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NUTRITIONAL AND DYSMETABOLIC FACTORS WITH POTENTIAL IMPACT ON TYPE 2 DIABETES: EPIDEMIOLOGICAL AND MOLECULAR STUDIES

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ABSTRACT

Diabetes is a group of diseases characterized by hyperglycaemia. Type 2 diabetes encompasses 85-95% of all diabetes with a prevalence that is expected to increase worldwide.

In search of nutritional and dysmetabolic factors with potential impact on type 2 diabetes this thesis focused on the risk of type 2 diabetes and prediabetes and association with wholegrain intake as well as effects of hyperglycaemia and dyslipidaemia on the function and survival of insulin secreting cells.

We found in a population-based prospective study (Stockholm Diabetes Prevention Program) that low consumption of wholegrain was associated with an increased risk of deteriorating glucose tolerance. The strongest association was seen for individuals who at baseline had normal glucose tolerance (NGT) and at follow-up (8-10 years later) had progressed to prediabetes. Furthermore we confirmed effect modifications by polymorphisms of the TCF7L2 gene.

An *in vivo* study of hyperglycaemic effects on beta cell mitochondrial morphology revealed that moderate hyperglycaemia induced larger, fewer and swollen mitochondria. These morphological effects on mitochondria could partially be inhibited by treatment with a K_{ATP}-opener. The morphological effects on mitochondria were reproduced *in vitro* and were linked to dysfunctional oxidative metabolism.

Since diabetes is often accompanied by dyslipidaemia we aimed to study the effects of increased uptake of fatty acids and low-density lipoprotein (LDL) in insulin secreting cells. By overexpressing CD36 in an insulin secreting cell line (INS-1) we found that CD36 increased uptake of fatty acids. Overexpression of CD36 reduced the acute potentiating effect of fatty acids on glucose induced insulin secretion. Moreover modest effects on fatty acid oxidation and on the activity of carnitine palmitoyl transferase 1 (CPT1) activity were found. CD36 overexpression also enhanced the uptake of oxidatively modified LDL (oxLDL) whereas the uptake of native LDL was not influenced. OxLDL dose-dependently decreased viability, however independently of CD36 overexpression. The result suggest that efficient cholesterol efflux counteracts potential toxicity by uptake of the lipoprotein and that extracellular signalling mediates the negative effects on viability by oxLDL

Keywords: Type 2 diabetes, wholegrain, hyperglycaemia, dyslipidaemia

LIST OF PUBLICATIONS

I. **Wirström T**, Hilding A, Gu HF, Östenson C-G, Björklund A. Low consumption of wholegrain products carries increased risk for deteriorating glucose tolerance, including progression to prediabetes. Submitted manuscript.

II. Ma Z, **Wirström T**, Borg LAH, Larsson-Nyrén G, Hals I, Bondo Hansen J, Grill V, Björklund A. Diabetes reduces beta cell mitochondria and induces distinct morphological abnormalities, which are reproducible by high glucose in vitro with attendant dysfunction. Islets 2012, 4(3), 233-242

III. **Wallin T**, Ma Z, Ogata H, Jørgensen IH, Iezzi M, Wang H, Wollheim CB, Björklund A. Facilitation of fatty acid uptake by CD36 in insulin-producing cells reduces fatty-acid-induced insulin secretion and glucose regulation of fatty acid oxidation. Biochim Biophys Acta. 2010, 1801(2), 191-197.

IV. **Wirström T**, Ketelhuth DFJ, Ma Z, Wang H, Grill V, Wollheim CB, Björklund A. Increased CD36-dependent uptake of oxidised LDL does not exert lipotoxicity in insulin secreting cells: indications of efficient cholesterol efflux. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
Acetyl-CoA	Acetyl-Coenzyme A
ACS	Acyl CoA synthase
ADP	Adenosine-diphosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ApoB100	Apolipoprotein B100
ATP	Adenosine-triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD36	Cluster of differentiation 36
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
C-peptide	Connecting peptide
CPT1	Carnitine palmitoyl transferase 1
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
Drp	Dynamin related protein
ER	Endoplasmic reticulum
FA	Fatty acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFQ	Food frequency questionnaire
FHD	Family history of diabetes
FITC	Fluorescein isocyanate
FTO	Fat mass and obesity associated protein
G6P	Glucose-6-phosphate
GIP	Glucose-dependent insulinotropic polypeptide
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GPR40	G-protein-coupled receptor 40
GTPase	Guanosine-triphosphate
HbA1c	Glycated haemoglobin
HBSS	Hank’s balanced salt solution
HDL	High density lipoprotein
HOMA	Homeostatic model assessment
HSL	Hormone sensitive lipase
IFG	Impaired fasting glycaemia
IGT	Impaired Glucose Tolerance
INS-1	Rat insulinoma cell line
IP3	Insositol 1,4,5-triphosphate

K_{ATP} channel	ATP sensitive potassium channel
KRB	Krebs-Ringer Bicarbonate
LADA	Latent Autoimmune Diabetes of the Adult
LDH	Lactate dehydrogenase
LDL	Low-density-lipoprotein
LPL	Lipoprotein lipase
MDA	Malondialdehyd
Mfn	Mitofusion protein
MODY	Maturity onset diabetes of the young
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NGT	Normal glucose tolerance
nLDL	Native low-density lipoprotein
N_{VM}	Number of mitochondria pre unit beta cell volume
OGTT	Oral glucose tolerance test
OPA1	Optic atrophy 1
OR	Odds ratio
oxLDL	Oxidised low-density lipoprotein
PAF	Paraformaldehyde
PBS	Phosphate buffer saline
PC	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCFA	Short chain fatty acid
SDPP	Stockholm Diabetes Prevention Program
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis gel
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SSO	Sulfo-N-Succinimidyl-oleate
SU	Sulfonylurea
SUR1	Sulfonylurea receptor 1
S_{VM}	Outer surface area of mitochondria per unit beta cell volume
T2D	Type 2 diabetes
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid
TCF7L2	Transcription factor 7-like 2
UCP-2	Uncoupling protein 2
VLDL	Very low-density lipoprotein
V_{VM}	Volume of mitochondria per unit beta-cell volume

1 BACKGROUND

1.1 DIABETES PREVALENCE, DIAGNOSIS AND CLASSIFICATION

In 2011 the estimated prevalence of diabetes worldwide was 8.3% or 366 million people. The prevalence is expected to increase to 9.9% or 552 million people in the year 2030 [1].

Approximately 5-10% of all diagnosed diabetes in adults is categorised as type 1 diabetes. Type 1 diabetes develops because of a loss of insulin production due to autoimmune reactions towards the pancreatic beta cells [2, 3]. Included in the type 1 diabetes category is Latent Autoimmune Diabetes of the Adult (LADA) which is regarded a slowly progressing autoimmune form of diabetes [4].

Type 2 diabetes accounts for 85-95% of all diagnosed diabetes [2, 3, 5]. Development of type 2 diabetes is due to the body's ineffective use of insulin caused by insulin resistance as well as deficient insulin secretion [5].

Other types of diabetes include monogenetic diseases (maturity onset diabetes of the young, MODY), mitochondrial diabetes [6] as well as gestational diabetes [7]. Diabetes can also result from other endocrine diseases, infections, medications, surgery and pancreatic disease [2, 8].

Complications of diabetes include damages to nerves and blood vessels; hence diabetes is a serious cause of kidney failure, amputation and blindness [5].

An intermediate stage between normal glucose regulation and diabetes is referred to as 'impaired glucose regulation'. This stage includes impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) [7]. Table 1 summarises the 2006 WHO recommendations for the diagnostic criteria for diabetes, and intermediate hyperglycaemia (IGT and IFG) [9].

IGT and IFG are often referred to as prediabetes since these conditions are often seen before a diabetes diagnosis is reached. Approximately 70% of subjects with prediabetes have been reported to eventually develop diabetes [10].

Inadequate insulin secretion and insulin resistance are the two conditions that separately or combined cause all forms of diabetes. An overview of insulin secretion is presented below, followed by a description of insulin action.

Table 1. Adapted from 2006 WHO recommendations for the diagnostic criteria for diabetes and intermediate hyperglycaemia

Diabetes	
Fasting plasma glucose	≥ 7.0 mmol/l or
2 h plasma glucose*	≥ 11.1 mmol/l
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose	< 7.0 mmol/l and
2 h plasma glucose*	≥ 7.8 and < 11.1 mmol/l
Impaired Fasting Glycaemia (IFG)	
Fasting plasma glucose	6.1 to 6.9 mmol/l and (if measured)
2 h plasma glucose*	< 7.8mmol/l

* Venous plasma glucose 2 h after a 75 g oral glucose load. If 2 h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded. Adapted from reference [9].

1.2 BETA CELL FUNCTION AND METABOLISM

1.2.1 Insulin biosynthesis

Biosynthesis of insulin starts with the synthesis of preproinsulin, a single-chain 86 amino acid precursor polypeptide, on the polyribosomes associated with the rough endoplasmic reticulum (RER) [11]. Preproinsulin is translocated into the RER lumen and proteolytic processing removes the amino-terminal signal peptide resulting in proinsulin. Proinsulin forms di-sulfide bounds to uphold tertiary structure and is transported through the Golgi where it chelates with Zn^{2+} (hexamers). Finally an immature granule is formed. Within the immature granule proinsulin is further cleaved into the connective-peptide (C-peptide) and the A and B chains of insulin generating the mature granule. It is these newly formed mature granules that are secreted upon stimulation.
Approximately 1 % of the total insulin content is secreted upon glucose stimulation per hour while the remaining pool is constantly turned over (half-life 3-5 days). If demands of insulin increase, (as in type 2 diabetes) more immature granules will be secreted and hence also increased amounts of unprocessed proinsulin. Proinsulin biosynthesis is regulated by the different secretagogues such as glucose, amino acids, fatty acids and glucagon-like peptide 1 (GLP-1) [11].

1.2.2 Insulin secretion

1.2.2.1 Glucose

A simplified schematic model of insulin secretion is illustrated in figure 1. Glucose enters the beta cells by facilitated diffusion through glucose transporters (GLUT 1, in humans, GLUT 2, in rodents) [8, 12]. Intracellular glucose is phosphorylated by glucokinase (GK) (rate limiting step) to glucose-6-phosphate (G6P). G6P is then further metabolised through glycolysis into pyruvate.

Pyruvate enters the mitochondria and is further metabolised by either pyruvate carboxylase (PC) for the anaplerosis/cataplerosis pathway into oxaloacetate or by pyruvate dehydrogenase (PDH) for the glucose oxidation pathway into acetyl-CoA.
Both these actions enhance mitochondrial tricarboxylic acid (TCA) cycle activity, thereby generating ATP. This causes the cytosolic ATP/ADP ratio to increase which in turn induces closure of ATP-sensitive potassium channels (K_{ATP}-channels) [13].

The K_{ATP}-channel is an octameric complex of two protein subunits; four regulatory proteins, sulfonylurea receptor 1 (SUR1), and four pore forming subunits (Kir 6.2) [14]. ATP binds to the Kir 6.2 subunit and closes K_{ATP}-channels [14]. Sulfonylureas, a category of drugs used in diabetes treatment, bind to SUR1 and increase the ATP-sensitivity of Kir 6.2. On the other hand K_{ATP}-channel openers such as diazoxide or tifenazoxide (used in the present thesis) inhibit insulin secretion by opening (thus inhibiting closure) of the channel [14].

Closure of K_{ATP}-channels leads to membrane depolarisation and opening of voltage dependent Ca^{2+}-channels which facilitates extracellular Ca^{2+} to enter the cell. In the secretory process insulin secretory granules are mobilised towards the plasma membrane, then fuses with it, thereby releasing insulin [8].

A rise in cytosolic Ca^{2+} initiates the exocytosis of insulin granules. Granules docking at the cell membrane and the exocytosis processes can be further influenced by glucose metabolism through activation of phospholipase C (PLC) which in turn promotes hydrolysis of phospholipids and formation of inositol 1,4,5-triphosphate (IP_3) which can stimulate Ca^{2+} release from the endoplasmic reticulum (ER). Activation of PLC also generates diacylglycerol (DAG) which stimulates protein kinase C (PKC) which in turn also increases Ca^{2+}. Glucose metabolism can also activate adenylate cyclase (AC) and further protein kinase A (PKA) through cyclic AMP (cAMP) [15].

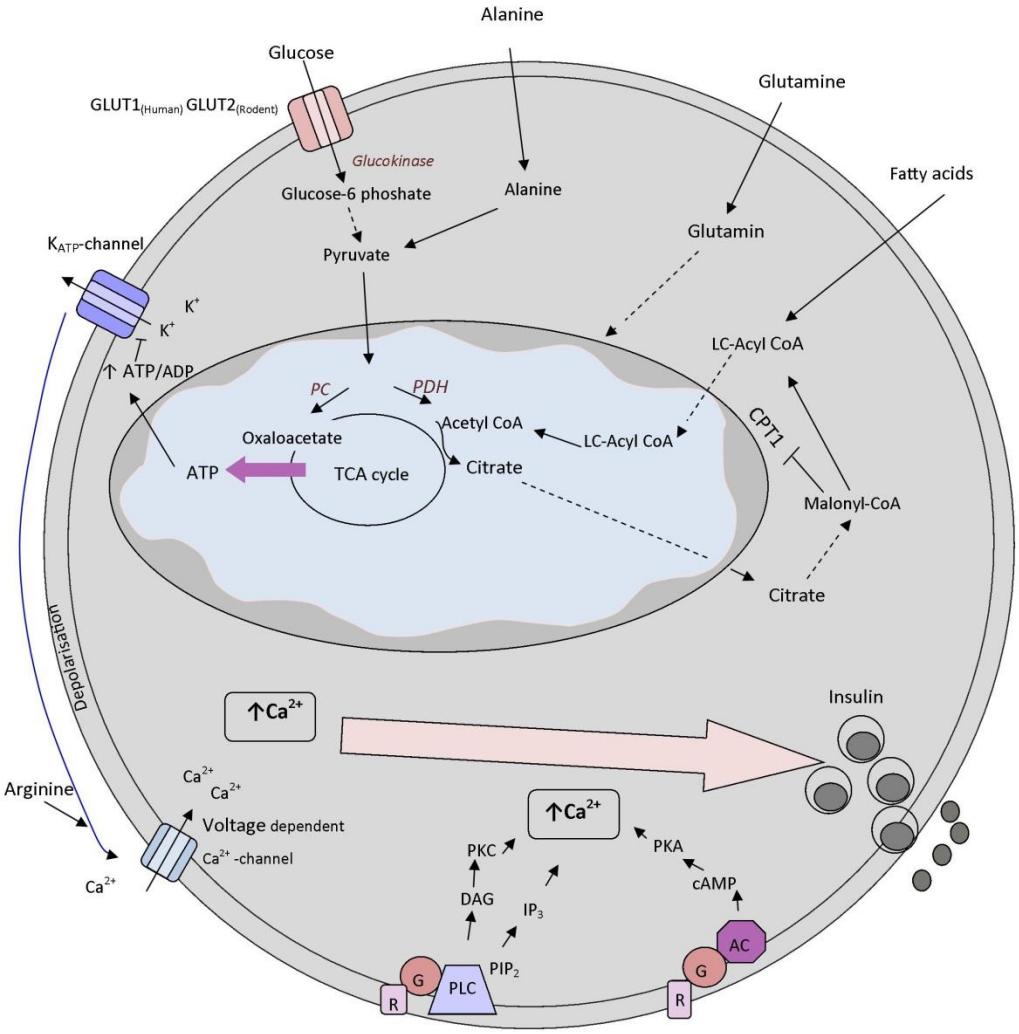

Figure 1. Schematic model of insulin release, for details see text.

1.2.2.2 Other secretagogues

Of the nutrients the beta cells are most sensitive to glucose. However other nutrients such as fatty acids and amino acids can also initiate or amplify insulin secretion, see figure 1.
Glutamine and alanine are quantitatively the most abundant amino acids in the blood. Amino acids at physiological concentrations do not individually induce insulin secretion. However supra-physiological concentrations or a combination of amino acids are able to induce insulin secretion and potentiate glucose-induced insulin secretion. The mechanisms for how amino acids stimulate insulin secretion are complex and partly include mitochondrial metabolism. Alanine (via pyruvate) increases production of ATP thus stimulating the K_{ATP}-channel dependent pathway. Alanine also acts directly on the depolarisation of the membrane as an electrogenic amino acid through co-transportation by Na^+ causing the influx of Ca^{2+}. Glutamine can in combination with leucine promote insulin secretion through several complex mitochondrial mechanisms. Arginine in supra-physiological concentrations can depolarise the membrane by acting as a cationic amino acid. Alanine and glutamine have also been shown to alter and regulate gene expression of several genes involved in metabolism [16, 17].

