IN VITRO AND *IN VIVO* STUDIES OF APOLIPOPROTEIN CIII IN DIABETES

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In vitro and *in vivo* studies of apolipoprotein CIII in diabetes

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To my family

ABSTRACT

Apolipoprotein CIII (apoCIII) is an important regulator of lipid metabolism and it is also known to have pro-inflammatory properties. From the point of view of diabetes it is interesting that both insulin deficiency and insulin resistance increase the expression of the apoCIII gene.

ApoCIII needs to be kept within a physiological window and this explains the different responses in islets from neonatal and adult rats exposed to the apolipoprotein. In the former the addition of apoCIII had an anti-inflammatory and protective role, while in the latter adult tissue the effects were negative. This is due to the fact that neonatal rats have sub-physiological levels of apoCIII and supplement of the apolipoprotein lifts up the concentration to the optimal range, where it is protective, in contrast to adult islets where the addition results in levels outside the protective range and here apoCIII is pro-inflammatory and apoptotic.

A previous study has demonstrated that lowering apoCIII with antisense oligonucleotides during a period of the pre-diabetic phase in BB rats, an animal model for type-1 diabetes (T1D), prolongs the time to onset of disease. In the same animal model treatment with polyphenol-containing red wines and fenofibrate, both PPARα agonists reported to lower apoCIII, were tested. However, these substances did not have any effect on apoCIII and consequently not on the debut of diabetes in BB rats.

ApoCIII is also increased in type-2 diabetes (T2D) and the apolipoprotein is produced within the pancreatic islets. It increases in parallel with the development of insulin resistance and it deteriorates islet function. Impeding the increase maintains a normal islet function.

Furthermore, HFD-induced obesity, insulin resistance and T2D can be prevented, and more interestingly reversed, by decreasing apoCIII, despite continuation on HFD.

In conclusion, apoCIII is an important diabetogenic factor and the aim for the future is to find safe ways to lower the apolipoprotein in individuals at risk of developing obesity, insulin resistance and diabetes.

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Manuscript

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[*] These authors contributed equally

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[*] These authors contributed equally

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LIST OF ABBREVIATIONS

ACE	Anterior Chamber of the Eye
Apo	Apolipoprotein
ASO	Antisense Oligonucleotides
AUC	Area Under Curve
BAT	Brown Adipose Tissue
BB rat	BioBreeding rat
IPGTT	Intraperitoneal Glucose Tolerance Test
IPITT	Intraperitoneal Insulin Tolerance Test
$[Ca^{2+}]_i$	Cytoplasmic free Ca^{2+}-concentration
Chl	Cholesterol
CM	Chylomicron
CVD	Cardiovascular Diseases
DM	Diabetes Mellitus
DPBB	Diabetes Prone BB rats
DRBB	Diabetes Resistant BB rats
FA	Fatty Acid
HDL	High-Density Lipoprotein
HFD	High Fat Diet
HL	Hepatic Lipase
HSPG1	Heparin Sulfate Proteoglycan 1 Receptor
IDL	Intermediate-Density Protein
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LRP1R	Low-Density Lipoprotein Related Protein 1 Receptor
mRNA	Messenger Ribonucleic acid
MTC	Methyl Cellulose
NAFLD	Non-Alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor Kappa B
OCR	Oxygen Consumption Rate

PPAR	Peroxisome Proliferator-Activated Receptor
RWP	Red Wine Polyphenols
SAT	Subcutaneous Adipose Tissue
Scr	Scrambled ASO
T1D	Type-1 Diabetes
T2D	Type-2 Diabetes
TG	Triglycerides
VAT	Visceral Adipose Tissue
VCO_2	Carbon Dioxide Production
VLDL	Very Low-Density Lipoprotein
VO_2	Oxygen Consumption
WAT	White Adipose Tissue

1 INTRODUCTION

1.1 DIABETES MELLITUS

1.1.1 Epidemiology

Diabetes Mellitus (DM) is a collection of pathological conditions all characterized by hyperglycemia. Diabetes is currently affecting over 400 million people worldwide and the number is continuously increasing according to the International Diabetes Federation (IDF) (1) and the World Health Organization (WHO) (2). In 2015, 1.6 million deaths were directly caused by DM making it among the leading causes of death worldwide. Apart from the huge suffering for the patients the economic burden on society is enormous.

1.1.2 Classification

Diabetes mellitus is divided into different subtypes where the major ones are type-1 (T1D) and type-2 (T2D).

T1D comprises about 5-10% of the total number of diabetic subjects (1, 2). The exact cause of T1D is not known, but the pathophysiology includes a combination of immunologic, genetic and environmental factors that drive an autoimmune destruction of the insulin secreting β-cells in the islets of Langerhans (3-5). The destruction is thought to be the result of an imbalance between regulating and pathogenic T-lymphocytes. CD8^{+} T-lymphocytes recognize β-cells as autoantigens, among which the most common are insulin, glutamic acid decarboxylase (GAD65), zinc transporter 8 (ZnT8) and transmembrane tyrosine phosphatase 2 (IA-2). Once activated the CD8^{+} T-lymphocytes can destroy β-cells by releasing granzyme or perforin containing granules, or via Fas-ligand interactions. The appearance of multiple autoantibodies is a strong predictor of onset of T1D (6-8).

WHO estimated in 2015 that the vast majority, around 90% of diabetic patients, have T2D. Insulin resistance and dysfunction of the pancreatic β-cell are hallmarks of the disease. According to WHO obesity is a major independent risk factor for developing the disease, and more than 90% of the subjects with T2D are overweight or obese (2, 9).

1.2   LIPID METABOLISM

1.2.1   Lipoproteins

Lipoproteins are particles that transport hydrophobic lipids [triglycerides (TG) and cholesterol] in the hydrophilic circulation (Figure 1). This is made possible by the surface-bound hydrophilic molecules such as phospholipids and unesterified cholesterol and by amphipathic apolipoproteins (10). The apolipoproteins can either be integrated in the monolayer of lipids, such as apoB, or reside on top of the lipoproteins, like apoCIII (11). The latter are exchangeable between the different classes of lipoproteins.
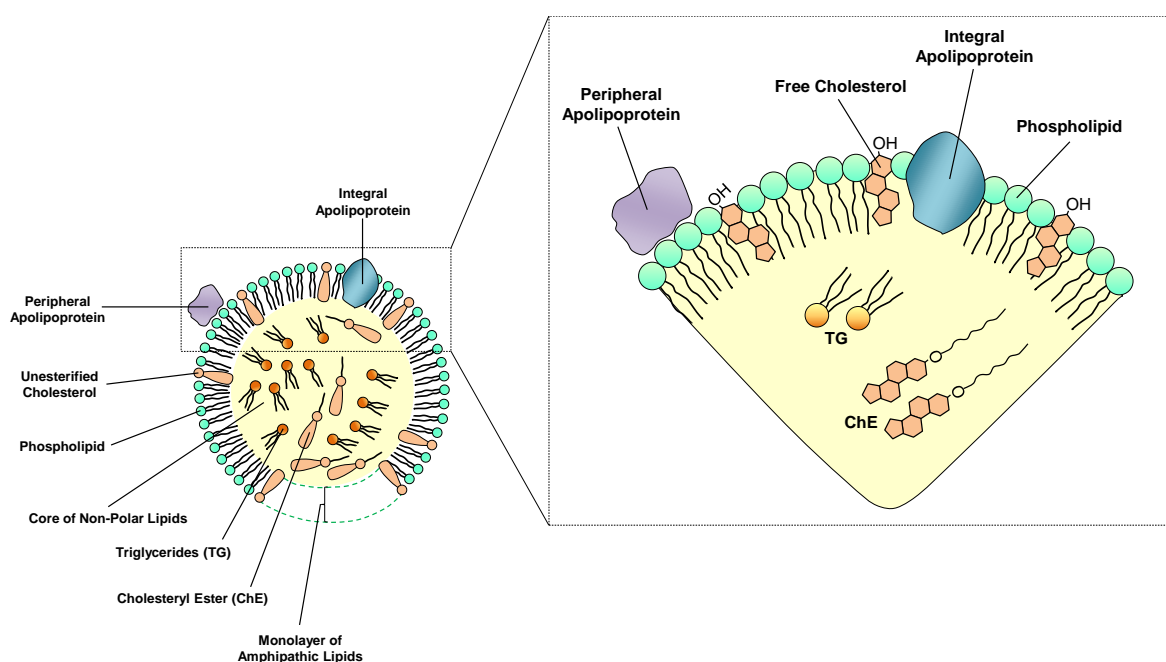
Figure 1. Schematic overview of a lipoprotein. A hydrophobic core with triglycerides and esterified cholesterol surrounded by a monolayer of phospholipids, unesterified cholesterol and apolipoproteins. The close-up shows the two kinds of apolipoproteins in a lipoprotein molecule. Integral apolipoproteins, like apoB, are deeply embedded in the phospholipid layer and peripheral apolipoproteins, like apoCI, CII and CIII, are located on the surface of the lipoprotein particle.

