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PO Box 117
221 00 Lund
+46 46-222 00 00



Apolipoprotein A-I in glucose control and amyloidosis

OKTAWIA NILSSON

FACULTY OF MEDICINE | LUND UNIVERSITY



“Barely did I know what happened, before Oktawia had become a natural part of my life. And I think she has that impact on many people. Bringing smiles to people’s faces and a lot of energy to any of the multitude of projects she takes on. You could imagine that with so many things going on, she would drop the ball once in a while, but no. Oktawia is also a person who gets things done. Not for a single moment did I doubt that she would be where she is today, with her Ph.D. thesis successfully completed. Throughout her academic career, from her double Bachelor’s degree in Chemistry and Biotechnology at the University of Warsaw to her move to Lund and her Master’s degree in Molecular biology and further to the doctoral program in Biomedicine, she has always persistently strived for high-quality research. However, it has not come at the expense of fun times too, with plenty of laughter, traveling, sightseeing, bicycle trips, and city escapes. Even when separated by distance, she keeps her friends and family close and makes sure that we are all doing all right. With Oktawia, there is always time for a ‘fika’, and often with a delicious cake that she made herself! As she is now embarking on new adventures you can be sure of one thing. You will meet her busy with exciting projects, and always with great enthusiasm and many laughs.”

Gustav Nilsson, **Proud Husband and Devotee**



*This is me in action. At this point, cleaning some very important cuvettes in Diamond, UK.
Photo by Rita Del Giudice.*

Apolipoprotein A-I in glucose control and amyloidosis

Oktawia Nilsson



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DOCTORAL DISSERTATION

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To be defended in the lecture hall Belfragesalen at BMC D15, Sölvegatan 19, Lund
on March 27th 2020 at 9.15.

Faculty opponent

Prof. Dr. med. Susanna Hofmann
Helmholtz Zentrum München

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Abstract The role of Apolipoprotein A-I (ApoA-I), the main protein component of HDL, in cholesterol transport and metabolism is well known and has been studied for more than four decades. More recently, ApoA-I protein has been shown to also have a positive role in glucose control by both stimulation of glucose uptake by muscles and by increasing glucose-stimulated pancreatic insulin secretion. Two of the four papers included in this thesis are focused on the role of ApoA-I in glucose control. In paper I , we discovered that pre-incubation of beta cells and isolated murine islets with ApoA-I augmented glucose stimulated insulin secretion. To dissect the cellular mechanisms of action, we used a variety of functional and microscopic approaches. We concluded that ApoA-I's positive action on beta cells involves ApoA-I internalization into beta cells, Pdx1 nuclear translocation, and increased levels of proinsulin processing enzymes. Altogether, these events lead to an increased number of insulin granules. In paper II , we addressed the impact of hyperglycemia on the function of ApoA-I in glucose control. Prolonged hyperglycemia in poorly controlled diabetes leads to an increase in reactive glucose metabolites that covalently modify proteins, including ApoA-I, by non-enzymatic glycation reaction. To investigate the impact of ApoA-I glycation on its functionality, we chemically glycated ApoA-I with two different metabolites and performed structural and functional studies. We concluded that site-specific, covalent modifications of ApoA-I alter the protein structure, reduce the lipid-binding capability as well as the ability to catalyze cholesterol efflux from macrophages. Glycation modifications eliminated the ApoA-I stimulatory effect on the <i>in vivo</i> and <i>in vitro</i> glucose clearance. Altogether, it was concluded that glycation modification of ApoA-I impairs the ApoA-I protein functionality in lipid and glucose metabolism. The two remaining papers included in this thesis are focused on another aspect of ApoA-I, its ability to aggregate in insoluble fibrils causing a disease known as ApoA-I related amyloidosis. So far, more than twenty known human amyloidogenic variants of the <i>APOA1</i> gene have been found to lead to progressive accumulation of ApoA-I protein in vital organs, causing their dysfunction and failure. ApoA-I amyloidogenic mutations are associated with low ApoA-I and HDL-cholesterol plasma levels, however, subjects affected by ApoA-I-related amyloidosis do not show a higher risk of cardiovascular diseases. In paper IV , we investigated the structural features, the lipid-binding properties and the functionality of four ApoA-I amyloidogenic variants. We found that these variants are characterized by a higher efficiency at catalyzing cholesterol efflux from macrophages. This finding can at least in part explain why the carriers of ApoA-I amyloidogenic variants do not have a higher risk of developing cardiovascular diseases despite lower levels of HDL-cholesterol. To further expand on these observations, in paper III , we examined the clinical plasma samples obtained from patients carrying two of the variants previously investigated <i>in vitro</i> and from matched control individuals. Patients displayed a unique HDL profile with a higher content of the smaller HDL particles was observed in samples from carriers as compared to controls. In line with previous observations, the HDL from the carriers had an improved cholesterol efflux capacity. Structural analysis revealed that ApoA-I variants in 8.4 nm HDL particles showed an increased protein dynamics in close proximity to the region of the mutations. This region-specific increased protein flexibility may contribute to improved functionality of the ApoA-I variants in catalyzing cholesterol efflux.		
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To my Ciocia

