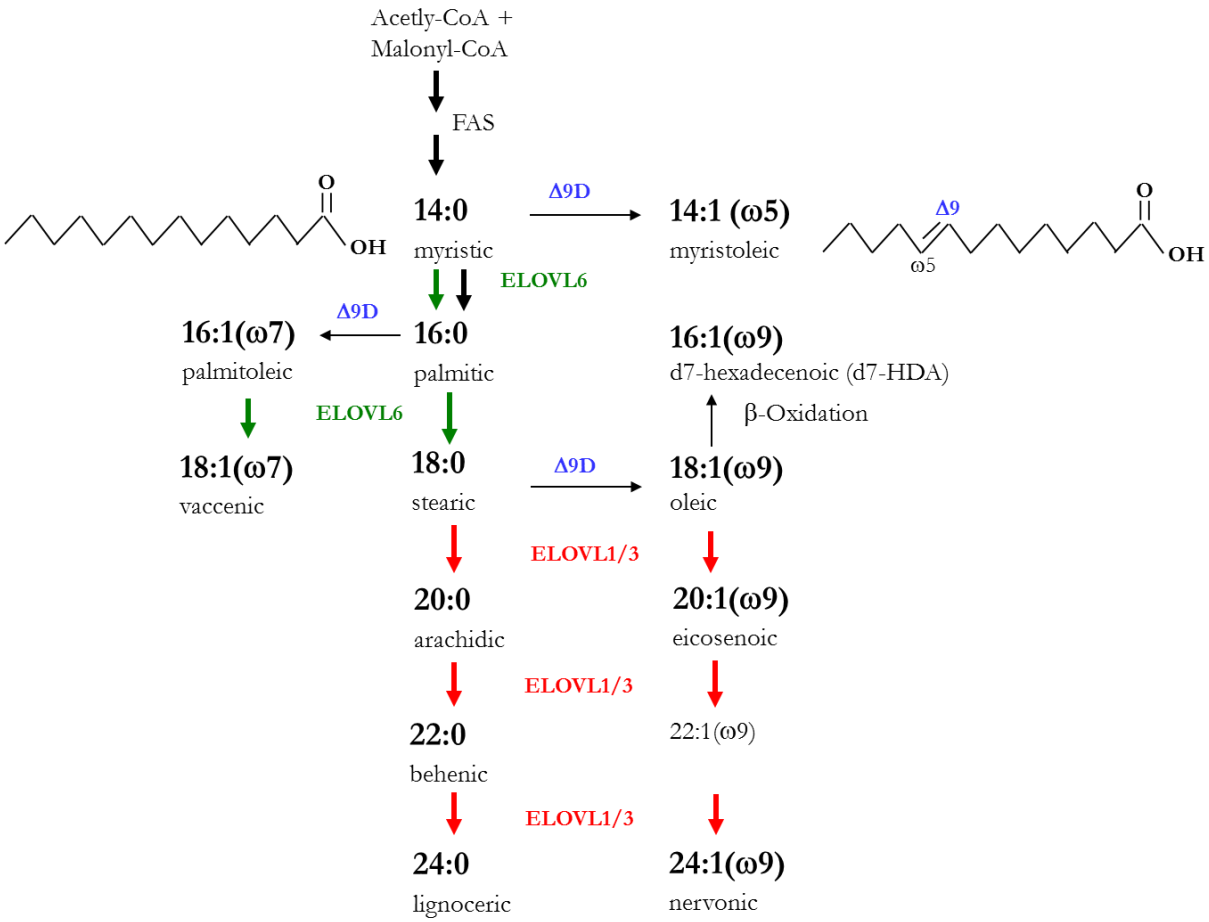

Figure 3. SFA and MUFA Pathway

SFA and MUFA are synthesized by chain-elongation, executed by ELOVL, and desaturation, performed by desaturases, from palmitic acid. Chemical structures are shown for myristic (C14:0) and myristoleic acid (C14:1). The desaturase Δ9D inserts a double-bond at the Δ9-carbon, which is the 9th C-atom counting from the carbonyl carbon toward the terminal methyl carbon. Myristoleic acid is a ω5 FA since it contains a double-bond at the 5th carbon-carbon bond. For simplicity reasons this double-bond is not shown as a cis-confirmation. Abbreviations are given in brackets and in Supplemental Table 3. Only FA which could be measured in the present study are depicted.

SFA and MUFA are synthesized from palmitic acid by chain-elongation and desaturation, which means that two carbon-atoms are added before the carbonyl carbon of the FA and that a double-bond is inserted in the chain, respectively. Several elongation-of-very-long-chain-FA (ELOVL) genes encoding distinct elongases (ELOVL1, ELOVL2, ELOVL3, ELOVL5, ELOVL6) have been identified in humans and are responsible for different FA conversions (Wang Y, 2006). So far, three desaturases have been found in humans, namely Δ5D, Δ6D, Δ9D which in-

sert double-bonds at the Δ5-, Δ6-, and Δ9-carbon, respectively (Cho HP, 1999; Cho HP, 1999; Sjögren P, 2008). The Δ9-carbon is the 9th carbon counting from the carbonyl carbon toward the terminal methyl carbon. For example, Δ9D convertes myristic acid (C14:0) into myristoleic acid by inserting a double-bond at the Δ9-carbon which is, in this case, also the 5th carbon-carbon bond, counting from the terminal methyl carbon toward the carbonyl carbon. Thus, myristoleic acid is a ω5-FA also denoted as C14:1.

Humans lack the ability to insert double-bonds beyond the Δ9-carbon. Hence, the ω6-FA linoleic acid (LA) and the ω3-FA α-linolenic acid (ALA) cannot be synthesized by the body. LA and ALA are essential FA and are precursors of a class of signaling molecules called eicosanoids of which some have pro- and others have anti-inflammatory properties (De Caterina R, 2001; Levin G, 2002; University of Kansas Medical Center, 2004). The chemical structure of LA and ALA and the pathways for the ω3-FA and ω6-FA are shown in Figure 4.

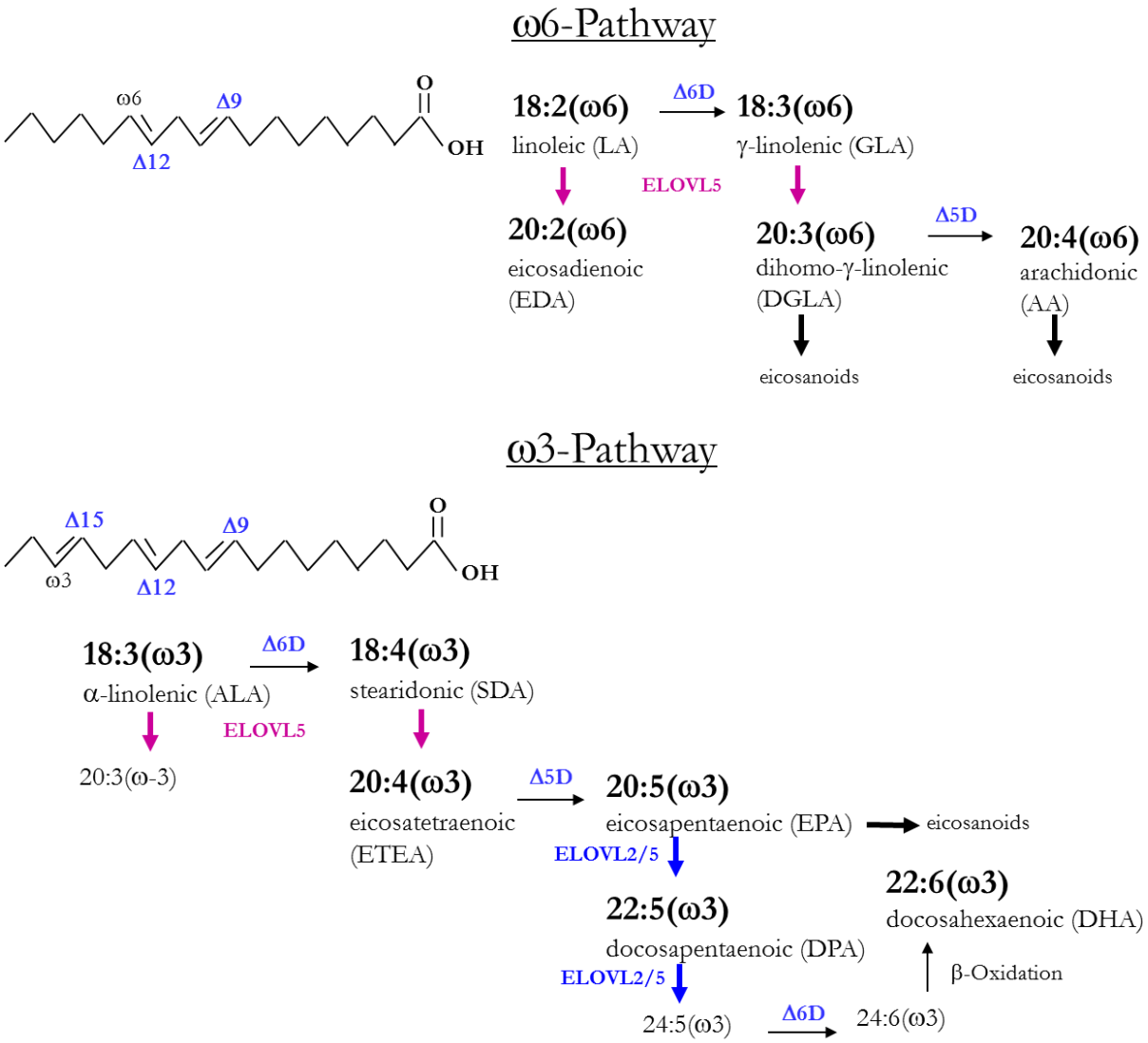

Figure 4. ω6-FA and ω3-FA Pathways

ω6-FA and ω3-FA are synthesized by chain-elongation, executed by ELOVL, and desaturation, performed by desaturases, from the essential FA LA and ALA, respectively. LA (18:2(ω6)) has 18 C-atoms and two double-bonds which are located on the 6th and 9th carbon-carbon bond, counting from the terminal methyl carbon toward the carbonyl carbon. The desaturase Δ5D inserts a double-bond at the Δ5-carbon, which is the 5th carbon counting from the carbonyl carbon toward the terminal methyl carbon. For simplicity reasons this double-bond is not shown as a cis-confirmation. Abbreviations are given in brackets and in Supplemental Table 3. Only FA which could be measured in the present study are depicted.

In the present study, 24 plasma FA were measured which are given in Supplemental Table 3. Their relative levels display a specific plasma FA pattern. Plasma FA patterns are altered early in the development of InsRes. Previous studies demonstrated that the relative levels of specific FA in plasma and tissues are associ-

ated with metabolic risk and that they might predict the development of metabolic disease. In a large prospective study, the plasma FA pattern measured at baseline of those subjects with MetSyn or T2D exhibited elevated proportions of SFA (myristic acid, palmitic acid) and MUFA (palmitoleic acid, oleic acid). From the ω6-FA LA was decreased whereas γ-linolenic acid (GLA), and dihomo-γ-linolenic acid (DGLA) were found to be increased in MetSyn and T2D (Vessby B, 1994; Warensjö E, 2005). No association of ω3-FA with an increased risk for T2D was found (Vessby B, 1994; Warensjö E, 2005). The association of higher palmitic acid and DGLA, and lower proportions of LA with the development of IGT or T2D was also found in other prospective (Laaksonen DE, 2002; Wang L, 2003) and cross-sectional (Pelikanova T, 2001; Salomaa V, 1990; Vessby B, 1994; Kotronen A, 2009) studies, in which various plasma compartments including cholesterol esters and phospholipids (PL) were investigated. The mechanisms underlying these alterations in plasma FA profiles are incompletely understood. Dietary fat amount and composition (see Nutritional Aspects of Cardiovascular Risk Factors), FA *de novo* synthesis and conversion by FA desaturases are all likely to play a role, whereas a potential role of FA elongases is not well understood (Vessby B, 2003). An important mechanistic role is played by the transcription factor SREBP1c which insulin-dependently stimulates *de novo* lipogenesis and induces at least some FA desaturases and elongases (Hegarty BD, 2005; Wang Y, 2006; Qin Y, 2009). In summary, consistent changes in FA patterns are observed in subjects with InsRes, indicating that FA species are risk markers and potentially also causal factors of this pathology.

Phospholipids

Lipids are a class of molecules, including for example cholesterol, cholesterol esters, PL, sphingolipids, and TAG, with a wide diversity in structure and biological function. They serve as energy sources, as structural components of cellular membranes and as first and second messengers in signal transduction and molecular

recognition processes (Van Meer G, 2008). PL and cholesterol are essential components of cell membranes (Dowhan W, 2008) and the latter is also a precursor for steroid hormones and bile acids. The diversity of lipids is extended by the head groups of PL. Phosphatidylcholine (PC), a major PL, and its derivate lysophosphatidylcholine (LPC) incorporate choline as a head group and are important components of cellular membranes (Van Meer G, 2008). PC and LPC species show a wide diversity by containing different FA. Each LPC comprises one and each PC comprises two distinct FA species (Van Meer G, 2008). TAG function mainly as energy sources stored in adipose tissue and can be mobilized for degradation and energy production in other tissues such as muscle cells. Due to their hydrophobic character, lipids are transported in plasma and other body fluids by water-soluble complexes called lipoproteins (Fielding CJ, 2008).

Lipoproteins

Lipoproteins are composed of lipids and proteins, most importantly apolipoproteins (apo) which assure that the lipoprotein is water soluble and are responsible for several metabolic functions (Fielding CJ, 2008). Lipoproteins are usually classified according to their density, namely from the lowest to the highest density: chylomicrons, very low density lipoproteins (VLDL), chylomicron remnants, intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) which is further divided into HDL2 and HDL3 (Havel RJ, 2001).

1.3 Postprandial Metabolism

In the present study, postprandial lipid metabolism is analyzed as well as its alterations occurring during the development of InsRes. An overview of the postprandial lipid metabolism is illustrated in Figure 5.

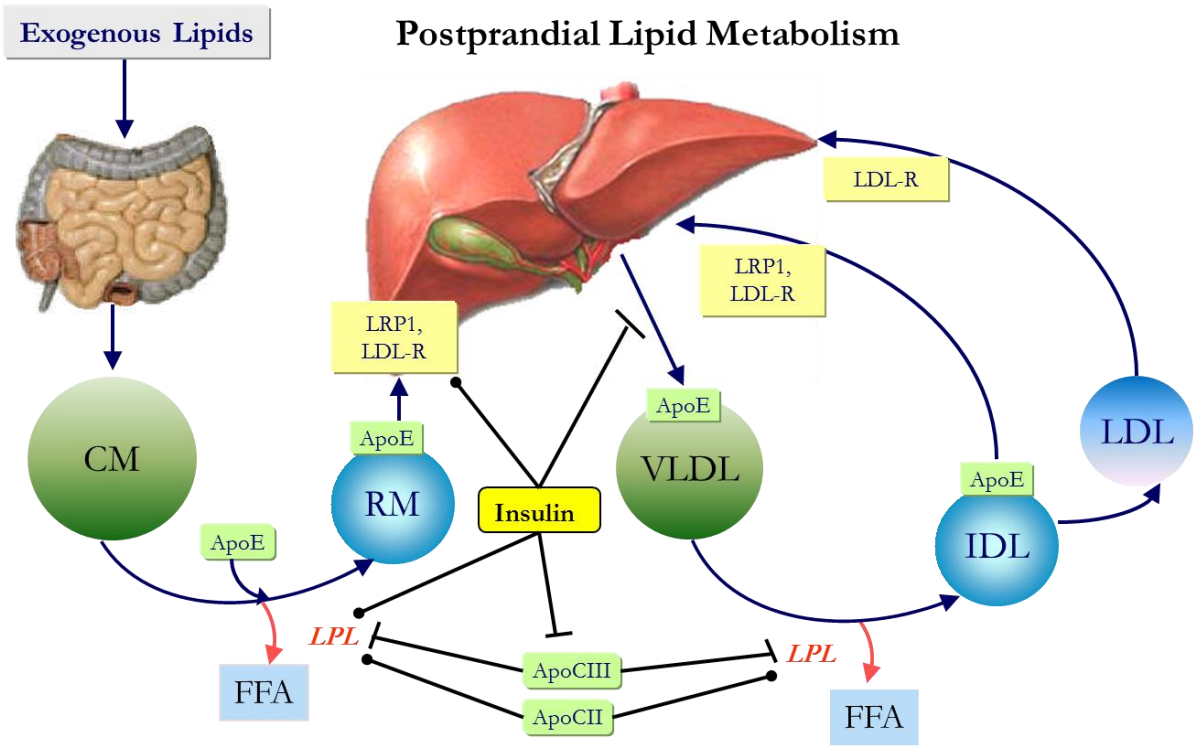

Figure 5. Postprandial Lipid Metabolism

Chylomicrons (CM) released by the intestine and VLDL secreted by the liver belong to the class of TAG-rich lipoproteins (TRL). Lipoprotein lipase (LPL) hydrolyzes CM and VLDL and thereby releases FFA and chylomicron remnants (RM) and IDL particles, respectively. RM and IDL are taken up by the liver via the interaction of apoE with LDL-receptor (LDL-R) and LDL-R-related protein 1 (LRP1). LPL is regulated by apoCII and apoCIII. Insulin is an important regulator of LPL, apoCII and apoCIII balance, VLDL release and RM uptake by the liver. Lines with a crossbar symbolize inhibition and lines with a bullet point symbolize activation.

Lipids from a consumed meal are degraded and absorbed as FA by the small intestine. Intracellular they are re-synthesized to TAG which are inserted into chylomicrons together with PL, cholesterol, cholesterol esters and apoliporoteins and then released into the plasma. TAG are mainly transported by chylomicrons and VLDL which are two classes of TAG-rich lipoproteins (TRL) (Heeren J, 2001). In contrast to chylomicrons which are secreted from the intestine and include apoB48 and apoA isoforms (apoAI, apoAII, and apoAIV), VLDL is secreted from the liver and contains apoB100 (Fielding CJ, 2008; Havel RJ, 2001). Components of HDL are secreted with chylomicrons and VLDL, as well as independently as HDL precursors (Havel RJ, 2001). HDL particles circulating in the plasma donate apoC in exchange for apoA to the newly released chylomicrons and VLDL. ApoCII activates

and apoCIII inhibits the lipoprotein lipase (LPL), an enzyme catalyzing the hydrolysis of TRL thereby forming chylomicron remnants and IDL and releasing FFA. During the formation of chylomicron remnants and IDL, HDL exchanges apoCII for apoE with the partially lipolyzed chylomicrons and VLDL particles (Fielding CJ, 2008). The chylomicron remnants and some VLDL remnants, namely IDL, return to the liver by binding with its apoE to the LDL-R and LRP1 (Heeren J, 2001). The liver packs several lipids and proteins into VLDL which is secreted again. The second half of IDL is further lipolyzed to LDL by the hepatic lipase (Havel RJ, 2001). During this process, apoE is released and apoB100 is now exposed. LDL delivers its cholesterol esters to the liver and to extra-hepatic tissue. It is endocytozed and absorbed by these cells and lysosomal acidic lipases hydrolyze cholesterol ester to cholesterol. The latter is available for the steroid hormone and bile acid synthesis and the integration in membranes. HDL is not only responsible for the delivery of apoC and apoE to chylomicrons and VLDL but also for the delivery of cholesterol and cholesterol esters to the liver and to steroid hormone producing glands, as part of the reverse cholesterol transport (Fielding CJ, 2008; De Grooth GJ, 2004). HDL and VLDL also exchange TAG and cholesterol esters mediated via the action of cholesterol ester transfer protein (De Grooth GJ, 2004). Insulin plays an important role in the postprandial lipid metabolism. Insulin regulates LPL either directly (Ginsberg HN, 2005) or indirectly by down-regulating apoCIII gene-expression (Waterworth DM, 2000) and balancing the ratio of apoCII and apoCIII (Ginsberg HN, 2005). Insulin can also activate LDL-R gene expression (Streicher R, 1996).

Insulin resistant and obese states are frequently accompanied by so-called atherogenic dyslipidemia which is characterized by elevated levels of TAG, low levels of HDL cholesterol (HDL-C) and changes in the composition of LDL particles (Howard BV, 1999). Higher TAG and lower HDL-C levels and the presence of small dense LDL particles are risk factors for CVD (Branchi A, 2006; Nakatsuji H, 2009). It is believed that central obesity and hyperinsulinemia lead to increased se-

cretion of VLDL especially in postprandial states (Howard BV, 1999; Ginsberg HN, 2005). Additionally, LPL is less efficiently regulated in insulin resistant states and further inactivated by a shift of the balance of apoCII to apoCIII which is usually regulated by insulin (Ginsberg HN, 2005). This results in a slowed lipoprotein catabolism and a decreased clearance of TRL. This slowed clearance and the fact that chylomicrons and VLDL are enriched with TAG lead to an increased exchange of cholesterol esters and TAG between VLDL and HDL. These TAG-rich HDL are cleared much faster, whereas the smaller VLDL particles are converted to smaller IDL and further to so-called small dense LDL particles. In the end, plasma contains more TRL, fewer HDL particles, and more small dense LDL particles (Howard BV, 1999). In addition to insulin as an important regulator of the postprandial TAG levels and chylomicron clearance, the long-term FA composition of the diet and especially its ω3-FA content may also play a role (Brown AJ, 1991; Harris WS, 1988; Park Y, 2003).

1.4 Pro-inflammatory Proteins and their Role in Insulin Resistance

In addition to the alterations in plasma FA, lipids and lipoproteins, serum concentrations of pro-inflammatory proteins are increased already before the onset of T2D and CVD, indicating chronic subclinical inflammation. One of these pro-inflammatory proteins is plasminogen activator inhibitor-1 (PAI1), the predominant inhibitor of the fibrinolytic system (Figure 6). The fibrinolytic system is an enzymatic system which can dissolve blood clots. It comprises an inactive pro-enzyme called plasminogen which can be converted to the active enzyme plasmin which degrades fibrin into soluble fibrin-degraded products. Two immunologically different types of physiological plasminogen activators are known: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). t-PA activates plasminogen in the circulation, whereas u-PA is responsible for the activation of cell-bound plasminogen. t-PA and u-PA can both be inactivated by PAI1 (Lijnen HR, 2005). PAI1 exists in two confirmations as an active and latent form. Active PAI1 (aPAI1) is secreted from the synthesizing cells and converts

spontaneously to a latent confirmation which can be partially re-activated (Lijnen HR, 2005).

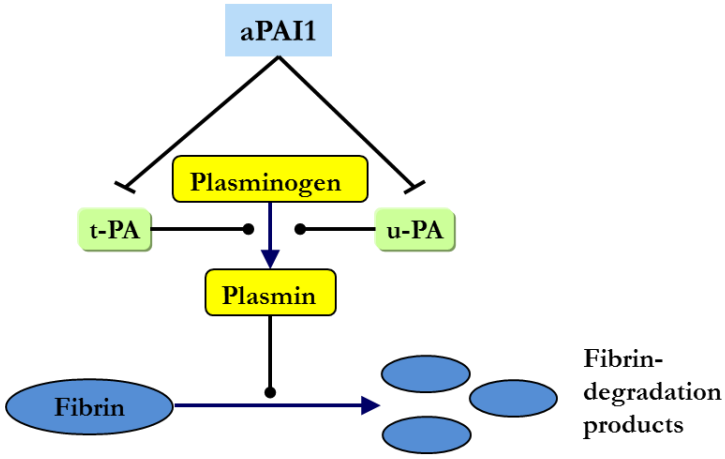

Figure 6. Simplified Fibrinolysis

During the fibrinolysis, t-PA and u-PA convert the inactive enzyme plasminogen to the active enzyme plasmin. Plasmin degrades fibrin to fibrin-degradation products. t-PA and u-PA can both be inactivated by aPAI1.