Acute exposure to fatty acids (FA) potentiates glucose induced insulin secretion both in vivo [18, 19] and in vitro [20]. The effect is dependent on the unbound fraction rather than the total concentration. Saturated fatty acids are more potent than unsaturated [21]. The mechanisms by which fatty acids stimulates insulin secretion could be several such as amplifying activation of the secretory machinery, activation of protein kinase C [22], or binding/activation to G coupled protein 40 (GPR40) and subsequent signalling [23]. The potentiating effect of acute FA in glucose induced insulin secretion is not persistent. Instead long term stimulation of fatty acids results in decreased glucose induced insulin secretion [18]. Multiple mechanisms, not fully elucidated have been proposed. Mechanism have been linked to ER-stress, apoptosis, effects on proinsulin biosynthesis and disturbed interplay between nutrient metabolism and other circulating factors influencing mitochondrial function [13, 24, 25].

1.2.2.3 Other signalling

Oral glucose administration amplifies insulin secretion compared to intravenous infusion of glucose in healthy subjects. This is due to the incretin effect. Incretins are hormones released from the gastro-intestinal tract into the circulation after food ingestion. For glucose homeostasis the most important incretins are GLP-1 which is secreted from the L-cells localized in the ileum and the colon and glucose-dependent insulinotropic polypeptide (GIP) which is secreted from the K-cells localized in the duodenum and proximal jejunum [26, 27]. GLP-1 agonists (used in the treatment of type 2 diabetes) slow gastric emptying, increase satiety and promote modest weight loss and are in preclinical studies suggested to improve beta cell function, lower blood pressure and improve cardiovascular outcomes [27].

Other signalling for insulin secretion operates through the parasympathetic nervous system which can enhance both insulin and glucagon secretion by stimulation of the vagus nerve. Activation of the sympathetic nervous system can inhibit basal and glucose-induced insulin secretion [26].

Other islet hormones also come into play for glucose regulation. Glucagon, which is secreted from the alpha cells of the islets of Langerhans, stimulates glucose production in the liver both by gluconeogenesis and glycogenolysis. These effects are important for maintaining glucose homeostasis during a fasting state. Somatostatin, secreted from the delta cells of the islet of Langerhans, can decrease the small intestinal transit time and also inhibit the secretion of insulin and glucagon. Furthermore other incretins or gastro intestine polypeptides can affect the secretion of glucagon and somatostatin [26, 27].

1.2.3 Role of mitochondria for beta cell function

The mitochondrion is a double-membrane organelle responsible for energy supply mainly in the form of ATP. It has a circular deoxyribonucleic acid (DNA) which is maternally inherited and contains 37 genes. These genes are transcribed within the mitochondria into some of the components of the respiratory chain complexes. Other components of respiratory chain complexes are synthesized in the cytosol by nuclear DNA (nDNA) and transported into the mitochondria [28].

Mutations in mitochondrial DNA (mtDNA) have been linked to maternally inherited forms of diabetes [29]. Mitochondria in the beta cells are especially important compared to other cell types since they are the ultimate sensor of glucose homeostasis and couples metabolism to insulin secretion. Beta cell glycolysis generates higher proportions of pyruvate compared to other cells because the lactic dehydrogenase enzyme levels are low. The replenishment of carbons both in the form of acetyl-CoA and in the form of oxaloacetate and subsequent oxidation activates the electron transfer in the respiratory chain with the final production of ATP. A rise in cytosolic ATP levels then acts as a signal for insulin secretion [28].

Mitochondria continuously divide and fuse with other mitochondria. Several proteins are known to be involved in these fission and fusion events. Of proteins involved there has been much focus on mitofusion protein 1 and 2 (Mfn1, Mfn2). These proteins are GTPases associated to the outer cell membrane which has been suggested to be involved in the outer membrane fusion. Optic atrophy 1 (OPA1), a dynamin GTPase located in the intermembrane space and also associated with the inner membrane has been suggested to be involved in the inner membrane fusion [30, 31]. For fission the most important protein may be the dynamin related protein 1 (Drp1) which is located in the cytosol and is thought to be involved in the constriction of mitochondria fission [30, 31]. Mitochondrial morphology depends partly on a balance between fusion and fission. Mutations in the OPA1 and Mfn2 have thus been associated with dysfunction. Additionally both fission and fusion are involved in early stages of apoptosis and in particular mitochondrial fragmentation [31].

In the transport of electrons in the respiratory chain there is always a small leakage of oxygen. This oxygen can form reactive oxygen species (ROS). It has been speculated that the ROS formation adjacent to the mtDNA could be involved in the accumulation of mtDNA damage including mutations. Mutations could subsequently lead to increased ROS formation, a notion which is part of the ageing hypothesis [31, 32]. Beta

cells have been shown to have less of the enzymes handling ROS such as catalase or glutathione peroxidase [33] and also lack protective histones [34]. Beta cells would then be particularly sensitive to ROS induced damage compared to other tissues. It is in any case clear that functioning mitochondria is a prerequisite for functioning insulin secretion and glucose homeostasis.
With the progression of type 2 diabetes when glucose homeostasis declines and metabolic control worsens, mitochondria are subjected to glucotoxicity, i.e. negative effects of hyperglycaemia (more detailed in 1.4.4). Abnormal mitochondria morphology has been found in islets from type 2 diabetes patients [35]. Potential mitochondrial effects of glucotoxicity remain to be fully elucidated.

1.2.4 Role of lipids for beta cell function

1.2.4.1 General function and transport

Lipids assist important biological functions in the body e.g. transport of lipid soluble vitamins. Furthermore phospholipids and cholesterol are important for maintaining cell integrity, as they are part of cell membranes. When transported in plasma lipids they also serve as energy substrates and in the exchange of energy storage places [36, 37]. They act as mediators via prostaglandins and leukotrienes, and act as ligands for gene transcription [36].
Transport of free fatty acids in plasma occurs to major part with fatty acids bound to albumin [38] although a small fraction is unbound. Fatty acids can enter cells by passive diffusion over the cell membrane or by facilitated transport with membrane transport proteins. Several of the transport proteins have been extensively studied in various tissues [39-41].
CD36, also called fatty acid translocase (FAT), is one of the most important proteins involved in fatty acid uptake by cells. CD36 is an 88 kDa trans-membrane protein which is heavily glycosylated, see figure 2 [42]. CD36 is involved in many functions because of its wide expression and broad specificity [43, 44]. It is thus also involved in binding of oxLDL, in phagocytosis and in toll-receptor signalling [45]. CD36 is expressed in many different cell types such as monocytes, macrophages, muscle cells, adipocytes and pancreatic beta cells etc. [40, 42, 43]. In non-beta cells it has been shown to facilitate the major uptake of long-chain fatty acids [46].
CD36 facilitated fatty acid uptake is suggested to take place in caveolae which are formed in lipid rafts however data are not conclusive [47]. In beta cells, CD36 was found in the plasma membrane as well as intracellular and co-localized with insulin granules [40]. Little is known about expression and trafficking of CD36 in cell organelles. Variations in CD36 expression have been found both in vitro and in vivo to influence fatty acid metabolism [48]. Since CD36 is widely expressed the interpretation of the regulation of its expression is complex. A number of cell lines and animal models have been used to study the effects of altered CD36 expression [48] but few studies have been made in insulin secreting cells.

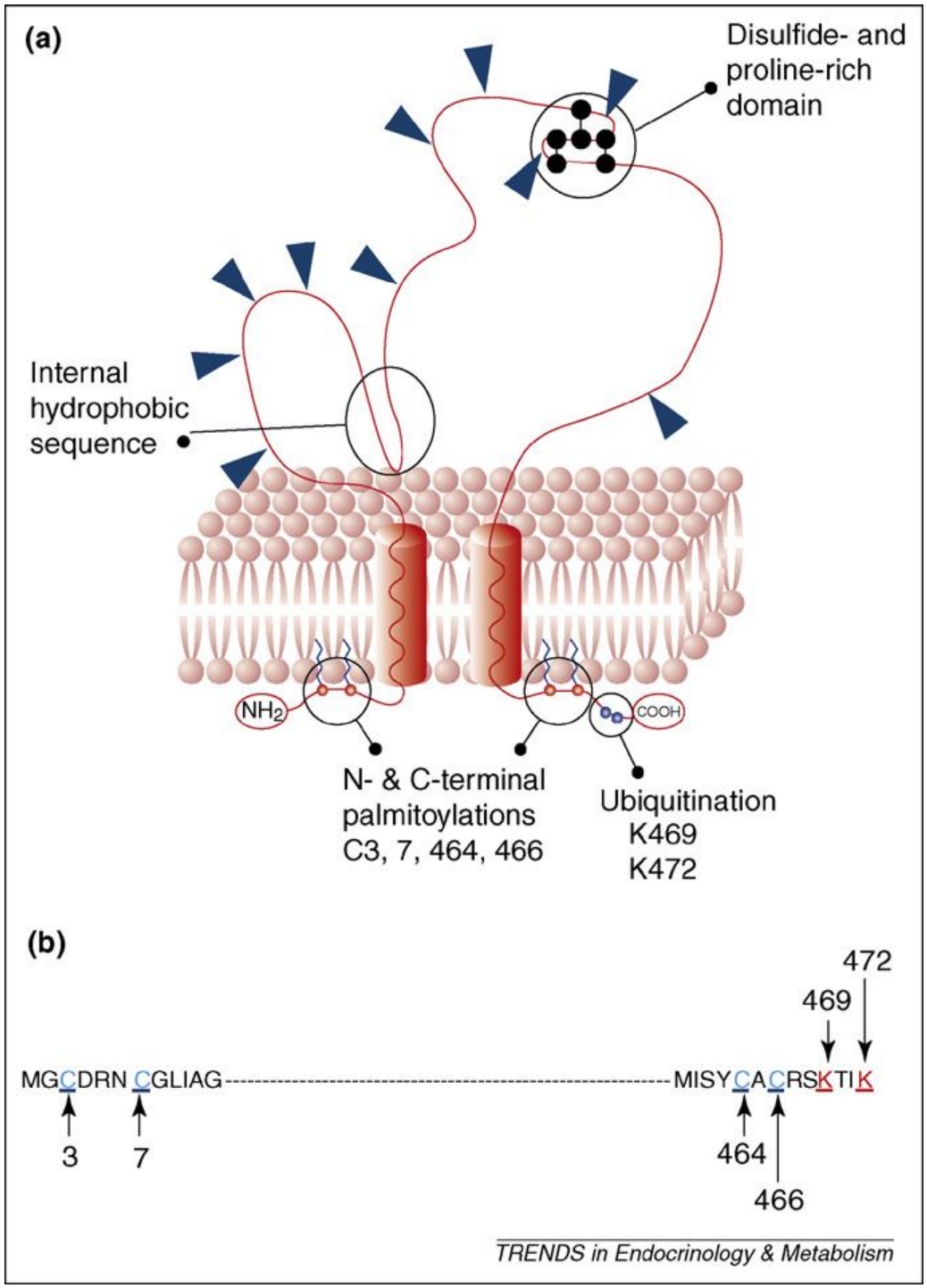

Figure 2. Predicted hairpin topography of CD36 protein in the plasma membrane. CD36 extracellular domain has multiple glycosylation sites, a proline-rich domain and an internal hydrophobic sequence. The N- and C-terminus in the cytosolic domain has several palmitoylation and ubiquitination sites. Palmitoylation are suggested to be involved in recruitment of CD36 and ubiquitination sites are sensitive for fatty acids and insulin suggested to play a role in the turnover of CD36.
Figure is from Su X and Abumrad N.A. Cellular fatty acid uptake: a pathway under construction. Trends in Endocrinology & Metabolism 2009:20(2):72-77 [42].

When fatty acids are transported into the beta cells they are transformed by acyl-CoA synthase (ACS) into long-chain acyl CoA. When glucose levels are low, long-chain acyl-CoA are transported into the mitochondria by carnitine palmitoyl transferase 1 (CPT1) for beta oxidation. When glucose levels are high, glucose metabolism in the tricarboxylic acid (TCA) cycle produces citrate which can be transported to the cytosol and converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits CPT1 and thus transport of long-chain acyl-CoA to mitochondria [13], see figure 1.
Furthermore malonyl-CoA formation from acetyl-CoA is inhibited by the AMP-activated protein kinase (AMPK) (activated when AMP increases, in low glucose levels) inhibiting the acetyl-CoA carboxylase [13].
In order to move water insoluble molecules, i.e. triglycerides and cholesterol, through the blood and inside and outside of cells, both proteins and lipids are assembled into structures called lipoproteins. Low-density-lipoprotein (LDL) consists of triglycerides and cholesteryl ester molecules in its core; unesterified cholesterol molecules, of which approximately one-third lies in the core and two-thirds reside on the surface; a surface monolayer of phospholipid molecules; and a single copy of apolipoprotein B-100 (ApoB100) [49].
The transport of lipids by lipoproteins in plasma is tightly regulated through hormonal and metabolic control that influences synthesis/assembly and catabolism/clearance of these particles in different organs. Furthermore carbohydrate and lipid metabolism are intertwined and the lipoprotein lipase (LPL) and hormone sensitive lipase (HSL) are partly regulated by insulin and catecholamines [8].

1.3 INSULIN ACTION

Insulin is transported in the circulation to peripheral tissues (most importantly muscle, fat and liver) where it stimulates the uptake of glucose, resulting in normalisation of blood glucose levels. This regulation of blood glucose levels is necessary since both hypoglycaemia (acute) and hyperglycaemia (long-term) are detrimental. Insulin resistance (a common feature in type 2 diabetes patients) in the peripheral tissues means that more insulin than normal is required for cells to take up glucose. Because of insulin resistance the demands for insulin grow higher until beta cells are unable to meet the demands. The tightly controlled blood glucose regulation is then lost and hyperglycaemia prevails and leads to further deterioration of insulin secretion [8].

1.4 RISK FACTORS AND DYSMETABOLIC FACTORS INVOLVED IN TYPE 2 DIABETES

Genetic, intra-uterine conditions as well as post-natal environmental factors are involved in the development of type 2 diabetes. Both insulin sensitivity and insulin secretion are affected [8].

1.4.1 Genetic factors and family history of diabetes

Individuals with a family history of diabetes have a two to four fold increased risk of developing diabetes compared to individuals without family history of diabetes. The risk is depending on number of relatives with diabetes and how close these relatives are [50]. Family history of diabetes as a risk factor can partly be explained by shared genes

but also by shared environment. However, there is no doubt that there is a strong genetic component in type 2 diabetes.
Genetic association studies have found a number of genes (36-44 genes) associated to type 2 diabetes however the effect size is low. Most of the diabetes-associated genes involve the function of beta cells rather than insulin resistance [51, 52].
One of the strongest associations was found for gene variants in TCF7L2 [51]. Two Scandinavian prospective cohort studies showed that carriers of risk allele T in single nucleotide polymorphism (SNP) rs7903146 increased the risk for diabetes by 60%. These studies found an association to enhanced expression of TCF7L2 in human islets, in addition to impaired insulin secretion [53]. Variation in TCF7L2 influences the efficacy of sulfonylureas (SU) which are drugs promoting insulin secretion; subjects with risk allele were less likely to respond to SU. TCF7L2 risk allele did not influence the efficacy of metformin (a drug enhancing the insulin sensitivity) [54].
TCF7L2 polymorphisms have been associated with certain phenotypic parameters such as impaired insulin secretion, beta cell function and GLP-1 potentiation of insulin secretion [55]. TCF7L2 has also been suggested to modify the risk for type 2 diabetes association to carbohydrate quality and quantity [55]. Such a modifying effect of TCF7L2 has however been disputed, [56] emphasizing the need for further studies.

1.4.2 Intra-uterine conditions

Intra-uterine risk factors are related to intra-uterine growth retardation and low birth weight [57, 58]. Intrauterine growth retardation was also associated with hypertension which could explain the association between hypertension and diabetes [57, 59]. Intrauterine environment caused by gestational diabetes is also a risk factor for diabetes in the offspring [58, 59].

1.4.3 Environmental factors

1.4.3.1 Overweight and physical inactivity

Overweight or obesity is considered one of the strongest predictors of type 2 diabetes [58, 60]. The risk of diabetes is strong whether measured by increased waist circumference, waist to hip ratio or BMI [60-63]. Obesity is a heterogeneous disease where several genes have been identified with susceptibility loci. One of the most studied is the ‘fat mass and obesity associated protein’ (FTO) [64]. Although there is a genetic component in obesity the risk can be modified by environmental factors such as physical activity [65]. In fact epidemiological studies indicate a 30-50% reduced risk of developing diabetes in physical active individuals (overweight and normal weight) compared to physical inactive individuals [66].

1.4.3.2 Dietary factors

The increasing incidence of type 2 diabetes occurs in the societies where there has been a major shift towards a more inactive lifestyle and rapid changes in the dietary patterns both with regards to availability and with more energy dense diet [59]. Numerous studies have looked for dietary risk factors for development of diabetes, yet convincing evidence for population based guidelines are scarce.
A high intake of saturated fat has been associated with a higher risk for impaired glucose tolerance in epidemiological observational studies [59, 67]. Higher levels of

saturated fatty acids in serum lipids and in muscle phospholipids were associated with increased levels of fasting glucose and decreased insulin action [59, 68, 69]. Interventions studies with replacement of saturated with unsaturated fatty acids improved glucose tolerance and enhanced insulin sensitivity [70].