*“Nothing in life is to be feared, it is only to be understood. Now it is the time
to understand more, so that we may fear less.”*

Marie Skłodowska-Curie

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Preface

Apolipoprotein A-I (ApoA-I) is a versatile protein, characterised by several beneficial effects. As it is the main protein component of HDL, the “good” cholesterol, it is mostly well-known and described for its atheroprotective properties.

The presented thesis is centred around two much lesser-explored functions of ApoA-I. The first part explores and explains the positive properties of ApoA-I to regulate glucose control, providing a possible new treatment avenue for type 2 diabetes and one of its main complications, cardiovascular diseases. Hyperglycemia, which is a common hallmark of diabetes, leads to the progression of the disease as well as to modification of many plasma proteins. The consequences of ApoA-I modification are addressed in this thesis, strengthening the need for plasma glucose control in diabetic people.

ApoA-I-related amyloidosis is a rare, genetic condition which leads to organ failure. In the second part, we found that these naturally occurring ApoA-I amyloidogenic variants have improved capacity to efflux cholesterol. Structure-function connection was determined providing an important insight into this novel finding.

The presented thesis is based on three published papers and one manuscript. In the following pages I will first introduce you to ApoA-I and its remarkable properties and then discuss our findings in the light of other research. I hope that you will enjoy this journey.

If you are looking for a short summary of my thesis, on page 73 you will find “Science for everyone”.

Original papers

- I. **Nilsson O**, Del Giudice R, Nagao M, Grönberg C, Eliasson L, Lagerstedt JO. Apolipoprotein A-I primes beta cells to increase glucose stimulated insulin secretion. *Biochim Biophys Acta Mol Basis Dis.* 2020;1866(3):165613.
- II. Domingo-Espin J, **Nilsson O**, Bernfur K, Del Giudice R, Lagerstedt JO. Site-specific glycations of apolipoprotein A-I lead to differentiated functional effects on lipid-binding and on glucose metabolism. *Biochim Biophys Acta Mol Basis Dis.* 2018;1864(9):2822-2834.
- III. **Nilsson O**, Lindvall M, Obici L, Ekström S, Lagerstedt JO*, Del Giudice R*. Size and molecular structure dynamics of amyloidogenic ApoA-I variants in HDL affect their ability to mediate cholesterol efflux. *Submitted manuscript.*
- IV. Del Giudice R, Domino-Espin J, Iacobucci I, **Nilsson O**, Monti D, Lagerstedt JO. Structural determinants in ApoA-I amyloidogenic variants explain improved cholesterol metabolism despite low HDL levels. *Biochim Biophys Acta Mol Basis Dis.* 2017;1863(12):3038-3048.

*RDG and JOL contributed equally to this article.