1.2.1.1   Classification

There are five classes of plasma lipoproteins, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), high-density lipoproteins (HDL) and chylomicrons (CM). The subtypes are determined based on molecular weight, composition, size and physical characteristics (10).

1.2.1.2   Production and function

CMs are produced in enterocytes in the intestine from dietary fat derived TGs and are secreted into the lymph before they enter the circulation. VLDL is synthesized primarily from

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TGs, but also from cholesterol in the liver. Once entered into the circulation CMs and VLDL transport energy to the peripheral target organs such as muscle and adipose tissue through lipolysis of TGs into free fatty acids (FA) by the action of lipoprotein lipase (LPL). Lipolysis of CM and VLDL result in CM remnants and IDL, respectively (12). These particles can be removed from the circulation by hepatic clearance through LDL receptors (LDLR), LDLR related protein 1 (LRP1) receptors and heparin sulfate proteoglycan receptors (HSPGs) (13, 14). IDL can also go through a second step of lipolysis and form LDL. LDL, popularly known as "the bad cholesterol" further circulates and delivers mainly cholesterol, but also TGs to the periphery before it is cleared from plasma by the liver. HDL, on the other hand, is formed in peripheral tissues and transport TGs and cholesterol to the liver and is traditionally known as "the good cholesterol". Recent studies have shown that HDL particles containing surface-bound apoCIII reduce the cholesterol efflux capacity of HDL and result in a more atherogenic phenotype (15, 16).

1.2.2 Dyslipidemia in obesity and T2D

Insulin has a key role for a normal lipid metabolism. Under conditions of insulin resistance, often seen in obesity and T2D, there are major changes such as impairment or loss of inhibition of hepatic VLDL production, no activation of LPL and no increase in the expression of LRP1 receptors or LDLR to facilitate hepatic clearance of VLDL remnants. The outcome is hypertriglyceridemia (17). Elevated TGs and small, dense LDL in combination with low HDL is a common dyslipidemic profile in T2D. Reports show that 72-85% of T2D patients have dyslipidemia (18, 19).

1.2.3 Apolipoproteins

Apolipoproteins contain six main classes, apoA-apoH, and numerous subclasses. They are attached to or incorporated into lipoproteins and regulate lipid transport in the circulation (Figure 1) (11, 20). Disturbances in the ratio of apolipoproteins are related to dyslipidemias, which are associated with obesity, insulin resistance, cardiovascular diseases (CVD) and T2D (21-23).

1.3 APOLIPOPROTEIN CIII

Apolipoprotein CIII (apoCIII) is a small (8.8kD, 79 amino acids) protein. The main production sites are the liver and the proximal part of the intestine. There are three important mechanisms whereby apoCIII promotes hypertriglyceridemia (24-28). The first is by acting as an inhibitor of LPL and at high concentrations also hepatic lipase (HL) (29-32). Second, it enhances the hepatic assembly and secretion of VLDL (33, 34). Thirdly, apoCIII inhibits hepatic receptor-mediated clearance of lipoproteins (35, 36). Besides its role in lipid metabolism apoCIII can also stimulate processes involved in vascular inflammation and atherogenesis (37, 38).

1.3.1 Genetics and structure

ApoCIII is located in the apoAI/CIII/AIV gene cluster on chromosome 11q23 in humans (39, 40). It consists of 6 amphipathic α-helixes that promote binding to the lipid interface of lipoproteins (41). ApoCIII is synthesized as a 99 amino-acid peptide. Thereafter a 20 amino-acid peptide is cleaved off resulting in the mature form of the protein (11).

1.3.1.1 Isoforms of apoCIII

Posttranslationally, apoCIII is glycosylated and exist in three different isoforms; $apoCIII_0$, $apoCIII_1$ and $apoCIII_2$, with 0, 1 or 2 sialic acids covalently bound to it (42). The different isoforms account for 10, 55 and 35 %, respectively, of total apoCIII in circulation (43). The different isoforms have similar half-life and metabolism (44-46).

1.3.1.2 Mutations in the human ApoCIII gene

There are humans with mutations in the apoCIII gene resulting in lifelong reduced levels of apoCIII. These individuals have favorable lipid profiles, lower blood pressure, increased insulin sensitivity and live longer (47-50). In contrast, there are carriers of gain-of-function mutations in the apoCIII gene resulting in increased apoCIII. This leads to hypertriglyceridemia, impaired TG clearance, non-alcoholic fatty liver disease (NAFLD) and insulin resistance (51). Another report showed that people with haplotypes in the apoCIII gene, resulting in increased apoCIII, had an increased risk of developing T1D (52).

1.3.2 Regulation of apoCIII

ApoCIII is regulated by a number of different pathways. So far it is known that insulin and activation of peroxisome proliferator-activated receptors (PPARs) or farnesoid X receptors (FXRs) inhibits (53-55) and insulin resistance, glucose, FA and activation of NFκB induce expression (56-60) (Figure 2).

Insulin

PPAR Activation —————| **apoCIII** ← **Insulin Resistance**

FXR Activation

Hyperglycemia

Free Fatty Acids

NF-KB Activation
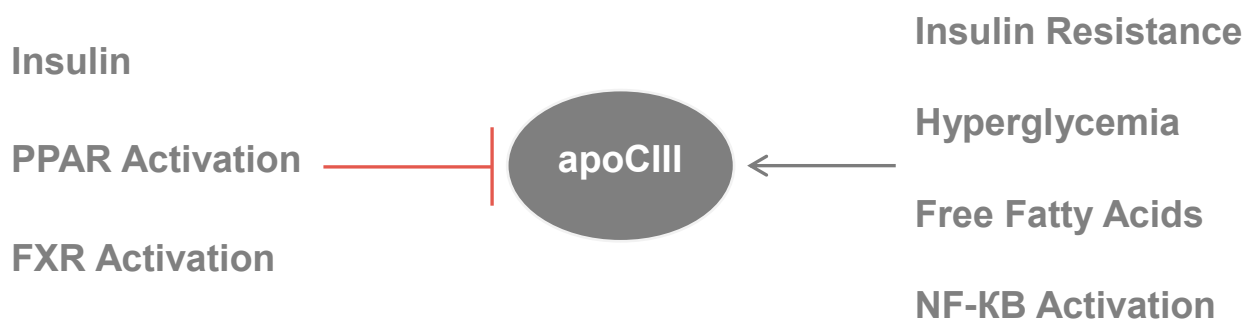
Figure 2. Factors known to regulate apoCIII. To the left, some of the known inhibitors and to the right some of the activators of apoCIII.