Among other dietary factors that can modify the risk for type 2 diabetes, dietary fiber and wholegrain have gained a lot of attention.

Definition of dietary fiber has varied over the years and in different countries. Previously the terms soluble and insoluble dietary fiber was used to explain physical effects. However these terms are less used today. Clarification of the current definition of fiber can be found in ref [71]. Briefly; non-starch polysaccharides (such as cellulose, hemicellulose, pectins and other hyrocolloids) are the main constituent of dietary fiber. Dietary fiber also includes resistant starch, resistant oligosaccharides and dietary associated lignin. These are all non-digestible carbohydrates, i.e. they are resistant to hydrolysis and absorption in the small intestine. Instead insoluble fibers, (mainly from wholegrain) undergo anaerobic fermentation in the colon by bacteria with the production of short-chain fatty acids (SCFA) which could then be used as energy substrate by the mucosa in the colon [71]. These SCFA could also have effect on hepatic glucose output [72, 73]. Soluble form of fibers (mainly from fruit and vegetables) have effects on gastric emptying and gastric transit time, and thereby the glucose response [73]. Average intake of dietary fiber in adults is 15-30 g/day while the recommended dietary intake varies between 25-45 g/day [71]. In Sweden only 5-10% of adults reach the recommended intake [74].

Wholegrain is not a nutrient as such, but generally considered as a food group. Wholegrain is an important contributor of fiber intake. Wholegrain is generally defined as grains that consist of the intact, ground, cracked or flaked caryopsis whose principal anatomical components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact caryopsis. Wholegrain foods or wholegrain products are however differently defined in different countries [75].

A cohort by Liese et al. 2005 [76] found that intake of fiber was positively associated with insulin sensitivity and negatively associated to fasting insulin levels. Dietary fiber was also negatively associated with the probability of having insulin resistance (measured by HOMA-IR) [77]. Prospective studies have found that increasing intake of whole grain [78, 79] and cereal fiber [73, 80-82] resulted in decreased risk of type 2 diabetes. Other prospective studies have showed low intake of fiber, mainly cereal fiber, to increase the risk of type 2 diabetes [83, 84]. Not all studies found an association of diabetes risk and total fiber intake. Therefore it is still to be established if the wholegrain or the cereal fiber (mostly insoluble fiber) or the fruit and vegetables (mostly soluble fibers) provides the beneficial effects [71]. Further studies on individuals with type 2 diabetes and whether fiber rich diets could improve glycaemic control are needed. Some intervention studies suggest an effect of the soluble fiber rather than the insoluble [58]. Of the two nutrients described, (saturated fat and dietary fiber), the effect of fiber is more documented. However more prospective studies are needed on the importance of wholegrain for effects on insulin resistance and development of diabetes. Wholegrain and possible gene interactions also warrant further studies as they are contradictory [56, 85].

1.4.4 Effects of hyperglycaemia

Chronic hyperglycaemia has been shown to have detrimental effect on insulin secretion and insulin action. Improving metabolic control in type 2 diabetic patients improves insulin secretion [86]. This provides one piece of evidence that chronic hyperglycaemia in itself exerts negative effects on glucose homeostasis. A reduced insulin resistance in type 2 diabetes patients after improvement in metabolic control has also been demonstrated [8].

Mechanisms studied implicate oxidative stress as a large contributor to these detrimental effects. Hyperglycaemia in vitro for 3 days increased rat islet apoptosis, cytoplasmic DNA fragmentation and caspase-3 activity. Furthermore gene expression of pro-apoptotic proteins increased while gene-expression of anti-apoptotic proteins decreased [87]. Another mechanism involves the impairment of insulin gene expression [88]. Glucotoxicity has also been shown to induce ER-stress [89]. Mechanism behind induction of insulin resistance include a number of biochemical disturbances in insulin signalling [8].

Long term hyperglycaemia is known to desensitize beta cells to glucose-induced insulin secretion as shown in glucose-infused rats [90]. This desensitization could be avoided if insulin secretion was blocked by K_{ATP} opener [91]. It was proposed that the desensitization effect of hyperglycaemia was secondary to overstimulation of the beta cells [91].

To separate the effects of hyperglycaemia (glucotoxic effects) from overstimulation a transplant study was made in moderately hyperglycaemic animals [92]. Transplants of islets to moderately hyperglycaemic animals were combined with treatment of K_{ATP} openers. This procedure resulted in beneficial effects on insulin secretion which could not be explained by effects of insulin content or beta cell number [92].

To follow up on these findings it seemed important to investigate effects on mitochondria of chronic hyperglycaemia and the modifying effects of K_{ATP} openers.

1.4.5 Dyslipidaemia

1.4.5.1 General

Diabetes confers an increased risk of cardiovascular disease and mortality [93]. Abnormalities of lipids and lipoproteins such as high triglycerides, low high density-lipoproteins (HDL) and increased concentration of LDL-cholesterol particles are often found in patients with diabetes [94]. Prevalence of hypercholesterolemia (total) is not increased in patients with type 2 diabetes, nevertheless mortality increases exponentially with serum cholesterol levels [94].

Causes for dyslipidaemia have both genetic and environmental components. There are a number of monogenic and polygenic disorders that influence lipid metabolism. Other genetic variants require environmental influence in order to achieve clinical manifestations [95]. Pathogenesis of diabetic dyslipidaemia is well studied and suggested to be coupled to insulin resistance. With insulin resistant fat cells the circulation of free-fatty acids increases which enhances the production of very low-density lipoprotein (VLDL) in the liver [94]. Hyperinsulinemia (secondary to insulin resistance) also affects lipid exchange between different lipoproteins, generating increased concentrations of small dense LDL particles and reduced levels of HDL

cholesterol [94]. Dyslipidaemia as in diabetes is also common in prediabetes. Hence dyslipidaemia can exist long before glycaemic control is lost [96].

1.4.5.2 Fatty acid and lipoprotein interaction with beta cells under normal and diabetic conditions

As mentioned previously, fatty acids influence beta cell function. Acute exposure to fatty acids thus potentiates insulin secretion, while long term exposure can reduce insulin secretion.

Lipoprotein involvement in cardiovascular diseases is well established and atherosclerotic effects of oxLDL have been in focus [97]. The effect of oxLDL in development of diabetes is however largely unknown. Patients with type 2 diabetes have been found to have higher levels of serum oxLDL than controls and these levels increased with duration of type 2 diabetes [98]. LDL was found to be taken up by rat and human beta cells [99] via an LDL receptor [100]. Uptake of oxLDLs has in other tissues been shown to occur via scavenger receptors such as CD36 [44]. OxLDL has been shown to reduce insulin secretion in insulin secreting cell lines caused by reduced preproinsulin mRNA expression [101, 102].

However, there is discrepancy on whether native LDL and oxLDL affects the function of beta cells or whether the lipoproteins cause necrosis or apoptosis [100, 102, 103]. Also the importance of cellular uptake of lipoproteins for negative the effects has not been elucidated. Thus further studies are warranted.

2 AIMS

The overall objectives of this thesis was first, to study the impact of certain nutritional risk factors on the progression to abnormal glucose tolerance and next to study the influences of type 2 diabetes associated conditions of dysmetabolism in insulin-secreting cells.

Specific aims for the individual studies

Study I
To test in a prospective population-based cohort for associations between intake of fiber and wholegrain and the risk of developing abnormal glucose tolerance, including prediabetes and type 2 diabetes, and to test for modulation by polymorphisms of the TCF7L2 gene.

Study II
To examine in beta cells the impact of a diabetic state with hyperglycaemia on morphometry of mitochondria and relate *in vivo* findings with glucose effects *in vitro* as well as modifying influence of K_{ATP} opener.

Study III
To investigate how overexpression of CD36 in insulin producing cells affects cellular localisation of CD36, uptake and efflux of fatty acids, insulin secretion and oxidative metabolism.

Study IV
To investigate if CD36 in insulin producing cells potentially scavenges oxLDL and further to study whether enhanced scavenging affects function.

3 MATERIAL AND METHODS

3.1 SUBJECTS AND STUDY DESIGN

The first study is based on a population-based cohort of Stockholm Diabetes Prevention Program (SDPP) illustrated in figure 3. All participating subjects gave their informed consent and the study has been approved by the ethics committee of Karolinska University Hospital.

3.1.1 Baseline study

Subjects (ages 35-56 years) were invited to the study in the years 1992-1994 (men) and 1996-1998 (women) from four (men) or five (women) municipalities in Stockholm County. Subjects answered a short questionnaire about country of birth and whether they or their relatives had diabetes. Response rate was 79% for men and 85% for women. From these 47% (men) and 50% (women) were excluded due to; prior diabetes, other country of birth, unclear or insufficient family history of diabetes (FHD). Positive FHD, which was self-reported by the subjects, was classified as follows; at least one first-degree relative (parent or sibling) or at least two second-degree relatives (grandparents, uncles or aunts) with diabetes onset later than 35 years of age.

The sample was enriched to approximately 50% with subjects having positive FHD and they were matched to subjects having negative FHD by age and municipality. These subjects were invited to the first health examination which included an oral glucose tolerance test (OGTT) (2 h, 75 g), body measurement such as length, weight, waist/hip circumference, blood pressure and an extensive questionnaire. The questionnaire included questions on tobacco and alcohol consumption, physical activity, education, socio-economic and psychosocial factors and dietary habits. Excluded after first health examination were subjects with incomplete examinations, pregnancy, breastfeeding, and certain medical conditions. Finally baseline study comprised of 3128 men and 4821 women.

3.1.2 Follow-up study

Baseline study was followed up 8-10 years later and subjects were invited to a second health examination. Excluded from this invitation were subject who either 1) moved out of Stockholm County, 2) who died during the follow-up period or 3) who already at baseline were diagnosed with diabetes. The invited subjects underwent a similar health examination and questionnaire as at baseline. Those who were diagnosed with diabetes during the follow-up period were excluded from the OGTT. Instead for these subjects a fasting blood sample was drawn and subjects were asked about year of diagnosis and type of treatment. In the end 2383 men and 3329 women representing 76% and 69% of the baseline subjects were included in the follow-up study.

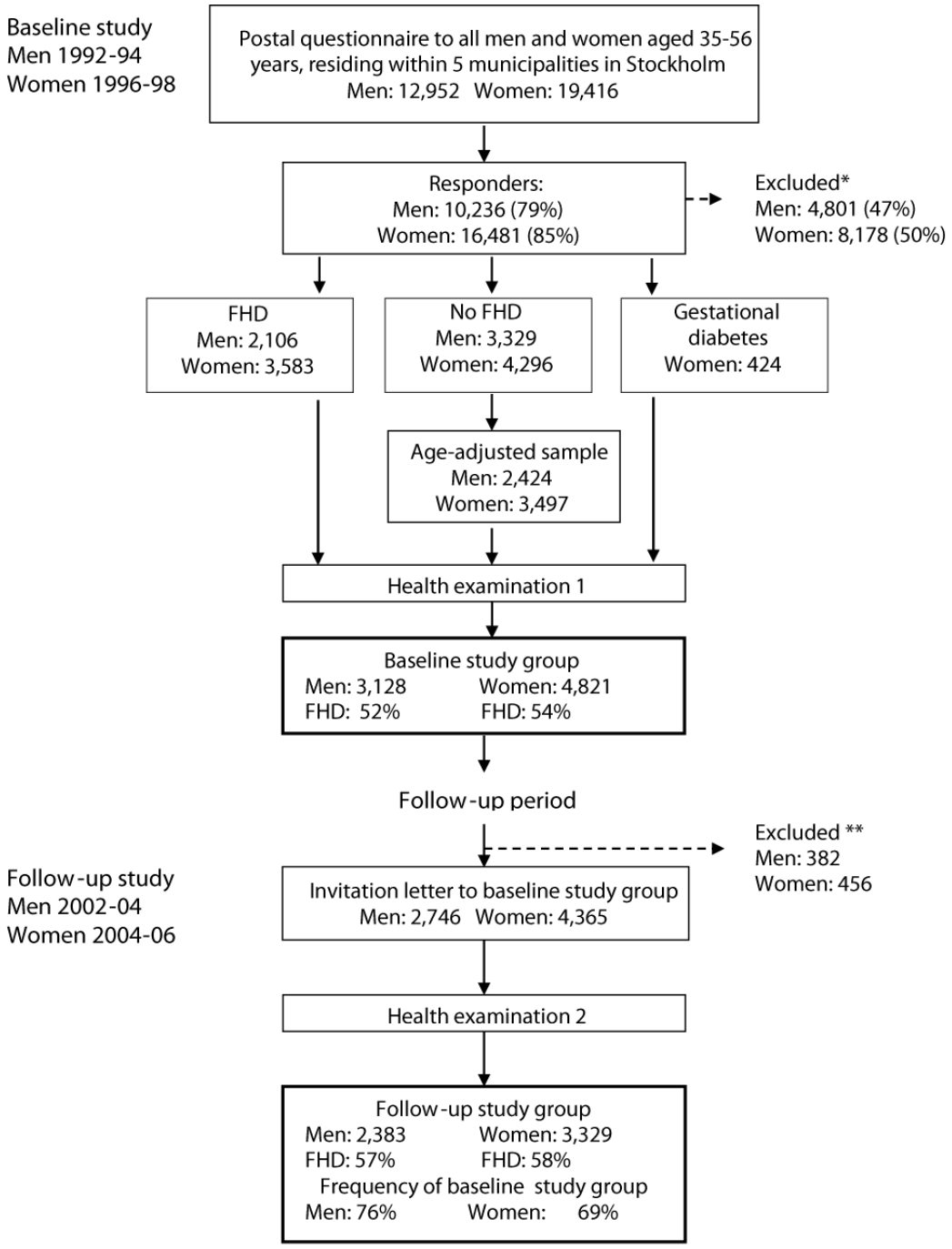

Figure 3. Study design. Baseline and follow-up study of men and women in Stockholm Diabetes Prevention Program. FHD (family history of diabetes). *Excluded due to already known diabetes, foreign origin, unclear or insufficient FHD. ** Excluded due to diagnosis of diabetes at baseline examination, moved outside Stockholm county or deceased.

3.1.3 Classification of glucose tolerance

Glucose tolerance was classified according to WHO statement (see Table 1) and subjects were identified as having either normal glucose tolerance (NGT), impaired fasting glucose, impaired glucose tolerance or type 2 diabetes [9]. Pre-diabetes was classified as either IFG or IGT or the combination of the two. Glucose was analysed by glucose oxidase method and insulin and proinsulin was assayed by radioimmunoassays

[104]. Proinsulin data at baseline was available for men only and levels (in pmol/L) and the ratio of proinsulin/insulin in per cent were calculated.

3.1.4 BMI, HOMA-IR and insulin response calculations

Relative change in body mass index (BMI) between baseline and follow-up was calculated in per cent. The median value of BMI change, based on the distribution among all subjects, was used to dichotomize the sample.

$$Relative\ change\ in\ BMI\ in\ percent = \left(\frac{BMIfollowup}{BMIbaseline}\right) * 100$$

The homeostasis model assessment [105] was used to assess insulin resistance (HOMA-IR) calculated as

$$HOMA - IR = \frac{fasting\ insulin * fasting\ glucose}{22.5}$$

Insulin response was estimated in two ways: as beta-cell function (HOMA- beta) calculated as

$$HOMA - beta = \frac{fasting\ insulin * 20}{fasting\ glucose - 3.5}$$

or from the difference between 2 h and 0 h plasma insulin values from the OGTT (insulin-response$_{(2h\text{-}0h)}$)

$$Insulin\ response = Insulin(2h) - Insulin(0h)$$

3.1.5 Dietary fiber and wholegrain

The dietary food-frequency questionnaire (FFQ) was primarily designed to evaluate fat and fiber intake. This questionnaire has previously been validated by a 7-day weighted record in a similar subgroup of men [106]. All food items in the FFQ contributing with fiber were analysed in our study. The eight frequency response options were; at least 4 times/day, 2-3 times/day, once/day, 4-6 times/week, 2-3 times/week, once/week, 1-3 times/month and seldom or never. To assess fiber intake in gram per day the food database (version 2011-07-18) at the Swedish National Food Agency [107] and a standard portion size/serving of the food items was used. As the FFQ was specifically aimed to assess fiber and fat we were not able to assess the total energy intake.

The food database also provides data on wholegrain content. This allowed us to calculate the total wholegrain intake. In these calculations we included all food items that contained at least 18 g wholegrain per serving, i.e. crisp bread, wholemeal bread, oatmeal and muesli.

To evaluate whether the intake of fiber and wholegrain varied over time we also calculated fiber and wholegrain intake from follow-up data. These calculations were performed in the same way as for baseline data.

Intake of wholegrain and fiber was categorized into tertiles according to the distribution among all included subjects or used as continuous variables, reported as a decrease by 30 g/day (wholegrain) or 10 g/day (fiber).