Additional papers by the author not included in this thesis

- I. Edmunds SJ, Liebana Garcia R*, Nilsson O*, Domingo-Espin J, Stenkula KG, Lagerstedt JO. ApoAI-derived peptide increases glucose tolerance and prevents formation of atherosclerosis in mice. *Diabetologia*. 2019; 62(7):1257-1267.
- II. Del Giudice R, Nilsson O, Domingo-Espin J, Lagerstedt JO. Synchrotron radiation circular dichroism spectroscopy reveals structural divergences in HDL-bound apoA-I variants. *Scientific Reports*. 2017;7(1):13540.
- III. Domingo-Espin J, Lindahl, Nilsson-Wolanin O, Cushman SW, Stenkula KG, Lagerstedt JO. Dual Actions of Apolipoprotein A-I on Glucose-Stimulated Insulin Secretion and Insulin-Independent Peripheral Tissue Glucose Uptake Lead to Increased Heart and Skeletal Muscle Glucose Disposal. *Diabetes*. 2016;65(7):1838-1848.

*RLG and ON contributed equally to this article.

Abbreviations

AApoA-I	ApoA-I-related amyloidosis
ABCA1	ATP-binding cassette (ABC) transporter A subfamily
ABCG1	ATP-binding cassette (ABC) transporter G subfamily
AGE	Advanced Glycation End Products
AMPK	5' adenosine monophosphate (AMP)-activated protein kinase
ApoA-I	Apolipoprotein A-I
B _{max}	Cholesterol Efflux Capacity
cAMP	Cyclic Adenosine Monophosphate
CE	Cholesteryl Esters
CETP	Cholesterylester Transfer Protein
CD	Circular Dichroism
CPE	Carboxypeptidase E
CVD	Cardiovascular Diseases
CytD	Cytochalasin D
DIO	Diet Induced Obesity
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
EL	Endothelial Lipase
FC	Free Cholesterol
GA	Glycolaldehyde
GLP-1	Glucagon-like Peptide 1
Glut4	Glucose transporter type 4
GSIS	Glucose-Stimulated Insulin Secretion
GTT	Glucose Tolerance Test

HDL	High Density Lipoproteins
HDL-C	HDL-Cholesterol
HDX	Hydrogen Deuterium Exchange (HDX)
HL	Hepatic Lipase
IDL	Intermediate Density Lipoproteins
IR	Insulin Resistance
K_d	Dissociation Constant, Cholesterol Efflux Efficiency
LCAT	Lecithin-Cholesterol Acyltransferase
LDL	Low Density Lipoproteins
LF	Lipid-Free
LPL	Lipoprotein Lipase
MDC	Monodansylcadaverine
MG	Methylglyoxal
MLV	Multilamellar Vesicles
MS	Mass Spectrometry
PC1/3	Prohormone Convertase enzyme PC3
PLTP	Phospholipid Transfer Protein
POPC	1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine
RCT	Reverse Cholesterol Transport
rHDL	Reconstituted HDL
SR-BI	Scavenger Receptor Class B type I
SRCD	Synchrotron Radiation Circular Dichroism
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TEM	Transmission Electron Microscopy
TG	Triglycerides
WB	Western Blot
WT	Wild Type
VLDL	Very Low Density Lipoproteins

Background

It wasn't up until 1901 when Josef Nerking observed that plasma contained fat bound to protein, leading to the discovery of lipoproteins¹. After treating horse plasma with pepsin and HCl, he discovered that there were significant amounts of fat released that were impossible to extract by ethyl ether alone. It took almost another thirty years until lipoproteins were successfully isolated for the first time, which was done by researchers from the Pasteur institute in 1929^{2,3}. Thanks to the development of electrophoresis and ultracentrifuge techniques, the researchers were able to isolate and classify different lipoproteins. Significant advancements have been made since then of which some of the core findings are presented in the brief summary below.

Lipoproteins

Lipoproteins are water-soluble, heterogeneous nanoparticles formed by specific proteins, called apolipoproteins, and lipid molecules. Plasma lipoproteins are secreted mostly by the liver or the intestine, whereas lipoproteins of the central nervous system are secreted mainly by the glial cells^{4,5}. Mature lipoprotein particle consists of an apolar lipid core containing mainly cholesterol esters (CE) and triglycerides (TG), and an amphipathic lipoprotein coat that contains a monolayer of phospholipids, free (un-esterified) cholesterol and apolipoproteins. The placement of apolipoproteins on the lipoprotein surface is vital for the particle structure and function in lipid transport and metabolism. Apolipoproteins contain both hydrophobic and hydrophilic regions which allow them for interactions in aqueous and lipid environment. Thanks to these amphipathic properties, apolipoproteins control the transport of water-insoluble lipids in aqueous environment, such as plasma. Additionally, they ensure lipid metabolism by interacting with enzymes, lipid transfer proteins, lipid transporters and lipoprotein receptors⁶⁻⁸.