Elevated plasma levels of PAI1 result in hypofibrinolysis, a state in which removal of thrombi from the vascular system is impaired. High levels of plasma PAI1 are associated with obesity, hypertriacylglyceridemia (Folsom AR, 2001; Kraja AT, 2007), hypertension (Kraja AT, 2007; Poli KA, 2000) and InsRes (Festa A, 2003). In large prospective studies, elevated plasma PAI1 concentrations have been identified as predictors for T2D (Festa A, 2002) and CVD (Poli KA, 2000; Folsom AR, 2001). Furthermore, PAI1 activity was higher in normal glucose tolerant offspring of T2D patients (Gürlek A, 2000). Several factors were suggested to influence PAI1 levels and its activity either directly or indirectly such as TNFα, unsaturated FFA (Dellas C, 2005), VLDL (Stiko-Rahm A, 1990), and leptin (Singh P, 2010), whereas the role of insulin is still a matter of debate (McCarty MF, 2005).

1.5 Nutritional Aspects of Cardiovascular Risk Factors

As mentioned earlier, the diet composition is supposed to have an impact on the risk for T2D and CVD. Currently, ω3-FA are in the special focus. However, the results regarding a possible effect of ω3-FA on risk factors are conflicting. The

beneficial health effects of high seafood diets containing a lot of ω3-FA were first reported in Greenland Eskimos in the 1960s and 1970s (Sagild U, 1966; Kromann N, 1980). Since then they have been extensively studied in connection with several diseases (Simopoulos AP, 2008; Vrablík M, 2009). Although various expert societies including the American Heart Association (Kris-Etherton PM, 2003), American Diabetes Association (Buse JB, 2007) and nine European societies (Graham I, 2007) recommended increases in the consumption of fish and vegetables containing ω3-FA as one part for the prevention of CVD, the results of supplementation studies in healthy individuals and T2D patients have been controversial (Friedberg CE, 1998; Hartweg J, 2009).

More precisely, ω3-FA treatment had no influence on fasting glucose and fasting insulin (Hartweg J, 2009) in some studies, whereas in others it even had a negative impact on fasting glucose levels (Friedberg CE, 1998) and glucose tolerance (Giacco R, 2007).

Moreover, whereas ω3-FA supplementation led to reduced fasting and postprandial TAG levels, its effect on LDL-C and HDL-C is debatable (Hartweg J, 2009; Harris WS, 1989).

Long-term supplementation of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) had no significant effect on PAI1 levels or activity in subjects having moderate hyperlipidemia (Finnegan YE, 2003), T2D and hypertension (Woodman RJ, 2003), whereas it led to increased PAI1 activity in healthy individuals (Moller JM, 1992). Since PAI1 levels decrease during the day and especially after a meal, postprandial PAI1 was extensively studied and reported to be slightly higher after acute ingestion of ω3-FA in men with MetSyn (Montegaard C, 2010) but not in healthy (Pacheco YM, 2006).

Taken together, the potential benefits of ω3-FAs in CVD risk reduction in healthy subjects and T2D patients remain inconclusive.

Test meals have extensively been used to examine postprandial lipid and PAI1 responses in relation to InsRes and risk of CVD. The test meals used in published studies vary considerably in their amount of fat, carbohydrates and proteins. Mixed meals containing carbohydrates, as the one described in the present study, are chosen when the insulin effect on the postprandial lipid metabolism is of interest. The presence of carbohydrate in a high-fat meal invoked an increased insulin response that significantly reduced and delayed postprandial TAG levels in healthy subjects (Westphal S, 2002; Kriketos AD, 2003) but failed to do so in InsRes (Kriketos A, 2005). The metabolic effects of a sole glucose load as assessed during an oral glucose tolerance test (OGTT) on lipids have so far not been studied extensively (Ogita K, 2008; Nakatsuji H, 2009).

2 Materials and Methods

2.1 Study Population

92 apparently healthy non-smoking subjects with one or two parents with T2D (T2D offspring) were recruited using a patients' database from the Hospital Bethanien, Hamburg, Germany, and newspaper advertisements. Subjects were admitted to the Hospital Bethanien. The exclusion criteria for the participants were as follows: 1) diabetes, 2) cancer, 3) renal disease, 4) previously experienced stroke or heart attack, 5) intake of glucocorticoids, 6) intake of anti-hypertensive or lipid-lowering medication, 7) pregnancy, and 8) age < 18 or > 70 years**.** One subject with fasting TAG concentration > 400mg/dl was excluded, resulting in 91 individuals for analyses. 52.7% of the subjects had a T2D father, 40.7% a T2D mother and 6.6% had two T2D parents.

The study protocol was approved by the local ethics committee and all participants gave written informed consent. At the time of enrollment, a short interview based food questionnaire was conducted. In addition, they underwent a medical examination conducted by Ronja Müller and Dr. Oliver Bargheer during which their resting blood pressure (diastolic, DBP, and systolic, SBP) was measured. Body mass index (BMI) was calculated as

$$\frac{weight\ [kg]}{height^2\ [m^2]}$$

and waist-to-hip ratio (WHR) by

$$\frac{waist\ circumference\ [cm]}{hip\ circumference [cm]}.$$

Blood samples were drawn by Ronja Müller and Dr. Oliver Bargheer in the morning after overnight fast and 2h after a subsequent 75g oral glucose load (300 ml Dextro ® O.G.-T., Roche Diagnostics) as an OGTT.

Overweight was defined as BMI higher than 25 kg/m² (National Cholesterol Education Program (NCEP) Expert Panel on Detection, 2002). Extreme obesity was defined as BMI ≥ 40kg/m². Definitions for the diagnosis of T2D and pre-

diabetic states with impaired glucose regulation are given in Table 1. MetSyn cut-offs were used as defined recently (Table 2), using waist circumference (waist) criteria recommended for Europeans (Alberti KG, 2009).

Category	0h-Glucose	2h-Glucose
Normal	< 100mg/dl	< 140 mg/dl
IFG	100-125 mg/dl	-
IGT	-	140-199mg/dl
T2D	≥ 126mg/dl	≥ 200mg/dl

Table 1. Criteria for Clinical Diagnosis of T2D, IFG, and IGT

Criteria for clinical diagnosis of T2D, impaired fasting glucose (IFG), and IGT as defined by (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Glucose needs to be measured in the fasting state (0h) and 2h into a standardized OGTT.

Measure	Categorial Cut Points	
	Men and Women	
elevated blood pressure	SBP ≥ 130 and/or DBP ≥ 85mmHg	
elevated fasting glucose	≥ 100 mg/dl	
elevated fasting TAG	≥ 150 mg/dl	
	Men	Women
elevated waist	≥ 102cm	≥ 88cm
reduced fasting HDL-C	< 40mg/dl	< 50mg/dl

Table 2. Criteria for Clinical Diagnosis of MetSyn

Criteria for clinical diagnosis of MetSyn as defined by (Alberti KG, 2009) using waist as recommended for Europeans. Patients meeting any three or more of these criteria are diagnosed with MetSyn. Medication influencing any of these parameters are also considered as criteria but were not relevant here since they were exclusion criteria.

A subgroup of the study (n=39, 24 females, 15 males) were studied again at a second visit. After an overnight fast, they had the choice between three fat-rich breakfasts (Supplemental Table 1) as an oral fat tolerance test (OFTT) with different protein sources (cheese with sausage or egg), matched for calories (1100 kcal), protein (12.2-13.3 % of total energy), carbohydrate (39.6-40.4 % of total energy), fat (46.6-47.9 % of total energy), FA composition (Supplemental Table 2) and glycemic load (60.5-62.0 per serving) (Foster-Powell K, 2002). Blood samples were taken by Ronja Müller before, two hours and four hours after the test meal. The postprandial results represented here were independent of meal choice.

2.2 Analysis of Insulin Resistance Parameters

Plasma glucose was determined by the hexokinase method using a clinical analyzer (Roche Diagnostics). Serum insulin was determined by chemiluminescence immunoassay on an Immulite Analyzer (DPC-Biermann). Plasma glucose and insulin were both measured in the department of clinical chemistry with fasting reference domains of 60-110mg/dl and 6 -27 µU/l, respectively. HOMA-IR was calculated using fasting glucose and insulin with the formula:

$$HOMA_{IR} = \frac{glucose\ \left[\frac{\mathrm{mg}}{\mathrm{dl}}\right] * insulin\left[\frac{\mu\mathrm{U}}{\mathrm{ml}}\right]}{405}$$

The cut-off value of 1.77 for HOMA-IR, as defined for subjects with MetSyn (Esteghamati A, 2010), was also used in the present study. HOMA-%B was calculated using fasting glucose and insulin with the formula (Matthews DR, 1985):

$$HOMA_{\%B} = \frac{20 * insulin\ \left[\frac{\mu\mathrm{U}}{\mathrm{ml}}\right]}{0.0555 * \mathrm{glucose}\ \left[\frac{\mathrm{mg}}{\mathrm{dl}}\right] - 3.5}$$

2.3 Analysis of Plasma Fatty Acids

FA methyl esters for gas chromatography were prepared by Ralf Uken under the supervision of Dr. Klaus Tödter from total plasma and from TAG fractions isolated from total plasma based on the method of Lepage and Roy (Lepage G, 1986), with modifications, as described previously (Scheja L, 2008). Analyses were performed by Ralf Uken under the supervision of Dr. Klaus Tödter using an HP 5890 gas chromatograph (Hewlett Packard) equipped with flame ionization detectors (Stationary phase: DB-225 30 m x 0.25 mm id., film thickness 0.25 µm; Agilent, Böblingen, Germany). Peak identification and quantification was performed by comparing retention times and peak areas, respectively, to standard chromatograms. All FA which could be determined including their abbreviations are listed in (Supplemental Table 3).

2.4 Analysis of Plasma Phospholipid Species

PC and LPC species were analyzed by Dr. Marian K. Mosior and Dr. David A. Peake of the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, USA, in organic plasma extracts by a flow injection electrospray ionization and precursor ion tandem mass spectrometry (ESI MS-MS) method, adapted from the work of Liebisch et al. (Liebisch G, 2004). The flow injection MS/MS system consisted of a Quattro Ultima triple quadrupole mass spectrometer and MassLynx 4.1 software (Waters, Milford, MA) interfaced to a 1100 binary HPLC (Agilent Technologies, Palo Alto, CA) and CTC PAL autosampler (LEAP Technologies, Carrboro, NC) equipped with active wash station.

Protonated molecular ions of phosphatidyl species were selected by two separate precursor ion scans of m/z 184. The ion intensities across the flow injection profile were summed together, and after isotope correction, the concentrations of each lipid species were then calculated relative to PC and LPC internal standards (m/z 450–650), using response factors obtained from serial dilutions of equimolar mixtures containing 12 different PC and 8 LPC synthetic standards (Avanti Polar Lipids, Alabaster, AL).

Plasma samples (10µl) were spiked with 2.5nmole each of LPC-15:0, PC-14:0 and PC-21:0 internal standards prior to extraction. Sample aliquots were diluted into a total of 250µl of water, containing 25µl 5N HCl, and 750µl of 2:1 methanol/chloroform. After mixing thoroughly, 250µl of water and 250µl of chloroform were added. The samples were then mixed again and centrifuged (4000 rpm, 5°C) for 10 min. The lower chloroform layer was removed, evaporated under a stream of warm nitrogen, and reconstituted in 0.50ml of 75% methanol, 25% chloroform (v/v), 10 mM ammonium acetate. For both PC and LPC measurements 10µl of plasma extract was analyzed in triplicate.

2.5 Analysis of Lipoproteins

Concentrations of plasma total TAG, total cholesterol (TC) and HDL-C, which was only used for clinical diagnosis of MetSyn, were determined by Anette Rosche using commercial kits (Roche/Hitachi). Direct measurements of plasma concentrations of TRL (comprising VLDL and chylomicrons), remnants (comprising IDL and remnants, RM), LDL, HDL2, and HDL3 were performed by Ronja Müller and Bülent Polat using fast-performance liquid chromatography (FPLC). For lipoprotein fractionation, 200 µl pooled plasma samples were separated by FPLC on a Superose 6 column (Amersham-Biosciences). 40 Fractions of 0.5 ml were collected and assayed for total TAG and TC, as described above. Concentrations of HDL2-C, HDL3-C (summed up as HDL-C), LDL-C, RM-C, and TRL-C resulted from the sum of the cholesterol levels of the corresponding fractions. TAG levels in the lipoprotein fractions were determined accordingly. The added total concentrations of the lipoproteins were then multiplied by 2.5 to yield the concentration relating to the whole plasma.

Concentrations of the apolipoproteins, apoAI, apoAII, apoB, apoCII, apoCIII, and apoE were determined using Lincoplex kits by Linco Diagnostics Inc. (Millipore).

2.6 Analysis of Pro-Inflammatory Parameters

Plasma levels of adiponectin, leptin, resistin, total PAI1 (tPAI1) and aPAI1, IL6, TNFα, MCP1, were determined using Lincoplex kits by Linco Diagnostics Inc. (Millipore). Reference values are not available since these parameters were not measured for diagnostics but for research only. Plasma concentrations of asymmetric dimethylarginin (ADMA) were measured by Dr. Edzard Schwedhelm of the department of experimental and clinical pharmacology using LC-MS/MS.

2.7 Statistical Analyses

In order to account for total lipid content of plasma, FA, PC and LPC values were normalized to total FA, PC or LPC concentration, respectively. Since it is rather complicated to measure the activities of hepatic enzymes like desaturases and elongases in vivo in humans, they are usually estimated as product/substrate ratios (Eva Warensjö, 2006; Pan DA, 1995). Therefore, estimated activities of ELOVL5 were calculated as the respective product/substrate ratios (DGLA/GLA, eicosadienoic acid (EDA)/LA).

Most parameters were not normally distributed but left-skewed. This is a common phenomenon if concentrations are measured. To receive approximately normal distributions, all data were log-transformed for correlation and factor analysis. In group comparisons, un-transformed data were used to simplify comparisons with reported reference ranges and medical interpretation.

Group Comparisons

Differences between women and men were assessed by Wilcoxon test since this can be used to compare skewed data. Median, minimum and maximum values could then be related to reported reference ranges for both sexes. When mean values and the standard error of the mean (SEM) were of interest and should be plotted, differences between two mean values were assessed by paired or unpaired t-test. Paired t-tests have a greater power than unpaired t-tests. Paired t-test are applied for example if parameters were measured before and after a treatment in one group. Consequently, differences between two time-points were assessed by paired t-test.

Pearson's Correlation Analysis

Pearson's correlation analyses can detect linear relationships between two normally distributed variables. Associations of FA, TAG, physical and biochemical variables with parameters associated with InsRes and MetSyn were explored by applying Pearson's correlations. Sex-, age- and waist-adjusted correlations were computed to

test if the detected relationships were independent of sex, age and obesity as assessed by waist.

Factor Analysis

The factor analysis aims at describing the variability among observed variables in terms of a potentially lower number of un-observed variables called factors. Thus, the factor analysis searches for joint variations of variables correlating strongly with each other possibly due to a common underlying regulatory mechanism or in response to un-observed latent variables. The gained information about the interdependencies between observed variables can later be used to reduce the number of variables in a dataset or to search for common underlying regulatory mechanisms.

As the first part of the factor analysis, a principal component analysis (PCA) was conducted to search for underlying patterns which explain most of the variance of the entire dataset. By this means, it is determined whether the different parameters studied can be replaced by a smaller number of underlying patterns or un-observed variables. For a better interpretation, principal components were rotated by Varimax rotation to yield factors similar to the directly measured variables. Missing values were replaced by means. Factor loadings (pattern) are coefficients of the factor and reveal the degree to which a given variable is represented in a particular factor. A factor loading > 0.50 was considered relevant. The sum of all squared factor loadings of the un-rotated solution (PCA) gives the eigenvalue of this factor. Thereby, the eigenvalue displays the amount of the total variance explained by this factor. Only factors having an eigenvalue higher than one were chosen for further analysis since it is believed that only these factors explain a significant amount of the total variance. Extracted factors can later be used to reduce the number of dimensions (variables) of a dataset.

Here, the factor analysis was used to identify the FA moieties in PC and LPC species. PC and LPC species loading on a factor together with one or several total plasma FA were supposed to contain the same FA moieties. A similar factor analysis was performed to reveal insulin resistant parameters and FA which changed in

line with postprandial aPAI1. This time, missing values were deleted pairwise. It is assumed that all parameters loading on one factor might be regulated by the same underlying mechanism.

Linear Regression Analysis

A linear regression approaches the relationship between a dependent variable and one or more independent variables. However, in contrast to a correlation analysis which assesses un-directed relationships, the direction of the relationship is of importance in a regression analysis. Thus, a linear regression analysis can be applied to quantify the strength of the linear relationship between the dependent (response) variable and one or several independent (predictor) variables and to assess which independent variable may have no relationship with the dependent variable at all. A multiple stepwise regression starts with a model containing no variables. Variables are tested one by one and are included if they are statistically significant. Every time a new variable is added, the method tests if some variables can be deleted without worsening the model. This procedure is continued until all significant variables are part of the model. A linear multiple stepwise regression was performed with the purpose to detect those insulin resistant parameters and FA which explained most of the variance of the postprandial change of aPAI. Missing values were excluded pairwise.

Software

The factor analyses and the linear regression were performed using SPSS 17.0 and 18.0 software (SPSS, Chicago, IL) whereas all other statistical analyses were conducted using the programming language R, version 2.8.0 and 2.11.0.

3 Results

3.1 Metabolic Characterization of the Study Population

The participants of the study were invited after an overnight fast. Plasma samples were collected and used to determine hormones, lipids, adipokines and inflammation markers for the metabolic characterization. The cohort characteristics including comparisons between women and men are shown in Table 3 and Table 4. Normal values for both sexes are also presented if they were available.

The first part of the table displays the anthropometric measurements. Of the 91 individuals 35 (38%) were overweight but no one was extremely obese. With 50% of the men but only 33% of the women being overweight, relatively more men than women were affected. The second section of the table presents parameters associated with MetSyn. In the study population, 11% of the women and 20% of the men met three or more of the criteria for the diagnosis of MetSyn. Parameters associated with InsRes are shown in the third section of Table 3.

Comparing men and women, established gender differences were confirmed in plasma levels of HDL-C (Alberti KG, 2009) (Table 3), adiponectin, and leptin (Cnop M, 2003; Plaisance EP, 2009) (Table 4), which were all higher in women, and anthropometric measures, namely lower abdominal obesity parameters in women (Alberti KG, 2009). Men also were more InsRes as indicated by higher HOMA-IR, and had higher SBP and TNFα levels (Table 3 and Table 4, respectively). Despite these gender differences, the correlation results were in most cases independent of sex (see below). Therefore, for further analyses women and men were combined.

Anthropometric parameters						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
Age [years]	43 (18 – 70)	47 (18 – 70)		38 (20 – 54)		0.161
BMI [kg/m²]	23.9 (18.3 – 35.2)	23.2 (18.3 – 35.2)	< 25 ‡	25.0 (20.0 – 33.4)	< 25 ‡	0.081
Waist [cm]	**84 (60 – 118)**	**77 (60 – 112)**	**< 88 #**	**94.3 (72 – 118)**	**< 102 #**	**<0.001**
WHR	**0.84 (0.67 – 1.11)**	**0.81 (0.67 – 0.97)**		**0.93 (0.84 – 1.11)**		**<0.001**
Overweight subjects (BMI≥ 25)	n=35 (38%)	n=20 (33%)		n=15 (50%)		

MetSyn associated parameters						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
Waist [cm]	**84 (60 – 118)**	**77 (60 – 112)**	**< 88 #**	**94.3 (72 – 118)**	**< 102 #**	**<0.001**
Glucose [mg/dl]	**95 (71 – 122)**	**91 (71 – 118)**	**< 100 #**	**103 (85 – 122)**	**< 100 #**	**<0.001**
TAG [mg/dl]	81 (23 – 321)	78 (35 – 248)	< 150 #	90 (23 – 321)	<150 #	0.285
HDL-C [mg/dl] *	**53.0 (22.0 – 93.0)**	**58.0 (36.0 – 93.0)**	**> 50 #**	**44.5 (22.0 – 69.0)**	**> 40 #**	**<0.001**
SBP[mmHg]	**126 (100 – 208)**	**124 (100 – 208)**	**< 130 #**	**131 (115 – 164)**	**< 130 #**	**0.020**
DBP [mmHg]	78 (54 – 108)	78 (54 – 108)	< 85 #	77 (58 – 100)	< 85 #	0.631
MetSyn #	n=13 (14%)	n=7 (11%)		n=6 (20%)		

InsRes associated parameters						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
Glucose [mg/dl]	**95 (71 – 122)**	**91 (71 – 118)**	**< 100 †**	**103 (85 – 122)**	**< 100 †**	**<0.001**
Insulin [µU/ml]	8.00 (3.10 – 27.5)	7.70 (3.10 – 27.5)	< 27 $	9.15 (3.50 – 22.5)	< 27 $	0.164
HOMA-IR	**1.94 (0.65 – 7.60)**	**1.72 (0.65 – 7.60)**	**< 1.77 ¥**	**2.21 (0.73 – 6.56)**	**< 1.77 ¥**	**0.019**
HOMA-%B	92.3 (35.4 – 486)	99.3 (35.4 – 486)		79.5 (37.2 – 176)		0.113

Table 3. Characteristics of the Study Population (Part I)

Data are presented as median (min – max). P-values were calculated using Wilcoxon-test to compare men vs. women. Significant differences ($p<0.05$) are shown in boldface. All values were measured in the fasting state. * HDL-C measured for clinical diagnostic of MetSyn. † (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003), # (Alberti KG, 2009), ‡ (National Cholesterol Education Program (NCEP) Expert Panel on Detection, 2002), $ (Clinical Chemistry, 2010), ¥ (Esteghamati A, 2010).

Lipids and lipoproteins						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
TAG [mg/dl]	81 (23 – 321)	78 (35 – 248)	< 150 #	90 (23 – 321)	< 150 #	0.285
TC [mg/dl]	181 (106 – 276)	185 (135 – 276)	< 200 ‡	173 (106 – 249)	< 200 ‡	0.052
LDL-C [mg/dl]	102 (48.7 – 185)	101 (66.3 – 185)	< 130 ‡	103 (48.7 – 158)	< 130 ‡	0.672
HDL-C [mg/dl]	**64.8 (26.1 – 101)**	**70.3 (38.4 – 101)**	**> 50 #**	**54.1 (26.1 – 83.6)**	**> 40 #**	**<0.001**

Hormones						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
Adiponectin [ng/ml]	**14936 (3346 – 47715)**	**20005 (5891 – 47715)**	$	**10252 (3346 – 26279)**	$	**<0.001**
Leptin [pg/ml]	**8287 (550 – 63372)**	**12658 (1853 – 63372)**	$	**4288 (550 – 14269)**	$	**<0.001**

Inflammation markers						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
tPAI1 [pg/ml]	10776 (2776 – 35786)	9911 (2776 – 32129)	$	13065 (5101 – 35786)	$	0.094
IL6 [pg/ml]	1.86 (0.55 – 27.45)	1.65 (0.55 – 17.51)	$	3.04 (0.58 – 27.45)	$	0.082
TNFα [pg/ml]	**1.73 (0.60 – 4.92)**	**1.67 (0.60 – 3.95)**	$	**1.88 (0.60 – 4.92)**	$	**0.033**
MCP1 [pg/ml]	114.1 (30.9 – 287.4)	112.5 (30.9 – 287.4)	$	124.8 (70.5 – 278.1)	$	0.219

Table 4. Characteristics of the Study Population (Part II)

Data are presented as median (min – max). P-values were calculated using Wilcoxon-test to compare men vs. women. Significant differences ($p<0.05$) are shown in boldface. All values were measured in the fasting state. # (Alberti KG, 2009), ‡ (National Cholesterol Education Program (NCEP) Expert Panel on Detection, 2002), $ parameters were not determined for clinical diagnosis but for research purposes only. Hence, normal and reference values do not exist or are not given.

In order to further characterize the cohort, the parameters listed in Table 3 and Table 4 were correlated with surrogate markers of obesity (BMI, waist), InsRes (HOMA-IR) and insulin secretion (HOMA-%B) (Matthews DR, 1985) (Table 5). BMI, waist and HOMA-IR showed strong association with each other, with parameters of the MetSyn, adipokines and inflammatory markers, most notably TNFα and tPAI1. HOMA-%B correlated strongly with markers for InsRes and leptin, however, not with measures of abdominal obesity. HOMA-%B also correlated with TAG and IL6 but not with the other lipids and inflammatory markers. All correlations were independent of age and apart from three exceptions also independent of sex. In many cases, significance did not change after the adjustment for waist. Taken together, the T2D offspring cohort has a high percentage of individuals with IFG and exhibits the well-established association of InsRes, obesity, low-grade inflammation and plasma dyslipidemia.