3.1.6 Established risk factors and potential confounders

Age was included as continuous variable. Family history of diabetes (FHD) was dichotomized as either positive or negative, (for classification se section on baseline study). BMI (kg/m^2) was used as a continuous variable or in three groups (<25.0, 25.0-29.9, and ≥30.0) for baseline characteristics. Physical activity was assessed based on the question on physical activity during leisure hours. Activity was categorized into three groups; low (sedentary), middle (moderate activity) and high (regular exercise and training). Smoking was categorized in three groups; never, former and current smoker. Education was categorized into three groups; low (elementary school), middle (senior high school, technical and vocational school) and high (university). Hypertension was categorized as "yes" in subjects having systolic blood pressure ≥140 and/or diastolic blood pressure ≥90 and/or on anti-hypertensive treatment, and as "no" in subjects having blood pressure <140/90 and no hypertension treatment.

3.1.7 Sample description and genotyping

All included subjects had complete data on FFQ as well as on potential confounders at baseline. The study was based on partly different samples. The first sample included all subjects participating in the follow-up study, thus having either NGT or prediabetes at baseline. Cases were subjects who had progressed regarding their glucose tolerance at follow-up; from NGT at baseline to either prediabetes (IGT or IFG or the combination of the two) or to type 2 diabetes at follow-up or from prediabetes at baseline to type 2 diabetes at follow-up. Controls were all other subjects, i.e. those who did not display deterioration of glucose tolerance from baseline to follow-up. The second sample was a subgroup of the first sample and included only subjects with NGT at baseline. Cases in the second sample were subjects who progressed to either prediabetes or to type 2 diabetes at follow-up, whereas controls were subjects displaying NGT at both baseline and follow-up.

In a third sample, a subgroup of the first sample, data on TCF7L2 gene variants were available. DNA extraction and genotyping had previously been performed. Briefly five SNP were genotyped [108]. A selection of gene variants rs7903146 and rs4506565 was made for further association analysis. This sample comprised only men and cases were those who, from NGT or prediabetes at baseline, developed type 2 diabetes at follow-up. Controls displayed NGT at both baseline and follow-up.

3.2 ANIMALS AND CELL LINES

All animal studies were performed in accordance with guidelines from the Swedish National Board for Laboratory Animals and approved by the Northern Stockholm or by the Northern Swedish Ethical Committee on Experimental Animal Care. For the transplant study (paper II) inbred Wistar-Furth rats were used. For in vitro experiments Sprague-Dawley rats (an outbred model), were used. All animals were obtained by Scanbur (Sollentuna, Sweden).

The rat insulinoma cell line INS-1 (paper III and IV), characterised by Asfari M et al. 1992 [109], was chosen as origin for cell model. This cell line has been used extensively to study the mechanisms involved in regulation of insulin secretion because of its responsiveness to glucose at levels found in vivo [110]. From the INS-1 a stable clone of INS-1r9 was established. INS-1r9 carries the reverse tetracycline/doxycycline-

dependent transactivator [111] which has been described elsewhere [112, 113]. The plasmid used in the secondary stable transfection was constructed by subcloning the complementary DNA (cDNA) encoding the rat CD36 into the expression vector PUHD10-3 [111]. The procedures for stable transfection, clone selection and screening were previously described by Wang 1997 [112]. The CD36*10 clone was chosen for further experiments.

3.2.1 Islet and cell line culture, and islet isolation

Islets of Langerhans (paper II) were isolated by collagenase digestion in Hank's balanced salt solution (HBSS) as previously described [114], followed by sedimentation. Islets were handpicked under stereomicroscope and transferred to petri dishes containing RPMI1640, 2 mmol/l glutamine, 10 % (v/v) fetal bovine serum (FBS), 100 U/ml benzylpenicillin, 0.1 mg/ml streptomycin and 11 mmol/l glucose and cultured free-floating overnight at 37°C in an atmosphere of 5% CO_2 in air. For in vitro experiments islets from Sprague-Dawley rats were isolated and cultured overnight with different glucose concentrations as indicated by each experimental protocol. In long-term culture (two and three weeks respectively) islets were cultured in 0.5 % BSA instead of fetal calf serum. Culture media were changed every 2-3 day. After culture, equal-sized islets were transferred to dishes with Krebs–Ringer bicarbonate (KRB) medium, 10 mmol/l Hepes, 0.2% BSA, and 3.3 mmol/l glucose, and pre-incubated for 30 min at 37°C. They were then collected for electron microscopy, batch incubation, Western blot and measurements of ATP, ADP and DNA.
Cells (paper III and IV) were cultured in RPMI 1640 with 11 mM glucose, 10 mM HEPES, 10 % heat inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol. Further, INS-1r9 media was supplemented with geneticin 150 mg/ml and INS-1 CD36 was additionally supplemented with 100 mg/ml hygromycin. Overexpression of CD36 was obtained by culturing cells with doxycycline (75–500 ng/ml). Cells were grown in monolayer, split weekly by trypsin and all experiments were performed between the passages of 60-100.

3.2.2 Glucose-induced insulin secretion measurements

Following culture and pre-incubation as described above, equal sized islets (paper II) were incubated in groups of three for 60 min at 37°C in KRB (3.3 or 16.7 mmol/l glucose). Each experimental condition consisted of three or four individual groups of three islets. The insulin accumulated in the KRB was measured as previously described [115]. Islet insulin contents were measured after acid-ethanol extraction [116] of islets retrieved from batch incubations.
Cells (paper III and IV) were cultured for 48 h ±doxycycline (500 ng/ml) (in order to achieve overexpression of CD36). For *long-term* experiments cells were cultured ±doxyxycline and ±palmitate or oleate (50 µmol/l) (paper III) or nLDL or oxLDL (20 µg/ml or 50 µg/ml) (paper IV) for another 48 h. Prior to glucose-induced insulin secretion cells were cultured for 2-3 h in RPMI without glucose, with 20 mM HEPES and with 1% FBS. Cells were further pre-incubated for 30 min in Krebs-Ringer bicarbonate buffer (KRB) without glucose and, with 10 mmol/l HEPES and 0.2% BSA. Final incubations were made in same KRB but with the indicated glucose concentrations of 2.5 and 21.5 mM for 30 min. For *acute* experiments cells were

cultured for 48 h and then precultured and preincubated as previously mentioned and at final incubation palmitate (100 µmol/l or 50 µmol/l) or oleate (100 µmol/l) (paper III) or nLDL or oxLDL (20 µg/ml or 50 µg/ml) (paper IV) was added. Aliquots of secretion into media were secured. Acid ethanol was added to cells to extract cellular insulin contents.

3.2.3 Transplant protocol

One day after birth male rats (paper II) were made diabetic by an intra-peritoneal injection of 90 mg/kg streptozotocin (Sigma). Diabetes was confirmed by blood glucose >10.0 mmol/l at day three. Female syngenic rats i.e. immunologically compatible for transplantation, were used as donors. Islets of Langerhans were isolated from 16-20 weeks old rats as previously described and cultured overnight. The following day 50-150 islets were transplanted under the left kidney capsule as previously described [92, 117]. To minimize inter-donor variation islets isolated from two to three rats were mixed and divided into three equal portions; one third was transplanted to a non-diabetic rat (later vehicle-treated), one third to diabetic rats (later vehicle-treated) and one third to diabetic rats (later treated with tifenazoxide). The number of islets transplanted to each rat was purposely kept low enough not to reverse diabetes in the recipients. Following transplantation, rats were administered vehicle (non-diabetic and diabetic recipients) or tifenazoxide (3 mg/kg, diabetic recipients) once daily between 08.00 and 10.00 hours by gavage. The vehicle component consisted of glycerol 5 vol %, gelatine 0.5 %, 40 vol % and carboxy-methyl-cellulose 2 %, 55 vol %. Tifenazoxide was dissolved in 2 % NaOH. The treatment period lasted for 63 days. It was followed by 7 days of wash-out, i.e. the cessation of vehicle or tifenazoxide administration. The transplants were cut out of from the under renal capsule. At all times the animals had free access to water and a standard laboratory chow for rats (Scanbur, Stockholm, Sweden). Blood glucose levels were measured non-fasting once every week between 08.00 and 10.00 hours (i.e. just before medication) throughout the experimental period.

3.2.4 Electron microscopy - ultrastructural morphometry

Transplants (paper II) were excised from under renal capsule and fixed in ice cold mixture of 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, with an osmolarity of 390 mOsmol/kg. After dehydration transplants were embedded in an epoxy resin. Ultra-thin sections giving a silver interference colour were prepared on an ultramicrotone and picked up on form coated one-hole grids. The sections were stained with uranyl acetate and lead citrate. The electron microscopy was performed with a Hitachi H-700 with 75 kV acceleration tension. After fixation and embedding, eight transplants from each experimental group were prepared for electron microscopy. Twenty-two electron micrographs at a primary enlargement of 7200x were made from each transplant. The micrographs were distributed over the sections areas by systematic random sampling of beta-cell-rich areas. The negative film was scanned and analysed. In the stereological analysis, beta-cells were identified by their typical appearance of secretory granules. A total of 176 sections were analysed per treatment group. Totally 528 electron micrographs sections were analysed including approximately 25.000 mitochondria. Next a grid was applied to the image and parameters analysed. The methods to calculate the parameters are previously described [118, 119]. Insulin

granules were calculated and classified as mature or immature. Mature granules were classified by the appearance of a dense core and wide halo whereas the immature granules were classified by the appearance of pale core and narrow halo. Also for insulin granules volume density, number of granules and average volume were calculated in a similar fashion as for mitochondria.

3.2.5 Subcellular fractionation and Western blots

Cells (paper III) were grown ±doxycycline (500 ng/ml). Cells were washed, harvested and homogenized with a pestle motor followed by 10 passages through a syringe in homogenization buffer. Homogenate was centrifuged at 700 x g for 15 min. Supernatant was mixed with sucrose (250 mM) and Percoll (15 %). Further centrifugation was performed at 48 000 x g for 25 min. The plasma membrane (top fraction) and insulin granule (bottom fraction) were collected. Fractions were washed two times in homogenization buffer by 150 000 x g centrifugation for 30 min. Cytosol fraction was obtained by centrifugation of homogenate at 150 000 x g for 60 min [120]. Plasma membrane fraction was confirmed by western blot with a Na^+ /K^+ ATPase antibody. Secretory granules were confirmed by measurement of insulin (measured by RIA as previously described).

Table 2. Western blot antibodies

Primary antibody	Type	Species	Dilution	Origin
Total OXPHOS Cocktail	Monoclonal	Mouse	1:5000	MitoSciences USA
OPA-1	Monoclonal	Mouse	1:1000	BD Biosciences Transduction Laboratories, Sweden
CD36	Polyclonal	Rabbit	1:500	Santa Cruz Biotechnology, USA
Na^+/K^+ ATPase	Monoclonal	Mouse	1:2000	Millipore, USA

After culture, islets (paper II) or cells (paper III and IV) were washed and protein concentration (for cells) was measured by DC protein assay (BIO-RAD). Samples were denatured in loading buffer at room temperature for 20 min or at 80°C for 10 min. Samples were analysed on 7.5-12% sodium dodecyl sulfate polyacrylamide electrophoresis gels (SDS-PAGE) run for 1 h at 150 V and were then transferred to nitrocellulose membrane for 1 h at 250 mA. Membranes were blocked for 2 h at room temperature with 5% (w/v) fat-free milk, 0.1% Tween 20 (Sigma) in Tris-buffered saline, pH 7.6 for 2 h at room temperature. Membranes were then incubated overnight at 4° C with primary antibody (see table 2). Membranes were washed in Tris-buffer for 1 h and then incubated in 2.5 % fat-free milk in 0.1% Tween 20 in Tris-buffered saline with secondary antibody. Secondary antibody incubations employed a HRP-linked anti-mouse or goat-anti rabbit (Pierce Biotechnology) for 1 h at room temperature. Bands

were visualized by chemiluminescence kit (Pierce Biotechnology), exposed to film (Amersham Biosciences) and documented with a flat-bed scanner and quantitation software (Kodak 1D).

3.2.6 Real-time reverse transcription PCR

We used PCR to quantify the relative amount of mtDNA (paper II) present per nDNA. DNA was extracted by DNeasy Blood & Tissue kit (Qiagen). Mitochondrial relative DNA copy number was determined by calculation of the mtDNA/nDNA to the control ratio (i.e. 11 mmol/l glucose). mtDNA-encoded NADH dehydrogenase 2 gene (ND-2) was used as a marker for total mtDNA and 18S ribosomal DNA for nDNA.
Cells (paper III) were treated as for long-term insulin secretion experiments. After that, cell total RNA, isolated using an RNeasy mini kit (Qiagen), was reverse-transcribed using random hexamer primers and SuperScript II (Invitrogen) according to the manufacturer's instructions. The real-time RT-PCR was monitored and analysed by the Sequence Detection System (Applied Biosystems). All genes were normalized to18S and beta-actin.

3.2.7 ATP, ADP and DNA measurements

ATP (paper II) was determined using a bioluminescence kit (Roche Diagnostics). ADP was measured after enzymatic removal of ATP, as described previously [121]. For DNA, islets (paper II) were washed twice with cold PBS and 1 µl PBS/islet was added followed by sonication. Fluorescent DNA Quantitation Kit (Bio-Rad) was used according to manufacturer’s instructions.

3.2.8 Respiring mitochondria and oxygen consumption

A cell suspension was prepared from islets (paper II) by trypsin digestion as described previously [122]. Cell suspension were directly plated on polylysine-coated cover glasses in Petri dishes and cultured for 48 hours in RPMI 1640 supplemented with 5.5 or 27 mmol/l glucose ± diazoxide (325 µmol/l). After culture cells preincubated in 3.3 mmol/l glucose for 30 min at 37°C and 70 nmol/l Red CMXRos, MitoTracker Red (MTR) (Molecular probes) was added for 10 min and then rinsed. MTR is a cell-permeable selective dye, which passively diffuses across plasma membranes and accumulates in active mitochondria. The dye covalently binds free sulfhydryls and does not fluorescence until it enter an actively respiring cell. To reduce potential artefacts and mitochondrial toxicity from overloading, the dye concentration was held as low as possible (70 nmol/l). Live cell images were collected using a Leica SP2 spectral laser scanning confocal microscopy system equipped with a 250 mW argon/krypton laser (Omnichrome Inc.). The technique for measurement of MTR labelled beta-cells has been described in detail [123].

The oxygen consumption (paper II) was measured by Clark-type polarographic oxygen sensors and high-resolution respirometry (Oxygraph-2k, OROBOROS). Samples of 400 islets (paper II) in cell culture medium were added to a chamber recording oxygen uptake at basal respiration during 20 min of stable oxygen consumption. Consumption rates were calculated as the negative time derivate followed by the addition of 2 µg/ml oligomycin of the oxygen concentration present in the chamber (pmol/s/400 islets).

3.2.9 LDL isolation, preparation and labelling

LDL (paper IV) (density = 1.019 – 1.063 g/ml) was isolated by ultracentrifugation from pooled plasma of healthy donors obtained from Blood Central of Karolinska University Hospital, Stockholm Sweden, as described [124]. 2 mmol/l Benzamidine, 0.5 mmol/l PMSF and 0.1 U/ml Aprotinin (all from Sigma-Aldrich), were added immediately after the plasma was prepared. After isolation, LDL was dialyzed extensively against PBS. 1 mmol/l EDTA (Sigma-Aldrich) was added to an aliquot of LDL to generate unmodified LDL. Oxidized LDL was obtained by incubating 1 ml of LDL (1 mg/ml) protein content, determined by Bradford assay, (BIO-RAD) in the presence of 20 µmol/l $CuSO_4$ (Sigma-Aldrich) for 18 h at 37°C. The oxidation of LDL leads to the formation of lipid peroxidation products, i.e. malondialdehyd (MDA), that are reactive to thiobarbituric acid (TBARS). The extent of oxidation was evaluated as described [125]. Lipoprotein preparations were sterilized by passage through a 0.22 µm filter.

LDL was labelled by fluorescein isothiocyanate (FITC) using a modification of a previously described method [126]. Briefly, LDL (1.5-2 mg/ml) was dialyzed overnight against 500 mmol/l $NaHC0_3$ pH 9.5. Next, 100 µg of FITC (Sigma-Aldrich), dissolved in DMSO (1 mg/ml), was added for each mg of LDL and incubated at room temperature for 2 h. After incubation conjugates were separated from the free fluorochrome by filtration on a PD 10 column using PBS for elution. FITC concentration in LDL preparations was measured by absorption spectroscopy against FITC standard at 495 nm. Protein concentration was determined by Bradford assay (BIO-RAD). Radiolabeled LDL was obtained with the incorporation of [1,2-^{3}H] cholesteryl oleate as previously described [127]. In brief, 1 mCi of [1,2-^{3}H] cholesteryl oleate was dried under N^2 and then re-dissolved in 100 mL of dimethyl sulfoxide. LDL (20 mg protein/mCi) was then added to the solution and allowed to incubate for 2 hours. At the end of the incubation period, free excess of [1,2-^{3}H] cholesteryl oleate was removed by PD10 gel-filtration (GE Healthcare).