The lipoprotein nomenclature, which was first introduced already in 1955⁹ and still in use today, classifies lipoproteins on the basis of their density. Particles containing more apolipoproteins are denser than those containing more lipids (Figure 1). There are five major classes of lipoproteins, “chylomicrons”, “very low density lipoproteins” (VLDL), “intermediate-density lipoproteins” (IDL), “low density lipoproteins” (LDL), and “high density lipoproteins” (HDL). Brief introductions to the different lipoprotein

classes are presented below followed by detailed description of organization and function of HDL particles, which is the main focus of the thesis.

Chylomicrons

Chylomicrons are large TG rich lipoproteins found only after a meal and formed in intestines. They were described in 1924 and ultimately named after their chylous properties, i.e. found associated with lipids, and for being in the micrometre- range size¹⁰. Chylomicrons are involved in the transport of dietary TG and cholesterol to peripheral tissues and the liver. The main structural protein of chylomicrons is one molecule of Apo B-48 accompanied by additional apolipoproteins such as ApoA-I, A-II, A-IV, A-V, Cs, and E. The size of chylomicrons varies between 75 to 1200 nm¹¹ and is dependent on the amount of fat consumed. A high fat meal leads to the formation of larger particles with high amounts of TG, whereas during the fasting state chylomicrons are small and low on TG¹².

Very low density lipoproteins VLDL

VLDLs are produced by the liver and are rich in triglyceride. Their main apolipoprotein is Apo B-100, one per particle, accompanied by ApoCs and ApoE. As in case of chylomicrons, the size of VLDLs depends on the quantity of triglyceride carried in the particle and it ranges between 40-100 nm. When hepatic production of triglyceride is increased, secreted VLDL particles are larger¹².

Intermediate density lipoproteins IDL

IDL particles are products of the degradation of VLDLs by lipoprotein lipase (LPL)¹³. They contain apolipoproteins ApoB-100 and ApoE which encircle the lipoprotein core enriched with TGs and CEs. The size range of IDLs is between 25-35 nm. They are pro-atherogenic¹².

Low density lipoproteins LDL (“bad cholesterol”)

LDL particles originate from VLDL and IDL particles and are further enriched in cholesterol. LDLs are the principal carriers of cholesterol in circulation. The main apolipoprotein of LDL particles is Apo B-100, one per particle¹². LDL particles vary in size (21-24 nm) and density. High blood levels of small, dense LDLs are associated with hypertriglyceridemia, low HDL levels, obesity, type 2 diabetes, and inflammatory states. They are also considered to be more pro-atherogenic as compared to bigger particles. This is due to decreased affinity for the LDL receptor which results in longer retention of the LDL particle in the circulations, as well as higher likelihood to enter the arterial wall where they are retained and may cause damage. Importantly, small dense LDL particles are more prone to oxidation, which increases their uptake by macrophages leading to the formation of foam cells and the development of atherosclerotic plaques¹².

High density lipoproteins HDL (“good cholesterol”)