	BMI	Waist	HOMA-IR	HOMA-%B
BMI		**0.82 (p<0.0001)** [s]	**0.58 (p<0.0001)** [s,w]	**0.29 (p=0.0054)** [s,w]
Waist	**0.82 (p<0.0001)** [s]		**0.54 (p<0.0001)** [s]	0.11 (p=0.30)
WHR	**0.56 (p<0.0001)** [s,w]	**0.87 (p<0.0001)** [s]	**0.41 (p<0.0001)** [s]	-0.03 (p=0.79)
SBP	**0.37 (p=0.0004)** [s]	**0.39 (p=0.0002)** [s]	**0.35 (p=0.0007)** [s]	0.11 (p=0.29)
DBP	**0.36 (p=0.0005)** [s]	**0.36 (p=0.0004)** [s]	**0.29 (p=0.0056)** [s]	0.08 (p=0.45)
Glucose	**0.27 (p=0.0099)** [s,w]	**0.43 (p<0.0001)** [s]	**0.36 (p=0.0005)** [s,w]	**-0.56 (p<0.0001)** [s,w]
Insulin	**0.54 (p<0.0001)** [s,w]	**0.46 (p<0.0001)** [s]	**0.97 (p<0.0001)** [s,w]	**0.74 (p<0.0001)** [s,w]
HOMA-IR	**0.58 (p<0.0001)** [s,w]	**0.54 (p<0.0001)** [s]		**0.56 (p<0.0001)** [s,w]
HOMA-%B	**0.29 (p=0.0054)** [s,w]	0.11 (p=0.30)	**0.56 (p<0.0001)** [s,w]	
TAG	**0.21 (p=0.041)**	0.19 (p=0.07)	**0.32 (p=0.0023)** [s,w]	**0.23 (p=0.025)** [s,w]
TC	-0.12 (p=0.27)	-0.17 (p=0.12)	-0.06 (p=0.57)	-0.09 (p=0.38)
HDL-C	**-0.49 (p<0.0001)** [s]	**-0.61 (p<0.0001)** [s]	**-0.41 (p<0.0001)** [s]	-0.12 (p=0.27)
LDL-C	0.05 (p=0.63)	0.08 (p=0.46)	0.00 (p=0.98)	-0.18 (p=0.096)
Adiponectin	**-0.43 (p<0.0001)** [s]	**-0.58 (p<0.0001)** [s]	**-0.45 (p<0.0001)** [s,w]	-0.16 (p=0.12)
Leptin	**0.45 (p<0.0001)** [s,w]	0.14 (p=0.20)	**0.45 (p<0.0001)** [s,w]	**0.50 (p<0.0001)** [s,w]
tPAI1	**0.39 (p=0.0001)** [s]	**0.35 (p=0.0009)** [s]	**0.33 (p=0.0015)** [s]	0.06 (p=0.55)
IL6	**0.21 (p=0.043)**	**0.26 (p=0.012)**	**0.25 (p=0.015)** [s]	**0.21 (p=0.046)** [s]
TNFα	**0.32 (p=0.0018)** [s]	**0.35 (p=0.0006)** [s]	**0.41 (p=0.0001)** [s,w]	0.15 (p=0.16)
MCP1	0.06 (p=0.57)	0.01 (p=0.92)	0.05 (p=0.64)	-0.07 (p=0.52)

Table 5. Correlations of Clinical and Biochemical Variables

Results are shown as Pearson's correlation coefficient, R, (p-value, p). All values were measured in the fasting state. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex and waist are marked with s and w, respectively. Significance did not change after adjustment for age. Positive and negative correlations are marked in green and red, respectively.

3.2 Lipids and Insulin Resistance

Fatty Acids

The question how individual FA species are related to InsRes was addressed. In this chapter, all measured FA are considered except for the ω3-FA which are examined in a separate chapter. The relative amounts of the individual total plasma FA species, quantified by gas chromatography, were correlated with glucose and insulin concentrations, as well as with HOMA-IR and HOMA-%B. All corresponding Pearson's correlation coefficients and p-values are shown in Table 6.

Only a few SFA species correlated significantly with these InsRes parameters. The strongest but still modest correlation was shown by stearic acid with glucose and HOMA-%B. Palmitic acid correlated positively with insulin and therefore also positively with HOMA-%B (Table 6).

Only a few MUFA species showed significant correlations with the tested InsRes parameters. Insulin correlated much better with MUFA than glucose. The strongest correlations were found for insulin with d7-hexadecenoic (d7-HDA) and oleic acid (Table 6).

Of the studied ω6-FA only LA correlated significantly negative with insulin and HOMA-IR. LA also showed a negative correlation with HOMA-%B. In contrast to LA, EDA and DGLA correlated negatively with glucose and positively with HOMA-%B (Table 6).

DGLA and EDA are products of the FA elongase ELOVL5 (Inagaki K, 2002; Leonard AE, 2000). The ratios of DGLA and EDA with their respective precursors (DGLA/GLA, EDA/LA) correlated significantly negative with glucose (Table 6), suggesting that individuals with high fasting glucose have a lower activity of this enzyme.

	Glucose	Insulin	HOMA-IR	HOMA-%B
SFA				
Myristic	0.06 (p=0.56)	0.21 (p=0.050)	**0.21 (p=0.048)** [a]	0.14 (p=0.17)
Palmitic	-0.09 (p=0.38)	**0.22 (p=0.037)**	0.18 (p=0.080)	**0.26 (p=0.013)** [s,w]
Stearic	**0.23 (p=0.030)** [s,w]	-0.16 (p=0.12)	-0.10 (p=0.35)	**-0.29 (p=0.0058)** [s,w]
Arachidic	-0.04 (p=0.72)	-0.03 (p=0.75)	-0.04 (p=0.70)	-0.01 (p=0.92)
Behenic	-0.10 (p=0.33)	-0.07 (p=0.49)	-0.09 (p=0.38)	0.04 (p=0.71)
Lignoceric	-0.13 (p=0.21)	-0.04 (p=0.72)	-0.07 (p=0.53)	0.06 (p=0.59)
MUFA				
Myristoleic	0.03 (p=0.76)	0.16 (p=0.14)	0.15 (p=0.14)	0.13 (p=0.23)
Palmitoleic	-0.09 (p=0.38)	0.09 (p=0.40)	0.06 (p=0.56)	0.18 (p=0.087)
Vaccenic	-0.03 (p=0.74)	0.06 (p=0.58)	0.05 (p=0.66)	0.15 (p=0.25)
d7-HDA	0.03 (p=0.77)	**0.23 (p=0.027)** [s]	**0.23 (p=0.032)** [a]	0.19 (p=0.070)
Oleic	0.07 (p=0.51)	**0.22 (p=0.034)**	0.23 (p=0.031)	0.14 (p=0.17)
Eicosenoic	0.01 (p=0.93)	-0.18 (p=0.085)	-0.17 (p=0.11)	-0.18 (p=0.096)
Nervonic	-0.12 (p=0.24)	0.01 (p=0.89)	-0.02 (p=0.89)	0.11 (p=0.31)
ω6-FA				
LA	-0.01 (p=0.89)	**-0.27 (p=0.0088)** [s, a]	**-0.26 (p=0.013)** [s,a]	**-0.24 (p=0.023)** [s,w]
EDA	**-0.31 (p=0.0028)** [s,a,w]	0.07 (p=0.49)	0.00 (p=0.98)	**0.29 (p=0.0050)** [s,a,w]
GLA	0.04 (p=0.69)	-0.03 (p=0.75)	-0.02 (p=0.83)	-0.08 (p=0.45)
DGLA	**-0.31 (p=0.0026)** [s,a,w]	0.19 (p=0.064)	0.11 (p=0.29)	**0.40 (p=0.0001)** [s,a,w]
AA	-0.09 (p=0.38)	-0.04 (p=0.72)	-0.06 (p=0.59)	0.01 (p=0.93)
ELOVL5				
EDA/LA	**-0.25 (p=0.017)** [s,a,w]	0.20 (p=0.053)	0.13 (p=0.21)	**0.37 (p=0.0004)** [s,a,w]
DGLA/GLA	**-0.25 (p=0.018)** [s,a,w]	0.16 (p=0.13)	0.09 (p=0.38)	**0.34 (p=0.0010)** [s,a,w]

Table 6. Correlations of Plasma FA with InsRes Parameters

Pearson's correlation between plasma FA [%], estimated ELOVL5 activity (DGLA/GLA, EDA/LA) and HOMA-IR, HOMA-%B, fasting glucose and insulin. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive and negative correlations are marked in green and red, respectively. FA abbreviations are listed in Supplemental Table 3.

ω3-Fatty Acids

The role of ω3-FA in InsRes is still a matter of debate. Hence, the associations of plasma ω3-FA with InsRes parameters are studied separately in this chapter. Similar to the analyses of all other plasma FA, relative amounts of plasma ω3-FA were correlated with fasting insulin, glucose, HOMA-IR and HOMA-%B. The results of the corresponding Pearson's correlation are shown in Table 7.

Surprisingly, several ω3-FA correlated positively with fasting glucose and negatively with HOMA-%B (Table 7). The strongest positive correlations were detected for EPA and docosapentaenoic acid (DPA) with glucose. This suggests that higher plasma ω3-FA concentrations, especially EPA and DPA, are associated with elevated fasting glucose.

	Glucose	Insulin	HOMA-IR	HOMA-%B
ALA	**0.21 (0.042)**	0.12 (p=0.27)	0.16 (p=0.13)	-0.06 (p=0.57)
SDA	0.12 (p=0.26)	0.07 (p=0.52)	0.09 (p=0.13)	-0.04 (p=0.71)
ETEA	0.06 (p=0.58)	0.04 (p=0.69)	0.05 (p=0.61)	0.03 (p=0.81)
EPA	**0.23 (p=0.031)** [s,w]	-0.16 (p=0.14)	-0.09 (p=0.38)	**-0.30 (p=0.0041)** [s,w]
DPA	**0.22 (p=0.038)**	-0.17 (p=0.11)	-0.11 (p=0.31)	**-0.31 (p=0.0032)** [s,a,w]
DHA	0.07 (p=0.51)	-0.02 (p=0.82)	-0.01 (p=0.95)	-0.08 (p=0.45)

Table 7. Correlations of Plasma ω3-FA with InsRes Parameters

Pearson's correlation between plasma ω3-FA [%] and HOMA-IR, HOMA-%B, fasting glucose and insulin. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive and negative correlations are marked in green and red, respectively. FA abbreviations are listed in Supplemental Table 3.

Phospholipids

In order to confirm the correlations of FA species with distinct InsRes parameters, and to account for variability in lipid class distribution among study subjects, the FA composition of the major plasma PL, PC, and its derivative LPC, which both have a high content of ω3- and ω6-FA were analyzed. Individual molecular PC and LPC species, defined by number of FA carbon atoms and double bonds, were

quantified by mass spectrometry. Since the identity of the two FA side chains of the PC molecules was not fully resolved by the mass spectrometry method, the predominant FA moieties in these PC and LPC species (Supplemental Table 4) were identified by a statistical factor analysis combining total plasma FA species, LPCs and PCs (Table 8).

The factor analysis extracted 13 factors with an eigenvalue higher than one which all together explained 83% of the variance of the dataset containing all total plasma FA, LPC and PC species. Table 8 comprises only the five most important factors each having an eigenvalue higher than five.

The ω3-FA EPA (C20:5n-3) and DHA (C22:6n-3) and their corresponding LPC species load on the first factor. The PC species 40:6, 38:6, 36:5 and 38:5 which also load on this factor exhibit the same number of double-bonds as EPA, DHA, LPC-20:5 and LPC-22:6. Consequently, it is assumed that these PC species contain a reasonable amount of EPA or DHA and a matching SFA or MUFA.

The fifth factor is also of importance since the ω6-FA DGLA (C20:3n-6) and EDA (C20:2n-6) and their corresponding LPC species load on it. At the same time, PC-36:3 and PC-38:3 loaded on this factor leading to the assumption that they are to a reasonable amount composed of DGLA or EDA with a matching SFA or MUFA.

	Loadings of variables by factor				
	1	2	3	4	5
PC-40:6	**0.94**				
PC-38:6	**0.91**				
LPC-22:6	**0.89**				
LPC-20:5	**0.78**				
DHA	**0.76**				
PC-36:5	**0.73**				
EPA	**0.64**				
PC-38:5	**0.60**				
PC-36:6	0.58				
PC-32:1		0.90			
PC-30:0		0.87			
LPC-16:1		0.79			
Myristoleic acid		0.79			
Myristic acid		0.74			
Palmitoleic acid		0.73			
PC-32:2		0.63			
PC-34:1		0.54			
PC-34:4		0.53			
LPC-20:4			0.91		
PC-36:4			0.91		
PC-38:4			0.84		
AA			0.80		
LPC-22:4			0.69		
PC-34:2	-0.51		-0.63		
LPC-18:2			-0.57		
PC-36:2			-0.54		
PC-36:0				0.79	
PC-36:1				0.77	
Stearic acid				0.76	
Vaccenic acid				-0.65	
LPC-18:0				0.64	
GLA				0.63	
PC-38:3					**0.84**
DGLA					**0.80**
LPC-20:3					**0.78**
PC-36:3	-0.56				**0.64**
EDA				-0.51	**0.60**
LPC-20:2					**0.59**
PC-32:0					-0.58
LA		-0.51			
Eigenvalues	7.91	7.88	6.37	5.15	5.04
% Total variance	11.29	11.26	9.10	7.36	7.20

Table 8. Factor Analysis with FA, LPC and PC Species

Only factors having eigenvalues larger than five are shown. Loadings < 0.5 were not recorded. Those FA, LPC and PC species showing the strongest correlations with InsRes parameters and their corresponding loadings are presented in boldface. FA abbreviations are listed in Supplemental Table 3.

The selected LPC and PC species were correlated with fasting glucose, insulin and HOMA-%B as shown in Table 9. HOMA-IR was not considered any more since it did not correlate with EDA, DGLA; EPA or DHA (Table 6, Table 7).

	Glucose	Insulin	HOMA-%B
ω6-FA			
EDA	**-0.31 (p=0.0028)** s,a,w	0.07 (p=0.49)	**0.29 (p=0.0050)** s,a,w
DGLA	**-0.31 (p=0.0026)** s,a,w	0.19 (p=0.064)	**0.40 (p=0.0001)** s,a,w
LPC-20:2	**0.31 (p=0.0032)** s,a,w	0.12 (p=0.26)	**0.35 (p=0.0009)** s,a,w
LPC-20:3	-0.19 (p=0.078)	**0.38 (p=0.0002)** s,a,w	**0.47 (p<0.0001)** s,a,w
PC-36:3	**-0.33 (p=0.0014)** s,a,w	0.09 (p=0.42)	**0.32 (p=0.0023)** s,a,w
PC-38:3	0.02 (p=0.83)	**0.34 (p=0.0010)** s,a	**0.30 (p=0.0044)** s,a,w
ω3-FA			
EPA	**0.23 (p=0.031)** s,w	-0.16 (p=0.14)	**-0.30 (p=0.0041)** s,w
DHA	0.07 (p=0.51)	-0.02 (p=0.82)	-0.08 (p=0.45)
LPC-20:5	**0.30 (p=0.0045)** s,a,w	0.10 (p=0.37)	-0.14 (p=0.19)
LPC-22:6	**0.25 (p=0.017)** s,a,w	0.13 (p=0.21)	-0.08 (p=0.47)
PC-36:5	**0.22 (p=0.039)**	0.01 (p=0.96)	-0.16 (p=0.14)
PC-38:5	**0.24 (p=0.022)** w	-0.07 (p=0.50)	**-0.26 (p=0.016)** s,w
PC-38:6	**0.25 (p=0.017)** s,w	**-0.03 (p=0.76)**	**-0.22 (p=0.038)** s,w
PC-40:6	**0.36 (p=0.0005)** s,aw,	0.10 (p=0.35)	-0.17 (p=0.11)

Table 9. Correlations of Important PL Species with InsRes Parameters

Plasma DGLA (C20:3n-6), EDA (C20:2n-6), EPA (C20:5n-3), DHA (C22:6n-3), LPC and PC species were correlated with HOMA-%B, glucose and insulin values. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive and negative correlations are marked in green and red, respectively.

The correlations of LPC species were very similar to and, mostly, stronger than those of the respective FA measured in total plasma (Table 9). For instance, like total plasma DGLA (C20:3n-6) and EDA (C20:2n-6), LPC-20:3 and LPC-20:2 showed a strong negative correlation with 0h-glucose (Table 9, Figure 7). LPC-20:5 and EPA (C20:5n-3) correlated positively with glucose (Table 9, Figure 7).

The PC species showed correlations consistent with the corresponding total plasma FA and LPC species. PC-36:3 and PC-38:3, species containing DGLA and EDA, showed negative correlations with glucose similar to those of total plasma DGLA, EDA and the corresponding LPC species LPC-20:3, LPC-20:2 (Table 9). EPA containing PC-36:5 and PC-38:5 species correlated positively with glucose similar to total plasma EPA and the corresponding LPC species LPC-20:5 (Table 9).

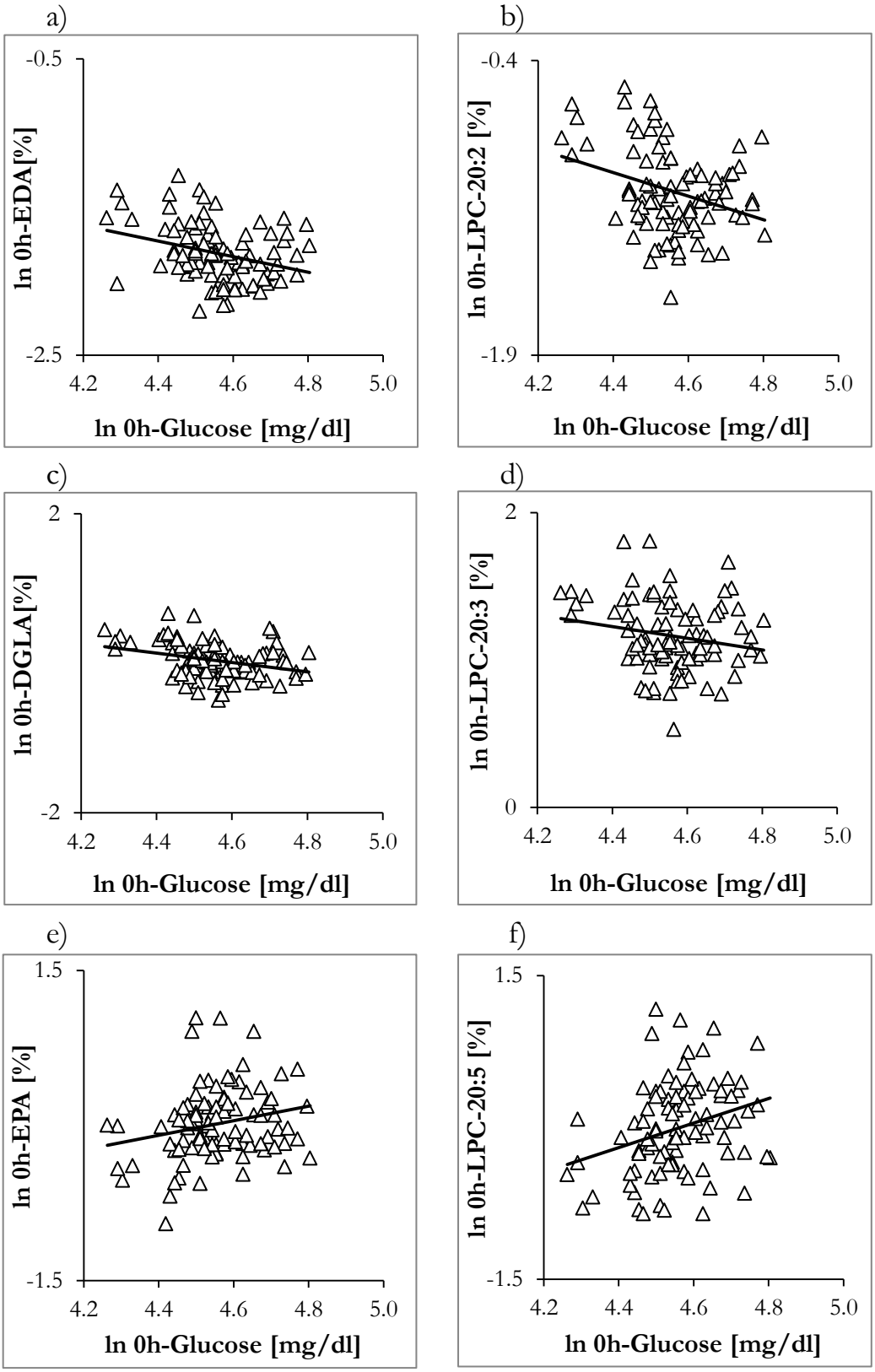

Figure 7. EDA, DGLA and EPA containing LPC Species and Glucose

Scatterplots showing the relationship between 0h-glucose and EDA (a), LPC-20:2 (b), DGLA (c), LPC-20:3 (d), EPA (e), and LPC-20:5 (f). Normalized FA values (% of total FA) were used for log-transformation. Correlation coefficients and p-values are listed in Table 9.

3.3 Metabolic Characterization of the Cohort during an OGTT

The participants of the study were subjected to an OGTT to assess glucose tolerance. Plasma samples were collected 2h after the glucose load. The cohort characteristics related to glucose tolerance including comparisons between women and men are shown in Table 10. Normal values for both sexes are also presented if they were available.

glucose tolerance associated parameters						
	Total cohort (n=91)	Women (n=61)	Normal values (women)	Men (n=30)	Normal values (men)	p
Glucose [mg/dl]	**90 (51 – 175)**	**100 (59 – 175)**	**< 140 †**	**81.5 (51 – 113)**	**< 140 †**	**0.001**
Insulin [µU/ml]	**48.9 (15.3 – 229)**	**52.9 (15.3 – 229)**		**33.5 (18.5 – 181)**		**0.006**
Normo-glycemic †	n=58 (64%)	n=46 (75%)		n=12 (40%)		
IFG †	n=31 (34%)	n=13 (21%)		n=18 (60%)		
IGT †	n=2 (2%)	n=2 (3%)		n=0 (0%)		

Table 10. Glucose Tolerance of the Study Population

Data are presented as median (min – max) and were measured 2h after the glucose load. P-values were calculated using Wilcoxon-test to compare men vs. women. Significant differences ($p<0.05$) are shown in boldface. † (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Of the subjects 58 (64%) were normoglycemic, 31 (34%) had IFG, and two (2%) had IGT. On average, women in the cohort were less glucose-tolerant, as indicated by higher average 2h-glucose and 2h-insulin (Table 10), whereas men had higher fasting glucose (Table 3).

When 2h-glucose and 2h-insulin were correlated with obesity and InsRes parameters, only 2h-insulin correlated positively with BMI, HOMA-IR and HOMA-%B (Table 11).

	BMI	Waist	HOMA-IR	HOMA-%B
2h-Glucose	0.10 (p=0.36)	-0.05 (p=0.66)	0.13 (p=0.24) [s]	0.11 (p=0.30)
2h-Insulin	**0.28 (p=0.0070)** [s,w]	0.08 (p=0.45)	**0.47 (p<0.0001)** [s,w]	**0.48 (p<0.0001)** [w]

Table 11. Correlations of 2h-Glucose and 2h-Insulin with Clinical Parameters

Results are shown as Pearson's correlation coefficient, R, (p-value, p). Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex and waist are marked with s and w, respectively. Significance did not change after adjustment for age. Positive correlations are marked in green.

3.4 Lipids during an OGTT

Fatty Acids

It was studied how individual FA species are related to glucose tolerance. The relative amounts of the individual total plasma FA species (except for the ω3-FA which are considered in a separate chapter), quantified by gas chromatography, were correlated with glucose and insulin concentrations at 2h. All corresponding Pearson's correlation coefficients and p-values are shown in Table 12.