1.3.3 Antisense oligonucleotides (ASO)

ASOs are short (12-20 nucleotides) nucleic acid sequences that hybridize to their complementary mRNA and inhibit translation. Most commonly this inactivation occurs through activation of ribonuclease H1 (RNaseH1), an endonuclease that degrades the mRNA. ASOs are chemically modified on the nucleotide backbone to avoid degradation and prolong their half-life (61).

ASO treatments have been extensively tested in different animal models such as mice, rats and non-human primates (62, 63) and are now also used in clinical trials in humans. An ASO targeting human apoCIII (Volanesorsen) has been tested in patients with T2D and hypertriglyceridemia. Although the study was small, the treatment resulted in lower TGs and VLDL and increased HDL. It also improved insulin sensitivity in the patients (64).

2 AIMS

The overall aim of this thesis was to investigate the role of apoCIII in T1D, insulin resistance and T2D.

The specific aims were to:

- Clarify why the effect of addition of apoCIII differs in neonatal and adult rats.

- Investigate if polyphenols in red wine or fenofibrate, both PPARα agonists, can affect the onset of TID by reducing apoCIII.

- Analyze whether apoCIII is expressed in pancreatic islets and if there is a link between its expression and the development of insulin resistance and β-cell failure.

- Investigate if lowering of apoCIII can prevent and/or reverse the harmful effects of high-fat diet-induced obesity and T2D in mice.

3 MATERIAL AND METHODS

3.1 ANIMALS

All animals were housed in a temperature- and humidity-controlled room with 12 hours light/dark cycles with food and water *ad libitum*. Animal care and experimentations were carried out according to the Animal Experiment Ethics Committee at Karolinska Institutet.

3.1.1 Bio Breeding (BB) rats

Diabetes prone (DP) and, as controls, Diabetes resistant (DR) BB rats on a Wistar background were obtained from our breeding colony at Karolinska Institutet. They were housed under specific pathogen free conditions.

3.1.2 C57Bl6/J mice

Male C57Bl6/J mice were purchased from Harlan laboratories (USA) or Charles River Laboratories (Germany).

3.1.3 Obese (ob/ob) mice

Ob/ob (Lep^{ob}/Lep^{ob}) and ob/lean (Lep^{ob}/Lep^{+}) mice on a C57Bl6/J background were obtained from our breeding colony at Karolinska Institutet, Stockholm, Sweden. Ob/ob mice are leptin deficient due to a point mutation in the leptin gene (65). The mice are obese and insulin resistant between 2-7 months of age (66).

3.1.4 ApoCIII$^{-/-}$ mice

ApoCIII$^{-/-}$ mice were originally generated at The Jackson Laboratory (USA) by replacing exon 4 of the apoCIII gene with a neomycin resistance cassette in the opposite direction to the gene resulting in a global knock-out of apoCIII (67).

3.2 TREATMENTS AND DIETS

3.2.1 Red wine polyphenols and fenofibrate treatment

An equivalent to one glass of red wine per day (3.2mL/kg) was administered by oral gavage to DPBB rats between the ages of 30-60 days. As control DPBB rats were given water. Two different kinds of wine were used: Sonovino Primitivo, Italy, consisting of 90% Primitivo and 10% Negroamaro grapes, containing medium amounts of polyphenols and Reserve du Vieux Noir, Malbec, France, made of Malbec grapes containing high levels of polyphenols. If a rat developed diabetes before the age of 60 days the treatment was terminated.

DPBB rats were given a daily dose of 100mg/kg fenofibrate (Lipanthyl, Abbott, France) dissolved in 1% methyl cellulose solution (MTC) according to the same treatment protocol as above. As controls DPBB rats were given the vehicle.

In both treatment groups rats were weighed daily until onset of diabetes. Blood samples were taken before and after 30 days of treatment, and at the debut of diabetes.

3.2.2 High Fat Diet (HFD)

C57Bl6/J mice were fed a HFD (Open Source Diets D12492, Research Diet, New Brunswick, NJ, USA) between the ages of 4-9 months, 2-8 months or 2-5.5 months. The HFD consisted of 60% fat, 20% carbohydrate, and 20% protein whereas the controls were fed normal diet (R70, Lantmännen, Sweden) that contained 11.4% fat, 62.8% carbohydrate and 25.8% protein.

3.2.3 Antisense oligonucleotide (ASO) treatment

Ob/ob mice were treated with apoCIII antisense (ASO) (ION 353982, 5'GAGAATATACTTTCCCCTTA-3') or a scrambled control ASO (scr) (ION 141923, 5'CCTTCCCTGAAGGTTCCTCC-3'). 25mg/kg body weight was administrated i.p twice per week between the ages of 8 to 16 weeks.

C57Bl6/J mice were treated with scr ASO (ION 141923) (scr), 12.5mg/kg/week or 25mg/kg /2x/week or apoCIII-ASO (ION 353982) (ASO), 25mg/kg/2x/week between the ages of 2-8 months, 2-5.5 months and 2.5-3.5 months.

3.3 SERUM AND PLASMA ANALYSIS

3.3.1 Insulin

3.3.1.1 ArcDia

ArcDia 2-photon fluorescence excitation microparticle fluorometry (TPX) assay for insulin (ArcDia Group, Finland) was used to determine serum insulin.

3.3.2 Plasma triglycerides

Triglycerides were measured in 10μL of plasma using the Triglyceride Colorimetric Assay Kit (Cayman Chemical, USA).

3.3.3 Lipid profiles

Lipid profiles were obtained by size-exclusion chromatography as previously described (68). Using a LaChrom Elite HPLC system (Hitachi, Germany) and Superose 6 PC 3.2/300 gel column (GE Helthcare, Sweden), lipoproteins were separated by size class. TG and cholesterol concentrations were determined using TG, glycerol-3-phosphate oxidase-phenol + aminophenazone (GPO-PAP) and cholesterol, cholesterol oxidase (CHOD)-PAP reagents (Roche, Switzerland). The TG and cholesterol content were measured as the area under the curve (AUC) using EZChrome Elite software (Aglient Technologies, Germany).

3.4 ANALYSIS OF LIVER TRIGLYCERIDES

Triglyceride content was determined in snap frozen liver tissue by carcass saponification in 0.1M KOH in 99% ethanol (69). Triglycerides were analyzed using Free Glycerol Reagent and compared to Glycerol Standards (Sigma-Aldrich, USA). The glycerol concentration (triolein equivalents) was measured by spectrophotometry (SAFAS-MONACO spectrophotometer) at λ =540nm. Total triglyceride content was expressed in mg/g of tissue.

3.5 HISTOLOGY

3.5.1 Hematoxylin and Eosin (H&E) staining

Liver, subcutaneous (SAT), visceral (VAT) and brown adipose tissue (BAT) were dissected out, snap frozen and kept at -80°C until use. Tissue sections (12-20 μm thick) were obtained with a cryostat (Microm HM500M/Cryostar NX70; Thermo Scientific, USA) and collected onto SuperFrost Plus microscope slides (VWR International). The sections were formalin-post-fixed during 30 min, stained with H&E (Histolab, Sweden) and mounted in DABCO (Sigma-Aldrich, USA) or VectaMount permanent mounting medium (Vector Laboratories, Inc.). Pictures were taken at 20 and 40X magnification in an optical microscope (Leica, Germany).