High density lipoproteins (HDL) are the smallest of lipoproteins (8-12 nm) and with the highest density. The composition of the HDL particle includes an outer amphipathic envelope consisting of phospholipids (mainly phosphatidylcholine), several molecules of un-esterified cholesterol and proteins, which together encircle the core of esterified cholesterol and TG¹⁴. As described in more detail below, the HDL population is highly heterogeneous and can be divided into several particle subclasses based on protein content, density and size. HDL are linked to a vast variety of cardioprotective properties, due to their pivotal role in the reverse cholesterol transport (RCT), process by which cholesterol is transported from peripheral tissues to the liver, where is catabolized and secreted out as bile¹⁵. Moreover, HDL were found to have anti-oxidative¹⁶, anti-inflammatory¹⁷, anti-thrombotic¹⁸, and anti-apoptotic properties¹⁹. There have been more than 200 proteins identified in the HDL proteome, and the different particle subclasses are characterised by a different protein compositions²⁰. The by far the most abundant HDL protein is Apolipoprotein A-I (ApoA-I), making up to 70% of the protein content. Second most abundant HDL-associated protein is apolipoprotein A-II (ApoA-II) and it accounts for around 15-20% of the total HDL protein content²¹. The remaining part is made up of amphipathic proteins such as ApoCs, ApoE, ApoD, ApoM, ApoA-IV, and many others²². ApoM, for example, is only found in 5% of the total HDL particle pool in human plasma^{23,24}.

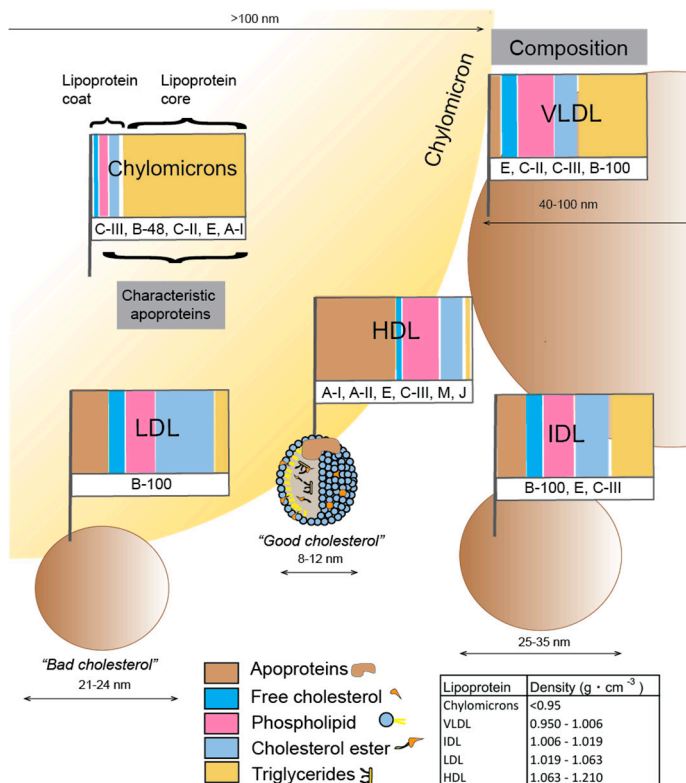


Figure 1. Representation of plasma lipoproteins. Major classes of plasma lipoproteins, including chylomicrons, very low, intermediate, low and high density lipoproteins (VLDL, IDL, LDL, HDL) are shown with indicated particle diameters. Lipoprotein composition and characteristic apolipoproteins are shown in the flags. The densities of the different classes of lipoproteins are shown in the table.

Lipoprotein metabolism

The western diet, highly enriched with saturated fats and sucrose, and low dietary intake of fibre, provides a health risk which contributes to the development of metabolic diseases²⁵. Lipids are insoluble in water therefore they must be transported in the circulation as lipoproteins. Lipoprotein metabolism starts in the intestine with an uptake of diet-derived lipids by chylomicrons. Once in the circulation, the TG associated with chylomicrons are metabolized in the peripheral tissue by LPL resulting in the release of free fatty acids which can be taken up by cells and used as an energy source. The remnant chylomicrons are taken up by the liver. TG-rich VLDLs are formed in the liver and secreted into circulation. Again, LPLs activity leads to the release of free fatty acids and subsequent formation of IDLs. The IDLs are further metabolised

to become LDL, which can be then taken up by multiple tissues, including liver, via the LDL receptor. The RCT facilitated by HDL, leads to the transport of cholesterol from peripheral tissues to the liver where it can be catabolised and secreted through the bile. This process is highly important as it accounts for multiple positive outcomes, including anti-atherogenic properties¹². HDL biogenesis, cholesterol efflux, as well as structural and functional aspects of HDL will be further discussed in the presented thesis.