3.2.10 Fatty acid uptake and efflux

Cells (paper III) were cultured ± doxycycline (500 ng/ml). For uptake experiments cells were counted in Bürker chamber and equal amount of cells were transferred to tubes and centrifuged for 2 min 1000 rpm. Cells were re-suspended in ice-cold KRB with 11 mmol/l glucose and 2.1 µCi (6 µmol/l) ^{14}C-oleate (GE Healthcare) and transferred in triplicates to micro-centrifugation tubes which were prepared with a bottom layer of 6 mol/l urea over layered by a 10:3 mixture of dibutyl-dinonylphtalate [128]. Uptake was stopped by centrifugation at 8000 rpm for 15 s. The urea layer containing the cells was cut off and put into scintillation vials. Scintillation liquid was added and samples were transferred to a gamma counter (Packard).

In some uptake experiments an irreversible inhibitor of CD36 Sulfo-N-Succinimidyl-oleate (SSO) was used (a kind gift from Dr N.A. Abumrad Washington University School of Medicine). SSO was prepared in 1 M stock solution in DMSO. Cells were preincubated for 25 min in 37°C in KRB without glucose and with or without 0.5 mmol/l SSO. DMSO concentration was kept below 0.05% and was added to control experiments. Cells were washed, and further treated as above. For efflux experiments cells were washed and then incubated with KRB and ^{14}C-oleate (same concentration as for uptake experiments) for 4 min in room temperature. Cells were then centrifuged and

re-suspended in ice-cold 2 % BSA-KRB and efflux stopped at different time points. Radioactivity in the separated cells was processed as above. Aliquots from efflux media were saved for thin layer chromatography (TLC).

3.2.11 Uptake and efflux of FITC / ^{3}H-cholesterol LDL

3.2.11.1 FITC-LDL

Cells (paper IV) were cultured for 36 h in 10% FBS ±doxycyline (75 or 500 ng/ml). They were then further cultured in media containing 1% FBS for 12 h ±doxycycline. Fresh culture media with 1 % FBS with or without LDLs (20 µg/ml) were added. After 30 min and onward time-points cells were washed twice with ice cold PBS. Cells were then flushed off by vigorous pipetting, transferred to tubes and centrifuged at 1000 rpm for 2 min at 4°C. Supernatants were discarded and cells re-suspended in 4 % formaldehyde (PAF). The uptake of FITC-labeled nLDL and oxLDL was analysed on a CyAnTM ADP flow cytometer (Dako). For each experiment 10,000 events were acquired. The fluorescence of cells without added lipoproteins or FITC was used as control. Uptake of FITC nLDL and oxLDL was also performed in low (2.5 mmol/l) or high (21.5 mmol/l) glucose. In some experiments the uptake of LDLs was also performed with INSr9 cells (which only harbour the doxycycline gene).
For efflux experiments uptake media were removed and efflux media with HDL as acceptor with or without doxycyline (500 ng/ml) was added to cells. They were then cultured to allow efflux for 4 h. From results of preliminary experiments with 10-250 µg/ml of HDL a concentration of 50 µg/ml was chosen for further experiments. Efflux was stopped and collection of cells was performed as described above.

3.2.11.2 ^{3}H-cholesterol LDL

CD36 overexpression (paper IV) was induced with doxycyline (500 ng/ml) as for FITC experiments. The uptake of ^{3}H-labelled LDLs was assessed after culture with ^{3}H-cholesterol-nLDL or ^{3}H-cholesterol-oxLDL (20 µg/ml) for 24 h in 1% FBS. Cells were then washed with PBS and lysed in acid ethanol. Aliquots were later counted in a scintillation counter.
Efflux of ^{3}H with HDL as acceptor was measured at different time points after uptake of ^{3}H. Media were then secured and cells were lysed in acid ethanol (to measure remaining radioactivity).

3.2.12 Thin layer chromatography

After incubation ± doxycycline (500 ng/ml) for 48 h, and then further incubation with ^{14}C-Oleic or ^{14}C-palmitic acid (1 µCi, 18.9 and 16.7 µmol/l respectively, GE Healthcare) for 30 min, 4 or 48 h cells (paper III) were washed in PBS, trypsinized and washed again. To the cell pellet chloroform/methanol (2:1) was added together with ^{3}H-triolein (0.05 µCi, internal standard, Perkin Elmer Life and Analytical Sciences). This was followed by 30 min of incubation with periodic vortexing at room temperature. After centrifugation (6000 rpm for 2 min), the phases were separated by adding NaCl (0.9%) followed by centrifugation (2000 rpm for 2 min). The upper phase was removed and the lower organic phase was dried under N_2-gas. The dried lipids were solubilised in chloroform/ethanol (9:1) and applied to a Silica gel TLC plate (Merck). TLC Mix 32 (Larodan Fine Chemicals) was used as a positive control.

Petrolium ether/Ether/Acetic acid (79:20:1) was used as mobile phase. The lipids were visualized by spraying with phosphomolybdic acid. Spots representing lipids were cut out, mixed with scintillation cocktail and counted for ^{3}H and ^{14}C radioactivity. Aliquots from efflux experiments were performed specifically for fractionation of oleate. The procedure was basically the same as for cells with the exception that unlabelled oleate was added to each sample before the TLC procedure.

3.2.13 Oil Red O and triglyceride measurement

Cells (paper III and IV) were cultured in the indicated concentration of doxycycline, palmitate, nLDL and oxLDL for 48h. Cells were fixed in 4 % PAF, rinsed and stained with oil red O and counterstained with hematoxylin.
Cellular triglycerides (paper III) were extracted according to Folch et al. [129] and measured using the Triglyceride (GPO-TRINDER) kit (Sigma).

3.2.14 Fatty acid oxidation and CPT1 activity

Cells (paper III) were cultured ±doxycycline (500 ng/ml) for 48 h and treated as for insulin secretion experiments. Cells were then incubated in 2.5 and 21.5 mmol/l glucose KRB in the presence of 0.084 μCi (14 μmol/l) of [1-^{14}C]-oleate (GE Healthcare). Incubations were carried out in a shaking water bath at 37 °C for 2 h in 1-ml glass tubes inside 20-ml scintillation vials. Incubation was stopped by injection of 0.1 ml of 0.1 mol/l HCl and hyamine was added to scintillation vials. The sealed scintillation vials were left overnight, at room temperature, to absorb $^{14}CO_2$ into the hyamine. Samples were put in counter and data were expressed as pmol [1-^{14}C]-oleate/2 h/1x10^6 cells.
For CPT1 activity, cells (paper III) were induced with doxycycline (500 ng/ml) for 48 h. During the last 24 h of this period the glucose concentration was changed from 11 to either 5.5 or 27 mmol/l glucose. CPT1 activity was determined by a radiometric method as described in Morillas et al. [130].

3.2.15 Oxidative stress

For measurement of oxidative stress cells (paper III) were cultured for 48 h ±doxycycline (500 ng/ml). Cells were then washed and re-suspended in PBS, and loaded with 5 μmol/l CM-H_2DCFH-DA (Molecular Probes) for 20 min at 37 °C. Following loading, cells were exposed to 40 or 400 μmol/l of H_2O_2. The production of ROS was assessed by measuring the fluorescent signal evoked by the deacetylated product (DCF) in the setting of a 488 nm argon laser and 550 ±20 band-pass filters.

3.2.16 Mitochondrial mass

Mitochondrial mass was assessed by fluorescence of the Mito Tracker Green (Molecular Probes M7514). This probe is taken up into mitochondria, producing a fluorescence signal which is reportedly not affected by the prevailing mitochondrial membrane potential, thereby providing a measure of mitochondrial mass. After culture, cells (paper III) were collected, washed and re-suspended in PBS. Cells were loaded with 0, 20, and 50 nmol/l Mito Tracker Green for 30 min at 37 ˚C. After loading, dead cells and fragments were gated-out by forward and side scatters. The mitochondrial

mass was assessed by measuring the fluorescent signal in the setting of a 488 nm argon laser and 550 ± 20 band-pass filters.

3.2.17 Viability assays

For the different tests of viability, cells (paper IV) were cultured in for 48 h ±doxycycline (500 ng/ml) and then further incubated for 48 h ±doxycycline and/or with 20, 50 or 100 µg/ml nLDL or oxLDL. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [131] media were removed and fresh media with MTT solution 0.5 mg/ml (Sigma-Aldrich) were added to the wells. The plates were incubated for 6 h at 37°C in an atmosphere of 95 % O_2 and 5 % CO_2. One half of the volume of the media was removed and 2-propanol, supplemented with HCl (3.3 ml/l), was added to solubilize the MTT formazan. The plates were then placed on a mechanical shaker for 1 h at room temperature to complete solubilisation. Absorbance was measured at 570 nm using Multiskan Ascent microplate photometer (Thermo Scientific).
Cytotoxicity was also evaluated by the release of lactate dehydogenase (LDH) into culture media using a LDH assay kit (Cayman Chemicals). Culture plates were centrifuged for 10 min at 200 x g. Culture media was removed and cells were lysed for 30 min and further analysed according to manufacturer's protocol.
For apoptosis measurements, cells (paper III) were exposed to test agents, oleate (100 µmol/l) and palmitate (50 µmol/l) ±doxycycline (500 ng/ml) in 11 mmol/l glucose and 10 % FCS for 48 h. Camptothecin (positive control) was added only during the last 12 h. Cells were detached by treating with 0.05 % EDTA in PBS and collected by centrifugation. Apoptotic and necrotic cells were dual stained with Annexin V and propidium iodide using a Vybrant® Apoptosis Assay Kit #2 (Molecular Probes). Flow cytometric analysis was performed using a 15 mW argon laser (488 nm), 550 nm diachronic long-pass filter and 550 ± 20 nm band-pass filter detecting Annexin V and 675 ± 15 nm band-pass filter detecting propidium iodide. The percentage of apoptosis and necrosis was calculated from 3 separate experiments.
Apoptosis was also measured in paper IV with the use of a cell death detection ELISA kit (Roche) according to manufactures instructions.
DNA fragmentation (paper III) was assessed by the Quick Apoptosis DNA Ladder Detection Kit (LabForce/MBL) following the manufacturer's protocol.

3.2.18 Confocal microscopy

After uptake of FITC-labelled LDLs for 24 h cells (paper IV) were transferred to slides by using Shandon Cytospin 2 at 500 rpm for 5 min. Nuclei were stained by DAPI (1:250000) (Sigma-Aldrich) in PBS for 10 min. Slides were then washed twice in PBS and mounted. Cell fluorescence was analysed with a Leica TCS SP5 (Leica Microsystems) microscope equipped with filters for the detection of DAPI and FITC. Images were acquired at x63 magnification using an immersion oil objective and the Leica Application Suite Advanced Fluorescence software.
Cells (paper III) were grown ±doxycycline 500 ng/ml. Cells were fixed in 4 % (PAF) phosphate buffer and then permeabilised for 1 h in PBS containing 0.1% saponin and 0.5% BSA. The cells were incubated with CD36 antibody (Cascade Bioscience, mouse monoclonal IgA, clone 63, 1:500). They were then exposed to insulin antibody after which FITC- or Rhodamine-conjugated secondary antibodies were applied. Samples

were analysed using a Zeiss laser confocal microscope. Images were taken with a 60× objective.

3.3 STATISTICAL ANALYSIS

Paper I: Data are given as mean with 95% confidence interval (CI) or numbers (proportions). Comparisons of continuous variables were performed by unpaired t-test or one-way ANOVA for independent groups, by paired t-test for dependent groups and by chi-square test for categorical variables. Odds ratios (OR), together with 95% CI were calculated by multiple logistic regression analysis. Intake of wholegrain and fiber was categorized into tertiles according to the distribution among all included subjects or used as continuous variables, reported as a decrease by 30 g/day (wholegrain) or 10 g/day (fiber). Since no statistical interaction was found between sex and intake of wholegrain or fiber, analyses are reported not only separately, but also for women and men combined. When categories of wholegrain or fiber were used in the logistic regression analyses, tests for linear trend were conducted by assigning median values in tertiles of wholegrain or fiber as a continuous variable. In analyses of genotype association both additive and dominant models were used. In the evaluation of the interaction between genotype and intake of fiber or wholegrain, multiplicative product terms were included in the logistic regression models. Differences between models with and without product term were evaluated by log-likelihood ratio test. Linear regression analysis was used to evaluate associations between intake of fiber or wholegrain and baseline values of HOMA-IR, HOMA- β, insulin-response$_{(2h\text{-}0h)}$ or proinsulin. In these analyses HOMA-IR, HOMA- β, insulin-response$_{(2h\text{-}0h)}$ and proinsulin were log transformed to improve skewness. P-values <0.05 were considered significant and we did not adjust p-values for multiple testing. The analyses were performed using the SAS Statistical Program version 9.2 (SAS Institute, Cary, NC, USA).

Paper II: Data in tables and figures are expressed as means ± SEM. For multiple comparisons, one-way ANOVA was performed. When normality test failed then Kruskal-Wallis ANOVA on ranks followed by Student-Newman-Keuls post-hoc test was performed. A *p* value of less than 0.05 was considered significant.

Paper III and IV: Results are expressed as mean ± SEM. Significant differences were tested using Student's paired t test (two-sided). A *P* value < 0.05 was considered significant.

4 RESULTS

4.1 PAPER I

At follow-up 12.8% of subjects had deteriorated in glucose tolerance, men more than women.
Results are focused on wholegrain since wholegrain generally revealed stronger associations than total fiber. A low intake of wholegrain was associated with an increased risk for deterioration in glucose tolerance (i.e. progress from NGT to prediabetes or type 2 diabetes, or from prediabetes to type 2 diabetes).

The subjects who at baseline had NGT and at follow-up had progressed to prediabetes showed the strongest association with intake of wholegrain. Analysing men and women separately, the association was stronger in men compared to women. However no interaction between sex and wholegrain intake could be documented.

Linear regression analysis revealed an inverse association between HOMA-IR at baseline and intake of wholegrain at baseline for all subjects. An inverse association was also found for intake of wholegrain at baseline and insulin response$_{(2h-0h)}$ for all subjects.

Subjects who had deterioration of glucose tolerance i.e. progressed to either prediabetes or type 2 diabetes were categorized into tertiles of their baseline values of HOMA-IR, HOMA-beta and insulin response$_{(2h-0h)}$. The majority of cases in the highest tertile of HOMA-IR were also found among those in the highest tertiles of HOMA-beta and of insulin response$_{(2h-0h)}$.

Linear regression analysis revealed an inverse association between proinsulin and intake of wholegrain among all men. Proinsulin levels for subjects who later deteriorated in glucose tolerance increased significantly by increasing tertiles of HOMA-beta.

Individual intake of wholegrain at baseline was similar to intake at follow-up.

Evaluations of associations between intake of wholegrain and fiber and TCF7L2 rs7903146 and rs4506565 were done in a subset of men. A significant association was found between baseline intake of wholegrain and development of type 2 diabetes at follow-up. When stratified on genotypes, no associations were found in subjects carrying the T-allele (risk allele for both SNPs), either in heterozygotic or homozygotic carriers, between decreased baseline intake of wholegrain at baseline and development of type 2 diabetes after adjustment for all confounders. In non-risk allele carriers i.e. CC (rs7903146) and AA (rs4506565) an increased risk to develop type 2 diabetes was found with decreasing intake of wholegrain. Assuming an additive model, interaction terms, genotype × intake of wholegrain, included in the regression model, were significant for both SNPs for wholegrain. Since individuals with heterozygous genotype conferred a significant risk of diabetes compared to non-risk allele carriers a dominant model of inheritance was also evaluated, and similar results were obtained.

4.2 PAPER II

4.2.1 In vivo results

Transplantation moderately but swiftly decreased blood glucose in the diabetic-vehicle treated rats, (presumably because of the added beta cell mass). Such a rapid decline was not apparent in tifenazoxide-treated rats, probably because of the blocking effect of this compound on insulin secretion. All in all, moderate hyperglycaemia prevailed. Body weight increased less in the vehicle-treated diabetic rats than in non-diabetic recipients. Body weight did not increase appreciably in tifenazoxide-treated diabetic rats.

The outer surface area of mitochondria per unit beta cell volume (S_{VM}) was decreased by 12 % by the diabetic state. Most importantly the number of mitochondria per unit beta cell volume (N_{VM}) was decreased (by 31 %) in vehicle-treated diabetic rats compared to non-diabetic rats.

Secondary parameters showed that the diabetic state led to larger mitochondria as demonstrated by an increase in average mitochondrial volume by 68 % (V_{VM}/N_{VM}), and average mitochondrial outer surface area by 38 % (S_{VM}/N_{VM}). Conversely, the mitochondrial outer surface area per unit mitochondrial volume (S_{VM}/V_{VM}) was 16 % smaller in diabetic vs. non-diabetic rats. This indicates that there was a change in shape from elongated to more round mitochondria in diabetic vs. non-diabetic vehicle-treated animals. We observed mitochondria of at least 2-3 fold increased size and with reduced density in many sections of grafts in vehicle-treated diabetic animals. The variation in average mitochondrial volume and outer surface area was larger in grafts from vehicle-treated diabetic vs. non-diabetic treated animals. Treatment with tifenazoxide partially corrects effects by the diabetic milieu. Previous tifenazoxide-treatment of the diabetic rats partly but significantly normalized the diabetes-induced differences in average mitochondrial volume and average mitochondrial outer surface area, $p < 0.05$. Tifenazoxide also reduced the variability of mitochondrial size by eliminating mega-mitochondria.