3.5.2 Oil Red `O´ (ORO) staining

Mice were anesthetized with isoflurane and transcardially perfused with PBS followed by freshly prepared 4% paraformaldehyde (PFA) in PBS. Livers were dissected out and post-fixed overnight. After fixation livers were processed with a sucrose gradient [10-30% (wt/vol) sucrose solution in PBS containing 0.01% (wt/vol) sodium azide and 0.02% (wt/vol) bacitracin], frozen in dry ice and preserved at -80°C until use. Liver cryosections (20 μm thick) were obtained in a cryostat (Microm HM500M/Cryostar NX70; Thermo Scientific, USA) and collected onto SuperFrost Plus microscope slides (VWR, Sweden). After 2 hours at room temperature, slides were rinsed in 60% (vol/vol) isopropylic alcohol for 1 min, stained in freshly prepared 0.1% (vol/vol) ORO (Sigma-Aldrich, USA) in 60% (vol/vol) isopropylic alcohol solution for 30 min, washed in distilled water and mounted in aqueous media. Liver sections from all experimental groups and controls were immediately imaged under a 20X magnification objective using an optical microscope (Leica, Germany). Imaging was performed in three non-consecutive liver sections separated by 100 μm each. For each section, 3 fields were imaged in 3 individual animals per experimental group and controls.

3.6 METABOLIC STUDIES

3.6.1 Intraperitoneal Glucose Tolerance Test (IPGTT)

Mice were fasted for 6 hours or overnight and their blood glucose measured before an intraperitoneal (i.p) injection of glucose (1.5 or 2.0g/kg body weight). Thereafter, blood glucose was measured 10, 30, 60, and 120 or 15, 30, 45, 60, 90 and 120 min post injection of

glucose with either a Freestyle lite (Abbott Diabetes Care, USA) or an Accu-Chek Advantage (Roche Diagnostics, USA) glucose meter.

3.6.2 Intraperitoneal Insulin Tolerance Test (IPITT)

Basal blood glucose was measured in mice fasted for 6 hours or overnight. Thereafter i.p injections of insulin (0.25U/kg body weight) and 10 min after, glucose (1 g/kg body weight) were given. Blood glucose was measured with a glucose meter (Freestyle lite, Abbott Diabetes Care, USA) at 15, 30, 60, 90 and 120 min after the glucose injection.

3.6.3 Metabolic cages

Mice were allowed to acclimatize for 24 hours in single cages. Thereafter, food intake, oxygen consumption (VO_2), carbon dioxide production (VCO_2), and movement were continuously measured during 4 days in an Oxymax Lab Animal Monitoring System (CLAMS, Columbus Instruments,USA). Mice had *ad libitum* access to food except for one night when they were fasting. Movement was reported as total beam breaks for the XYZ axis.

3.6.4 Body composition

C57Bl6/J mice fed control diet or HFD and treated with scr or apoCIII ASO for 14 weeks were subjected to an EchoMRI-100 system (Echo Medical Systems, USA) to assess total lean and fat mass in unanesthesized, fed mice. The measurement is based on nuclear magnetic resonance (NMR) that takes advantage of the difference in density of the hydrogen nuclei in adipose tissue, water and bone (70).

3.6.5 Oxygen consumption rate (OCR)

OCR in tissue fragments from BAT was measured based on a protocol previously described by Vernochet et al (71). Intrascapular BAT from control and HFD fed mice treated with scr or apoCIII ASO was isolated. Tissues were rinsed thoroughly with an unbuffered Krebs-Henseleit solution (KH) containing 110mM NaCl, 4.5mM KCl, 2mM $MgSO_4$, 1.2mM Na_2HPO_4, 0.5mM Carnitine and 5mM glucose. Tissues were cut into ~10 mg pieces, placed inside the wells of Seahorse XF24 Islet Capture Microplates (#101122-100, Agilent Technologies, USA) and covered with the provided islet capture screen. 500µL of KH solution (pH 7.2) was added to each well. Plates were incubated at 0% CO_2 for 30 min and subsequently analyzed on a Seahorse XF24-3 (Seahorse Biotechnology, USA). The measurement cycle was 3 min mix, 2 min wait, 3 min measure. Four separate measurements were averaged to yield baseline OCR, followed by injection of 10mM sodium pyruvate and 10µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and four additional separate measurements which were averaged to yield a representation of state 3 respiration.

3.7 STUDIES IN ISLETS OF LANGERHANS AND MIN6M9 CELLS

3.7.1 Human islets

Human islets were obtained within the Nordic Network for Islet Transplantation and were approved by the Regional Ethical Review Boards in Uppsala and Stockholm. Islets were cultured in CMRL-1066 (ICN Biomedicals) supplemented with HEPES (10mM), L-glutamine (2mM), Gentamycin (50mg mL^{-1}), Fungizone (0.25mg mL^{-1}), Ciprofloxacin (20mg mL^{-1}), nicotinamide (10 mM) and 10% FBS.

3.7.2 Mouse islets

Mice were sacrificed by cervical dislocation and pancreas perfused with 3ml of 1mg/ml collagenase A or P (Roche, USA) in Hank's balanced salt solution (HBSS) (Sigma, Sweden) buffer supplemented with 0.2% bovine serum albumin (BSA) and 25mM HEPES. Pancreas was thereafter extracted and digested in a water bath at 37°C for 12-20 min. Islets were handpicked in ice-cold HBSS containing 0.2% BSA and 25mM HEPES. Islets were either immediately used for mRNA or protein analysis or were cultured overnight. For overnight culture of islets RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2mM glutamine as well as 100U/ml and 100µg/ml of penicillin and streptomycin, respectively, were used.

3.7.3 MIN6m9 cells

Mouse insulinoma cell line (MIN6m9 cells), between passage 35 and 42, were cultured at 5% CO_2 and 37°C in DMEM containing 11.1mM glucose and supplemented with 100U/ml Penicillin, 100µg/ml Streptomycin, 2mM glutamine, 10% FCS, and 75µM β-mercaptoethanol. Medium was changed every second day.

3.7.4 Biosensor construction and transduction

pENTR2A.RIP1.FoxO1GFP was generated by replacing the cDNA for EGFP in pENTR2A.RIP1.EGFP with that of FoxO1-GFP obtained from pEGFP.N1.hFOXO1 IRES-3Tomato was generated by introducing a cDNA encoding three copies of the red fluorescent protein dTomato downstream of the IRES sequence in pIRES (Clontech, USA). The IRES-3Tomato cassette was then introduced into pENTR2A.RIP1.FoxO1GFP thus creating pENTR2A.RIP1.FoxO1GFP-IRES-3Tomato. pENTR2A.RIP1.FoxO1(H215R)GFP-IRES-3Tomato was generated by replacing histidine (CAT) 215 by arginine (CGT) by site-directed mutagenesis by employing the QuikChange mutagenesis kit (Stratagene, USA) and respective oligonucleotides purchased from Sigma. All constructions were verified by DNA sequencing.

The expression cassette was transferred into the promoterless adenovirus plasmid pAd/PL-DEST (Invitrogen, USA) by the Gateway technique. The ViraPower Adenoviral Expression System (Invitrogen, USA) was used to generate a replication-deficient adenovirus, which was

used for transduction of cells and islets. Islets from ob/lean and ob/ob mice were transduced with 107 pfu/ml of the biosensor encoding adenovirus.