Apolipoprotein A-I (ApoA-I)

ApoA-I is the main protein component of HDL and is primarily produced by the liver, and to a smaller extent in the intestines²⁶. The most important function of ApoA-I is mediating cholesterol efflux from peripheral tissues, which constitutes for its cardioprotective properties²⁷. Moreover, ApoA-I creates a scaffolding that enables packaging of lipids in HDL particle and promotes interaction with accessory proteins (lipases, enzymes, transfer proteins, cell surface proteins), responsible for remodelling and maturation of lipoproteins throughout their lifespan²⁰. These vital properties as well as structural features of the ApoA-I protein are the focuses of the following chapter.

Structural features of ApoA-I

ApoA-I (28 kDa) is a 243 amino acids single polypeptide that lacks glycosylation and disulfide bridges. It makes up around 70% of the total HDL protein content. The concentration of ApoA-I in human plasma is around 130 mg/dL (around 50 μ M) with plasma HDL/ApoA-I turnover of around five days²⁸. Lipid-free (LF) ApoA-I accounts for 5-10% of total ApoA-I (around 2 μ M), and is mostly monomeric^{29,30}. ApoA-I consists of an N-terminal domain (amino acids 1-43), a C-terminal domain (amino acids 44-243), and a central domain (amino acids 123-166)³¹. ApoA-I has 10 amphipathic α -helical repeats (eight 22-mers and two 11-mers) typically interspersed by proline residues that create a kink in the helical structure³². The *APOA1* gene is located on the 11th chromosome and is encoded by two regions of the gene. The initial translation product of ApoA-I contains an N-terminal extension, a 24-amino acid long pre-pro-sequence. The 18-amino acid signal peptide that directs the protein to the rough endoplasmic reticulum where the signal peptide is cleaved off, resulting in the formation of a pro-protein. The pro-protein, which now contains the remaining 6 additional amino acids, is secreted in plasma where the extra amino acids are removed and mature ApoA-I is formed³³. The final translation product of the ApoA-I first region, encoded by exon-3, is named G-helix and consists of residues 1-43. It is divided into proline (Pro)-rich G0 motif (residues 1-10) and three 10/11-mer motifs G1-G3 (residues 11-43). The second 44-243-region is encoded by exon-4 and consists of ten

Pro-interspersed 11/22-mer tandem repeats, named H1-H10. This region has a high tendency to form amphipathic α -helices, which are considered to be the main lipid surface binding motifs in apolipoproteins^{34,35}(Figure 2).