	2h-Glucose	2h-Insulin
SFA		
Myristic	0.11 (p=0.30)	0.16 (p=0.14)
Palmitic	0.14 (p=0.18) [s]	0.20 (p=0.058)
Stearic	0.01 (p=0.90)	-0.15 (p=0.15)
Arachidic	0.07 (p=0.49)	0.07 (p=0.52)
Behenic	-0.04 (p=0.68)	-0.01 (p=0.92)
Lignoceric	-0.03 (p=0.81)	0.01 (p=0.96)
MUFA		
Myristoleic	0.15 (p=0.15)	0.15 (p=0.16)
Palmitoleic	0.17 (p=0.11)	**0.23 (p=0.026)** [s,a,w]
Vaccenic	**0.22 (p=0.039)** [s,a,w]	**0.27 (p=0.0090)** [s,a,w]
d7-HDA	0.07 (p=0.50)	**0.24 (p=0.024)** [s,a,w]
Oleic	-0.02 (p=0.83)	0.03 (p=0.79)
Eicosenoic	-0.18 (p=0.082)	-0.17 (p=0.10)
Nervonic	0.11 (p=0.32)	0.11 (p=0.30)
ω6-FA		
LA	**-0.24 (p=0.024)** [s,a,w]	**-0.30 (p=0.0038)** [s,a,w]
EDA	0.06 (p=0.58)	0.18 (p=0.090)
GLA	**-0.24 (p=0.023)** [s,a,w]	**-0.21 (p=0.042)** [w]
DGLA	0.04 (p=0.72)	**0.28 (p=0.0080)** [s,a,w]
AA	0.05 (p=0.66)	0.15 (p=0.15)
ELOVL5		
EDA/LA	0.17 (p=0.10)	**0.30 (p=0.0033)** [s,a,w]
DGLA/GLA	**0.25 (p=0.019)** [s,a,w]	**0.38 (p=0.0002)** [s,a,w]

Table 12. Correlations of Plasma FA with Glucose Tolerance Parameters

Pearson's correlation between plasma FA [%], ELOVL5 activity (DGLA/GLA, EDA/LA) and 2h-glucose and 2h-insulin. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state except for glucose and insulin which were measured 2h after the glucose load. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive and negative correlations are marked in green and red, respectively. FA abbreviations are listed in Supplemental Table 3.

None of the SFA and only a few MUFA species showed significant correlations with the testes glucose tolerance parameters. Insulin correlated much better with MUFA than glucose. The strongest correlations were found for 2h-insulin with palmitoleic, vaccenic and d7-HDA (Table 12).

Of the studied ω6-FA LA and GLA showed negative correlations with 2h-glucose and 2h-insulin. A strong positive correlation was also observed for DGLA with 2h-insulin (Table 12).

The ratios of EDA and DGLA with their respective precursors (EDA/LA, DGLA/GLA) estimating ELOVL5 activity correlated significantly positive with 2h-insulin (Table 12). The DGLA/GLA ratio also correlated positively with 2h-glucose, suggesting that individuals with low glucose tolerance (as indicated by high 2h-glucose levels) have a higher activity of ELOVL5.

ω3-Fatty Acids

The associations of plasma ω3-FA with glucose tolerance parameters are studied separately in this chapter. For this purpose, Pearson's correlations between ω3-FA and 2h-glucose and 2h-insulin were performed (Table 13).

	2h-Glucose	2h-Insulin
ALA	0.08 (p=0.43)	-0.10 (p=0.33)
SDA	-0.02 (p=0.88)	-0.09 (p=0.41)
ETEA	-0.03 (p=0.75)	-0.07 (p=0.52)
EPA	0.06 (p=0.60)	-0.07 (p=0.51)
DPA	-0.03 (p=0.78)	-0.12 (p=0.26)
DHA	**0.28 (p=0.0066)** ^{s,a,w}	**0.27 (p=0.0085)** ^{s,a,w}

Table 13. Correlations of Plasma ω3-FA with Glucose Tolerance Parameters

Pearson's correlation between plasma ω3-FA [%] and 2h-glucose and 2h-insulin. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state except for glucose and insulin which were measured 2h after the glucose load. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive correlations are marked in green. FA abbreviations are listed in Supplemental Table 3.

Surprisingly, only one ω3-FA correlated with the tested glucose tolerance parameters (Table 13). DHA showed a positive correlation with plasma 2h-glucose and 2h-insulin concentrations. 2h-Glucose is an OGTT parameter closely linked to defects in peripheral glucose disposal (Abdul-Ghani et al. 2006). Consequently, it is indicated that a high plasma DHA content is related to peripheral InsRes.

Phospholipids

The previously selected LPC and PC species were correlated with glucose tolerance parameters as shown in Table 14.

	2h-Glucose	2h-Insulin
ω6-FA		
EDA	0.06 (p=0.58)	0.18 (p=0.090)
DGLA	0.04 (p=0.72)	**0.28 (p=0.0080)** s,a,w
LPC-20:2	0.15 (p=0.15)	**0.25 (p=0.016)** a,w
LPC-20:3	0.06 (p=0.58)	**0.36 (p=0.0006)** s,a,w
PC-36:3	-0.14 (p=0.21)	-0.01 (p=0.90)
PC-38:3	-0.01 (p=0.94)	0.19 (p=0.076)
ω3-FA		
EPA	0.06 (p=0.60)	-0.07 (p=0.51)
DHA	**0.28 (p=0.0066)** s,a,w	**0.27 (p=0.0085)** s,a,w
LPC-20:5	0.13 (p=0.23)	0.08 (p=0.44)
LPC-22:6	**0.34 (p=0.0011)** s,a,w	**0.34 (p=0.0010)** s,a,w
PC-36:5	0.10 (p=0.35)	0.05 (p=0.67)
PC-38:5	0.07 (p=0.51)	0.01 (p=0.94)
PC-38:6	**0.28 (p=0.0070)** s,a,w	**0.21 (p=0.048)** s,a,w
PC-40:6	**0.29 (p=0.0052)** s,a,w	**0.24 (p=0.022)** s,a,w

Table 14. Correlations of PL Species with Glucose Tolerance Parameters

Plasma DGLA (C20:3n-6), EDA (C20:2n-6), EPA (C20:5n-3), DHA (C22:6n-3), LPC and PC species were correlated with 2h-glucose and 2h-insulin values. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting state except for 2h-glucose and 2h-insulin which were measured 2h after a glucose load. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive correlations are marked in green.

The correlations of LPC species were very similar to and, mostly, stronger than those of the respective FA measured in total plasma (Table 14). For instance, LPC-20:3 showed a strong positive correlation with 2h-insulin similar to total plasma DGLA (C20:3n-6) (Table 14). In addition, LPC-22:6 and DHA (C22:6n-3) correlated positively with 2h-glucose and 2h-insulin (Table 14, Figure 8).

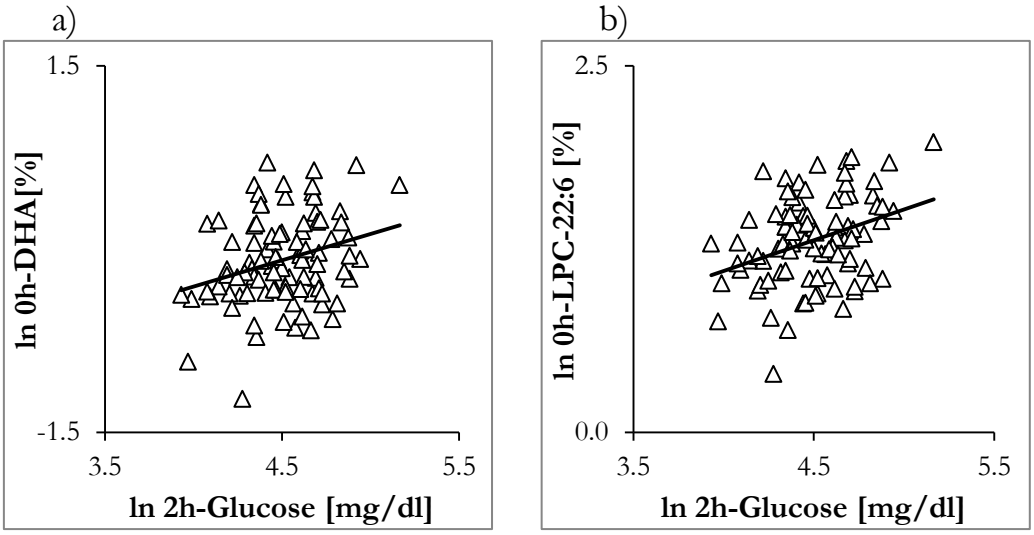

Figure 8. DHA containing LPC Species and Glucose

Scatterplots showing the relationship between 2h-glucose and DHA (a), LPC-22:6 (c). Normalized FA values (% of total FA) were used for log-transformation. Correlation coefficients and p-values are listed in Table 14.

The PC species showed correlations consistent with the corresponding total plasma FA and LPC species. PC-38:3 species containing DGLA showed a trend towards a positive correlations with 2h-insulin similar to that of total plasma DGLA and the corresponding LPC species LPC-20:3 (Table 14). The positive correlations of DHA and LPC-22:6 with 2h-glucose and 2h-insulin were also found for the DHA-containing PC species PC-38:6 and PC-40:6 (Table 14).

In summary, specific FA as well as PL species containing these FA showed distinct associations with parameters of InsRes and glucose tolerance. The ω6-FA LA correlated negatively with all InsRes and glucose tolerance parameters except of fasting glucose (Table 6, Table 12). DGLA, EDA and their corresponding LPC and PC species showed negative correlations with 0h-glucose (Table 9, Figure 7). Unexpectedly, positive correlations were found for 0h-glucose and several ω3-FA (ALA, EPA and DPA) (Table 7). In the case of EPA, these correlations were also found in the corresponding LPC and PC species (Table 9, Figure 7). Moreover, DHA, which also belongs to the ω3-FA family, and its corresponding LPC and PC species showed strong and positive correlations with 2h-glucose (Table 14, Figure 8).

This indicates that several ω3-FA, most importantly EPA and DPA, are positively associated with fasting glucose and that high levels of the longest ω3-FA, DHA, are related to peripheral InsRes.

3.5 Postprandial Triacylglycerol and Insulin Resistance

Lipid Response during an OFTT

It is well-established that the postprandial lipid metabolism is impaired early during the development of InsRes and MetSyn. With the purpose to study the association of InsRes and CVD with postprandial plasma lipids, a subgroup of the healthy T2D offspring cohort (n=39, 24 females, 15 males) received a mixed fat-rich breakfast used as an OFTT at a second test day (see Study Population). Plasma samples were collected in the fasting state (0h) as well as 2h and 4h after the breakfast. Total plasma TAG responses during the OFTT were used to define postprandial phenotypes.

The TAG responses during the OFTT varied profoundly among the participants. The majority of subjects (37/39) had increasing TAG between 0h and 2h (from now on referred to as first postprandial phase). Of these subjects 21 (12 females, 9 males) exhibited a further increase of TAG, whereas 15 (11 females, 4 males) showed decreasing TAG between 2h and 4h (second postprandial phase). According to the hypothesis that a delayed postprandial decline of TAG is associated with InsRes and cardiovascular risk, those subjects with increasing (group 1) and decreasing (group 2) TAG in the second postprandial phase were compared in relation to risk. Average and individual TAG curves of the two groups are represented in Figure 9.

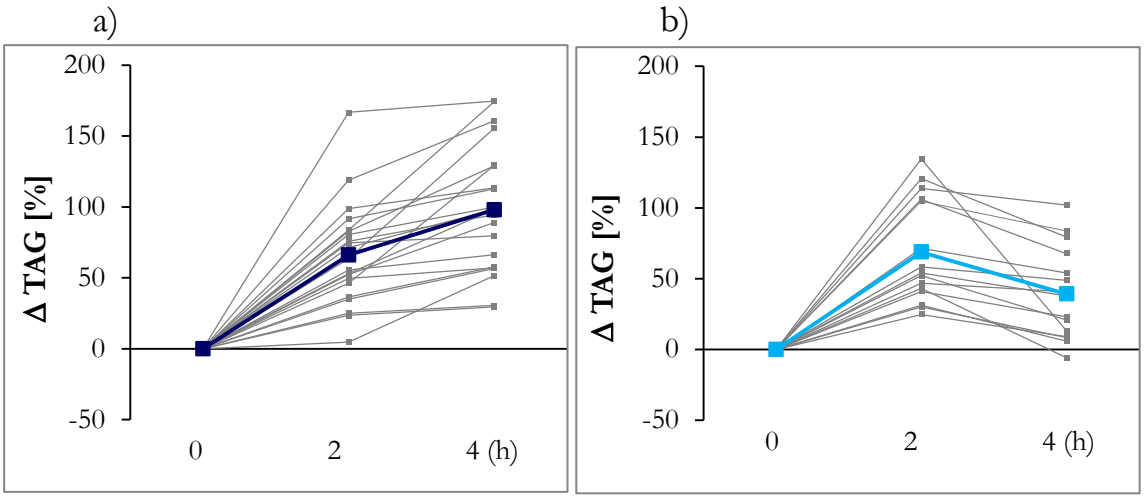

Figure 9. Changes of TAG during an OFTT

Changes of TAG during an OFTT for those subjects with a) increasing TAG and with b) decreasing TAG in the second postprandial phase. Mean changes are shown in percent based on the respective fasting values (mean ± SEM: 0h-TAG = 89.7 ± 7.7 mg/dl and 0h-TAG = 81.1 ± 8.8 mg/dl) and are marked with dark and light blue lines, respectively.

Lipoprotein metabolism was compared between the two groups by analysing total lipid and lipoprotein concentrations at different time points. Figure 10 presents the time course of total TAG, TAG associated with TRL (TRL-T) and remnants (RM-T). TAG, TRL-T, and RM-T showed a similar pattern. They did not differ significantly at 0h and 2h, whereas they were significantly higher at 4h in group 1 (more precisely, 4h total TAG (174.1 ± 14.2 mg/dl vs. 109.3 ± 11.1 mg/dl, p=0.0010), 4h-TRL-T (104.6 ± 13.2 mg/dl vs. 53.8 ± 8.4 mg/dl, p=0.0026), and 4h-RM-T (22.8 ± 1.8 mg/dl vs. 17.2 ± 2.1 mg/dl, p=0.046)). Moreover, TRL-T and RM-T changed in line with TAG by increasing in both groups in the first postprandial phase and further increasing only in group 1 while decreasing in group 2 during the second postprandial phase (Table 15).

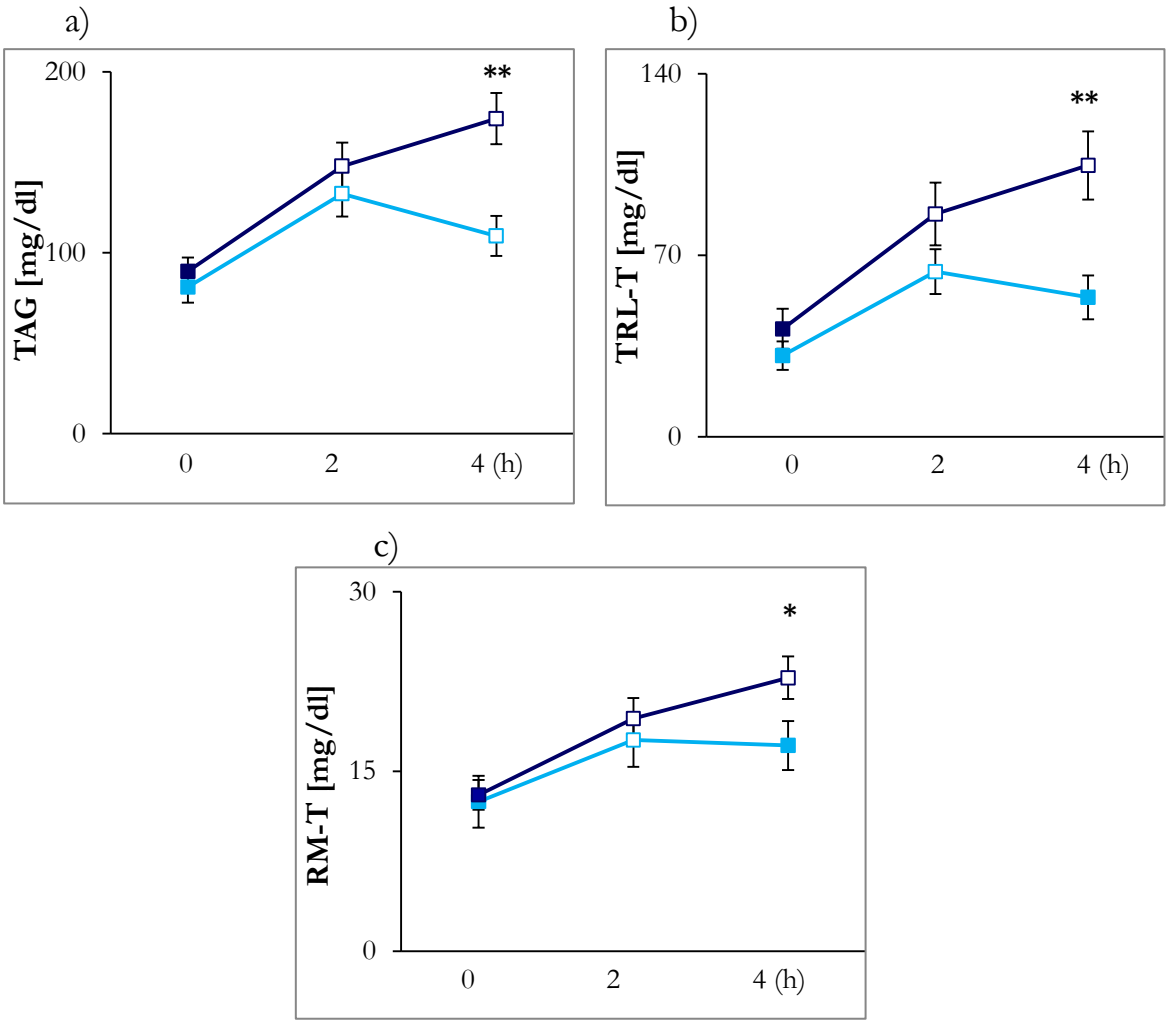

Figure 10. Plasma total TAG, TRL-T, and RM-T during an OFTT

Mean±SEM postprandial plasma total TAG (a), TRL-T (b), and RM-T (c) levels for the groups with increasing TAG (dark blue) versus decreasing TAG (light blue) in the second postprandial phase. Significant differences between the groups as detected with a t-test are marked with * for $p<0.05$ and ** for $p<0.01$. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Figure 11 presents the time course of HDL2-C which was not different between the two groups at any time point. Moreover, it did not change between the time points in group 1. However, a significant increase was observed in group 2 in the second postprandial phase (Table 15). TRL-C behaved very similar to total TAG with the 4h concentration being significantly higher in group 1 (13.1 ± 1.6 mg/dl vs. 6.58 ± 0.98 mg/dl, $p=0.0013$). HDL3-C on the other hand was not different between the groups at any time point or phase.

	First Postprandial Phase			Second Postprandial Phase		
[mg/dl]	↑ TAG n=21	↓ TAG n=15	p	↑ TAG n=21	↓ TAG n=15	p
TAG						
TAG	58.1±6.9	51.5±6.9	0.50	**26.2±4.5**	**-23.3±5.3**	**<0.0001**
TRL-T	44.3±5.7	32.3±5.2	0.13	**18.7±2.8**	**-9.91±6.9**	**0.0011**
RM-T	6.38±0.8	5.16±0.8	0.30	**3.39±0.5**	**-0.473±1.1**	**0.0037**
LDL-T	1.00±0.6	0.378±0.4	0.37	1.68±0.4	1.68±0.3	1.00
HDL2-T	1.48±0.3	1.02±0.2	0.25	1.27±0.2	0.802±0.2	0.14
HDL3-T	**1.44±0.3**	**0.150±0.4**	**0.0091**	0.571±0.2	0.438±0.2	0.69
Cholesterol						
TC	-1.90±1.5	1.87±1.9	0.13	**4.52±1.6**	**-1.73±1.3**	**0.0041**
TRL-C	3.90±0.6	0.224±0.6	0.71	**3.38±0.4**	**-0.82±0.5**	**<0.0001**
RM-C	-0.191±0.3	1.04±0.3	0.29	0.813±0.2	0.964±0.2	0.61
LDL-C	-2.63±1.0	-2.08±1.5	0.76	1.03±1.2	3.48±1.2	0.15
HDL2-C	-0.529±0.4	-0.577±0.5	0.94	**0.165±0.4**	**1.67±0.5**	**0.034**
HDL3-C	-0.916±0.4	-0.662±0.4	0.67	-0.457±0.5	0.363±0.4	0.20

Table 15. Changes of Lipoproteins during an OFTT

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two groups. Significant differences ($p<0.05$) are shown in boldface.

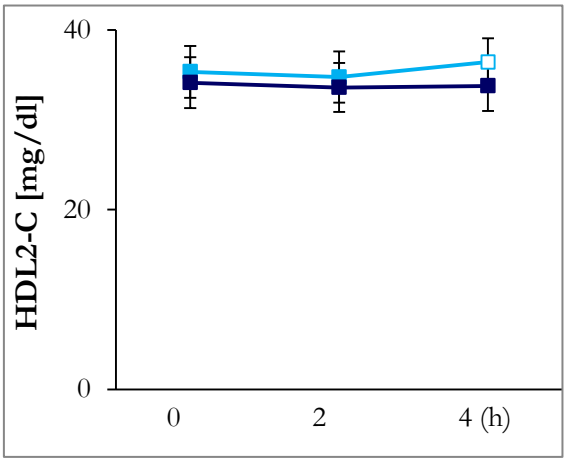

Figure 11. Plasma HDL2-C during an OFTT

Mean±SEM postprandial plasma HDL2-C levels for the groups with increasing TAG (dark blue) versus decreasing TAG (light blue) in the second postprandial phase. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Apolipoprotein concentrations were determined because they are important regulators of lipoprotein metabolism and clearance. Of the tested lipoproteins (apoAI, apoAII, apoB, apoCII, apoCII, apoE) only apoAII differed significantly in the fasting state by being higher in group 1 (1195823 ± 38492 ng/ml vs. 1001834 ± 56577 ng/ml, p=0.0087). Moreover, the fasting ratio apoE/apoCIII (0.306 ± 0.02 vs. 0.390 ± 0.1, p=0.048) was lower in in the same group.

Fatty Acids and Postprandial Triacylglycerol

It was asked whether delayed postprandial TAG decline was associated with specific FA pattern in plasma TAG. A comparison of the postprandial TAG groups revealed that those subjects with delayed postprandial decline had slightly lower fasting ω3-FA (1.7 ± 0.13 % vs. 2.3 ± 0.27 %, p=0.053) and ω6-FA (15.2 ± 0.69 % vs. 17.9 ± 1.14 %, p=0.054). When only the women were analyzed, the fasting and postprandial FA patterns differed even more pronouncedly with significantly higher SFA (stearic and palmitic acid) and at the same time significantly or slightly lower ω3- and ω6-FA in females with delayed postprandial decline (Table 16). Figure 12 presents fasting stearic, DHA and DGLA in females with increasing and decreasing TAG in the second postprandial phase.

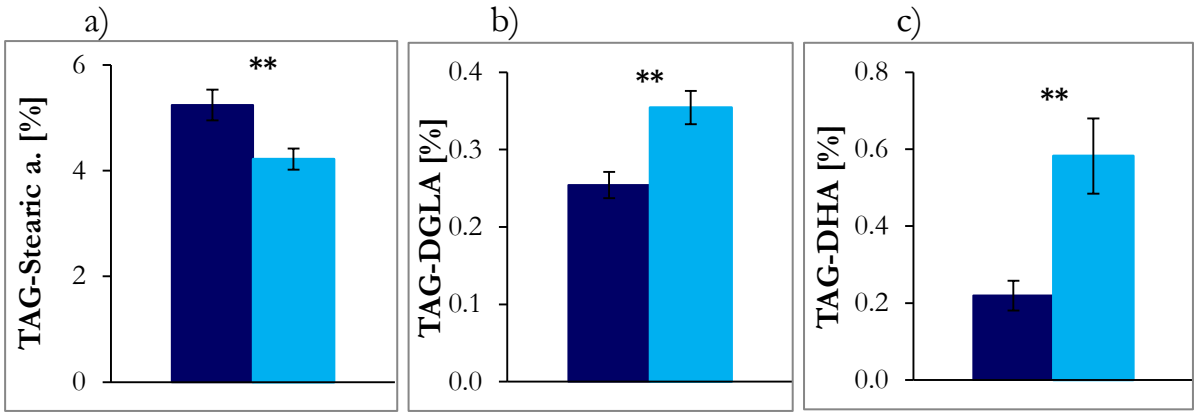

Figure 12. TAG-FA of OFTT Female TAG Groups

Mean ±SEM proportions of TAG-stearic acid (a), TAG-DGLA (b) and TAG-DHA (c) are shown for the females with increasing and decreasing TAG in the second postprandial phase in dark and light blue, respectively. Significant differences as detected with a t-test are marked with ** for p<0.01. Mean±SEM of the groups and p-values are listed in Table 16.