Insulin granules were quantified according to volume of granules per unit beta cell volume V_{VM}, number of granules per unit beta cell volume N_{VM} and average volume V_{VM}/N_{VM}. All parameters were decreased in beta cells from grafts in diabetic animals for the mature granules.

Qualitative ultra-structural findings from vehicle treated non-diabetic animals showed that mitochondrial profiles were round or elongated and the mitochondria had matrix of moderate density with distinct cristae in the internal membrane. Compared to vehicle-treated diabetic animals which had low density and disrupted cristae with irregular pattern. Observations of fusion or fission of mitochondria were also present in the vehicle-treated diabetic animals. Tifenazoxide-treatment of the diabetic rats resulted in a beta cell morphology that seemed of that in transplants to non-diabetic recipients.

4.2.2 In vitro results

Long-term in vitro culture in high glucose reproduced quantitative changes in mitochondrial morphology obtained in vivo. Abnormal morphometry associates with mitochondrial dysfunction. Glucose-stimulated insulin-secretion was decreased after long term culture in high glucose.

Long-term culture in high glucose also reduced constituents of mitochondrial complexes I-IV and OPA1 and reduced the ATP and ADP levels. Basal oxygen consumption was higher in islets cultured in 11 mmol/l glucose compared to islets cultured in 27 mmol/l. Basal oxygen consumption was also more reduced after addition of oligomycin (an inhibitor of ATP synthase activity) in 11 mmol/l compared to 27 mmol/l glucose. Likewise, was a measurement of active respiring mitochondria (measured by MTR) higher for the islets cultured in 11 mmol/l glucose compared to 27 mmol/l glucose.

4.3 PAPER III

CD36 protein was found in the plasma membrane in a doxycycline dose dependent manner (shown by immunofluorescence and western blot on subcellular fractions).

Cells with higher levels of CD36 protein had increased uptake of fatty acids but also increased efflux of fatty acids (demonstrated by efflux and TLC). Visual inspection (oil-red O) suggested an increased accumulation of lipids with increasing CD36 protein levels. Total cellular triglyceride content revealed a 20±5% increased accumulation with increased levels of CD36 protein. Yet the incorporations of isotope-labelled fatty acids were similar regardless of the CD36 protein levels.

Fatty acids acutely potentiated the glucose-induced insulin secretion however this effect was abolished in cells with increased protein levels of CD36. After 48 h culture with fatty acids glucose-induced insulin secretion was reduced in cells with higher levels of CD36 protein. Fatty acid oxidation was inhibited by high glucose. This inhibition by glucose was moderately supressed in cells with increased levels of CD36 protein. The inhibiting effect of high glucose on CPT1 enzyme activity was also repressed in cells with increased levels of CD36 protein.

Increased CD36 protein did not change the UCP-2 mRNA nor did it decrease the GPR40 mRNA. Increased CD36 protein did not induce apoptosis measured by DNA-laddering and Annexin V nor was increased ROS formation seen.

4.4 PAPER IV

An increased level of CD36 protein dose-dependently enhanced the cellular uptake of FITC-labelled oxLDL while no enhanced uptake was seen for FITC-labelled nLDL. Cells without increased levels of CD36 also displayed increased uptake of oxLDL compared to nLDL however not to the same degree as the cells with increased CD36 protein. Accumulation of FITC-labelled LDLs were visualised by confocal microscopy and an increased accumulation was seen for FITC-oxLDL cultured cells and especially

in cells with increased levels of CD36. However no accumulation of lipids could be detected by oil-Red O.

Increased uptake of oxLDL in cells with overexpression of CD36 did not induce additional toxicity tested by apoptosis, LDH release and MTT.

Culture for 48 h with oxLDL decreased glucose-induced insulin secretion. The decrease was similar regardless of levels of CD36 protein. OxLDL also reduced cellular insulin contents irrespective of levels of CD36 protein.

^{3}H-cholesterol-oleate labelled oxLDL were taken up more than ^{3}H-cholesterol-oleate labelled nLDL. But compared to FITC-labelled oxLDL there was no effect of increased levels of CD36 protein.
Labelling cells for 24 h with ^{3}H-cholesterol-oleate labelled LDLs and then performing efflux experiment showed that the LDLs are quickly effluxed from cells independently of CD36 protein levels. Increased HDL concentrations (work as acceptor) increased efflux of LDLs dose-dependently. However for FITC-labelled LDLs there was no increased efflux with increasing HDL concentrations. Also FITC-labelled oxLDL was retained in cells with increased levels of CD36 protein more than the ^{3}H-cholesterol-oleate labelled oxLDL.
OxLDL up-regulated CD36 after 48 h culture. Also culture with nLDL increased CD36 protein.

5 DISCUSSION

5.1 PAPER I

Because OGTT was performed both at baseline and follow-up we were able to identify subjects with prediabetes, i.e. IGT and/or IFG, and analyse them separately from type 2 diabetes subjects. The clinical relevance of studying prediabetes has received more attention in the last years. A study from the US suggest that approximately 35% of US adults (over the age of 20 years) and 50% (over the age of 65 years) had prediabetes defined by fasting plasma glucose or HbA1c concentrations [132]. IGT is projected to increase according to International Diabetes Federation, furthermore American Diabetes Association expert panel estimates that 70% of individuals with prediabetes will eventually develop diabetes [132]. In our SDPP study, about 30-40% of individuals with prediabetes developed type 2 diabetes within 8-10 years [133].

In this prospective population-based cohort study we show a negative association between intake of dietary wholegrain (and to a lesser extent dietary fiber) and the development of prediabetes, i.e. IGT and/or IFG.

We found only one population-based prospective study outside the US that has evaluated the influence of fiber and wholegrain on the development of type 2 diabetes: the Finnish study by Montonen et al. [82]. In the Montonen study wholegrain consumption was associated with reduced risk of type 2 diabetes. In the Finnish study, the participants were retrieved from health care examinations and the outcome (type 2 diabetes) was assessed from the medical certificates needed for reimbursement of drug costs. Individuals with type 2 diabetes treated only with diet and changes in life-style were thus not identified. Furthermore, the Finnish study did not have the necessary data to adjust for FHD and physical exercise, both which are possible confounders. The Finnish study was rather small with 4316 participants; however an advantage was that they studied both men and women. Several US studies have shown inverse associations of wholegrain intake and risk of type 2 diabetes [134]. Compared to US studies [78, 135] the intake of wholegrain was much higher in Montonen study [82]. It is known that the intake of wholegrain is much higher in Scandinavian countries, yet only 16-35% of the population meet the recommendation of 75 g/day per 10 MJ [136].

A recent meta-analysis showed that wholegrain and dietary fiber intake was inversely associated with the risk for type 2 diabetes [134]. In our study, a negative association of wholegrain intake was found for type 2 diabetes when adjusted for age and sex: however it was not significant after full adjustment for confounders. This could probably be explained by the low number of individuals in the type 2 diabetes group.

Although there was no significant difference between sexes (as tested by interaction term) the adverse association of low intake of wholegrain and fiber was stronger in men. One reason may be that our FFQ was validated in a group of middle aged men [106] but not in women. Hence, we cannot rule out the possibility that the questionnaire (which was the same for men and women) reflected somewhat less the actual intake in women than in men. Another important difference is the higher incidence of

deteriorating glucose tolerance in men vs. women in our study, which is in accordance with other studies [137]. Also the time to follow-up for women was somewhat shorter than for men. In any case our findings are in line with the difference by gender for the risk of type 2 diabetes in [82, 134].

To investigate the mechanisms behind the effect of wholegrain we tested associations of wholegrain intake with measurements of insulin resistance and beta cell function. We found an inverse association; low intake of wholegrain and fiber corresponded to increased insulin resistance. This finding is in line with cross-sectional studies [76, 138] and suggests that insulin sensitivity play a role in the protective effects.

Elevated proinsulin levels are a feature of abnormal beta-cell function in type 2 diabetes. Linear regression analysis revealed a negative association between proinsulin and intake of wholegrain among all men. In subjects deteriorating in glucose tolerance, the level of proinsulin was significantly increased in higher vs. lower tertiles of HOMA-beta at baseline. However, the proinsulin/insulin ratios remained unchanged. Thus, our data probably reflect the increased demands on beta-cell secretion that are induced by insulin resistance.

Associations were stronger with wholegrain than with total fiber intake. This is in line with other studies, [134]. We therefore presume that fiber content per se does not altogether explain the favourable association to wholegrain intake. Many theories on mechanisms of wholegrain antidiabetic effects have been presented; they include increased mineral, vitamin and antioxidant content, beneficial effects of insoluble fiber through fermentation and formation of short chain fatty acids, gastric transit time/emptying, intestinal transit time and more [139]. The elucidations of which primary effects that are operative await further studies.

We confirm effect modifications by polymorphisms of the TCF7L2 gene that were reported previously [85]. Our data show that the protective effect of wholegrain intake on the risk of developing type 2 diabetes was undetectable in subjects carrying the TCF7L2 rs7903146 T-allele. This finding is in line with a previous report [85] but is in disagreement with a meta-analysis [56], which did not find an influence of the TCF7L2 rs 4506565 genotype. However, the meta-analysis did not test for association with type 2 diabetes as in [85] but for an association with fasting glucose or fasting insulin measured in subjects without diabetes. It has been suggested that the diabetogenic variant of the TCF7L2 gene decreases GLP-1 expression and actions [140]. Since GLP-1 is known to slow gastric emptying we speculate (in agreement with) [85] that loss of this effect in carriers of diabetogenic risk alleles could cancel out a similar effect exerted by wholegrain. Considering the fairly low number of subjects in our study for these analyses, confirmatory studies are needed.

For the results based on our entire study population we acknowledge that it was rather small (5477 subjects) compared to the US prospective studies [134]. However our study was population-based and included both men and women. Several of the prospective studies from US [134] included health professionals who could be considered a heterogeneous group; hence generalizability from these studies may be a concern.

Strength of this study was that misclassification of type 2 diabetes and prediabetes was probably low since OGTT was performed in all participants thus providing us with direct measurements of glucose and insulin concentrations from which we could calculate HOMA for insulin resistance and beta cell function.
A further positive feature was the possibility to check for changes in intake of wholegrain and fibers between baseline and follow-up. Since the intake was similar it is less likely that changes over time influence the results.
The calculated mean intake of fiber among men corresponded well with both the FFQ-validation study [106], and the most recent national food data survey results in Sweden by Becker and Pearson Riksmaten 1997-98 [74]. Women had higher total intake of fiber compared to men which also corresponds well with data from national food data survey [74]. These similarities strengthen the validity of our estimations of total fiber intake. However, the FFQ did not allow us to calculate total energy intake. This is a limitation of our study.

In conclusion, low wholegrain intake associates with increased risk of deteriorating glucose tolerance especially progression from NGT to prediabetes. The adverse associations are primarily seen in men and linked to insulin resistance. Furthermore, we add evidence for effect modification by polymorphisms of the TCF7L2 gene.

5.2 PAPER II

Chronic moderate hyperglycaemia induces fewer, larger, swollen and more variably sized mitochondria, demonstrated by morphological abnormalities found in islet transplants from in vivo experiments. It seems important that these morphological changes could be reproduced by long term hyperglycaemia in vitro. These similarities indicate that the abnormalities are, at least in part caused by direct and/or indirect effects of hyperglycaemia.

The morphological changes found in vitro were associated with dysfunctional oxidative phosphorylation. After culture in hyperglycaemia there was a reduction in energy production. Hence there was a reduction in levels of ATP, of oxygen consumption tied to ATP production, signalling by Mito Tracker Red (a marker of active respiring mitochondria) as well as proteins of oxidative phosphorylation i.e. mitochondrial complexes I-IV. Interestingly complex V, (ATP synthase), the final enzyme in oxidative phosphorylation, was not affected and furthermore mitochondrial DNA in relation to nuclear DNA was increased and not decreased. This suggests that the effects of hyperglycaemia did not reflect unspecific toxicity.

Culture in vitro at low glucose concentrations has shown adverse effects on islets [141]. Therefore we cultured islets in 11 or 27 mmol/l glucose.

Mitochondrial morphology has been studied in other animal models of diabetes. In Goto-Kakizaki (GK) rats, a spontaneous non-obese type 2 diabetes animal model, an increased number of mitochondria have been reported, coupled with a decrease in size [142]. These differences compared to our results could be explained by beta cell specific genetic factors in GK rats compared to the hyperglycaemia effects per se that

was studied by us. However when GK rats were challenged with sucrose [143], the morphological abnormalities were similar to the ones found in the present study.
Another diabetic animal model, without beta cell specific genetic influence, is the insulin resistant MKR mouse model. Studies in this animal model show (similar to us) a decreased number and increased size of mitochondria [144]. Perhaps the increased demands of insulin in the insulin resistant animal model and the hyperglycaemia in our study are comparable with regard to the morphological effects we detect on mitochondria.

In light of our observation of megamitochondria we looked at a key protein involved in the fusion process of mitochondria, namely OPA1 [30, 31]. We observe a decrease rather than an increase in OPA1 in hyperglycaemia. This would seem negate OPA1 as a driver in the process that results in megamitochondria. Possibly megamitochondria are instead formed by the inhibition of fission, however we did not study fission related proteins.

The beneficial effects of tifenazoxide in our in vivo diabetes model are in line with beneficial ones on insulin secretion using a very similar protocol [92]. K_{ATP}-channel openers (tifenazoxide or diazoxide) can protect glucose induced insulin secretion by alleviating mitochondrial dysfunction. Further studies on the molecular mechanisms behind the beneficial effect of tifenazoxide have been hampered by unavailability of the drug for supplemental in vitro investigations. Also experiences with diazoxide indicate that the drug is unsuitable for longer term of culture in vitro (results not shown). Hence the molecular mechanisms behind the drug induced beneficial effects remain to be investigated.

The clinical relevance and significance of our findings is that even moderately increased hyperglycaemia (similar to type 2 diabetes patients) can exert marked influence on mitochondria.

5.3 PAPER III

This study shows that the CD36 transporter in insulin-producing cells is functioning for facilitated fatty acid transport. Overexpression of CD36 induces functional effects on glucose-induced insulin secretion and on fatty acid metabolism.

We find (by immunofluorescence and western blot on subcellular fractions) that overexpression of CD36 protein was mainly localised in the plasma membrane. This is in line with studies in other cells which show that CD36 can be recruited to the plasma membrane from intracellular depots [145-147]. From the subcellular fractions we detected a week signal for CD36 (by western blot) in secretory granules which would agree with data from Noushmehr et al. [40] which detected CD36 in insulin granules in human islets of Langerhans. At the time of publication of paper III we were unable to detect a signal for CD36 by western blot in non-induced cells. However, as shown in results of paper IV, improvements of western blot technique now allow us to have a signal also for the non-induced cells. Nevertheless, our findings of localisation of CD36 were limited to CD36 overexpressing cells, hence confirmatory studies on CD36 localisation in non-induced cells, native beta-cells or INS-1 cells could be of interest.

Even with our considerable overexpression of CD36 protein we found only modest effects on fatty acid metabolism and glucose-induced insulin secretion. Possibly cellular adaptations come into play which reduces the impact of increased uptake of fatty acids. Overexpressing CD36 increased fatty acid uptake by approximately 40%. However, overexpression of CD36 also enhanced fatty acid efflux which could be one of these cellular adaptations. Such enhanced efflux was shown in another cell type with overexpression of CD36 [148]. This could be by mechanisms of passive diffusion.

The most apparent functional effect by CD36 overexpression was the loss of normal potentiation of glucose-induced insulin secretion by acute addition of fatty acids. Palmitate enhanced glucose-induced insulin secretion more than oleate in the non-induced cells, which is in line with previous studies [21]. Therefore the inhibited potentiation was more apparent in experiments with palmitate than oleate. Evidence for the specificity of a CD36 overexpression effect was strengthened by normal fatty acid potentiation of glucose-induced insulin secretion in tet-on cells lacking the coupling to CD36.
Mechanisms behind the effects of CD36 overexpression on insulin secretion may partly be related to the modest alterations in fatty acid oxidation that were observed. Normally glucose, via malonyl-CoA inhibits fatty acid oxidation by allosteric inhibition of CPT1 [149]. CD36 overexpression attenuated the inhibitory effect of glucose on fatty acid oxidation and CPT1 activity. Hence a deficient accumulation of malonyl-CoA or alternatively a reduced effect of this CPT1 inhibitor could play a role for the CD36-induced metabolic abnormality. This could lead to reduced accumulation of fatty acid derived signalling molecules [150]. It has been shown that a mutation in the CPT1 enzyme rendered the enzyme resilient to malonyl-CoA inhibition which led to decreased glucose induced insulin secretion [151].