3.7.5 Intraocular islet transplantation

Isolated islets from ob/ob, apoCIII $^{-/-}$ and C57Bl6/J mice were transplanted into the anterior chamber of the eye (ACE) of ob/ob or C57Bl6/J mice. Biosensor transduced islets from ob/lean and ob/ob mice were transplanted into the ACE of 8 weeks old syngeneic littermate recipients. Mice were anesthetized with 2% Isolflurane (Baxter, USA) air mixture. Thereafter they were placed on a heating pad, the head restrained by a head holder and the eye fixed. Islets were aspirated into a cannula attached by 0.4mm polyethylene tubing to a 1mL Hamilton syringe. The cannula was inserted into the ACE through an incision made by a 27G needle. Once inside the ACE the islets were implanted on the iris. Thereafter the mice were injected subcutaneously with Temgesic (0.1mg/kg) as an analgesic and monitored until complete recovery.

3.7.6 *In vivo* and *in vitro* imaging of islets and image analysis

The islet grafts were imaged *in vivo* as previously described (72) using a TCS-SP5 II laser scanning confocal microscope (Leica microsystems, Germany) with water-dipping objectives (Leica HCX IRAPO L 25.0x0.95 and HXC-APO10x/0.30 NA). The mice were anesthetized by 2% isoflurane/air (Baxter, USA) and placed as for the transplantation (3.7.5). Viscotears (Novartis, Switzerland) was used as immersion liquid between the eye and the objective.

3.7.6.1 *Bright field images*

Bright field images of islet grafts were obtained by acquiring a z-stack of images through the transplanted islets using a DFC 295 digital camera (Leica Microsystems, Germany) attached to the TCS-SP5 II laser scanning confocal microscope (Leica Microsystems, Germany).

3.7.6.2 *Confocal imaging of islet reflected light, blood vessels and macrophages*

Islet morphology was imaged at 488nm and reflected light was detected at the same wavelength. Blood vessels and macrophages were visualized by tail vein injection of 100μL of 2.5ng/μL of Texas red fluorescently labeled Dextran (Life Technologies, USA). Blood vessels were imaged directly after injection whereas macrophages were imaged 72 hours later.

3.7.6.3 *Nicotinamide adenine dinucleotide phosphate (NAD(P)H)Flavin adenine dinucleotide (FAD)*

Measuring the endogenous fluorophores in the electron transport chain, NAD(P)H and FAD, provide information about metabolic rate (73). Islet NAD(P)H and FAD autofluorescence was determined by 2-photon laser-scanning microscopy. Excitation wavelengths were 760 nm for NAD(P)H and 900 nm for FAD. Fluorescence was detected after band-pass filters 460/50 and 525/50 for NAD(P)H and FAD, respectively.

3.7.6.4 *In vitro imaging*

In the transduced islets, green fluorescence protein (GFP) fluorescence was excited at a 488nm and fluorescence detected at 505 to 536nm. Tomato was excited at 561nm and fluorescence detected at 580 to 650nm. Backscatter signal from the 561nm excitation was collected at 555 to 565nm. Imaging was performed with an inverted Leica TCS-SP2 with a PL APO CS 20x/0.7NA objective. Human islets were imaged with a Leica TCS-SP5 II laser scanning confocal microscope (Leica microsystems, Germany) with a water-dipping objective.

3.7.6.5 *Image analysis*

The redox ratio NAD(P)H/FAD and image analysis of transduced cells and islets were performed using LAS AF software (Leica Microsystems, Germany). Analysis of islet volume, vessel volume and macrophage infiltration were performed with Volocity image analysis software (Perkin Elmer, USA).

3.7.7 Measurements of cytoplasmic free Ca2+ concentration ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were recorded in islets after a 16 hour incubation period with 0.07mg/mL apoCIII antisense or a scrambled control (Ionis Pharmaceuticals, USA) in RPMI 1640 medium or in islets immediately after micro-dissection from ACE two weeks post-transplantation. The basal medium used for islet perifusion experiments was a HEPES (25 mM) buffer at pH7.4 containing NaCl (125mM), KCl (5.9mM), $MgCl_2$ (1.2mM), $CaCl_2$ (1.28mM) and 1mg/mL BSA supplemented with either 3mM glucose, 11mM glucose or 25mM KCl. Islets, loaded with 2μM fura-2 acetoxymethyl ester (Molecular Probes, USA) for 50-60 minutes, were attached to coverslips using Puramatrix Hydrogel (BD Biosystem, USA), and mounted on an inverted epifluorescence microscope (Zeiss, Axiovert 135) connected to a Spex Industries Fluorolog system for dual-wavelength excitation fluorimetry. The measurements were performed as previously described (74).

3.8 MEASUREMENT OF APOPTOSIS

SensoLyte Homogeneous Rh110 Caspase 3/7 assay kit (AnaChem, USA) was used to determine cell death in ob/ob islets from 4-, 8- and 12-week old mice. Islets were placed in lysis buffer (AnaChem, USA) and protein measured with BCA method. 10μg of protein was loaded in wells of a black 384 well plate and assay buffer containing Rh110 caspase 3/7 substrate was added. The plate was incubated for 1 hour at 24°C in the dark. Fluorescence was detected at excitation/emission wavelengths λ=490/520nm to determine caspase activity.

3.9 WESTERN BLOTTING

3.9.1 Islets and MIN6m9 cells

Islets or MIN6m9 cells were washed and lysed in RIPA buffer (50mM Tris pH7.5, 150mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, EDTA-free protease inhibitor cocktail, 1μg/ml pepstatine and leupeptin). Protein amount was determined by the BCA method

(Thermo Scientific Pierce, USA). Equal amounts of protein (25-50μg) were separated over a 4-12% Bis-Tris gel with MES buffer system (Invitrogen, USA). Proteins were subsequently electrotransferred to a polyvinylidene fluoride (PVDF) membrane and incubated overnight with primary antibodies against Akt, phospho(p)-Akt, p-FOXO1, γ-tubulin, c-Myc, c-peptide and apoCIII (Table 1). Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Amersham, USA). Values were normalized to γ-tubulin.

3.9.2 Serum and plasma

For determination of serum or plasma apoCIII the samples were albumin depleted using AlbuSorb according to the manufacture's protocol (Biotech Support Group LLC, USA), freeze-dried and resuspended in RIPA buffer. Protein concentration was determined by BCA method (Thermo Scientific, USA) or Bradford method (BioRad, USA). Equal amounts of protein (15-25μg) were separated over a 4-12% Bis-Tris gel (Invitrogen, USA) and thereafter electrotransferred to a PVDF membrane. After blocking, membranes were probed with anti-apoCIII antibody (Table 1) overnight. Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Amersham, UK) (GE Healthcare, Sweden). Ponceau S or Coomassie Blue was used to stain the membrane after reading to provide a loading control.