	OFTT 0h			OFTT 4h		
TAG [%]	↑ TAG n=12	↓ TAG n=11	p	↑ TAG n=12	↓ TAG n=11	p
SFA						
Myristic	2.97±0.5	2.71±0.4	0.68	6.12±0.5	5.07±0.4	0.11
Palmitic	30.1±1.0	27.2±1.2	0.083	32.8±0.9	30.6±0.9	0.10
Stearic	**5.24±0.3**	**4.22±0.2**	**0.0094**	6.34±0.3	**5.58±0.2**	**0.031**
Arachidic	0.06±0.01	0.05±0.004	0.17	0.07±0.01	0.06±0.004	0.13
Behenic	0.02±0.01	0.01±0.002	0.14	0.01±0.02	0.01±0.002	0.20
SFA	34.2±1.7	38.4±1.8	0.063	45.3±1.3	**41.4±1.1**	**0.030**
MUFA						
Myristoleic	0.19±0.05	0.16±0.03	0.6	0.55±0.08	0.41±0.04	0.16
Palmitoleic	3.94±0.4	3.92±0.3	0.97	3.53±0.3	3.37±0.2	0.66
Vaccenic	2.39±0.2	2.44±1.4	0.83	2.50±0.1	2.48±0.1	0.88
Oleic	39.0±1.2	38.2±0.8	0.57	34.9±1.1	35.3±0.8	0.76
Eicosenoic	0.28±0.02	0.3±0.01	0.38	0.28±0.02	0.31±0.02	0.40
MUFA	45.8±1.3	45.0±0.8	0.60	41.8±1.1	41.9±0.8	0.93
ω6-FA						
LA	**12.9±0.6**	**16.6±1.4**	**0.031**	**10.4±0.5**	**13.1±0.6**	**0.0038**
EDA	0.09±0.01	0.12±0.01	0.052	0.10±0.01	0.11±0.01	0.35
GLA	0.30±0.04	0.34±0.05	0.59	0.26±0.04	0.32±0.06	0.37
DGLA	**0.25±0.02**	**0.35±0.02**	**0.0016**	**0.21±0.02**	**0.28±0.01**	**0.0026**
AA	0.83±0.07	1.04±0.1	0.16	**0.65±0.05**	**0.98±0.1**	**0.015**
ω6-FA	**14.3±0.6**	**18.3±1.4**	**0.019**	**11.6±0.5**	**14.8±0.6**	**0.0004**
ω3-FA						
ALA	0.74±0.07	1.07±0.1	0.061	0.76±0.05	0.91±0.09	0.16
ETEA	**0.07±0.01**	**0.12±0.01**	**0.0080**	**0.06±0.01**	**0.10±0.01**	**0.0030**
EPA	0.21±0.03	0.34±0.07	0.084	**0.15±0.02**	**0.28±0.05**	**0.030**
DPA	**0.22±0.02**	**0.34±0.05**	**0.045**	**0.15±0.01**	**0.28±0.03**	**0.0042**
DHA	**0.22±0.04**	**0.58±0.1**	**0.0043**	**0.15±0.02**	**0.40±0.06**	**0.0014**
ω3-FA	**1.46±0.1**	**2.45±0.3**	**0.015**	**1.28±0.07**	**1.97±0.2**	**0.0092**

Table 16. TAG-FA Pattern of OFTT TAG Female Groups

Proportional FA measured in TAG species. Data are presented as mean±SEM. P-values were calculated using t-test to compare the two groups. Significant differences ($p<0.05$) are shown in boldface. FA abbreviations are listed in Supplemental Table 3.

Risk for Type 2 Diabetes and Postprandial Triacylglycerol

It was investigated whether the individuals with a delayed TAG decline would exhibit signs of InsRes or be more obese. Surprisingly, neither insulin nor glucose differed in fasting, postprandial states or their response in the first or second postprandial phase (Figure 13). The subjects also had similar waist levels (Figure 13).

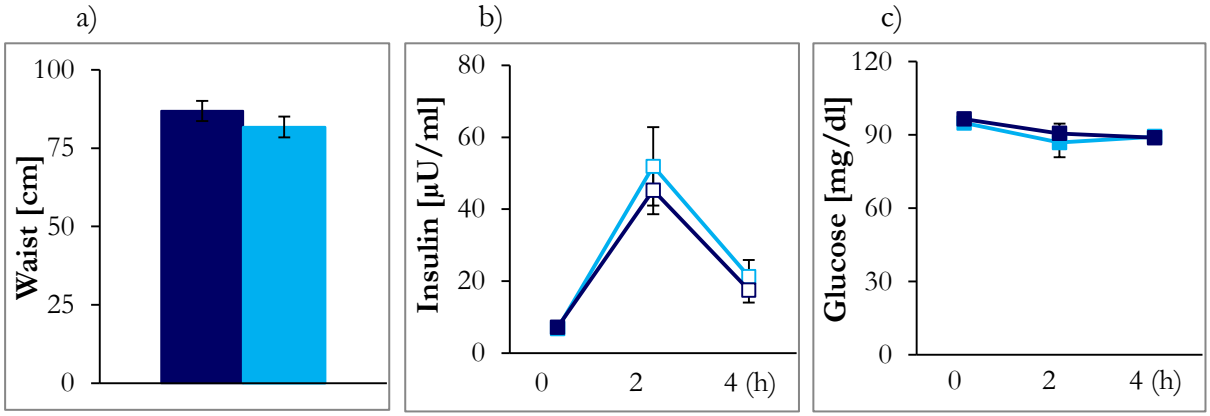

Figure 13. Waist, Insulin and Glucose of OFTT TAG Groups

Mean±SEM Waist (a) and postprandial plasma insulin (b) and glucose (c) levels for the groups with increasing TAG (dark blue box and line) versus decreasing TAG (light blue box and line) in the second postprandial phase. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Taken together, those individuals exhibiting a delayed postprandial TAG decline after the consumption of a mixed fat-rich breakfast had a similar fasting lipoprotein profile compared to subjects with fast decline. However, they had no increase in HDL2-C in the second postprandial phase (Figure 11) and exhibited a more unfavourable apolipoprotein and FA profile (Table 16). Unexpectedly, no association was found for TAG decline with InsRes or obesity parameters (Figure 13).

Lipid Response during an OGTT

In order to analyze the effect of insulin on the lipid metabolism independent of a fat-stimulus and to search for a better risk marker for InsRes and CVD than postprandial TAG changes, an OGTT was performed in the whole cohort of 91 subjects, which included those individuals studied in the OFTT (see Study Population). Blood samples were collected after an overnight fast (0h) and 2h after glucose load.

Correlations with plasma risk factors for T2D revealed that, surprisingly, the TAG response during the OGTT (G_Δ-TAG) correlated strongly and in several cases even better than fasting or 2h-TAG with obesity parameters and fasting glucose (Table 17). Since the TAG response during an OGTT seemed to hold additional information, I studied it more closely.

	0h-TAG (n=91)	2h-TAG (n=91)	G_Δ-TAG (n=91)
MetSyn			
Waist	**0.22 (p=0.037)** [a]	**0.30 (p=0.0038)** [s,a]	**0.43 (p<0.0001)** [s,a]
Glucose	0.13 (p=0.22)	**0.22 (p=0.034)** [s,a,w]	**0.31 (p=0.0032)** [s,a]
TAG		**0.95 (p<0.0001)** [s,a,w]	-0.12 (p=0.27)
HDL-C	**-0.36 (p=0.0005)** [s,a,w]	**-0.46 (p<0.0001)** [s,a,w]	**-0.35 (p=0.0007)** [s,a]
DBP	**0.24 (p=0.024)** [s,a]	**0.25 (p=0.0159)** [s,a]	0.06 (p=0.55)
SBP	**0.22 (p=0.039)** [a]	**0.26 (p=0.0124)** [s,a]	0.16 (p=0.14)
Obesity			
BMI	0.20 (p=0.054)	**0.30 (p=0.0038)** [s,a]	**0.33 (p=0.0014)** [s,a]
WHR	**0.21 (p=0.042)** [a]	**0.35 (p=0.0008)** [s,a]	**0.44 (p<0.0001)** [s,a]
InsRes			
Insulin	**0.40 (p=0.0001)** [s,a,w]	**0.49 (p<0.0001)** [s,a,w]	**0.33 (p=0.0013)** [s,a]
HOMA-IR	**0.41 (p=0.0005)** [s,a,w]	**0.53 (p<0.0001)** [s,a,w]	**0.40 (p=0.0001)** [s,a, w]

Table 17. Correlations of TAG Parameters with Risk Factors

Results are shown as Pearson's correlation coefficient, R, (p-value, p). Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. None of the data was log-transformed. All values, except for 2h-TAG which was measured 2h after a glucose load, were measured in the fasting state. G_Δ-TAG: Change of TAG during the OGTT. Positive and negative correlations are marked in green and red, respectively.

The TAG responses during the OGTT varied largely with the majority of subjects (70/91, 50 females, 20 males) having decreasing and 20 subjects (10 females, 10 males) having increasing TAG between 0h and 2h. It was explored whether the different TAG responses during the OGTT were associated with InsRes or CVD risk. To this end, subjects with increasing TAG were compared with those sex-matched individuals most efficiently decreasing TAG (n=20). Only individuals with fasting TAG < 200 mg/dl were considered to avoid biased results due to outliers, resulting in 10 women and 8 men for the first group and 9 women and 9 men for the second group (Figure 14). These groups overlapped only partially with the groups compared for the OFTT, to some extent due to the reason that only 39 of the 91 sub-

jects participated in the OFTT. Group 1 of the OFTT comprised 3 females and 3 males of the first OGTT group and group 2 of the OFTT shared 4 females and 1 male with the second OGTT group.

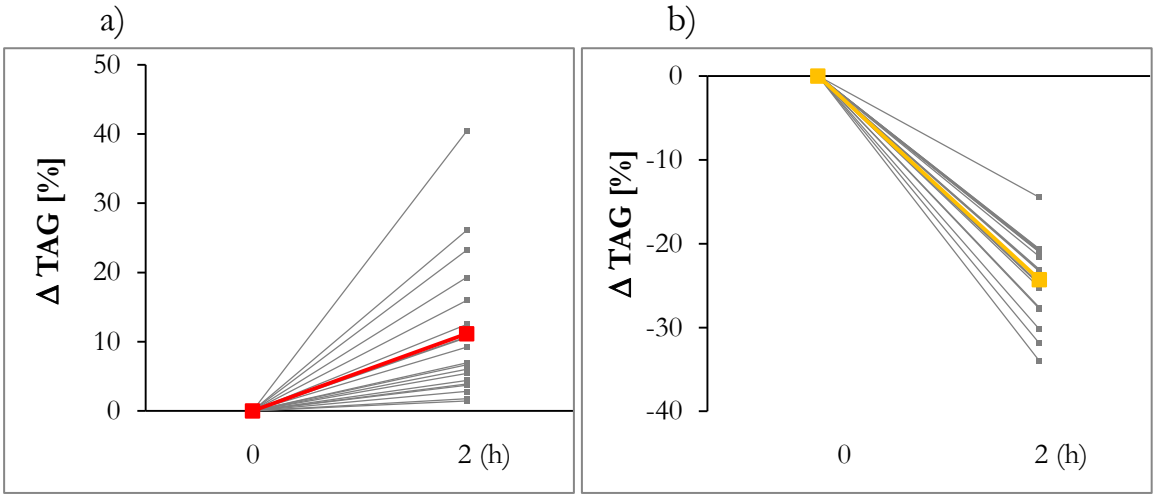

Figure 14. Changes of TAG during an OGTT

Changes of TAG during an OGTT for those subjects with a) increasing TAG and with b) decreasing TAG. Mean changes are shown in percent based on the respective fasting values (mean ± SEM: 0h-TAG = 79.7 ± 6.3 mg/dl and 0h-TAG = 108.9 ± 6.8 mg/dl) and are marked with red and yellow lines, respectively.

In consistence with the results of the OFTT, lipoprotein-associated TAG (TRL-T, RM-T, HDL2-T, HDL3-T) changed in line with total TAG during the OGTT (Table 18). However, surprisingly and in contrast to the results of the OFTT showing no differences in fasting states, fasting total TAG and lipoprotein-associated TAG were significantly lower in those subjects with increasing TAG during an OGTT (Table 18). Time curves of total TAG, TRL-T, and RM-T for both OGTT groups are presented in Figure 15. HDL2-C differed only slightly between the two groups (Table 18). Time curves of HDL2-C for both OGTT groups are presented in Figure 16.

	OGTT 0h			OGTT 2h			OGTT 2h-0h		
[mg/dl]	↑ TAG n=18	↓ TAG n=18	p	↑ TAG n=18	↓ TAG n=18	p	↑ TAG n=18	↓ TAG n=18	p
TAG									
TAG	**79.7±6.3**	**108.9±6.8**	**0.0033**	88.1±7.0	82.7±5.7	0.56	**8.33±2.0**	**-26.2±1.8**	**<0.0001**
TRL-T	33.0±4.6	45.2±6.5	0.14	40.4±5.7	33.6±5.9	0.41	**7.40±2.6**	**-11.7±3.0**	**<0.0001**
RM-T	**12.8±1.5**	**17.2±1.4**	**0.042**	14.5±1.5	14.4±1.4	0.97	**1.71±0.5**	**-2.77±0.5**	**<0.0001**
LDL-T	**26.3±1.9**	**32.0±1.6**	**0.030**	25.2±1.8	30.0±1.7	0.065	-1.07±0.6	-2.01±0.6	0.26
HDL2-T	**4.68±0.3**	**7.80±0.7**	**0.0008**	**4.95±0.4**	**6.92±0.7**	**0.029**	**0.26±0.2**	**-0.88±0.2**	**0.0006**
HDL3-T	**4.41±0.4**	**6.98±0.6**	**0.0007**	4.90±0.5	6.29±0.6	0.094	**0.49±0.2**	**-0.69±0.2**	**0.0005**
Cholesterol									
TC	186.2±10.2	190.9±8.3	0.72	186.3±9.4	186.4±8.0	1.00	0.17±1.9	-4.56±2.7	0.16
TRL-C	5.18±0.8	6.60±0.8	0.21	6.21±0.9	5.16±0.8	0.40	**1.02±0.3**	**-1.44±0.3**	**<0.0001**
RM-C	8.32±1.0	10.7±1.0	0.10	8.17±0.9	9.75±0.9	0.23	**-0.15±0.2**	**-0.92±0.3**	**0.042**
LDL-C	110.0±8.4	110.0±6.1	1.00	109.7±8.1	111.9±6.5	0.84	-1.22±1.3	0.90±1.2	0.25
HDL2-C	28.0±2.2	33.7±2.4	0.16	28.9±2.2	34.6±2.4	0.090	-0.09±0.4	0.86±0.4	0.10
HDL3-C	30.2±1.6	32.1±1.7	0.41	31.1±1.8	33.2±1.8	0.41	0.90±0.5	1.12±0.5	0.76

Table 18. Lipoproteins during an OGTT

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two groups. Significant differences ($p<0.05$) are shown in boldface.

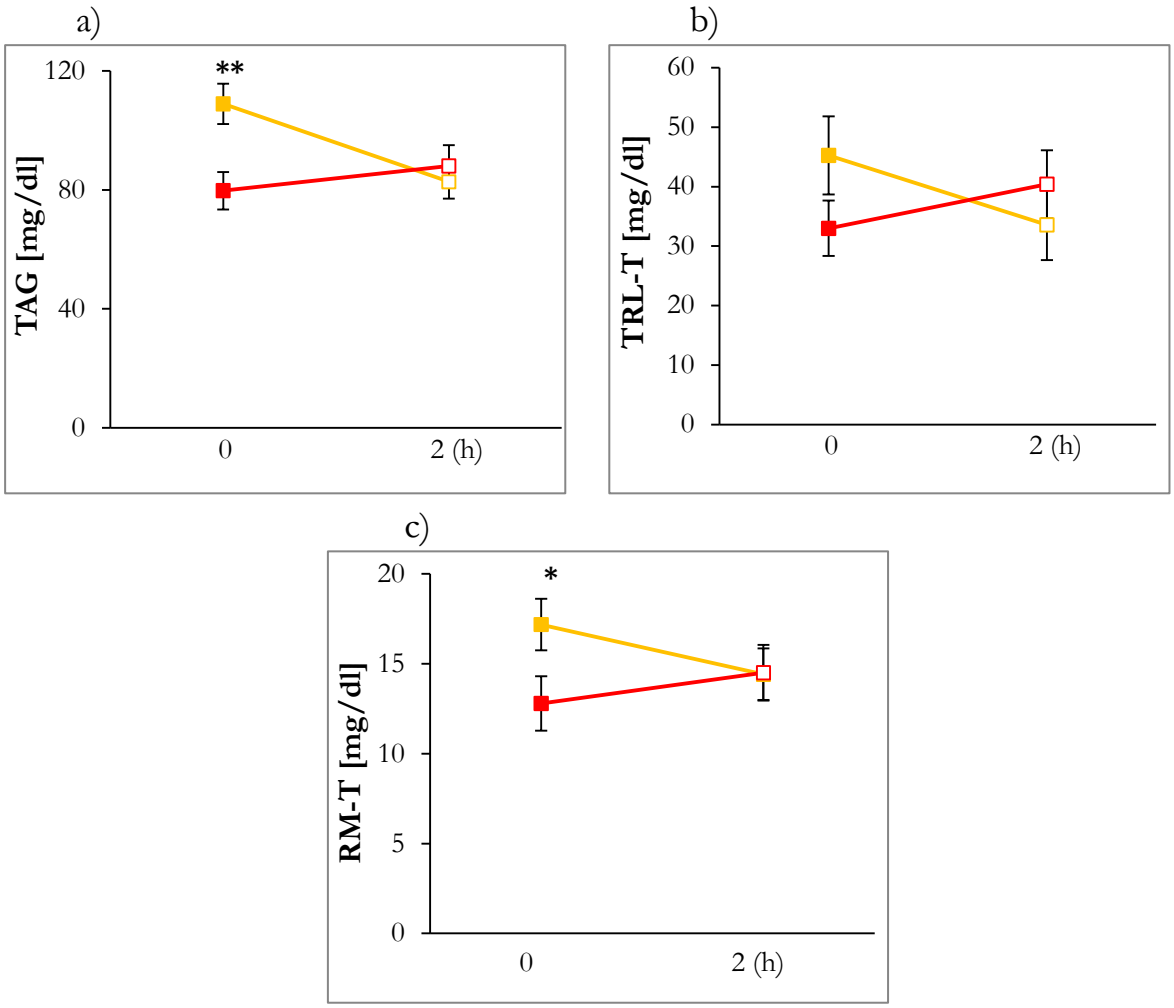

Figure 15. Plasma total TAG, TRL-T, and RM-T during an OGTT

Mean±SEM plasma total TAG (a), TRL-T (b), and RM-T (c) levels for the groups with increasing TAG (red line) versus decreasing TAG (yellow line) during an OGTT. Significant differences between the groups as detected with a t-test are marked with* for $p<0.05$ and ** for $p<0.01$. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

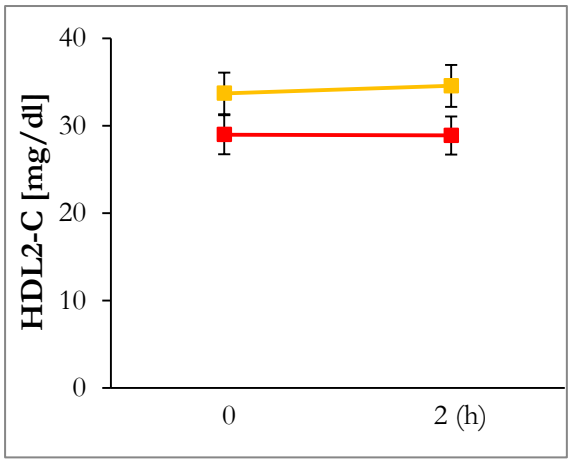

Figure 16. Plasma HDL2-C during an OGTT

Mean±SEM plasma HDL2-C levels for the groups with increasing TAG (red line) versus decreasing TAG (yellow line) during an OGTT.

Risk for Type 2 Diabetes and Post-Glucose Triacylglycerol

It was asked whether the OGTT groups differed concerning their risk for T2D. Subjects with increasing TAG during the OGTT were more obese, more insulin resistant as assessed by HOMA-IR, had higher fasting insulin and leptin levels but had at the same time lower fasting TAG than those individuals with most efficiently decreasing TAG (Table 19). 2h-Insulin (75±13.3µU/ml vs. 47±5.9µU/ml, p=0.063) and the insulin response (63±12µU/ml vs. 39±5.4µU/ml, p=0.094) were also slightly higher in subjects with increasing TAG during the OGTT. There were no differences detected in fasting glucose, HDL-C, BP, glucose tolerance as assessed by 2h-glucose (p=0.79), or the glucose response (p=0.70). Figure 17 shows the differences in waist, insulin and glucose for the two OGTT groups.

	OGTT			OFTT		
	↑ TAG n=18	↓ TAG n=18	p	↑ TAG n=21	↓ TAG n=15	p
Sex [n] (m/w)	8/10	9/9		9/12	4/11	
Age [years]	44.7±2.8	40.9±3.2	0.37	44.4±2.4	44.3±3.7	0.98
BMI	**26.5±0.9**	**23.5±0.8**	**0.029**	25.7±1.0	24.9±1.2	0.60
Waist	**93.1±2.8**	**84.3±2.8**	**0.034**	86.9±3.2	81.7±3.3	0.27
WHR	0.901±0.02	0.849±0.02	0.067	0.84±0.02	0.81±0.02	0.24
Glucose [mg/dl]	102±2.2	96.9±3.0	0.20	96.5±2.3	94.9±2.2	0.60
Insulin [µU/ml]	**12.4±1.4**	**7.71±0.8**	**0.0081**	7.18±1.3	6.76±0.9	0.79
HOMA-IR	**3.09±0.4**	**1.84±0.2**	**0.0056**	1.77±0.4	1.61±0.2	0.72
TAG [mg/dl]	**79.7±6.3**	**109±6.8**	**0.0033**	89.7±7.7	81.1±8.8	0.46
HDL-C [mg/dl]	59.2±3.7	65.8±4.0	0.23	59.0±3.9	60.9±3.6	0.72
DBP [mmHg]	79.9±2.4	77.3±2.6	0.47			
SBP [mmHg]	135±5.3	131±3.9	0.52			
Leptin [pg/ml]	**18521±4237**	**7491±1564**	**0.023**	16464±4748	17347±5145	0.90

Table 19. Characteristics of TAG Groups

Data are presented as mean±SEM. P-values were calculated using t-test to compare the corresponding TAG groups. Significant differences ($p<0.05$) are shown in boldface. Number (n) of men (m) and women (w) is given for sex. All values were measured in the fasting state.