Measurements of triglyceride stores and visual inspection of Oil-red O showed only moderate effects of CD36 on lipid accumulation. Furthermore, TLC data showed no increased incorporation of lipids by CD36 overexpression, suggesting that lipid accumulation would not cause the functional effects that we found.

Further, we found no structural damage (by the parameters; mitochondrial mass, ROS, or apoptosis) linked to increased uptake of fatty acids which could explain the functional effects.

Conclusively, CD36 overexpression increase uptake of fatty acids but also increases efflux of fatty acids. This futile transport of fatty acids could be thought to negatively affect the functional interplay between glucose and fatty acids on insulin secretion and fatty acid metabolism.

5.4 PAPER IV

This study shows that overexpressing CD36 in an insulin secreting cell line specifically enhances the uptake of FITC-labelled oxLDL and that this effect correlates with the extent of overexpression. In other cell types CD36 showed the highest affinity for oxLDL compared to other scavenging receptors that internalized oxLDL [152]. While

CD36 appears to be an important receptor for scavenging of oxLDL the importance of other scavenging receptors of oxLDL *in vivo* in beta cells is as yet uncertain.

OxLDL levels are elevated in type 2 diabetes patients and increase with duration of the disease [98]. Our results that oxLDL up-regulated the CD36 protein levels and that there was a dose-dependent relationship between degree of CD36 overexpression and resulting FITC-oxLDL accumulation suggests importance of the transporter during physiological and pathophysiological conditions. Our finding that also nLDL up-regulated CD36 can possibly be explained by formation of a significant amount of oxLDL during the 48 h period of culture [153].

We found that oxLDL dose-dependently decreases viability, measured by MTT, apoptosis and LDH release. Notably, these effects were independent of CD36 protein levels and uptake of FITC-oxLDL. Similarly, oxLDL dose-dependently decreased function, as assessed from glucose-induced insulin secretion, independently of CD36 protein levels and uptake of FITC-oxLDL. This discrepancy between FITC-oxLDL uptake and effect on viability and function can be explained by only modestly increased intracellular content of cholesterol, revealed by experiments of the uptake of ^{3}H cholesterol labelled oxLDL. Furthermore, there were no histological signs of lipid accumulation visualised by oil red O. Nor was the cell size increased as assessed from FACS data. Taking into account that FITC labels the protein part of LDL i.e. ApoB100, and that tritium (^{3}H) is incorporated into the LDL's cholesterol part, our results suggest that CD36 overexpressing cells on the one hand continuously enhance the uptake of oxLDL, recorded as the increased FITC accumulation over time, while on the other hand cells efficiently efflux cholesterol. Our data thus suggest that these insulin producing cells have efficient cholesterol efflux machinery that counteracts extensive lipid intracellular accumulation.

We found evidence of oxLDL toxicity with increasing concentrations of this lipoprotein, which is in line with studies using other types of cells [154]. Previous studies in rat pancreatic islet beta cells found no toxicity with increasing oxLDL concentration in the range of 0-25 µg/ml [100]. This discrepancy could be explained by concentrations tested, different cell types (INS-1 vs. primary rat beta cells), differences in culture conditions (FBS vs. BSA) or differences in oxLDL preparations. Our data indicate that toxic and functional effects of oxLDL do not require internalisation, and suggest that negative effects may come about due to extracellular receptor interactions. In fact, others have shown that deleterious effects of oxLDL may occur on the cell surface, such as formation of cytotoxins [155]. Extracellular effects could be exerted by a multitude of receptors which have been demonstrated in other tissues [156]. Interactions with such receptors can have negative effects [156].

We note that FITC-labelled products remain intracellular over a prolonged period of time. This suggests that the protein part of LDL, ApoB100, is not rapidly processed by INS-1 cells. It has been shown recently that ApoB100 derived peptides can influence innate and adaptive immune responses [157, 158]. Whether intracellular accumulation of ApoB100 can influence beta cell function remains to be investigated.

Some limitations to this study should be stated. The preparation method for oxLDL that we have used has been standardized and used in numerous studies during the last decades. However we cannot rule out that other modification pathways, i.e. by HOCl, chloramines, phenoxyl radical intermediates and peroxynitrate may generate other forms of oxLDL with different bioactivities. Moreover, this study is performed with INS-1 cells; hence ideally results should be confirmed in native beta cells. Furthermore oxidative modifications *in vivo* may differ from those that are here induced by copper.

5.5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Type 2 diabetes is a disease affecting many people in the world causing suffering and serious complications. With the extensive on-going research in vast different areas the knowledge about the disease is increasing; however the complexity of the disease has proven to be a difficult puzzle to solve.

Diabetes is thus a disease influenced by many factors and multiple genes. Therefore there are numerous mechanisms that can disturb the normally fine-tuned balance of glucose homeostasis. The loss of metabolic control that occurs in type 2 diabetes is detrimental for the progression of the disease. Hence early diagnose and treatment is important for preventing progression and complication of the disease. This thesis aimed to highlight some of the nutritional and dysmetabolic factors that potentially play a role in type 2 diabetes.

The adverse effects of hyperglycaemia are well studied but not fully elucidated. Beta cell metabolism appears to be a major player in these effects. Hyperglycaemia is often accompanied by dyslipidaemia which has emerged as an important target to treat as the risk for cardiovascular disease is increased in prediabetes and diabetes subjects.

We show that low intake of wholegrain increases the risk for developing abnormal glucose tolerance. Interesting studies for the future would be an intervention study to test if prediabetes subjects can reverse to normal glucose tolerance by increasing their intake of wholegrain. Other interesting studies would of course be to find molecular mechanisms for the effects of wholegrain intake.

Dyslipidaemia is a dysmetabolic factor with potential effects on type 2 diabetes. With our CD36 overexpressing INS-1 cell line we wanted to study the effects of increased uptake of fatty acids and oxLDL. Increased uptake of fatty acids and oxLDL shows negative effects on glucose-induced insulin secretion. The accumulation of lipids was however modest which would suggest that an efficient efflux system is present in the cells. Future studies in regulation of the efflux system could be of interest.

6 CONCLUSIONS

- Low consumption of wholegrain products carries increased risk for deteriorating glucose tolerance, especially progression from NGT to prediabetes. Furthermore, we add evidence for effect modification by polymorphisms of the TCF7L2 gene.
- Moderate hyperglycaemia induces morphological aberrations of beta cell mitochondria which can be replicated by long-term exposure to elevated glucose in vitro. These aberrations are then associated with dysfunction of oxidative phosphorylation.
- CD36 overexpression increases uptake of fatty acids but also increases efflux of fatty acids. This seemingly futile transport of fatty acids may affect the functional interplay between glucose and fatty acids on insulin secretion and fatty acid metabolism.
- CD36 specifically scavenges oxLDL into INS-1 cells; however the increased CD36 dependent uptake of oxLDL did not enhance toxicity and did not aggravate negative functional effects. Instead our data indicate that efficient intracellular cholesterol efflux machinery is present to balance increased uptake.

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8 REFERENCES

[1] Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract, 2011:311-321

[2] CDC. Centers for Disease Control and Prevention. National Diabetes Fact Sheet: national estimates and general information on diabetes and prediabetes in the United States. Atlanta GA, USA.: U.S. Department of Health and Human Services, 2011

[3] International, Diabetes, Federation. *IDF Diabetes Atlas, 5th edn.* Brussels, Belgium: International Diabetes Federation http://www.idf.org/diabetesatlas. In: Unwin N, Whiting D, Guariguata L, Ghyoot G, Gan D, eds., 2011

[4] Fourlanos S, Dotta F, Greenbaum CJ, *et al.* Latent autoimmune diabetes in adults (LADA) should be less latent. Diabetologia. 2005; 48: 2206-2212

[5] WHO. Diabetes Fact sheet Nº 312. 2011

[6] Maassen JA, Janssen GM, t Hart LM. Molecular mechanisms of mitochondrial diabetes (MIDD). Ann Med, 2005:213-221

[7] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med. 1998; 15: 539-553

[8] Agardh C-D, Berne C. Diabetes: Liber AB, 2009.

[9] WHO/IDF. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. WHO Document Production Services, Geneva, Switzerland, 2006

[10] Nathan DM, Davidson MB, DeFronzo RA, *et al.* Impaired fasting glucose and impaired glucose tolerance: implications for care. Diabetes Care. United States, 2007:753-759

[11] Uchizono Y, Alarcon C, Wicksteed BL, Marsh BJ, Rhodes CJ. The balance between proinsulin biosynthesis and insulin secretion: where can imbalance lead? Diabetes Obes Metab. England, 2007:56-66

[12] De Vos A, Heimberg H, Quartier E, *et al.* Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest. 1995; 96: 2489-2495

[13] Newsholme P, Gaudel C, McClenaghan NH. Nutrient regulation of insulin secretion and beta-cell functional integrity. Adv Exp Med Biol. 2010; 654: 91-114

[14] Mannhold R. KATP channel openers: structure-activity relationships and therapeutic potential. Med Res Rev. 2004; 24: 213-266

[15] Haber EP, Procopio J, Carvalho CR, Carpinelli AR, Newsholme P, Curi R. New insights into fatty acid modulation of pancreatic beta-cell function. Int Rev Cytol, 2006:1-41

[16] Newsholme P, Brennan L, Bender K. Amino Acid Metabolism, beta-Cell Function, and Diabetes. Diabetes. 2006; 55: S39

[17] Nolan CJ, Prentki M. The islet beta-cell: fuel responsive and vulnerable. Trends Endocrinol Metab. United States, 2008:285-291

[18] Sako Y, Grill VE. A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. Endocrinology. 1990; 127: 1580-1589

[19] Crespin SR, Greenough WB, 3rd, Steinberg D. Stimulation of insulin secretion by infusion of free fatty acids. J Clin Invest. 1969; 48: 1934-1943

[20] Malaisse W, F M-L. Stimulation of insulin secretion by noncarbohydrate metabolites. J Lab Clin Med. 1968; 72: 438-448
[21] Warnotte C, Nenquin M, Henquin JC. Unbound rather than total concentration and saturation rather than unsaturation determine the potency of fatty acids on insulin secretion. Mol Cell Endocrinol. 1999; 153: 147-153
[22] Yaney GC, Corkey BE. Fatty acid metabolism and insulin secretion in pancreatic beta cells. Diabetologia. 2003; 46: 1297-1312
[23] Itoh Y, Kawamata Y, Harada M, *et al.* Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature. England, 2003:173-176
[24] Cunha DA, Hekerman P, Ladriere L, *et al.* Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci. England, 2008:2308-2318
[25] Bollheimer LC, Skelly RH, Chester MW, McGarry JD, Rhodes CJ. Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. J Clin Invest. 1998; 101: 1094-1101
[26] Efendic S, Portwood N. Overview of incretin hormones. Horm Metab Res. 2004; 36: 742-746
[27] Mudaliar S, Henry RR. The incretin hormones: from scientific discovery to practical therapeutics. Diabetologia. 2012:
[28] Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. Nature. England, 2001:807-812
[29] Maassen JA, Jahangir Tafrechi RS, Janssen GM, Raap AK, Lemkes HH, t Hart LM. New insights in the molecular pathogenesis of the maternally inherited diabetes and deafness syndrome. Endocrinol Metab Clin North Am. United States, 2006:385-396, x-xi
[30] Westermann B. Molecular machinery of mitochondrial fusion and fission. J Biol Chem. United States, 2008:13501-13505
[31] Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. Cell. United States, 2006:1241-1252
[32] Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev. 1998; 78: 547-581
[33] Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes. 1997; 46: 1733-1742
[34] Maechler P, Li N, Casimir M, Vetterli L, Frigerio F, Brun T. Role of mitochondria in beta-cell function and dysfunction. Adv Exp Med Biol. 2010; 654: 193-216
[35] Anello M, Lupi R, Spampinato D, *et al.* Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. Diabetologia. 2005; 48: 282-289
[36] Kremmyda LS, Tvrzicka E, Stankova B, Zak A. Fatty acids as biocompounds: their role in human metabolism, health and disease: a review. part 2: fatty acid physiological roles and applications in human health and disease. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011; 155: 195-218
[37] Tvrzicka E, Kremmyda LS, Stankova B, Zak A. Fatty acids as biocompounds: their role in human metabolism, health and disease--a review. Part 1: classification, dietary sources and biological functions. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011; 155: 117-130
[38] Richieri GV, Kleinfeld AM. Unbound free fatty acid levels in human serum. J Lipid Res. 1995; 36: 229-240

[39] Schwenk RW, Holloway GP, Luiken JJ, Bonen A, Glatz JF. Fatty acid transport across the cell membrane: regulation by fatty acid transporters. Prostaglandins Leukot Essent Fatty Acids. Scotland: 2010 Elsevier Ltd, 2010:149-154
[40] Noushmehr H, D'Amico E, Farilla L, *et al.* Fatty acid translocase (FAT/CD36) is localized on insulin-containing granules in human pancreatic beta-cells and mediates fatty acid effects on insulin secretion. Diabetes. 2005; 54: 472-481
[41] Abumrad N, Harmon C, Ibrahimi A. Membrane transport of long-chain fatty acids: evidence for a facilitated process. J Lipid Res. 1998; 39: 2309-2318
[42] Su X, Abumrad NA. Cellular fatty acid uptake: a pathway under construction. Trends Endocrinol Metab. United States, 2009:72-77
[43] Febbraio M, Silverstein RL. CD36: implications in cardiovascular disease. Int J Biochem Cell Biol. 2007; 39: 2012-2030
[44] Collot-Teixeira S, Martin J, McDermott-Roe C, Poston R, McGregor JL. CD36 and macrophages in atherosclerosis. Cardiovasc Res. Netherlands, 2007:468-477
[45] Stewart CR, Stuart LM, Wilkinson K, *et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol. United States, 2010:155-161
[46] Ibrahimi A, Abumrad NA. Role of CD36 in membrane transport of long-chain fatty acids. Curr Opin Clin Nutr Metab Care. 2002; 5: 139-145
[47] Hajri T, Abumrad NA. Fatty acid transport across membranes: relevance to nutrition and metabolic pathology. Annu Rev Nutr. United States, 2002:383-415
[48] Febbraio M, Guy E, Coburn C, *et al.* The impact of overexpression and deficiency of fatty acid translocase (FAT)/CD36. Mol Cell Biochem. 2002; 239: 193-197
[49] Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta. Netherlands, 2000:189-210
[50] Heideman WH, Middelkoop BJ, Nierkens V, *et al.* Changing the odds. What do we learn from prevention studies targeted at people with a positive family history of type 2 diabetes? Prim Care Diabetes. England: 2011 Primary Care Diabetes Europe. Published by Elsevier Ltd, 2011:215-221
[51] Herder C, Roden M. Genetics of type 2 diabetes: pathophysiologic and clinical relevance. Eur J Clin Invest. 2011; 41: 679-692
[52] Wheeler E, Barroso I. Genome-wide association studies and type 2 diabetes. Brief Funct Genomics. England, 2011:52-60
[53] Lyssenko V, Lupi R, Marchetti P, *et al.* Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. J Clin Invest. 2007; 117: 2155-2163
[54] Pearson ER, Donnelly LA, Kimber C, *et al.* Variation in TCF7L2 influences therapeutic response to sulfonylureas: a GoDARTs study. Diabetes. United States, 2007:2178-2182
[55] Cornelis MC, Qi L, Kraft P, Hu FB. TCF7L2, dietary carbohydrate, and risk of type 2 diabetes in US women. Am J Clin Nutr. United States, 2009:1256-1262
[56] Nettleton JA, McKeown NM, Kanoni S, *et al.* Interactions of dietary whole-grain intake with fasting glucose- and insulin-related genetic loci in individuals of European descent: a meta-analysis of 14 cohort studies. Diabetes Care. United States, 2010:2684-2691
[57] Hales CN, Barker DJ, Clark PM, *et al.* Fetal and infant growth and impaired glucose tolerance at age 64. BMJ. 1991; 303: 1019-1022