3.9.3 Liver

Liver samples were homogenized in ice-cold buffer containing 0.42mM NaCl, 20mM HEPES (pH 7.9), 1mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, 1mM DTT, 20% (vol/vol) glycerol, 1μg/mL aprotinin, 1μg/mL leupeptin, 20mM sodium fluoride, 1mM trisodium orthovanadate, and 2mM phenylmethylsulfonyl fluoride. Tubes containing homogenates were exposed to thermal shock at -80°C in liquid nitrogen and thawed to 37°C three consecutive times, and centrifuged at 10 000 × g for 20 min. Protein levels were measured by Bradford method (BioRad, USA) and equivalent amounts of proteins (20μg) were loaded to a 4-12% Bis-Tris gel (Invitrogen, USA). For immunoblotting, membranes were blocked with 5% (wt/vol) BSA. Primary antibodies against Akt, p-Akt, Erk and p-Erk were applied overnight (Table 1). After incubation with secondary antibodies, blots were incubated in commercial enhanced chemiluminescence reagents (ECL-Prime; GE Healthcare, Sweden), and membranes were exposed to a luminescent image analyzer (Las-1000 Plus; Fuji). Obtained images were quantified using ImageJ software. Values for all proteins were normalized to β-actin.

Primary antibodies	Species	Dilution	Company
Akt	Rabbit	1:1000	Cell Signaling
apoCIII	Rabbit	1:1000	Cell Signaling
c-Myc	Rabbit	1:1000	Cell Signaling
c-peptide	Rabbit	1:1000	Cell Signaling
Erk	Rabbit	1:200	Santa Cruz
Insulin	Rabbit	1:1000	Cell Signaling
p-Akt	Mouse	1:1000	Abcam
p-Erk	Rabbit	1:1000	Abcam
p-FoxO1	Mouse	1:1000	Santa Cruz
β-Actin	Mouse	1:1000	Sigma-Aldrich
γ-tubulin	Guinea-Pig	1.1000	DAKO

Table 1. Antibodies used in experiments.

3.10 RNA ISOLATION AND QUANTITATIVE RT-PCR ANALYSIS

RNeasy Micro Kit was used to isolate total RNA from islets according to the manufactures protocol (Qiagen, Sweden). For adipose tissue mRNA extraction, we used the RNeasy Mini Lipid kit and for liver RNeasy Mini kit. Reverse transcription of total RNA was performed at 37°C using the High Capacity cDNA Reverse Transcription kit (Life technologies, USA). Expression of all genes was measured by real-time semi-quantitative PCR with Taq SYBR Supermix with ROX (Invitrogen, USA) on an ABI7900HT instrument (Applied Biosystems, USA) or by real-time semi-quantitative PCR with Maxima SYBR Green qPCR Master Mix with ROX (Thermo scientific,USA) on a QuantStudio 7 instrument (Life technologies, USA). The primer sequences are shown in table 2. β-actin was used as an endogenous control.

Gene	Forward primer sequence	Reverse primer sequence
apoCIII	CGCTAAGTAGCGTGCAGGA	TCTGAAGTGATTGTCCATCCAG
Cd137	GCTCCTCCACTCTTGCTACC	CAGCAAGTCAGTGGTGCTTC
FoxO1	TAGCGGGGGTCATAAATCGC	CTCCTTGAAGCTCGGGTGC
Gck	CTATGCCAGCATCAGCTTCC	TTGCTGAGGTCATTTAGGTCTTC
Irs1	GTCCAGGCACTGGAGCTTT	GCGCTTCACTCTTTCACGA
Irs2	GTTGCCCAACCAAAGCTTCC	ATGTAGCCTGCTCACTAACTCTT
Pgc1α	GTCGACCCTGGACGAACTGCTCT	CCTCTGGAGTCACAGAAATGGTGGTA
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Snap25	GAAACCAGTATTGCAGCACCCAAT	AATATTAGCAGGAGTGTTTGGTGGA
Tmem26	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Ucp1	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Vamp2	CCGAGCTGATGAGGGAGA	GAGGCTTGTTCCATGTAGCC
β-actin	CTGAACATTGGCGTTCGCAG	TCAAGGGGACAACCAGAGGA
apoCIII	TGCACCTCCTCCAAACCTTA	AGCTCCGACAACTTCTGGTC
PPARα	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
β-actin	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT

Table 2. Primer sequences used in gene expression studies. In white, primers used for mouse and in grey primers used for rats.

3.11 IMMUNOCYTOCHEMISTRY

3.11.1 Human islets

Human islets were fixed for 30 minutes in 4% PFA and thereafter incubated overnight at 4°C with primary antibodies against insulin and apoCIII (Table 1) in 10% goat serum blocking buffer containing 0.25% Triton X-100. After washing the islets were incubated with the secondary antibodies Alexa 488-labeled anti-guinea pig and Alexa 647 anti-rabbit (Life Technologies, USA).

3.11.2 Mouse islets

Islets were fixed with 4%PFA in PBS for at least 48 hours. They were incubated with primary antibodies anti-FoxO1 (Cell Signaling, USA) and anti-insulin (DAKO/Agilent, USA) (Table 1) in the presence of 0.1% Triton X-100 and 2% BSA for blocking for 48 hours at room temperature. Islets were then washed three times in PBS and incubated with the secondary antibodies Alexa 488-labeled anti-rabbit and Alexa 633-labeled anti-guinea pig (Life Technologies, USA) in the same buffer.

3.11.3 Myc-tagged apoCIII in MIN6m9 cells

Post-transfected cells were cultured on glass cover slips prior to 3% PFA fixation for 30 min. Cells were thereafter incubated with antibodies specific for c-Myc and c-peptide (Table 1) in 10% goat serum blocking buffer containing 0.25% Triton X-100, overnight at 4°C with gentle shaking. Cells were incubated with the secondary antibodies Alexa 488 anti-mouse (Santa-Cruz, USA) and Alexa 546 anti-rabbit (Cell Signaling, USA) in blocking buffer for 1 hour followed by washing and mounting using VectaShield mounting media with DAPI (Vector Labs, USA).

3.12 STATISTICAL ANALYSIS

Statistical analyzes were performed with GraphPad Prism 5 or 7 or IBM SPSS Statistics. Student's t-test, one-way ANOVA (Tukey's post-hoc) or Mann-Whitney U test were used when appropriate. P values <0.05 were considered statistically significant. In Paper III, figure 6J a Fisher's exact test was performed. All results are expressed as mean ± standard error of the mean (sem).

4 RESULTS AND DISCUSSION

4.1 PAPER I: THE YIN AND YANG OF APOLIPOPROTEIN CIII

Previous studies from our group have shown that serum from T1D patients hyperactivates the voltage-gated Ca^{2+}-channel. This results in increased $[Ca^{2+}]_i$ and β-cell apoptosis (75). The serum factor responsible for these effects was identified to be apoCIII (76). Patients with T1D have increased levels of apoCIII as the expression of the lipoprotein is inversely regulated by insulin (53). *In vivo* studies in the BB rat, an animal model for T1D, showed that there was a delayed onset of diabetes if apoCIII was reduced with an ASO against the apolipoprotein in pre-diabetic animals (77). Other studies have shown that apoCIII exhibit pro-inflammatory properties in endothelial cells (37, 38).

However, in contrast to these data, addition of apoCIII reduced apoptosis in pancreatic islets from neonatal rats exposed to pro-inflammatory cytokines. Thus, in this biological context apoCIII had a protective function opposite to the observation in β-cells from adult animals where the apolipoprotein exhibited pro-apoptotic effects (78). The aim of this study was to find an explanation to these seemingly inconsistent results.

Neonatal (5 days old) and adult (55 days old) Wistar rats were sacrificed and the livers were removed and snap frozen. mRNA was extracted and the expression of apoCIII was measured by qRT-PCR.

The expression of hepatic apoCIII was fivefold higher in the adult compared to the neonatal rats. These data are in line with previous reports where the levels of apoCIII were low at birth with a gradual increase reaching adult levels at about one month of age (79). The conclusion is that there is a physiological range that apoCIII needs to be kept within. In neonatal rats apoCIII is low and the addition increases it to normal levels that are protective, while in islets from adult rats addition of T1D sera or apoCIII increases the levels above the physiological window where the apolipoprotein is pro-inflammatory.