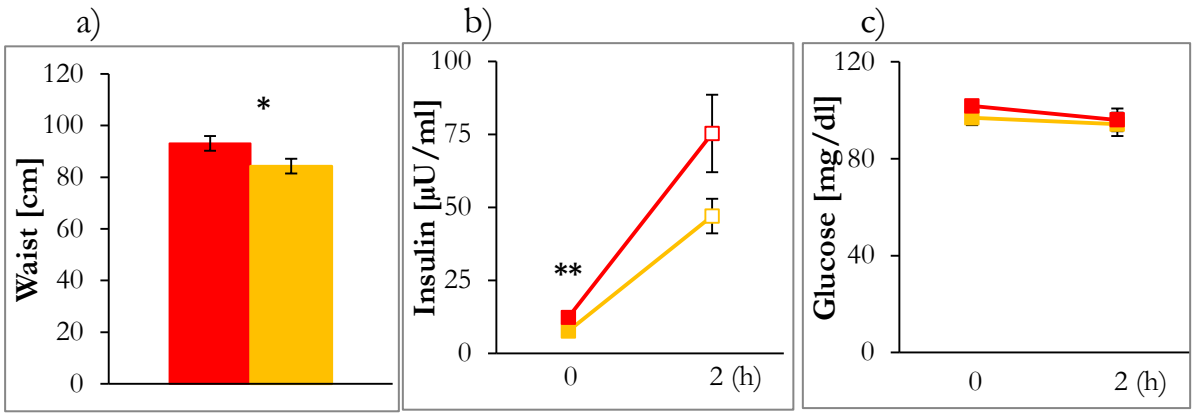

Figure 17. Waist, Insulin and Glucose of OGTT TAG Groups

Mean±SEM Waist (a) and plasma insulin (b) and glucose (c) levels for the groups with increasing TAG (red box and line) versus decreasing TAG (yellow box and line) during an OGTT. Significant differences between the groups as detected with a t-test are marked with * for $p<0.05$ and ** for $p<0.01$. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

As reported in Table 19, the experimental groups defined by TAG change in the OGTT differed with regard to obesity, InsRes and fasting TAG, whereas this was not the case for groups defined by delayed versus rapid TAG decline in an OFTT. The OGTT groups represented approximately the sex-matched upper and lower quartile of TAG change of the whole cohort. In contrast, the OFTT groups comprised roughly the upper and lower half of those individuals who took part in the OFTT so that in the end all four groups were composed of 18 to 20 subjects. With the aim to test if this difference in group design let to the different outcomes with regard to metabolic risk factors, a correlation analysis of TAG change was performed for all individuals of the OGTT and the OFTT, respectively.

Table 20 displays correlations of TAG changes during the second postprandial phase of the OFTT (F_Δ-TAG) with parameters associated with MetSyn, obesity and InsRes. F_Δ-TAG did not show a significant correlation with metabolic parameters, except for a weak, negative correlation with 0h-TAG.

	F_Δ-TAG (n=39)
MetSyn	
Waist	-0.02 (p=0.91)
Glucose	0.07 (p=0.67)
TAG	**-0.35 (p=0.027)** [s,a,w]
HDL-C	0.16 (p=0.33)
DBP	
SBP	
Obesity	
BMI	0.01 (p=0.94)
WHR	0.03 (p=0.87)
InsRes	
Insulin	0.03 (p=0.84)
HOMA-IR	0.05 (p=0.77)

Table 20. Correlations of Postprandial TAG Change with Risk Factors

Results are shown as Pearson's correlation coefficient, R, (p-value, p). Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. None of the data was log-transformed. All values were measured in the fasting state. F_Δ-TAG: Change of TAG during the second postprandial phase of the OFTT. Negative correlations are marked in red.

In contrast, G_Δ-TAG, 0h and 2h-TAG (Table 17) correlated significantly with metabolic parameters but G_Δ-TAG did not correlate with fasting TAG. G_Δ-TAG correlated better with parameters of abdominal obesity (waist, WHR) and with fasting glucose than 0h and 2h-TAG (Figure 18). Fasting TAG correlated better with fasting insulin (Figure 18) and BP (Table 17). Both 0h-TAG and G_Δ–TAG correlated equally well with HDL-C and with HOMA-IR. Surprisingly, when 0h and 2h-TAG were compared, 2h-TAG showed stronger correlations with all tested metabolic parameters (Table 17). However, G_Δ-TAG correlated even stronger with fasting glucose and parameters for abdominal obesity (Table 17).

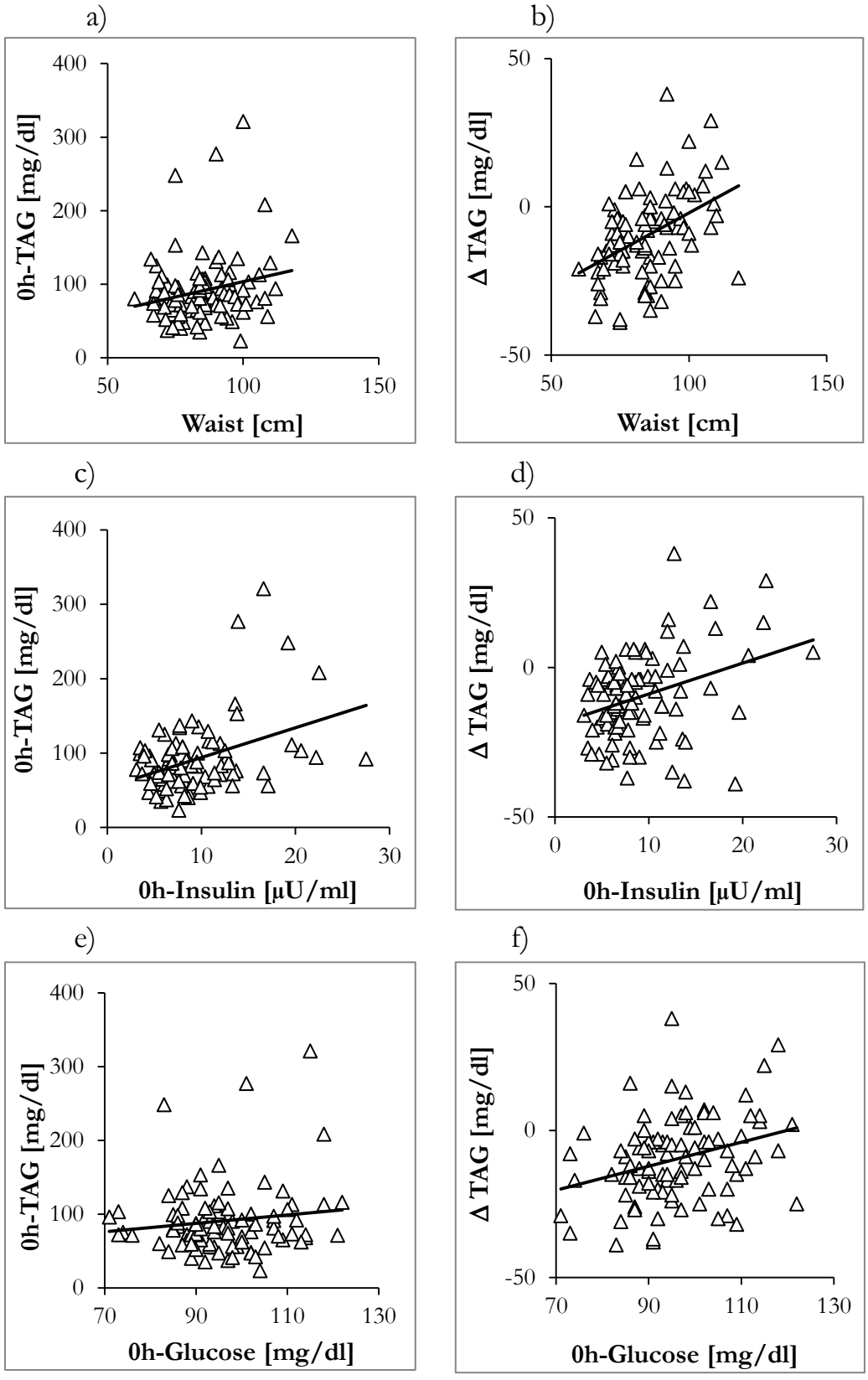

Figure 18. Correlations of TAG with Waist, Insulin and Glucose

Scatterplots showing the relationship between 0h-TAG and Δ-TAG and waist (a, b), 0h-insulin (c, d), 0h-glucose (e, f), respectively. Correlation coefficients and p-values are listed in.

In summary, fasting TAG and changes of TAG during an OGTT and in the second postprandial phase of an OFTT showed distinct associations with InsRes, CVD risk parameters and lipoprotein kinetics. Fasting and especially 2h-TAG correlated most strongly with fasting insulin and BP, whereas G_Δ-TAG was most significantly correlated with obesity and fasting glucose. In contrast, F_Δ-TAG was not associated with InsRes or obesity but instead with postprandial lipid metabolism in the second postprandial phase.

3.6 Active PAI1 and Cardiovascular Risk

The fourth part of the present work dealt with the question how aPAI1 as a marker for lowered fibrinolysis in CVD is associated with InsRes and MetSyn. aPAI1 was measured in fasting and postprandial plasma of a subgroup of 39 subjects who participated in the OFTT. Fasting aPAI1 was then correlated with distinct InsRes and MetSyn markers. The corresponding results are presented in Table 21.

Significant correlations were observed for aPAI1 with surrogate markers of adiposity (BMI, WHR, waist), InsRes (HOMA-IR, 0h-insulin), leptin and age. Almost all significant correlations were independent of age and sex. The correlations with leptin and age also remained significant after the adjustment for waist. None of the other measured inflammation markers (IL6, TNFα and MCP1) correlated significantly with aPAI1.

	aPAI1
Age	**0.34 (p=0.036)** [s,w]
Obesity	
BMI	**0.45 (p=0.0039)** [s,a]
Waist	**0.54 (p=0.0004)** [s,a]
WHR	**0.33 (p=0.038)** [s]
InsRes	
Glucose	0.16 (p=0.33)
Insulin	**0.39 (p=0.013)** [s,a]
HOMA-IR	**0.39 (p=0.014)** [s,a]
Lipids	
TAG	0.19 (p=0.24)
TC	0.11 (p=0.49)
LDL-C	0.31 (p=0.054)
HDL-C	0.27 (p=0.097)
Hormones	
Adiponectin	-0.14 (p=0.41)
Resistin	-0.11 (p=0.51)
Leptin	**0.46 (p=0.0034)** [s,a,w]
Inflammation Marker	
IL6	-0.07 (p=0.66)
TNFα	-0.01 (p=0.94)
MCP1	0.29 (p=0.072)

Table 21. Correlations of aPAI1 with MetSyn Parameters

Pearson's correlation between plasma aPAI1 and InsRes and MetSyn Parameters. Results are shown as correlation coefficient, R, (p-value, p). All values were measured in the fasting. Significant correlations ($p<0.05$) are shown in boldface. Significant correlations after adjustment for sex, age and waist are marked with s, a and w, respectively. Positive correlations are marked in green.

When the fasting aPAI values of the study cohort were sorted according to size, it was remarkable that nine individuals (six females, three males) were outliers by having levels above 25,000pg/ml (Figure 19).

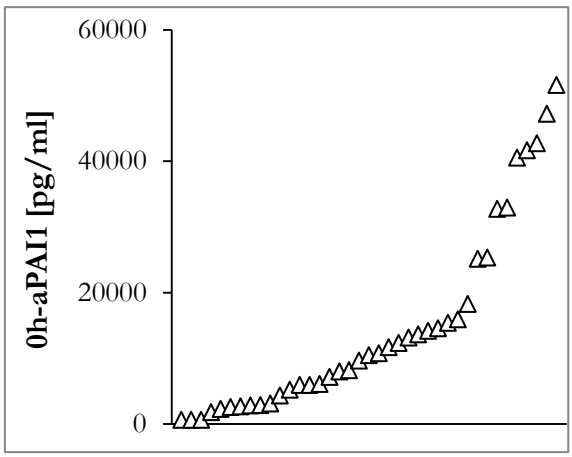

Figure 19. Fasting aPAI1 Levels

Fasting aPAI1 levels sorted according to size for all 39 subjects.

In order to test whether these subjects with elevated aPAI1 differed from those individuals with low aPAI with respect to their InsRes and CVD risk, those nine individuals with elevated aPAI1, representing the highest quartile, were compared with nine age- and sex-matched subjects exhibiting the lowest aPAI1 levels of this cohort (lowest quartile). Table 22 displays the characteristics of the two quartiles and the results of their comparison.

The two quartiles showed significant differences in accordance with the correlations listed in Table 21. More precisely, subjects with high aPAI1 were more obese (higher BMI and waist) and more insulin resistant (higher 0h-insulin and HOMA-IR) than those with low aPAI1, whereas leptin was only tendentially higher in the highest aPAI1 quartile. Fasting ADMA, which inhibits the NO synthase and is a marker for increased CVD risk (Anderssohn M, 2010), was also significantly higher in individuals with high aPAI1 than in those with low aPAI1 (0.60±0.03µmol/l vs. 0.52±0.03µmol/l, p=0.040). Unfortunately, BP was not measured at the second test day before the OFTT. However, DBP measured at the first test day before the OGTT was significantly higher in those individuals with higher aPAI1 (85.7±4.0mmHg vs. 74.8±1.3mmHg ,p=0.028). In conclusion, higher aPAI1 was related to higher parameters of adiposity, InsRes and CVD risk in healthy offspring of T2D patients.

	Highest Quartile	Lower Quartile	p
Age [years]	50.3±1.9	49.7±2.0	0.82
Obesity			
BMI [kg/m²]	**28.1±1.5**	**22.6±0.9**	**0.008**
Waist [cm]	**92.8±4.0**	**78.3±3.8**	**0.019**
WHR	0.883±0.03	0.818±0.03	0.15
InsRes			
Glucose [mg/dl]	96.9±3.3	95.6±2.0	0.73
Insulin [µU/ml]	**9.87±1.6**	**4.68±0.58**	**0.013**
HOMA-IR	**2.37±0.39**	**1.12±0.15**	**0.013**
Lipids			
TAG [mg/dl]	101±9.9	93.8±26	0.80
TC [mg/dl]	179±13	178±10	0.92
LDL-C [mg/dl]	124±9.9	113±7.5	0.39
HDL-C [mg/dl]	64.7±7.0	58.7±4.0	0.46
Hormones			
Adiponectin [ng/ml]	15948±2761	18895±3055	0.48
Resistin [pg/ml]	9083±761	10390±669	0.22
Leptin [pg/ml]	29691±11364	6478±1757	0.077
Inflammation Marker			
aPAI1 [pg/ml]	**37757±3096**	**5064±1070**	**<0.0001**
IL6	7.41±1.1	12.8±4.2	0.24
TNFα	2.39±0.3	2.09±0.3	0.51
MCP1	138±17	104±13	0.14

Table 22. Characteristics of aPAI1 Quartiles

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two quartiles. Significant differences ($p<0.05$) are shown in boldface. All values were measured in the fasting state.

Fatty Acids and Active PAI1

Since both aPAI1 and the fasting plasma FA pattern play a potential role during the development of T2D and CVD risk, it was tested whether they are associated with each other. This was studied using the same aPAI1 quartiles as before. The results of the comparison are presented in Table 23.

[%]	Highest Quartile	Lowest Quartile	p
SFA			
Myristic	1.40±0.11	1.33±0.17	0.74
Palmitic	22.6±0.39	22.3±0.54	0.57
Stearic	7.17±0.12	7.33±0.26	0.57
Arachidic	0.297±0.01	0.314±0.02	0.48
Behenic	0.792±0.03	0.846±0.05	0.38
Lignoceric	0.631±0.02	0.684±0.03	0.19
MUFA			
Myristoleic	0.127±0.02	0.153±0.03	0.43
Palmitoleic	2.44±0.24	2.06±0.21	0.25
Oleic	20.2±0.58	19.8±1.2	0.77
Vaccenic	1.69±0.09	1.76±0.08	0.61
Eicosenoic	0.167±0.01	0.178±0.02	0.66
Nervonic	1.53±0.09	1.62±0.13	0.58
ω6-FA			
LA	26.4±1.2	29.8±1.6	0.10
EDA	0.174±0.01	0.205±0.03	0.31
GLA	**0.808±0.10**	**0.463±0.10**	**0.025**
DGLA	1.90±0.12	1.61±0.15	0.16
AA	6.28±0.35	5.79±0.51	0.44

Table 23. Plasma FA Pattern of aPAI1 Quartiles

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two quartiles. Significant differences ($p<0.05$) are shown in boldface. All values were measured in the fasting state. FA abbreviations are listed in Supplemental Table 3.

None of the SFA or MUFA differed between the two aPAI1 quartiles. Among ω6-FA, only GLA differed significantly and was higher in individuals with higher aPAI1 (Table 23, Figure 20).

ω3-Fatty Acids and Active PAI1

Plasma proportions of ω3-FA were compared for the two aPAI1 quartiles (Table 24). Surprisingly the proportions of the ω3-FA eicosatetraenoic acid (ETEA), EPA and DPA, which are normally considered to be anti-atherogenic, were significantly higher in individuals with higher aPAI1 (Table 23, Figure 20).

	Highest Quartile	Lowest Quartile	p
ALA	0.652±0.06	0.507±0.06	0.11
ETEA	**0.127±0.01**	**0.059±0.01**	**0.0002**
EPA	**1.54±0.16**	**0.77±0.16**	**0.0036**
DPA	**0.623±0.02**	**0.535±0.03**	**0.047**
DHA	2.38±0.21	1.86±0.18	0.078

Table 24. Plasma ω3-FA Pattern of aPAI1 Quartiles

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two quartiles. Significant differences ($p<0.05$) are shown in boldface. All values were measured in the fasting state.

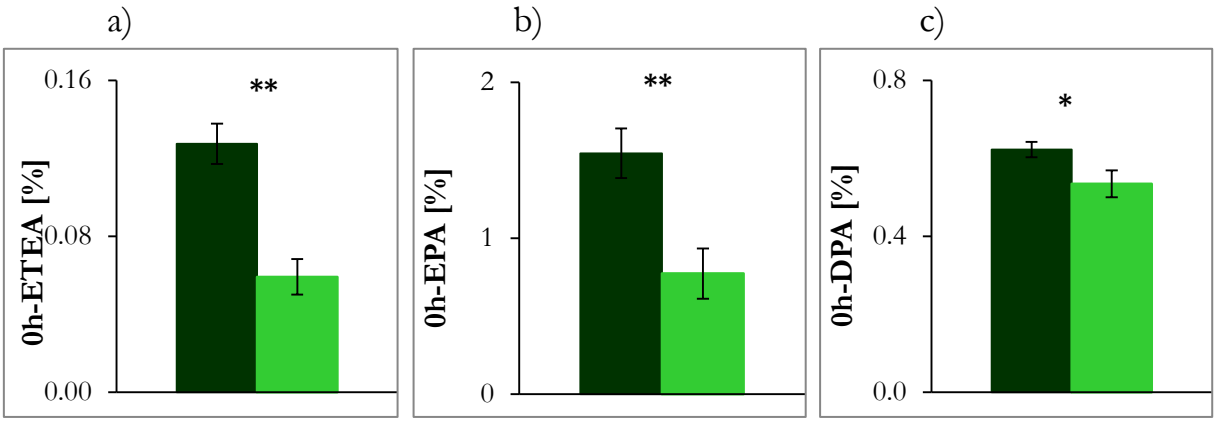

Figure 20. ω3-FA of aPAI1 Quartiles

Mean ±SEM proportions of the ω3-FA ETEA (a), EPA (b) and DPA (c) are shown for the highest and lowest aPAI1 quartiles in dark and light green, respectively. Significant differences as detected with a t-test are marked with * if $p<0.05$ and ** if $p<0.01$. Mean±SEM of the groups and p-values are listed in Table 24.

Postprandial Active PAI1

It is well described that plasma PAI1 concentrations decrease during the day and especially after the consumption of a meal. Therefore, the question arose whether these postprandial changes depend on the fasting PAI1 levels and if they are related to InsRes and postprandial changes of total plasma FA. For this purpose, the 39 subjects of the subgroup consumed a mixed fat-rich breakfast after an overnight fast as an OFTT (Study Population). Postprandial plasma data determined 2h and 4h after the consumption of the breakfast were compared for the same aPAI1 quartiles as used before.

The aPAI1 concentrations of the highest quartile decreased significantly in the first and the second postprandial phase (p=0.0022 and p=0.0043, respectively), whereas the aPAI1 levels of the lowest quartile decreased significantly only in the second postprandial phase (p=0.0032) (Figure 21). Beside the significantly steeper decrease of aPAI1 in those subjects with elevated fasting aPAI1 compared with those individuals with low fasting aPAI (p=0.0004 in the first postprandial phase and p=0.020 in the second postprandial phase), the aPAI1 levels exhibited in the highest quartile remained significantly higher in the postprandial state than aPAI1 concentrations in subjects of the lowest quartile (at 2h: 18170±2019pg/ml vs. 4329±764pg/ml, p<0.0001 and at 4h: 10412±1777pg/ml vs. 2294±442pg/ml, p=0.0016) (Figure 21).

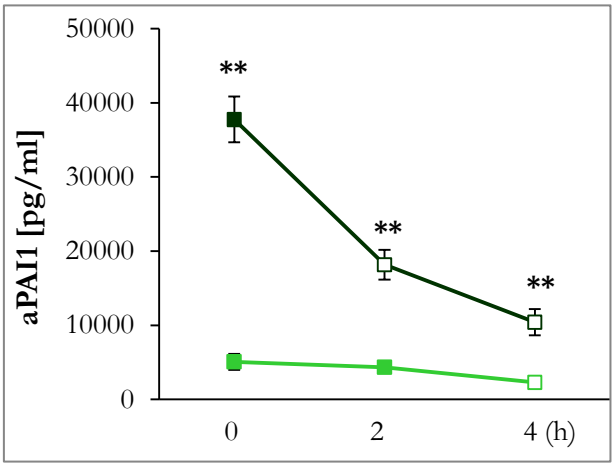

Figure 21. Postprandial aPAI1 of aPAI1 Quartiles

Mean±SEM postprandial plasma aPAI1 levels for the highest (dark green) versus lowest (light green) aPAI1 quartiles. Significant differences between the groups as detected with a t-test are marked with ** for p<0.01. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Postprandial Active PAI1 and Insulin Resistance

In order to analyze if the postprandial aPAI1 response was associated with InsRes, postprandial changes of insulin were compared for the aPAI1 quartiles. Individuals with elevated fasting and postprandial aPAI1 experienced a significantly steeper increase of insulin in the first postprandial phase and a slightly steeper decrease of insulin in the second postprandial phase (p=0.045 and p=0.090, respectively) (Figure 22). At the same time, their postprandial insulin levels were significantly

higher (at 2h: 76.0±16.7µU/ml vs. 33.19±3.7µU/ml, p=0.034 and at 4h: 27.9±6.39µU/ml vs. 8.29±1.17µU/ml, p=0.022) (Figure 22). Taken together with the higher HOMA-IR and fasting insulin levels, these results indicate that individuals with elevated aPAI1 are more insulin resistant.

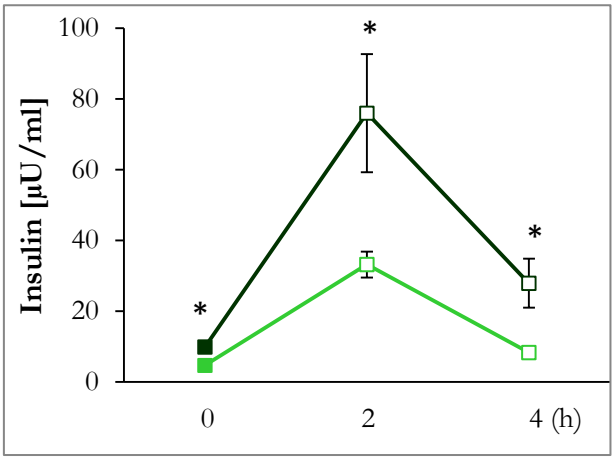

Figure 22. Postprandial Insulin of aPAI1 Quartiles

Mean±SEM postprandial plasma Insulin levels for the highest (dark green) versus lowest (light green) aPAI1 quartiles. Significant differences between the groups as detected with a t-test are marked with * for p<0.05. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Postprandial Fatty Acids and Active PAI1

Dietary FA were suggested to influence plasma PAI1 levels. Since specific fasting FA pattern were associated with elevated fasting aPAI1 in the present work, it was analyzed whether also postprandial FA profiles and changes were related to postprandial aPAI1 levels and changes. As displayed in Table 25, it was detected that postprandial plasma FA profiles were similar to those in the fasting state when compared for the aPAI1 quartiles.

None of the SFA and MUFA differed significantly between the two aPAI1 quartiles. Of the ω6-FA, 2h-EDA was significantly lower, whereas 2h- and 4h-GLA were significantly and 4h-DGLA was slightly higher in the highest quartile.