[58] Steyn NP, Mann J, Bennett PH, *et al.* Diet, nutrition and the prevention of type 2 diabetes. Public Health Nutr. England, 2004:147-165
[59] WHO. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser. 2003; 916: i-viii, 1-149
[60] Colditz GA, Willett WC, Stampfer MJ, *et al.* Weight as a risk factor for clinical diabetes in women. Am J Epidemiol. 1990; 132: 501-513
[61] Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care. 1994; 17: 961-969
[62] Carey VJ, Walters EE, Colditz GA, *et al.* Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. Am J Epidemiol. 1997; 145: 614-619
[63] Vazquez G, Duval S, Jacobs DR, Jr., Silventoinen K. Comparison of body mass index, waist circumference, and waist/hip ratio in predicting incident diabetes: a meta-analysis. Epidemiol Rev. United States, 2007:115-128
[64] Frayling TM, Timpson NJ, Weedon MN, *et al.* A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. United States, 2007:889-894
[65] Kilpelainen TO, Qi L, Brage S, *et al.* Physical activity attenuates the influence of FTO variants on obesity risk: a meta-analysis of 218,166 adults and 19,268 children. PLoS Med. United States, 2011:e1001116
[66] Bassuk SS, Manson JE. Epidemiological evidence for the role of physical activity in reducing risk of type 2 diabetes and cardiovascular disease. J Appl Physiol. United States, 2005:1193-1204
[67] Feskens EJ, Virtanen SM, Rasanen L, *et al.* Dietary factors determining diabetes and impaired glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study. Diabetes Care. 1995; 18: 1104-1112
[68] Folsom AR, Ma J, McGovern PG, Eckfeldt H. Relation between plasma phospholipid saturated fatty acids and hyperinsulinemia. Metabolism. 1996; 45: 223-228
[69] Vessby B, Tengblad S, Lithell H. Insulin sensitivity is related to the fatty acid composition of serum lipids and skeletal muscle phospholipids in 70-year-old men. Diabetologia. 1994; 37: 1044-1050
[70] Vessby B, Gustafsson IB, Boberg J, Karlstrom B, Lithell H, Werner I. Substituting polyunsaturated for saturated fat as a single change in a Swedish diet: effects on serum lipoprotein metabolism and glucose tolerance in patients with hyperlipoproteinaemia. Eur J Clin Invest. 1980; 10: 193-202
[71] EFSA Panel on Dietetic Products NaA. Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. EFSA Journal. 2010; 8: 1462-1539
[72] Thorburn A, Muir J, Proietto J. Carbohydrate fermentation decreases hepatic glucose output in healthy subjects. Metabolism. 1993; 42: 780-785
[73] Schulze MB, Schulz M, Heidemann C, Schienkiewitz A, Hoffmann K, Boeing H. Fiber and magnesium intake and incidence of type 2 diabetes: a prospective study and meta-analysis. Arch Intern Med. United States, 2007:956-965
[74] Becker W, Pearson M. Avdelningen för information och nutrition Livsmedelsverket Riksmaten 1997-98 Kostvanor och näringsintag i Sverige. Metod och resultat analys. 2002
[75] EFSA Panel on Dietetic Products NaA. Scientific Opinion on the substantiation of health claims related to whole grain (ID 831, 832, 833, 1126, 1268, 1269, 1270, 1271, 1431) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal. 2010; 8: 1766-1781

[76] Liese AD, Schulz M, Fang F, *et al.* Dietary glycemic index and glycemic load, carbohydrate and fiber intake, and measures of insulin sensitivity, secretion, and adiposity in the Insulin Resistance Atherosclerosis Study. Diabetes Care. United States, 2005:2832-2838
[77] Lau C, Faerch K, Glumer C, *et al.* Dietary glycemic index, glycemic load, fiber, simple sugars, and insulin resistance: the Inter99 study. Diabetes Care. United States, 2005:1397-1403
[78] de Munter JS, Hu FB, Spiegelman D, Franz M, van Dam RM. Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. PLoS Med. United States, 2007:e261
[79] Fung TT, Hu FB, Pereira MA, *et al.* Whole-grain intake and the risk of type 2 diabetes: a prospective study in men. Am J Clin Nutr. 2002; 76: 535-540
[80] Murakami K, Okubo H, Sasaki S. Effect of dietary factors on incidence of type 2 diabetes: a systematic review of cohort studies. J Nutr Sci Vitaminol (Tokyo). 2005; 51: 292-310
[81] Krishnan S, Rosenberg L, Singer M, *et al.* Glycemic index, glycemic load, and cereal fiber intake and risk of type 2 diabetes in US black women. Arch Intern Med. United States, 2007:2304-2309
[82] Montonen J, Knekt P, Jarvinen R, Aromaa A, Reunanen A. Whole-grain and fiber intake and the incidence of type 2 diabetes. Am J Clin Nutr. 2003; 77: 622-629
[83] Salmeron J, Ascherio A, Rimm EB, *et al.* Dietary fiber, glycemic load, and risk of NIDDM in men. Diabetes Care. 1997; 20: 545-550
[84] Salmeron J, Manson JE, Stampfer MJ, Colditz GA, Wing AL, Willett WC. Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. JAMA. 1997; 277: 472-477
[85] Fisher E, Boeing H, Fritsche A, Doering F, Joost HG, Schulze MB. Whole-grain consumption and transcription factor-7-like 2 (TCF7L2) rs7903146: gene-diet interaction in modulating type 2 diabetes risk. Br J Nutr. England, 2009:478-481
[86] Hidaka H, Nagulesparan M, Klimes I, *et al.* Improvement of insulin secretion but not insulin resistance after short term control of plasma glucose in obese type II diabetics. J Clin Endocrinol Metab. 1982; 54: 217-222
[87] Del Prato S. Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies. Diabet Med. England, 2009:1185-1192
[88] Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev. United States, 2008:351-366
[89] Marchetti P, Bugliani M, Lupi R, *et al.* The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. Diabetologia. 2007; 50: 2486-2494
[90] Leahy JL, Cooper HE, Deal DA, Weir GC. Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic in vivo glucose infusions. J Clin Invest. 1986; 77: 908-915
[91] Sako Y, Grill VE. Coupling of beta-cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats. Diabetes. 1990; 39: 1580-1583
[92] Bjorklund A, Bondo Hansen J, Falkmer S, Grill V. Openers of ATP-dependent K+-channels protect against a signal-transduction-linked and not freely reversible defect of insulin secretion in a rat islet transplantation model of Type 2 diabetes. Diabetologia. 2004; 47: 885-891

[93] Sarwar N, Gao P, Seshasai SR, *et al.* Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. England: 2010 Elsevier Ltd, 2010:2215-2222
[94] Mooradian AD. Dyslipidemia in type 2 diabetes mellitus. Nat Clin Pract Endocrinol Metab. England, 2009:150-159
[95] Durrington P. Dyslipidaemia. Lancet. England, 2003:717-731
[96] Goldberg IJ. Clinical review 124: Diabetic dyslipidemia: causes and consequences. J Clin Endocrinol Metab. 2001; 86: 965-971
[97] Mitra S, Goyal T, Mehta JL. Oxidized LDL, LOX-1 and atherosclerosis. Cardiovasc Drugs Ther. 2011; 25: 419-429
[98] Nakhjavani M, Morteza A, Asgarani F, *et al.* Metformin restores the correlation between serum-oxidized LDL and leptin levels in type 2 diabetic patients. Redox Rep. 2011; 16: 193-200
[99] Grupping AY, Cnop M, Van Schravendijk CF, Hannaert JC, Van Berkel TJ, Pipeleers DG. Low density lipoprotein binding and uptake by human and rat islet beta cells. Endocrinology. 1997; 138: 4064-4068
[100] Cnop M, Hannaert JC, Grupping AY, Pipeleers DG. Low density lipoprotein can cause death of islet beta-cells by its cellular uptake and oxidative modification. Endocrinology. 2002; 143: 3449-3453
[101] Okajima F, Kurihara M, Ono C, *et al.* Oxidized but not acetylated low-density lipoprotein reduces preproinsulin mRNA expression and secretion of insulin from HIT-T15 cells. Biochim Biophys Acta. 2005; 1687: 173-180
[102] Abderrahmani A, Niederhauser G, Favre D, *et al.* Human high-density lipoprotein particles prevent activation of the JNK pathway induced by human oxidised low-density lipoprotein particles in pancreatic beta cells. Diabetologia. 2007; 50: 1304-1314
[103] Roehrich ME, Mooser V, Lenain V, *et al.* Insulin-secreting beta-cell dysfunction induced by human lipoproteins. J Biol Chem. 2003; 278: 18368-18375
[104] Grill V, Dinesen B, Carlsson S, Efendic S, Pedersen O, Ostenson CG. Hyperproinsulinemia and proinsulin-to-insulin ratios in Swedish middle-aged men: association with glycemia and insulin resistance but not with family history of diabetes. Am J Epidemiol. 2002; 155: 834-841
[105] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985; 28: 412-419
[106] Persson PG, Carlsson S, Grill V, *et al.* Food frequency questionnaire versus 7-day weighed dietary record information on dietary fibre and fat intake in middle-aged Swedish men. Scand J Soc Med. 1998; 26: 75-80
[107] SLV. National Food Agency Sweden.
[108] Nordman S, Ostenson CG, Efendic S, Gu HF. Loci of TCF7L2, HHEX and IDE on chromosome 10q and the susceptibility of their genetic polymorphisms to type 2 diabetes. Exp Clin Endocrinol Diabetes. 2009; 117: 186-190
[109] Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology. 1992; 130: 167-178
[110] Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes. 2000; 49: 424-430

[111] Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science. 1995; 268: 1766-1769
[112] Wang H, Iynedjian PB. Modulation of glucose responsiveness of insulinoma beta-cells by graded overexpression of glucokinase. Proc Natl Acad Sci U S A. 1997; 94: 4372-4377
[113] Wang H, Maechler P, Ritz-Laser B, *et al.* Pdx1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem. 2001; 276: 25279-25286
[114] Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes. 1967; 16: 35-39
[115] Herbert V, Lau KS, Gottlieb CW, Bleicher SJ. Coated charcoal immunoassay of insulin. J Clin Endocrinol Metab. 1965; 25: 1375-1384
[116] Grill V, Rundfeldt M, Efendic S. Previous exposure to glucose enhances somatostatin secretion from the isolated perfused rat pancreas. Diabetologia. 1981; 20: 495-500
[117] Korsgren O, Jansson L, Andersson A. Effects of hyperglycemia on function of isolated mouse pancreatic islets transplanted under kidney capsule. Diabetes. 1989; 38: 510-515
[118] Weibel ER, Gomez DM. A principle for counting tissue structures on random sections. J Appl Physiol. 1962; 17: 343-348
[119] Borg LA, Andersson A, Berne C, Westman J. Glucose dependent alterations of mitochondrial ultrastructure and enzyme content in mouse pancreatic islets maintained in tissue culture: a morphometrical and biochemical study. Cell Tissue Res. 1975; 162: 313-321
[120] Walz HA, Wierup N, Vikman J, *et al.* Beta-cell PDE3B regulates Ca2+-stimulated exocytosis of insulin. Cell Signal. England, 2007:1505-1513
[121] Schultz V, Sussman I, Bokvist K, Tornheim K. Bioluminometric assay of ADP and ATP at high ATP/ADP ratios: assay of ADP after enzymatic removal of ATP. Anal Biochem. United States, 1993:302-304
[122] Cavallari G, Zuellig RA, Lehmann R, Weber M, Moritz W. Rat pancreatic islet size standardization by the "hanging drop" technique. Transplant Proc. United States, 2007:2018-2020
[123] Gustavsson N, Abedi G, Larsson-Nyren G, Lindstrom P. Timing of Ca2+ response in pancreatic beta-cells is related to mitochondrial mass. Biochem Biophys Res Commun. United States, 2006:1119-1124
[124] Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest. 1955; 34: 1345-1353
[125] Puhl H, Waeg G, Esterbauer H. Methods to determine oxidation of low-density lipoproteins. Methods Enzymol. 1994; 233: 425-441
[126] Schmitz G, Wulf G, Bruning T, Assmann G. Flow-cytometric determination of high-density-lipoprotein binding sites on human leukocytes. Clin Chem. 1987; 33: 2195-2203
[127] Faust JR, Goldstein JL, Brown MS. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured mouse adrenal cells. J Biol Chem. 1977; 252: 4861-4871
[128] Wollheim CB, Kikuchi M, Renold AE, Sharp GW. Somatostatin- and epinephrine-induced modifications of 45Ca++ fluxes and insulin release in rat pancreatic islets maintained in tissue culture. J Clin Invest. 1977; 60: 1165-1173
[129] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957; 226: 497-509

[130] Morillas M, Clotet J, Rubi B, *et al.* Identification of the two histidine residues responsible for the inhibition by malonyl-CoA in peroxisomal carnitine octanoyltransferase from rat liver. FEBS Lett. Netherlands, 2000:183-186
[131] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65: 55-63
[132] Tabak AG, Herder C, Rathmann W, Brunner EJ, Kivimaki M. Prediabetes: a high-risk state for diabetes development. Lancet. England: 2012 Elsevier Ltd, 2012:2279-2290
[133] Alvarsson M, Hilding A, Ostenson CG. Factors determining normalization of glucose intolerance in middle-aged Swedish men and women: a 8-10-year follow-up. Diabet Med. England, 2009:345-353
[134] Ye EQ, Chacko SA, Chou EL, Kugizaki M, Liu S. Greater whole-grain intake is associated with lower risk of type 2 diabetes, cardiovascular disease, and weight gain. J Nutr. United States, 2012:1304-1313
[135] Jensen MK, Koh-Banerjee P, Franz M, Sampson L, Gronbaek M, Rimm EB. Whole grains, bran, and germ in relation to homocysteine and markers of glycemic control, lipids, and inflammation 1. Am J Clin Nutr. United States, 2006:275-283
[136] Kyro C, Skeie G, Dragsted LO, *et al.* Intake of whole grain in Scandinavia: intake, sources and compliance with new national recommendations. Scand J Public Health. Sweden, 2012:76-84
[137] Eliasson M, Bostrom G. Chapter 5.2: major public health problems - diabetes. Scand J Public Health Suppl. Sweden, 2006:59-68
[138] McKeown NM, Meigs JB, Liu S, Wilson PW, Jacques PF. Whole-grain intake is favorably associated with metabolic risk factors for type 2 diabetes and cardiovascular disease in the Framingham Offspring Study. Am J Clin Nutr. 2002; 76: 390-398
[139] Fardet A. New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? Nutr Res Rev. England, 2010:65-134
[140] Hansson O, Zhou Y, Renstrom E, Osmark P. Molecular function of TCF7L2: Consequences of TCF7L2 splicing for molecular function and risk for type 2 diabetes. Curr Diab Rep. 2010; 10: 444-451
[141] Bensellam M, Van Lommel L, Overbergh L, Schuit FC, Jonas JC. Cluster analysis of rat pancreatic islet gene mRNA levels after culture in low-, intermediate- and high-glucose concentrations. Diabetologia. 2009; 52: 463-476
[142] Serradas P, Giroix MH, Saulnier C, *et al.* Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes. Endocrinology. 1995; 136: 5623-5631
[143] Mizukami H, Wada R, Koyama M, *et al.* Augmented beta cell loss and mitochondrial abnormalities in sucrose-fed GK rats. Virchows Arch. 2008; 452: 383-392
[144] Lu H, Koshkin V, Allister EM, Gyulkhandanyan AV, Wheeler MB. Molecular and metabolic evidence for mitochondrial defects associated with beta-cell dysfunction in a mouse model of type 2 diabetes. Diabetes. United States, 2010:448-459
[145] Bonen A, Luiken JJ, Arumugam Y, Glatz JF, Tandon NN. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. J Biol Chem. United States, 2000:14501-14508
[146] Luiken JJ, Koonen DP, Willems J, *et al.* Insulin stimulates long-chain fatty acid utilization by rat cardiac myocytes through cellular redistribution of FAT/CD36. Diabetes. 2002; 51: 3113-3119

[147] Luiken JJ, Dyck DJ, Han XX, *et al.* Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. Am J Physiol Endocrinol Metab. 2002; 282: E491-495
[148] Bastie CC, Hajri T, Drover VA, Grimaldi PA, Abumrad NA. CD36 in myocytes channels fatty acids to a lipase-accessible triglyceride pool that is related to cell lipid and insulin responsiveness. Diabetes. United States, 2004:2209-2216
[149] Nolan CJ, Madiraju MS, Delghingaro-Augusto V, Peyot ML, Prentki M. Fatty acid signaling in the beta-cell and insulin secretion. Diabetes. United States, 2006:S16-23
[150] Newsholme P, Keane D, Welters HJ, Morgan NG. Life and death decisions of the pancreatic beta-cell: the role of fatty acids. Clin Sci (Lond). England, 2007:27-42
[151] Herrero L, Rubi B, Sebastian D, *et al.* Alteration of the malonyl-CoA/carnitine palmitoyltransferase I interaction in the beta-cell impairs glucose-induced insulin secretion. Diabetes. United States, 2005:462-471
[152] Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem. 1993; 268: 11811-11816
[153] Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci U S A. 1984; 81: 3883-3887
[154] Napoli C. Oxidation of LDL, atherogenesis, and apoptosis. Ann N Y Acad Sci. 2003; 1010: 698-709
[155] Asmis R, Begley JG, Jelk J, Everson WV. Lipoprotein aggregation protects human monocyte-derived macrophages from OxLDL-induced cytotoxicity. J Lipid Res. United States, 2005:1124-1132
[156] Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol. United States, 2006:1702-1711
[157] Ketelhuth DF, Rios FJ, Wang Y, *et al.* Identification of a danger-associated peptide from apolipoprotein B100 (ApoBDS-1) that triggers innate proatherogenic responses. Circulation. United States, 2011:2433-2443, 2431-2437
[158] Nordin Fredrikson G, Hedblad B, Berglund G, Nilsson J. Plasma oxidized LDL: a predictor for acute myocardial infarction? J Intern Med. England, 2003:425-429