4.2 PAPER II: NEITHER POLYPHENOL-RICH RED WINE NOR FENOFIBRATE AFFECT THE ONSET OF TYPE 1 DIABETES MELLITUS IN THE BB RAT

There are several studies where the beneficial effects of polyphenols in red wine have been discussed (80, 81). Polyphenol-rich grape products have been suggested to increase the activation of PPARs that antagonize inflammatory transcription factors (81). Fibrates are used in the treatment of hypertriglyceridemia and have, among several other actions, been reported to have anti-inflammatory effects through the activation of PPAR. Our interest in red wine and fibrates, in relation to apoCIII, evoked from data showing down-regulation of apoCIII by activation of PPARs (54, 82, 83).

We therefore decided to investigate if a daily dose of red wine or fenofibrate to pre-diabetic DPBB rats could influence the progression of the disease.

Pre-diabetic DPBB rats were treated daily with either the equivalent to one glass of red wine or with fenofibrate between 30-60 days of age. Two wines with medium or high concentrations of polyphenols, but without alcohol, were used.

None of the treatments prevented or prolonged the time to onset of diabetes. Wine with alcohol was also tested and was without effect (Figure 3). Analysis at the end of the experiment revealed that neither the red wines nor fenofibrate had any effect on the expression of apoCIII.
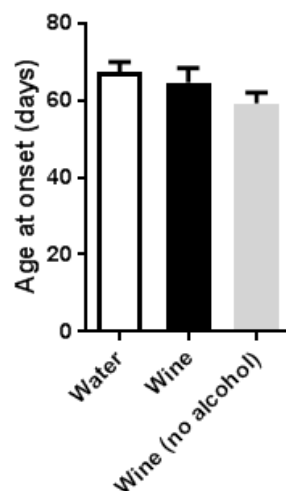
Figure 3. Age at onset of T1D in DPBB rats given a daily oral dose of red wine with high concentration of polyphenols with or without alcohol. Water was given as control.

This study demonstrates that in this rat model for T1D fenofibrate or polyphenol containing red wines cannot be used to lower apoCIII. As it is well known that there are differences between species our data do not exclude that these substances can be used to lower apoCIII in other animal models or humans.

4.3 PAPER III: APOLIPOPROTEIN CIII LINKS ISLET INSULIN RESISTANCE TO BETA-CELL FAILURE IN DIABETES

ApoCIII has since long been of interest within the field of CVD. It is a known pro-inflammatory risk factor promoting dyslipidemia and atherogenesis (24, 84, 85). The transcription of the apoCIII gene is increased by insulin deficiency and insulin resistance (53, 56). Humans, with reduced levels of apoCIII due to gene mutations, have a beneficial situation from general health aspects (47-50). On the contrary, apoCIII gene variants with increased levels of apoCIII are associated with the development of NAFLD, hepatic insulin resistance and T2D (51).

Our previous studies have identified apoCIII as a target in T1D, but as insulin resistance also increases apoCIII we have broadened our investigations to include insulin resistance and T2D.

In this study the aims were to clarify if apoCIII is produced within the pancreatic islet and if there is a link between the apolipoprotein and the development of insulin resistance and T2D. To answer these questions two different animal models were used. The first was the leptin deficient ob/ob mouse that between the ages of 4 to 12 weeks progressively gains weight becomes insulin resistant and hyperglycemic. The second model for insulin resistance and T2D was C57Bl6/J mice fed HFD.

We could show that apoCIII is produced within the islet and it increases in parallel with the development of insulin resistance. Interestingly, we could demonstrate that by decreasing the levels, using ASO against apoCIII, the mice had a normal age-related weight gain, glucose tolerance and intracellular Ca^{2+}-handling.

To further prove that apoCIII indeed was the factor that impaired the function of the pancreatic β-cell, islets from apoCIII$^{-/-}$ mice were transplanted into one eye of ob/ob or C57Bl6/J mice. In the other eye islets from ob/ob or C57Bl6/J mice, respectively, were transplanted (Figure 4). One month later, after vascularization and engraftment of the islets, the C57Bl6/J mice were put on HFD. Thus, all islets in these two diabetic mouse models were exposed to high systemic levels of apoCIII. However, the apoCIII$^{-/-}$ islets, that could not increase their intra-islet levels of apoCIII despite being in a diabetogenic milieu, maintained, in contrast to the transplanted ob/ob and C57Bl6/J islets, a normal size and function with no signs of inflammation.
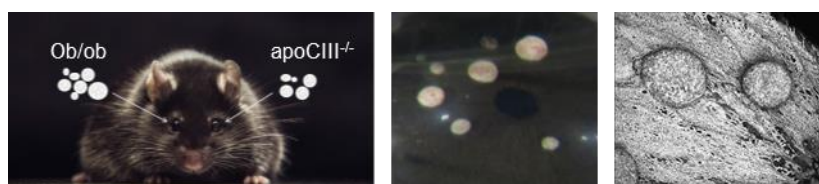
Figure 4. Ob/ob mouse transplanted with ob/ob islets into the right eye and apoCIII$^{-/-}$ islets into the left eye (left). Islets are engrafted and vascularized on top of the iris (middle). Confocal image of islets in the ACE (right).

We have also shown that apoCIII is present in human islets (Figure 5) and that islets from a T2D patient had more apoCIII positive cells than islets from healthy subjects.
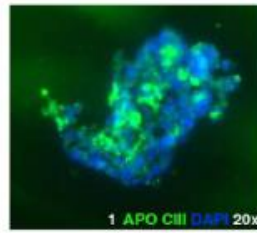
Figure 5. Human islet stained for apoCIII (green) and counterstained with DAPI for visualization of cell nuclei (blue).

In conclusion our data provides evidence that apoCIII is present in the pancreatic islet, increases under conditions of insulin resistance and is a link to β-cell failure and T2D. Decreasing apoCIII *in vivo* results in improved glucose tolerance and apoCIII$^{-/-}$ islets transplanted into diabetic mice, with high systemic levels of the apolipoprotein, demonstrate a normal intracellular Ca^{2+} response pattern and no hallmarks of inflammation. Hence, under conditions of islet insulin resistance, locally produced apoCIII within the islet is an important diabetogenic factor responsible for β-cell incapacitation.

4.4 PAPER IV: LOWERING APOCIII PREVENTS DIET-INDUCED DIABESITY

The estimated number of obese children and adults in the world 2015 were 108 and 604 million respectively (86). In parallel there has been an increase in obesity-related diseases with CVD as number one followed by T2D. An observational study in teenagers and young adults with T1D and T2D revealed that the prevalence of complications was higher in those with T2D. An alarming finding was that complications could be diagnosed already after a relatively short mean duration of 8 years of diabetes in these young subjects (87).

In this study the focus has been to elucidate if reducing apoCIII can influence obesity, insulin resistance and the development of diabetes. To address this question, C57Bl6/J mice were fed a HFD and the control group was given normal chow. Three different experimental protocols were used. In the first, mice were fed HFD for 10 weeks and thereafter treated with ASO to decrease the HFD-induced increase in apoCIII. They continued on HFD with the addition of ASO for 14 weeks. In the second protocol, they again started with HFD for 10 weeks and thereafter a short-term treatment with ASO and HFD for 4 weeks. In the third protocol the mice were directly from start given ASO in parallel with HFD for 14 weeks.