	2h			4h		
[%]	Highest Quartile	Lowest Quartile	p	Highest Quartile	Lowest Quartile	p
SFA						
Myristic	2.49±0.24	2.25±0.19	0.45	2.46±0.22	2.48±0.19	0.96
Palmitic	24.6±0.44	24.0±0.57	0.37	24.6±0.59	24.2±0.54	0.59
Stearic	7.56±0.13	7.68±0.20	0.63	7.64±0.14	7.65±0.21	0.98
Arachidic	0.267±0.010	0.288±0.015	0.26	0.269±0.012	0.282±0.012	0.44
Behenic	0.687±0.032	0.733±0.039	0.38	0.695±0.029	0.727±0.024	0.41
Lignoceric	0.537±0.025	0.577±0.025	0.27	0.540±0.020	0.571±0.021	0.31
MUFA						
Myristoleic	0.234±0.027	0.208±0.023	0.48	0.250±0.028	0.249±0.031	0.97
Palmitoleic	2.39±0.20	2.06±0.19	0.25	2.29±0.19	2.03±0.20	0.36
Oleic	20.9±0.63	20.8±1.0	0.94	20.2±0.56	20.4±0.72	0.88
Vaccenic	1.76±0.099	1.85±0.071	0.45	1.77±0.073	1.82±0.054	0.56
Eicosenoic	0.187±0.010	0.209±0.017	0.31	0.182±0.008	0.202±0.009	0.12
Nervonic	1.28±0.068	1.39±0.12	0.45	1.44±0.12	1.47±0.12	0.82
ω6-FA						
LA	24.0±1.2	27.2±1.3	0.097	24.3±1.20	27.2±1.1	0.096
EDA	**0.174±0.009**	**0.223±0.019**	**0.042**	0.197±0.017	0.237±0.020	0.15
DGLA	1.71±0.12	1.44±0.11	0.12	1.73±0.088	1.45±0.099	0.056
GLA	**0.731±0.098**	**0.421±0.088**	**0.032**	**0.712±0.091**	**0.414±0.084**	**0.029**
AA	5.63±0.37	5.23±0.43	0.49	5.79±0.41	5.24±0.36	0.33

Table 25. Postprandial Plasma FA Pattern of aPAI1 Quartiles

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two quartiles. Significant differences ($p<0.05$) are shown in boldface. FA abbreviations are listed in Supplemental Table 3.

Postprandial ω3-Fatty Acids and Active PAI1

The ω3-FA were again analyzed separately. As indicated in Table 26, all of the ω3-FA were significantly or at least slightly higher in postprandial plasma of individuals with elevated fasting aPAI1. The most significant differences were detected for ETEA and EPA (Figure 23). When the changes of the proportional FA were compared in the first and second postprandial phase, only the change of ETEA in the first postprandial phase differed significantly between the two quartiles with a steeper decrease in subjects of the highest aPAI1 quartile ($p=0.033$) (Figure 23).

	2h			4h		
[%]	Highest Quartile	Lowest Quartile	p	Highest Quartile	Lowest Quartile	p
ALA	0.633±0.049	0.514±0.039	0.078	0.592±0.041	0.492±0.035	0.082
ETEA	**0.113±0.009**	**0.055±0.008**	**0.0003**	**0.114±0.008**	**0.056±0.008**	**<0.0001**
EPA	**1.38±0.13**	**0.713±0.16**	**0.0049**	**1.41±0.16**	**0.727±0.16**	**0.0081**
DPA	0.579±0.022	0.519±0.036	0.18	**0.609±0.024**	**0.523±0.029**	**0.037**
DHA	2.13±0.15	1.69±0.18	0.079	2.14±0.18	1.66±0.18	0.084

Table 26. Postprandial Plasma ω3-FA Pattern of aPAI1 Quartiles

Data are presented as mean±SEM. P-values were calculated using t-test to compare the two quartiles. Significant differences ($p<0.05$) are shown in boldface.

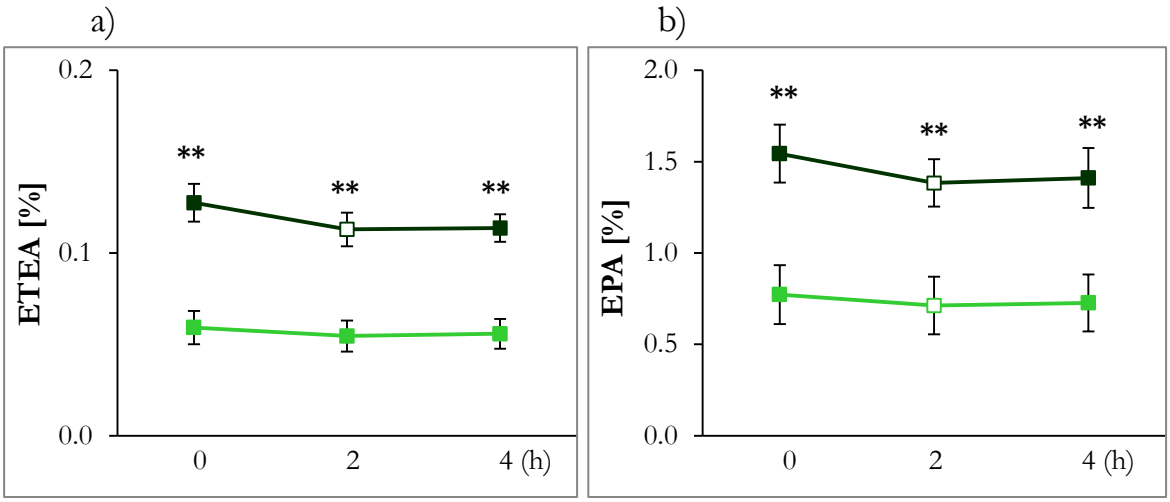

Figure 23. Postprandial ω3-FA of aPAI1 Quartiles

Mean±SEM postprandial plasma ETEA (a) and EPA (b) levels for the highest (dark green) versus lowest (light green) aPAI1 quartiles. Significant differences between the groups as detected with a t-test are marked with ** for $p<0.01$. Unfilled symbols represent significant differences from the previous time point as detected with a paired t-test.

Postprandial Changes of Active PAI1

To reveal which of the proportional FA and InsRes parameters changed in line with aPAI1 in the first postprandial phase, a factor analysis was performed for all 39 subjects (Table 27). Nearly all FA proportions loaded on the first two factors which is not surprising since an increase in one FA leads to a decrease in another FA when both are measured in percent. Furthermore, the changes of glucose and insulin in the first postprandial phase loaded together on the one factor. Leptin loaded inversely on the same factor which means that the changes of glucose and insulin were associated with each other and correlated inversely with the changes of leptin. The change of aPAI1 in the first postprandial phase loaded on the same factor as the changes of adiponectin, ETEA [%] and EPA [%] in the first postprandial phase leading to the assumption that in the early postprandial phase aPAI1 is regulated by the same mechanism as adiponectin, ETEA [%] and EPA [%].

	Loadings of variables by factor						
	1	2	3	4	5	6	7
Behenic [%]	0.93						
Oleic [%]	-0.92						
Lignoceric [%]	0.90						
AA [%]	0.85						
DGLA [%]	0.77						
Vaccenic [%]	-0.77						
Arachidic [%]	0.75						
Nervonic [%]	0.73						
ALA [%]	-0.72						
LA [%]	0.71	0.60					
Eicosenoic [%]	-0.68				0.53		
DHA [%]	0.58						
Myristic [%]		-0.80					
Myristoleic [%]		-0.77					
Palmitic [%]	-0.55	-0.73					
DPA [%]		0.56					
Leptin			0.85				
Insulin			-0.80				
Glucose			-0.72				
aPAI1				**0.81**			
Adiponectin				**0.73**			
EPA [%]				**0.63**			
ETEA [%]				**0.52**			
Stearic [%]					0.86		
Palmitoleic [%]	-0.51				-0.68		
EDA [%]						0.89	
ADMA							0.92
Eigenvalues	8.73	3.86	2.64	2.57	2.23	1.39	1.31
% Total variance	31.18	13.79	9.43	9.20	7.97	4.97	4.68

Table 27. Factor Analysis with aPAI1, FA and InsRes Parameters

All values are changes in the first postprandial phase. Loadings < 0.5 were not recorded. Those parameters showing the strongest correlations with aPAI1 and their corresponding loadings are presented in boldface. GLA did not load on any of the factors. FA abbreviations are listed in Supplemental Table 3.

Afterwards, a linear multiple stepwise regression analysis using the same parameters as above was performed for 39 individuals to detect those parameters explaining most of the variance of the change of aPAI1 in the first postprandial phase. The results in Table 28 show that individuals with the strongest decrease of EPA [%], adiponectin and increase of insulin in the first postprandial phase had the steepest decrease of aPAI1 at the same time.

Independent Variable	% of variance explained	p	β
EPA [%]	40.1	<0.001	0.531
Adiponectin	9.5	0.010	0.315
Insulin	5.4	0.049	-0.236
	55.0		

Table 28. Regression for the Postprandial Change of aPAI1

Linear multiple stepwise regression with change of aPAI1 in the first postprandial phase as dependent variable. Changes of glucose, leptin, ADMA, and all other FA [%] were used as independent variables however not included in the model.

Taken together, healthy offspring of T2D patients exhibiting elevated fasting aPAI1 had elevated fasting and postprandial proportions of ω3-FA, especially ETEA and EPA. They also showed a steeper postprandial decrease of aPAI1, especially in the first postprandial phase, which was associated with a more prominent decrease of EPA [%] and adiponectin and a greater increase of insulin at the same time. In conclusion, the behavior of aPAI1 is strongly linked to InsRes and the levels and changes of EPA [%].

4 Discussion

In the present study, 91 healthy offspring of T2D patients participated in an OGTT and a subset of the cohort underwent an OFTT with a mixed fat-rich meal as a fat load. The extensive lipid dataset, generated using fasting and post-challenge plasma samples from OGTT and OFTT, was analyzed here using univariate and multivariate statistical methods. The present study was an explorative study to find potential new associations and biomarkers and generate hypotheses regarding the underlying mechanisms. In the past, T2D risk and cardiovascular risk factors derived from clinical parameters have been addressed in large prospective studies focusing on risk of T2D and CVD, for example the Framingham heart study (Framingham Heart Study, 2010) and the Prospective Cardiovascular Münster Study (PROCAM, 2010). The potential role of plasma FA as risk factors has also been investigated in cross-sectional studies, as summarized by Vessby (Vessby B, 2003), as well as in prospective studies, the Uppsala Longitudinal Study of Adult Men (ULSAM, 2010), the Kuopio ischaemic heart disease risk factor study (KIDH, 2010) and in the Atherosclerosis Risk in Communities Study (ARIC, 2010). In these studies it was established that specific FA measured in fasting plasma correlate with metabolic disease parameters. The incentive to perform the present analysis was that the mechanisms and the metabolic implications causing these FA correlations are only incompletely understood (Vessby B, 2003), and that FA and other lipids have been studied less frequently in postprandial samples.

4.1 Lipids and Insulin Resistance

Fatty Acids

In the first part of the present analysis, the association of plasma FA with parameters of InsRes was investigated. Some of the results, especially for SFA and MUFA, have been observed in previous studies. For instance, palmitic acid was elevated and LA was reduced in subjects with high plasma insulin (Vessby B, 2003). As discussed in these publications, the increase in palmitic acid may be caused by higher

dietary intake of SFA or by increased *de novo* synthesis in insulin resistant individuals (Petersen KF, 2007; Hudgins LC, 2000). Similarly, the negative correlation of LA with InsRes may be a result of both low consumption of vegetable oil or increased enzymatic conversion of LA into its derivates. That the conversion of FA can be an important determinant of MetSyn and CVD is supported by recent publications demonstrating a strong effect of genetic polymorphisms in FA converting enzymes on plasma FA patterns (especially ω6-FA) (Williams ES, 2007; Lattka E, 2010).

The present study was not specifically designed to distinguish between nutritional factors and endogenous FA synthesis. The analysis focused on associations of plasma FA proportions with InsRes in the fasting state. Most notably, some novel associations of FA with fasting plasma glucose are reported here. This is interesting because elevated fasting plasma glucose can be used as a marker of hepatic glucose production (Abdul-Ghani MA, 2006). Together with other results, these findings suggest that quantitative alterations in individual FA can be used as biomarkers to dissect changes in metabolic pathways taking place during the development of hyperinsulinemia and InsRes.

ω6-Fatty Acids

One key finding of the present work was that the ω6-FA DGLA and EDA correlated negatively with fasting glucose (Table 6). At the same time, DGLA correlated strongly with HOMA-%B, due to the negative correlation with fasting glucose and a positive correlation with fasting insulin (Table 9). DGLA was described to be increased in plasma of metabolic risk subjects in many previous studies (Pelikanova T, 2001; Salomaa V, 1990; Vessby B, 1994). The mechanism responsible for DGLA elevation in metabolic disease is likely to include alterations in the FA conversion pathway leading from LA via GLA to DGLA (Figure 4). In the present work, the estimated activity of the FA elongase synthesizing DGLA (Moon Y, 2009) and also EDA (Inagaki K, 2002) (ELOVL5) correlated negatively with fasting glucose and positively with HOMA-%B (Table 6), suggesting a rise of the enzyme in individuals

with low fasting glucose. The concept that ELOVL5 regulates DGLA levels is consistent with a recent study reporting elevated plasma DGLA after adenoviral overexpression of ELOVL5 in livers of mice (Wang Y, 2008). Interestingly, the mice with increased liver ELOVL5 also had reduced fasting plasma glucose, increased liver glycogen content and improved hepatic insulin signaling. Another recent study in obese mice detected that increasing hepatic ELOVL5 activity restored plasma glucose and insulin, HOMA-IR and glucose tolerance to normal values (Tripathy S, 2010). The same study also revealed that elevated ELOVL5 activity suppressed levels of several enzymes and transcription factors involved in the hepatic glucose production.

These data suggest that the negative correlation of plasma DGLA with fasting glucose observed in the present cohort reflects a beneficial effect of DGLA on hepatic glucose production. Interestingly, eicosanoids derived from DGLA were shown to exert anti-inflammatory effects (Fan YY, 1998). Since inflammation is an important contributor to InsRes (Hotamisligil GS, 2006), it is possible that DGLA exerts insulin-sensitizing effects through reduction of inflammation.

The mechanism causing the positive correlation of DGLA with insulin levels observed here and in previous studies may involve insulin-dependent activation of the transcription factor SREBP1c (Hegarty BD, 2005). SREBP1c binds directly to and trans-activates the ELOVL5 gene (Qin Y, 2009).

In summary, the following model is proposed to explain the reciprocal correlation of insulin and glucose with DGLA (Figure 24). Hyperinsulinemia resulting from peripheral InsRes leads to elevation of liver ELOVL5 activity through SREBP1c activation. DGLA improves liver glucose metabolism through an unidentified mechanism. In other words, ELOVL5 induction is a (but not the only) mechanism whereby insulin suppresses hepatic glucose production.

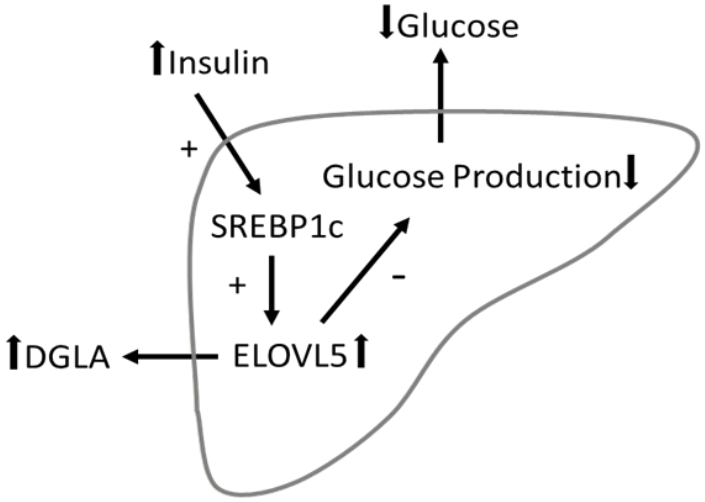

Figure 24. Model for Relation of DGLA with 0h-Glucose and 0h-Insulin

Hyperinsulinemia stimulates ELOVL5 in the liver through activation of SREBP1c, leading to higher DGLA levels. ELOVL5 rise leads to a decrease in hepatic glucose production and fasting blood glucose through an unknown mechanism.

The observed negative correlation of DGLA with fasting plasma glucose has not been described before. However, similar to the results presented here, one study (Salomaa V, 1990) reported a negative correlation of fasting glucose with estimated ELOVL5 activity. In contrast, another study, including healthy volunteers and T2D patients, detected a positive correlation of plasma DGLA with both fasting glucose and insulin (Lovejoy JC, 2001). One reason for the variation among the studies might be the different composition of the cohorts. The subjects of the present study were selected based on genetic predisposition, included a high number of subjects with IFG (37%), and exhibited a higher variation of fasting plasma glucose levels (female mean ± SD: 92.7 ± 10.1 mg/dl; male mean ± SD: 102.9 ± 9.3 mg/dl) compared to the cohort studied by Lovejoy et al. (Lovejoy JC, 2001). Another reason might be that in this offspring cohort fasting insulin and glucose did not correlate significantly with each other ($R=0.13$, $p=0.21$). In cohorts exhibiting a significant positive correlation of the two parameters a causal relationship of DGLA with glucose may be present which, however, could be masked by a stronger, positive correlation of DGLA with insulin. Clearly, the association of DGLA with fasting glucose needs to be confirmed in other cohorts. Also, the hypothesis

that DGLA is related to a mechanism reducing hepatic glucose production needs to be tested experimentally in glucose clamp and tracer infusion experiments.

ω3-Fatty Acids

One unexpected finding of this work was that fasting plasma proportions of ω3-FA were positively associated with plasma glucose levels in offspring of T2D patients. More precisely, plasma proportions of EPA and DPA were positively correlated with fasting glucose (Table 7). In addition, DHA correlated specifically with 2h-glucose and 2h-insulin (Table 13). In fact, only 2h-insulin and fasting leptin but no other FA or metabolic parameter showed a stronger correlation with 2h-glucose ($R=0.60$, $p<0.0001$ and $R=0.42$, $p<0.0001$, respectively). Whereas fasting glucose can be used as a marker for the hepatic glucose production, 2h-glucose can be used as a marker for the insulin-dependent peripheral glucose disposal (Abdul-Ghani MA, 2006). Thus, EPA and DPA were positively associated with hepatic InsRes and DHA was positively associated with peripheral InsRes.

The associations of EPA, DPA and DHA with glucose tolerance and InsRes reported in the literature are controversial. Some studies found no association of these ω3-FA with IGT, T2D or MetSyn (Warensjö E, 2005; Wang L, 2003; Salomaa V, 1990; Zhou YE, 2009), whereas others found moderately higher (Pelikanova T, 2001; Hodge AM, 2007) or lower (Yang J, 2004) EPA, DPA, and DHA in plasma of T2D patients. However, these studies did not explicitly distinguish between fasting and 2h-glucose.

It is not clear, why the plasma proportions of long-chain ω3-FA in the present and other studies correlate with IGT and InsRes. It may be that an elevation of ω3-FA is simply caused by lower intake of LA, a ω6-FA linked to InsSen and metabolic health (Summers LK, 2002). Since ω3- and ω6-FA compete for the same elongases and desaturases (Figure 4), low levels of LA result in enhanced conversion of ALA to longer-chain ω3-FA (Vessby B, 2002). In line with this hypothesis, a reciprocal

correlation pattern was observed for 0h-glucose with DGLA (Table 6), a major ω6-FA conversion product, compared with the corresponding ω3-FA, EPA and DPA (Table 7). Furthermore, this reciprocal correlation pattern was also detected when data of an interview-based dietary questionnaire in the present study were analyzed. For instance, fish consumption correlated positively with the ω3-FA ETEA ($R=0.29$, $p=0.064$) and EPA ($R=0.46$, $p<0.0001$) and negatively with the ω6-FA EDA ($R=-0.42$, $p<0.0001$) and DGLA ($R=-0.35$, $p=0.0008$). Unfortunately, the dietary data were not precise enough and did not include consumed quantities. Thus, they supported the measured plasma FA pattern but could not be used for further more detailed analyses.

However, DHA and other ω3-FA may also cause IGT and IFG by themselves to some degree, as suggested by some ω3-FA supplementation studies. More precisely, whereas studies including healthy individuals did not find an effect of ω3-FA supplementation on glucose tolerance, InsSen and secretion (Giacco R, 2007; Egert S, 2008), different results were reported for T2D patients who had decreased TAG and increased fasting glucose levels after the supplementation of DHA (Friedberg CE, 1998; Woodman RJ, 2002). It was further shown that fish oil supplementation led to a reduction in plasma TAG levels and an increase in glycerol gluconeogenesis (Puhakainen I., 1995) which might influence plasma glucose levels. Self-assessed fish consumption of the offspring cohort studied here correlated positively with fasting plasma glucose levels ($R=0.23$, $p=0.026$) further supporting the positive association of the plasma EPA and DPA with 0h-glucose. Taken together the present analysis and previously published papers suggest that ω3-FA supplementation does not improve InsRes and may actually worsen glucose homeostasis.

Phospholipids

Most FA species are more frequent in some lipid classes than in others. For example, oleic acid is enriched in TAG, whereas arachidonic acid (AA) is enriched in PL

such as PC. The analysis of total plasma FA patterns carries, therefore, the risk of reporting differences in lipid class profile rather than regulation of FA pathways. In order to rule out such effects, and also to test the suitability of PL as potential plasma biomarkers, plasma PC and LPC species were profiled and in the present study, their associations with InsRes and glucose tolerance were determined.

Those species containing EDA and DGLA, EPA or DHA showed a correlation pattern very similar to total plasma EDA, DGLA, EPA and DHA, respectively (Table 9, Table 14). In general, the statistical association of the above-mentioned PC and LPC species were better compared to the total plasma FA. Although they reflect the total FA pattern, they appear to be better suited as biomarkers to establish metabolic risk.

4.2 Postprandial Triacylglycerol and Insulin Resistance

Lipid Response during an OFTT

Another part of this work dealt with postprandial lipid changes in an OFTT, using a mixed fat-rich breakfast as a physiological lipid load, and their associations with InsRes and MetSyn. It was found that among the postprandial lipid parameters studied, total plasma TAG showed most significant correlations. The most profound differences between the study subjects, and most correlations, were found for TAG responses in the second postprandial phase, between two and four hours after the fat load. Whereas in some subjects TAG and TRL already decreased, they further increased in other individuals (Figure 9).

Individuals with InsRes (Branchi A, 2006), IFG, IGT or T2D (Rubin D, 2010) often exhibit higher postprandial TAG responses. Several previous studies discussed potential influences and mechanisms leading to elevated postprandial TAG levels in InsRes, T2D and obesity (Branchi A, 2006; Harris WS, 1989; Westphal S, 2002; Kriketos AD, 2003) including higher synthesis of VLDL and slower clearance of TRL particles. The suggested primary mechanism underlying the rise in fasting TAG concentrations is increased hepatic synthesis and secretion of VLDL (Nestel

PJ, 1984). This mechanism was proposed to also play a role in postprandial states (Brown AJ, 1991; Harris WS, 1988) although it was doubted if this mechanism takes place in normotriacylglyceridemic subjects (Harris WS, 1988). More precisely, it was hypothesized that at increased levels VLDL compete with CM for LPL-mediated lipolysis (Bjorkegren J, 1996) resulting in a slowed TRL clearance. The present study was not designed to analyze VLDL secretion or LPL turn-over rates, which is a laborious method not suited for epidemiological studies. However, since neither the fasting nor the 2h lipoprotein patterns differed in plasma of the two OFTT groups (Figure 10), one can argue that differences in TAG decline in the second postprandial phase are unlikely to be caused by differences in VLDL secretion and competition for LPL.

A second hypothesis states that enhanced LPL activity and therefore, TAG clearance, is present in individuals with increased decline of TAG. When the study design does not allow to measure LPL activity directly, the ratio apoCII/apoCIII is often used as an estimator for it since apoCII stimulates and apoCIII inhibits LPL (Ginsberg HN, 2005; Shachter NS, 2001). Analysis of the present data set found that neither apoCII and apoCIII alone nor their ratio differed between the OFTT groups, arguing that apoCII and/or apoCIII are not responsible for the different TAG decline in the second postprandial phase. ApoE is an apolipoprotein needed for the hepatic uptake of TRL via receptor-mediated endocytosis (Heeren J, 2001). Thus, ApoCIII and apoE have opposite roles on the hepatic uptake and clearance rate of TRL so that the overall removal of TRL from the plasma can estimated as the balance of apoCIII and apoE (Olivieri O, 2007; Taskinen MR, 2003). During the OFTT, the ratio apoE/apoCIII was lower in group 1 indicating that lower clearance might be the reason for the increasing TAG of these subjects in the second postprandial phase. This result is in line with a previous study detecting lower apoE/apoCIII in individuals with MetSyn (Olivieri O, 2007). Postprandial lipids were not measured in that study. However, it is known that MetSyn is accompanied with postprandial dyslipidemia. The only apolipoprotein which was significantly

different by itself between the OFTT groups was ApoAII, an apolipoprotein previously shown to be associated with higher postprandial TAG and TRL responses (Delgado-Lista J, 2007). ApoAII was shown to impair LPL-mediated TRL lipolysis in mice and men (Julve J, 2010), suggesting that impaired LPL-mediated TRL clearance is part of the mechanism leading to delayed TAG decline in Group 1.

Fatty Acids and Postprandial Triacylglycerol

In addition to the unfavorable apolipoprotein pattern, the FA pattern in fasting and postprandial plasma TAG of group 1 was marked by higher SFA and lower ω3-FA and ω6-FA proportions (Table 16). It has been established in several previous studies that dietary intake of ω3-FA decreases not only fasting but also postprandial TAG (Harris WS, 1988; Harris WS, 1989; Brown AJ, 1991). The mechanism underlying the postprandial effect of ω3-FA has not been fully dissected. A stimulatory effect of dietary ω3-FA on LPL gene expression and activity or clearances rates of fat emulsion was found in some studies (Park Y, 2003; Khan S, 2002) but not in others (Harris WS, 1993; Harris WS, 1988). This suggests that ω3-FA-dependent alteration in LPL activity may be one of the mechanisms involved in the TAG differences in the second phase of the OFTT.

Risk for Type 2 Diabetes and Postprandial Triacylglycerol

Surprisingly, InsRes or obesity did not seem to play a role in postprandial lipid responses since waist, insulin and glucose values were similar in both OFTT groups (Figure 13). This is in contrast to results of other studies which showed that insulin resistant individuals (Branchi A, 2006) and subjects with IFG, IGT or T2D (Rubin D, 2010) have higher postprandial TAG. However, the present cohort was in general healthier (less obese, less InsRes) and younger. It might be that the observed postprandial dyslipidemia occurs prior to other metabolic changes associated with MetSyn and InsRes. Furthermore, it is possible that ω3-FA-related mechanisms, as discussed above, are underlying the different TAG kinetics in OFTT group 1 and group 2. In this case, one would not necessarily expect a strong correlation of post-

prandial TAG kinetics with InsRes parameters, since ω3-FA supplementation in humans appears not to improve InsRes (Giacco R, 2007; Egert S, 2008).

Lipid Response during an OGTT

In order to analyze the effect of insulin on the lipid metabolism independent of a fat-stimulus and to search for a better marker than postprandial TAG in an OFTT, an OGTT was performed. Group analyses revealed that those individuals with increasing TAG during the OGTT had lower fasting TAG and TAG content in lipoproteins which increased in line with TAG during the OGTT (Table 18, Figure 15). These subjects also had slightly lower HDL2-C which tended to decrease during the OGTT (Table 18, Figure 16). Apolipoproteins were not measured at the first test day so that conclusions concerning the TAG lipolysis and clearance could not be done as in the OFTT analysis. However, lower HDL2-C levels indicate a lower clearance in those subjects with increasing TAG and TAG-containing lipoproteins during the OGTT.

Risk for Type 2 Diabetes and Post-Glucose Triacylglycerol

Although standardized OGTTs like the one used here are routinely performed in clinical diagnosis and research, only a few studies have reported the change of TAG during an OGTT (Nakatsuji H, 2009; Ogita K, 2008; Moore MC, 2000). These studies showed that, on average, the total plasma TAG levels decreased during the two hours after the glucose load. This is in agreement with the TAG decline found for the majority of individuals analyzed in the present study.

The mechanism which is responsible for the decline of TAG during an OGTT is likely dominated by insulin. In subjects, plasma insulin levels increased in response to a rise in plasma glucose levels during an OGTT (Abdul-Ghani MA, 2006; Moore MC, 2000; Ogita K, 2008). The rising insulin concentrations suppressed VLDL-T and apoB production in healthy subjects (Lewis GF, 1993) and could activate LPL (Ginsberg HN, 2005) and LDL-R (Streicher R, 1996) thereby leading to an in-

creased TAG clearance. The lower VLDL-T production and higher TAG clearance resulted in decreasing plasma TAG concentrations during an OGTT.

However, in the present study, plasma TAG levels increased in a minority of subjects during an OGTT (Figure 10). Similar results were obtained in obese subjects during an OGTT (Nakatsuji H, 2009) and obese and T2D individuals during acute hyperinsulinemia (Lewis GF, 1993; Pelikánová T, 1991; Soriguer F, 2008). Whereas TAG production declined equally in obese and control subjects, insulin was unable to inhibit apoB production in obese individuals (Lewis GF, 1993). It was further suggested that InsRes in obese subjects prevented the sufficient activation of LPL (Lewis GF, 1993; Nakatsuji H, 2009; Pelikánová T, 1991; Soriguer F, 2008). In the present study, subjects with increasing TAG during an OGTT were more obese and insulin resistant, as shown by higher BMI, WHR, waist, HOMA-IR, fasting insulin and leptin, than those subjects most pronouncedly decreasing TAG after an oral glucose load (Table 19). Thus, obesity and InsRes seemed to prevent the successful decline of TAG following an oral glucose load.

This association of the TAG change in the OGTT (G_Δ_TAG) with obesity and insulin resistance was also apparent when the whole study cohort was included in a correlation analysis. Surprisingly, the changes of TAG during the OGTT correlated significantly and in some cases even better with parameters of InsRes and MetSyn than fasting TAG which was in contrast to the results of the OFTT. It seems that G_Δ_TAG is better associated with obesity and fasting glucose, whereas fasting and especially 2h-TAG correlate a little bit better with fasting insulin and BP. Remarkably, fasting TAG and G_Δ_TAG did not correlate with each other further demonstrating that these two TAG parameters hold different metabolic information. None of the other studies reported the association of TAG change during an OGTT with several obesity and InsRes parameters. Only Nakatsuji et al. observed an association with abdominal obesity (Nakatsuji H, 2009).

These results indicate that TAG changes during an OGTT might be valuable marker for the early detection of InsRes which seems to be superior to fasting TAG. In addition, the measurement of TAG during OGTT is easy to achieve and unexpensive. However, larger prospective studies are necessary to verify these results.

4.3 Active PAI1 and Cardiovascular Risk

Active PAI1 and Insulin Resistance

Elevated plasma levels of tPAI1 are well-known predictors for T2D (Festa A, 2002) and CVD (Poli KA, 2000; Folsom AR, 2001). Moreover, PAI1 antigen concentrations and PAI1 activity were reportedly higher in offspring of T2D patients in comparison to controls (Gürlek A, 2000). In addition to its associations with T2D and CVD risk, tPAI1 was also associated with age, obesity, InsRes, dyslipidemia (higher TAG and LDL-C, lower HDL-C concentrations), inflammation (IL6), and hypertension (Kraja AT, 2007; Folsom AR, 2001). Similar correlations with age, obesity and InsRes were also found for aPAI1 in the present analysis (Table 21). However, associations between aPAI1 and dyslipidemia or inflammation were not significant or only detected as trends. A possible explanation for this discrepancy is that the number of subjects was too small (n=39) to detect significant correlations. However, it is also possible that aPAI1 and tPAI1 show different associations with T2D and CVD parameters. Supporting this hypothesis, tPAI1 correlated stronger with obesity and InsRes than aPAI1 (Table 5 vs. Table 21). In addition, tPAI1 correlated positively with fasting TAG and glucose ($R=0.42$, $p=0.0083$ and $R=0.36$, $p=0.023$, respectively). Since aPAI1 was less studied than tPAI1 in the past and is probably more important for the fibrinolysis, it was analyzed more closely in the present work.

Fatty Acids and Active PAI1

Since both aPAI1 and the fasting plasma FA pattern play a potential role during the development of T2D and CVD risk, it was tested whether they are associated with each other. This work demonstrates a clear positive association between fasting

aPAI1 and plasma proportions of PUFA (Table 23) and especially ω3-FA in healthy offspring of T2D patients (Table 24). On the other hand, SFA and MUFA proportions are unrelated to aPAI1 levels (Table 23). Similar associations were also found for fasting aPAI1 and postprandial FA (Table 25, Table 26).

Analysis of the aPAI1 changes following a mixed fat-rich breakfast revealed that elevated fasting aPAI1 levels led to a steeper postprandial decrease of aPAI1 (Figure 21). This was especially observed in the first postprandial phase. This steeper decrease is associated with a more prominent decrease of ETEA [%], EPA [%] (Figure 23) and adiponectin (Table 27) and greater increase of insulin (Figure 22) at the same time.

These results are contradictory in the sense that elevated PAI1 levels are used as a risk factor for CVD whereas higher intake and plasma proportions of ω3-FA are generally believed to be protective. The reciprocal association observed in the present analysis might be explained mechanistically by an increased production of PAI1 after the stimulation with DGLA and especially DHA in vitro (Karikó K, 1995). More precisely, it was shown that supplementation of cultured human endothelial cells with DGLA and DHA resulted in an increase of PAI1 mRNA levels. Hence, elevated concentrations of PUFA and especially ω3-FA may trigger an increase in PAI1 transcription levels and thereby also in plasma aPAI1 concentrations.

ω3-Fatty Acids and Active PAI1

So far, the relationship between PAI1 and plasma FA was studied only rarely in humans. One study found no association of tPAI1 antigen with the FA composition of erythrocyte PL, whereas the proportion of ω3-FA was inversely associated with tissue-type plasminogen activator (tPA) antigen (Scarabin PY, 2001). The aPAI1 conformation is able to form a complex with tPA and uPA (Dellas C, 2005; Lijnen HR, 2005). The PAI1-PA complex is then rapidly cleared via LRP1 (Dellas C, 2005). Almost all assays measure only the amount of unbound tPA or PAI1 an-

tigen. Therefore, an increase in PAI1 activity is accompanied by a decrease in t-PA antigen levels (Dellas C, 2005). Another study reported significant positive correlations after adjustment for metabolic confounders between PAI1 activity and GLA, EPA and DHA proportions measured in serum cholesterol esters (Byberg L, 2001), confirming the results of the present analysis. The differences between the study by Scarabin et al. and the present study might have occurred due to the different sites at which the FA were measured (erythrocyte PL in Scarabin et al. and total plasma in the present study). The participants of the study by Scarabin et al. comprised only men, several smokers and they were on average slightly older and more obese than the population studied in the present work (Table 3, Table 22). Furthermore, Scarabin et al. presented only relationships adjusted for BMI. All these factors could have contributed to the discrepant results.

The association between ω3-FA and aPAI1 suggests that increased ω3-FA consumption can be a cause for high plasma aPAI1 levels. Supplementation studies using long-chain ω3-FA or increased fish intake reported a rise in PAI1 activity, despite a reduction in plasma TAG (Emeis JJ, 1989; Boberg M, 1992). However, other studies did not find an effect of ω3 supplementation on PAI1 activity (Finnegan YE, 2003; Hansen JB, 2000) which led to a dispute (see summaries (Mutanen M, 2001; Hansen JB, 2000)). In some cases control groups received ω6-FA instead of ω3-FA. It was previously suggested (Byberg L, 2001) that the reason for the lack of difference in these studies might be a stimulatory effect of not only ω3-FA but also ω6-FA on PAI1 production as seen in vitro (Karikó K, 1995). This idea is supported by previous findings (Byberg L, 2001) and might explain the positive association of plasma GLA and several ω3-FA with aPAI levels in the present analysis. However, the association of ω3-FA with aPAI was more prominent here and the fish consumption, as stated at the previous test day, correlated significantly with fasting aPAI1 ($R=0.54$, $p=0.0004$, for $n=39$) also after adjustment for age, sex, waist, HOMA-IR.

Postprandial Active PAI1

Previous studies looked at effect of ω3-FA supplementation not only on fasting but also postprandial PAI1 concentrations. Some of these studies did not observe long-term (Delgado-Lista J, 2007; Jellema A, 2004) or acute (Montegaard C, 2010; Pacheco YM, 2006) effects of diets or meals containing ω3-FA on postprandial tPAI1, tPA or PAI1 activity. However, some studies need to be interpreted with caution since control groups might have consumed only water or ω3-FA were consumed together with carbohydrates or vegetables which might mimic the effect of ω3-FA in comparison to MUFA and SFA. The present study was not designed to analyze dietary effects on postprandial aPAI1 for two reasons. First of all, the FA pattern of the consumed meals did not differ (Supplemental Table 2). Secondly, the dietary questionnaire used here was not precise enough and did not include consumed quantities to allow a more detailed analysis of the long-term FA consumption.

Instead, the present work was the first which investigated postprandial aPAI1 in relation to postprandial plasma FA pattern and changes. A factor analysis was performed to analyze which FA proportions and metabolic parameters changed in line with aPAI1 in the first postprandial phase. The second postprandial phase was not considered since aPAI1 changed less prominently (Figure 21). It was revealed that the ω3-FA ETEA and EPA changed in line with aPAI in the first postprandial phase. Hence, the question arises whether aPAI1 is regulated together with ETEA and EPA in the postprandial state. Also, it was shown that adiponectin changed in a similar manner in the first postprandial phase, whereas insulin behaved in an opposite way to aPAI1, ETEA and EPA.

In contrast to adiponectin change, fasting adiponectin levels did not differ between the aPAI1 quartiles (Table 22). However, fasting insulin and postprandial insulin (Figure 22) suggest a lower InsSen in those subjects with high fasting aPAI1. This is supported by previous findings in pre-diabetic subjects in whom elevated tPAI1

levels were related to increased InsRes (Festa A, 2002). Elevated fasting adiponectin predicts higher InsSen and affects insulin-stimulated glucose disposal and lipoprotein metabolism (Tschritter O, 2003).

A potential role of insulin in this context is, however, still unclear. The results of one study showing that acute insulin infusion led to a decrease in PAI1 activity in healthy men (Landin K, 1992) are in line with the present results. It is well-known that the plasma concentrations of tPAI are highest in the morning and lowest in the late afternoon and evening, whereas t-PA activity exhibits an opposite diurnal variation (Lijnen HR, 2005). However, it is unknown whether this diurnal variation of tPAI is mainly due to a variation in PAI1 production or clearance. It was further shown that adiponectin also decreased in acute hyperinsulinemia (Möhlig M, 2002), during an OGTT and postprandially in overweight (Rubin D, 2008) and postprandially in T2D subjects (Annuzzi G, 2010). Annuzzi et al. also found decreasing adiponectin mRNA levels in adipose tissue of T2D subjects in the postprandial state. However, the mechanisms responsible for the postprandial decrease of adiponectin levels in plasma and mRNA in adipose tissue are unknown but might be the same or at least similar to the mechanism which are involved in the postprandial decrease of aPAI1.

For the present analysis this could mean that, despite the fact that those subjects with high fasting aPAI are more insulin resistant, their higher postprandial insulin levels might trigger the strong decrease of aPAI1 observed especially during the first postprandial phase.

However, other studies reported that insulin stimulated PAI1 expression in normal subjects (Carmassi F, 1999) as well as PAI1 biosynthesis and mRNA expression in cultured human hepatocytes (Kooistra T, 1989). These results are not necessarily contradictory. In the present study, acute postprandial effects on plasma aPAI1 concentrations were analyzed which are not only dependent on gene expression and protein synthesis but also on clearance rates. Moreover, it cannot be ruled out that the steep decrease in postprandial aPAI1 in one group of the present analysis is

caused by the higher fasting aPAI1 levels exhibited by the same group. Nevertheless, the present data are not sufficient enough to give an explanation for the differences in the postprandial changes of aPAI1 observed for the two aPAI1 quartiles.

It has been hypothesized that the stimulatory effect of ω3-FA on PAI1 levels and activity may be compensatory for a reduced thrombotic tendency in Greenland Eskimos naturally consuming a high seafood diet rich in ω3-FA (Schmidt EB, 1989). At the same time, these Greenland Eskimos had remarkably lower incidences of CVD and T2D compared to the general Danish population (Kromann N, 1980; Sagild U, 1966). As a result, the stimulatory effect of ω3-FA on PAI1 may not increase the risk for CVD because ω3-FA have a beneficial effect on the lipid and lipoprotein metabolism. This hypothesis is supported by findings in healthy men showing that fish oil supplementation decreases plasma TAG but does not affect other CVD risk factors (Damsgaard CT, 2008).

Taken together, aPAI1 is positively associated with InsRes and several risk factors for CVD but at the same time also with plasma ω3-FA. This somehow surprising relationship between aPAI1 and especially the plasma proportions of ETEA and EPA seems to play an important role concerning the changes occurring during the first postprandial phase. However, further studies in larger cohorts need to confirm these associations and investigate the underlying mechanisms.

5 Conclusion

Data from a healthy cohort of T2D offspring were analyzed to investigate relationships of lipid and FA pattern in an OGTT and OFTT with risk factors for T2D and CVD. Well-known associations of SFA and MUFA with metabolic risk markers could be confirmed. Interestingly, plasma proportions of the ω6-FA DGLA correlated positively with fasting insulin and negatively with fasting glucose. The ω3-FA EPA and DPA correlated inversely and were positively related to fasting glucose. Additionally, DHA was positively associated with 2h-glucose. This suggests that DGLA versus EPA and DPA are differently related to novel, not yet identified mechanisms of hepatic InsRes, whereas DHA is linked to mechanisms of peripheral InsRes.

Although postprandial plasma TAG levels were previously suggested as potential early risk markers for T2D and CVD, data of the present study could not confirm this hypothesis as TAG changes in the OFTT were associated with ω3-FA but not with other, well-established risk markers. In contrast, TAG changes in the OGTT were strongly linked to risk factors, indicating that TAG measurements during an OGTT and not during an OFTT might be valuable future marker for the early detection of InsRes and CVD risk.

Plasma aPAI1 levels exhibited well-known positive associations with InsRes parameters and several other risk factors for CVD. However, aPAI1 simultaneously correlated positively with several ω3-FA and changed in line with them during the first postprandial phase suggesting a common, underlying mechanism. This unknown mechanism needs further investigation and might reveal new potential therapeutic targets.

In summary, this leads to the hypothesis that the anti-atherogenic properties proposed for ω3-FA are mainly based on their beneficial effects on lipid metabolism but not on glucose metabolism and fibrinolysis. Furthermore, TAG changes during an OGTT are proposed as potential risk markers for InsRes and CVD.

Appendix

List of Supplemental Tables

Supplemental Tables

Breakfast A	Fat [g]	Carbohydrates [g]	Protein [g]
2 wheat rolls at 60g	2.2	60	9.6
10g butter	8.3	-	-
50g "Cervelatwurst" (sausage)	18	0.5	8
50g Maasdammer cheese 45% fat in dry matter	13.5	-	12.5
150g creamy yoghurt	12	22.2	4.2
150g grapes	-	23.3	1
sum	54	106	35
calories [kcal]		1078	
% of total energy	46.59	40.32	13.31
Breakfast B	Fat [g]	Carbohydrates [g]	Protein [g]
2 wheat rolls at 60g	2.2	60	9.6
25g butter	20.8	-	-
1 egg (60g)	7.2	-	7.8
50g Maasdammer cheese 45% fat in dry matter	13.5	-	12.5
150g creamy yoghurt	12	22.2	4.2
30g honey	-	22.5	-
sum	56	105	34
calories [kcal]		1088	
% of total energy	47.87	39.57	12.81
Breakfast C	Fat [g]	Carbohydrates [g]	Protein [g]
2 wheat rolls at 60g	2.2	60	9.6
13g butter	10.8	-	-
50g liver sausage	17.4	0.5	7.1
50g Camembert 50% fat in dry matter	13	-	10
150g creamy yoghurt	12	22.2	4.2
150g grapes	-	23.3	1
sum	55	106	32
calories [kcal]		1075	
% of total energy	47.58	40.43	12.20

Supplemental Table 1. Composition of Three Breakfast Choices

	breakfast choice		
FA [%]	**A**	**B**	**C**
Myristic	8.8	11.9	8.6
Palmitic	31.4	34.5	31.8
Stearic	12.7	12.0	11.7
Arachidic	0.239	0.195	0.255
Behenic	0.057	0.058	0.049
Lignoceric	0.040	0.061	0.047
SFA	**53.2**	**58.7**	**52.5**
Myristoleic	0.8	1.2	0.8
Palmitoleic	2.1	2.2	2.3
Oleic	33.0	31.6	34.2
Vaccenic	2.2	1.4	2.5
Eicosenoic	0.457	0.185	0.742
Nervonic	0.008	0.015	0.011
MUFA	**38.6**	**36.6**	**40.55**
ALA	0.982	0.777	0.868
EPA	0.045	0.055	0.045
DPA	0.136	0.120	0.172
DHA	0.036	0.070	0.032
ω3-PUFA	**1.20**	**1.02**	**1.12**
LA	7.3	5.0	7.1
GLA	0.009	0.006	0.004
EDA	0.256	0.057	0.318
AA	0.214	0.376	0.372
ω6-PUFA	**7.78**	**5.44**	**7.79**

Supplemental Table 2. FA Composition of Three Breakfast Choices

FA abbreviations are listed in Supplemental Table 3.

SFA	Formula	Abbreviation
Myristic acid	C14:0	
Palmitic acid	C16:0	
Stearic acid	C18:0	
Arachidic acid	C20:0	
Behenic acid	C22:0	
Lignoceric acid	C24:0	

MUFA	Formula	Abbreviation
Myristoleic acid	C14:1n-5	
Palmitoleic acid	C16:1n-7	
Vaccenic acid	C18:1n-7	
d7-Hexadecenoic acid	C16:1n-9	d7-HDA
Oleic acid	C18:1n-9	
Eicosenoic acid	C20:1n-9	
Nervonic acid	C24:1n-9	

ω3-FA	Formula	Abbreviation
α-linolenic acid	C18:3n-3	ALA
Stearidonic acid	C18:4n-3	SDA
Eicosatetraenoic acid	C20:4n-3	ETEA
Eicosapentaenoic acid	C20:5n-3	EPA
Docosapentaenoic acid	C22:5n-3	DPA
Docosahexaenoic acid	C22:6n-3	DHA

ω6-FA	Formula	Abbreviation
Linoleic acid	C18:2n-6	LA
Eicosadienoic acid	C20:2n-6	EDA
γ-Linolenic	C18:3n-6	GLA
Dihomo-γ-linolenic acid	C20:3n-6	DGLA
Arachidonic acid	C20:4n-6	AA

Supplemental Table 3. Nomenclature of FA

LPC species	**Predominant FA**
LPC-20:2	EDA (C20:2n-6)
LPC-20:3	DGLA (C20:3n-6)
LPC-20:5	EPA (C20:5n-3)
LPC-22:6	DHA (C22:6n-3)
PC species	**Predominant FA**
PC-36:3	a) C20:3n-6 + C16:0 b) C20:2n-6 + C16:1
PC-38:3	a) C20:3n-6 + C18:0 b) C20:2n-6 + C18:1
PC-36:5	a) C20:5n-3 + C16:0 b) C22:5n-3 + C14:0
PC-38:5	a) C20:5n-3 + C18:0 b) C22:5n-3 + C16:0
PC-38:6	a) C22:6n-3 + C16:0 b) C20:5n-3 + C18:1
PC-40:6	a) C22:6n-3 + C18:0 b) C20:5n-3 + C20:1n-9

Supplemental Table 4. Predominant FA Combinations in LPC and PC Species

Data based on the results of the factor analysis, the abundance of the FA and the correlation patterns of FA, LPC and PC species with InsRes and glucose tolerance parameters. The combinations a) are more abundant than the respective combinations b) based on plasma FA abundance and literature (Vance DE, 2008).

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