The results were very encouraging. Not only could the negative effects of HFD on body weight and glucose metabolism be prevented by the ASO treatment, the most interesting observation was that the metabolic phenotype could be reversed by lowering apoCIII. The short-term ASO treatment revealed that the improvement of the metabolism came before the weight reduction. These beneficial changes were not attributable to a lower food intake or a higher physical activity in the ASO treated mice.

In line with previous studies our data provides evidence that by lowering apoCIII there is an improved hepatic clearance of TG-rich particles and a decreased production and secretion of VLDL from the liver (33-35, 88,89). Furthermore, it is known that apoCIII inhibits LPL and by changing the activity of the enzyme the delivery of FA to adipose tissue will be affected (Figure 6) (90).

Another important contributing mechanism to the positive effects of decreasing apoCIII is the activation of BAT. This was verified in our study by increased O_2 consumption (VO_2) and CO_2 production (VCO_2), up-regulation of uncoupling protein 1 (*Ucp1*) and a higher body temperature in the ASO treated mice on HFD. Analysis of white adipose tissue (WAT) showed that also in this tissue there was an up-regulation of *Ucp1* and in addition other thermogenic genes, a sign of adipose tissue browning, indicating a capability of increased energy expenditure (Figure 6).

From these data it can be concluded that apoCIII is an interesting target in the battle against obesity, insulin resistance and diabetes.
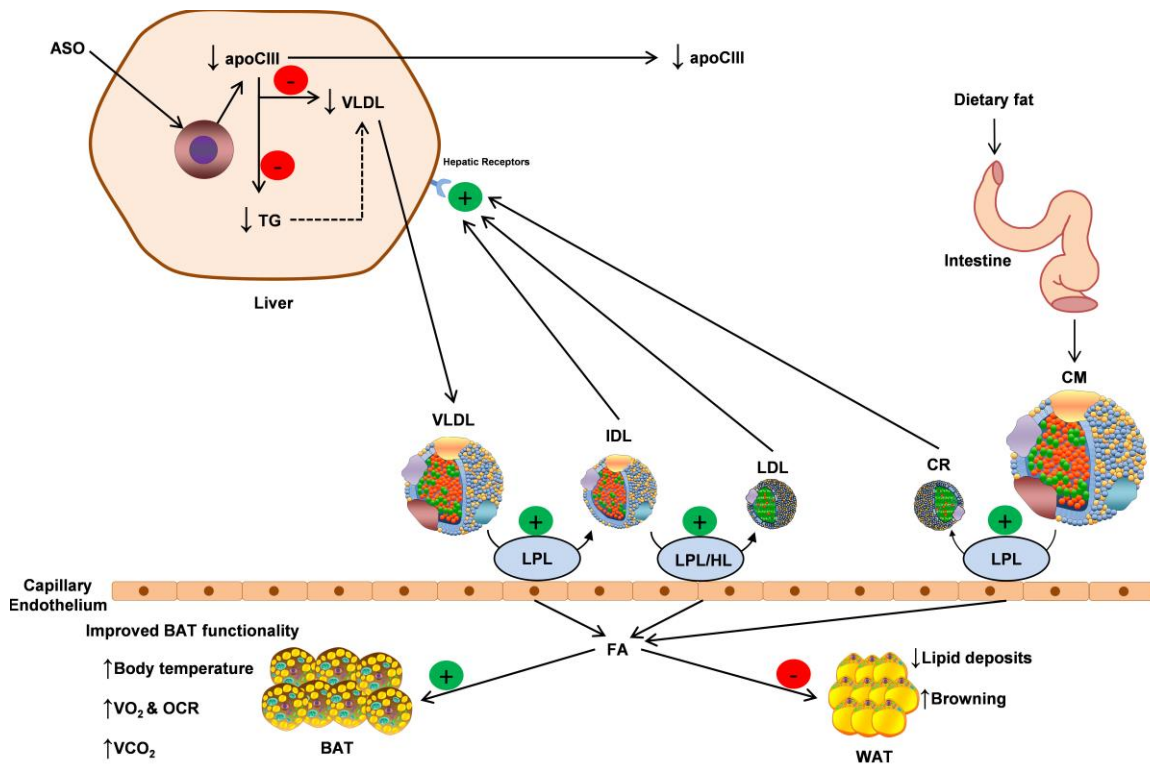
Figure 6. Overview of the changes in metabolism upon lowering apoCIII under high fat diet-conditions. Antisense (ASO) treatment decreases apoCIII and this activates LPL and the hydrolysis of VLDL, IDL and CM. The lipoprotein remnants can readily be taken up by hepatic receptors as lower levels of apoCIII improve this clearance. FAs derived from breakdown of lipoproteins are taken up by adipose tissue. The more efficient hepatic clearance, due to low apoCIII levels, shunts the FAs to BAT. This leads to lower lipid deposits and browning of WAT. To counteract for the increase in TGs, non-shivering thermogenesis is induced in BAT and the energy is dissipated as heat. A more functional BAT means less TGs in circulation. Normalized hepatic TGs, in combination with low apoCIII, results in decreased VLDL production and secretion. Together this results in a normolipidemic, lean and insulin sensitive phenotype.

4.5 FUTURE PERSPECTIVES

This thesis, together with several other publications, has started to unravel the complex role that apoCIII plays in physiology and pathophysiology.

We have found that ASO treatment to lower apoCIII is efficient in preventing obesity, insulin resistance and T2D in mice. However, the long-term effects of ASO treatment are not known, and it would be desirable to be able to decrease apoCIII using for example antibodies or nanobodies. We will, in collaboration with pharmaceutical industry and biotech companies, try to develop monoclonal apoCIII antibodies.

Another approach is the development of aptamers against apoCIII in collaboration with POSTECH (Pohang, South Korea). While both antisense and antibodies at present only allow a systemic reduction of apoCIII, the aptamer-based approach has the potential serving as a cell-type specific, targeted treatment. These experiments will provide proof-of-concept that targeting apoCIII-induced islet inflammation offers a potential intervention strategy in the prevention or treatment of individuals at high risk of developing T2D with its complications and will lay the foundation for pre-clinical studies.

To further investigate the role of islet specific apoCIII it would be interesting to transplant a larger number of apoCIII$^{-/-}$ islets into C57Bl6/J mice and thereafter put them on HFD. If these islets are protected from HFD-induced inflammation the transplanted mice would maintain a normal glucose tolerance and insulin sensitivity despite being on this diabetogenic diet.

In this thesis it is shown that apoCIII is expressed at very low levels at birth. However, it would be interesting to study whether apoCIII changes during aging and if there is a gender difference.

5 CONCLUSIONS

The overall conclusion from this thesis is that apoCIII is intimately involved in the maintenance of a normal β-cell function and survival and thereby adequate glucose homeostasis. The following specific conclusions can be made;

- There is a physiological window within which apoCIII needs to be kept.

- Polyphenol-rich red wine and fibrates have been reported to downregulate apoCIII by activating PPARα. However, in our animal model for T1D, the BB rat, these substances had no effect on the expression of apoCIII thereby leaving the onset of diabetes unaffected.

- ApoCIII is produced locally within the pancreatic islet. The development of insulin resistance is paralleled by an increase in islet apoCIII. Preventing this increase in apoCIII maintains a normal islet function.

- HFD increases apoCIII which leads to obesity, insulin resistance and T2D. By preventing the increase, or reducing the levels of apoCIII in already obese animals, this metabolic phenotype can be normalized, despite continuing on a HFD.

6 ACKNOWLEDGEMENTS

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