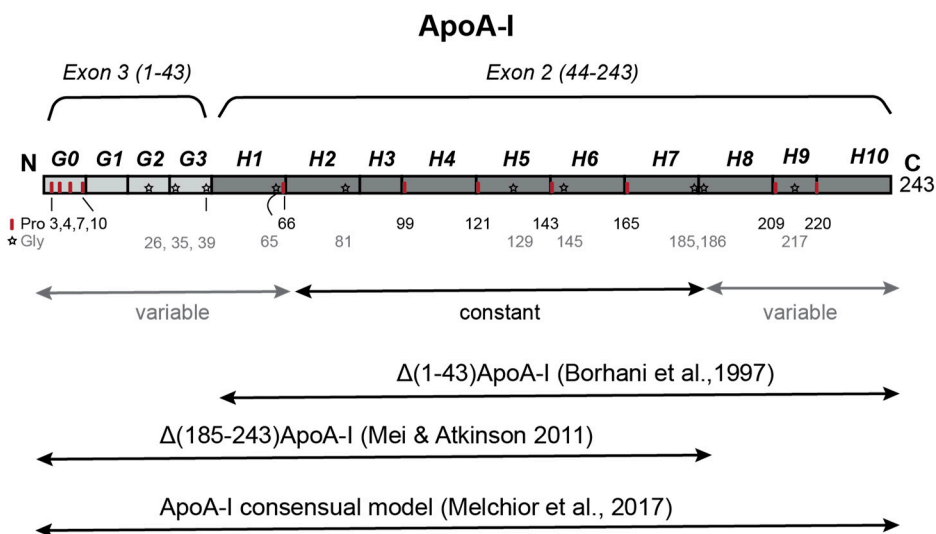


Figure 2. Schematics of ApoA-I domains. Segment 1-43 is encoded by exon 3 forming four 10/11-mer tandem repeats G0-G3 (light grey). Segment 44-243 is encoded by exon 4 forming ten 11/22-mer repeats, H1-H10 (dark grey). Pro (red lines) and Gly (stars) positions are shown by residue number. Segments, proposed to have a variable or constant structure in a double belt model, are depicted below³⁵. Region coverage for X-ray cristal structure of N-temrmmal truncated $\Delta(1-43)$ ApoA-I with a resolution of 4Å³², C-terminal truncated $\Delta(185-243)$ ApoA-I with a resolution of 2.2Å³⁶, and consensual model of the full-length ApoA-I, are depicted.

ApoA-I transiently dissociates and re-attaches to the lipid surface, adapting to the increasing lipid load. However, the mechanisms governing this phenomenon are not understood³⁵. In the discoidal model of the HDL with a diameter of 9.6 nm, there are two ApoA-I molecules per particle³⁷. The discs are composed of stacked anti-parallel protein rings, in the organisation known as the “double belt model”³⁸. This model proposes two antiparallel copies of ApoA-I closely wrapped around the HDL border via amphipathic α -helices. An H5/H5 registry is described although cross-linking studies revealed that these ApoA-I rings can adopt at least two different antiparallel registries with respect to each other³⁹, which was shown to have impact on HDL functionality⁴⁰. HDL formed with ApoA-I molecules artificially linked by their fifth and second helices (H5/H2) was shown to impair lecithin-cholesterol acyltransferase (LCAT, introduced in detail in the next chapter) esterification activity as compared to H5/H5 registry or the WT particle⁴⁰. This provides compelling evidence for structural properties being a determinant of the HDL function. Thanks to its flexible and

adaptable structure, ApoA-I exists in multiple states: LF, lipid-poor, and multiple lipid-bound states⁴¹. The flexible nature, tendency to self-associate and to bind hydrophobic substances, brings a challenge for high-resolution structural studies. Multiple approaches were undertaken to crystallize ApoA-I in its LF, monomeric state, providing crystal structures of truncation mutants, one lacking N-terminal 44 amino acids³², and the other lacking C-terminal 58 amino acids³⁶. The most up-to-date consensus model of ApoA-I in LF monomeric state, based on published data, was proposed by Melchior and colleagues⁴².

HDL subclasses

The HDL fraction in human plasma is made out of many subpopulations of particles which are being constantly remodelled by various plasma factors. The heterogeneity of the HDL population has been linked to its atheroprotective function⁴³. Nondenaturing gradient electrophoresis allows for separation of HDL into two major HDL subpopulations, HDL₂ and HDL₃ (Figure 3). The first one contains ApoA-I but no ApoA-II, whereas the latter contains both apolipoproteins. Therefore, HDL₃ particles are denser and smaller as compared to HDL₂^{44,45}. HDL can also be classified based on their surface charge into particles that migrate to either pre- β -position or α -position during agarose gel electrophoresis. Pre- β -position corresponds to LF ApoA-I, lipid-poor ApoA-I, and most of the discoidal HDL. Spherical HDL migrate to α -position⁴⁶, as presented in Figure 3.

HDL remodelling plasma factors

Once in the circulation, the C-terminal domain of ApoA-I interacts with an extracellular loop of the ATP-binding cassette (ABC) transporter A subfamily (ABCA1) receptor expressed by peripheral cells (Figure 4). Consequently, free cholesterol and phospholipids are transferred to the lipid-free/lipid-poor ApoA-I leading to the formation of **discoidal HDL**⁴⁷. The discoidal HDL consist of a phospholipid bilayer containing cholesterol that is enclosed by two ApoA-I molecules⁴⁸. The interaction with ABCA1 receptor is clearly of importance in HDL biogenesis. In humans with Tangier disease, a loss-of-function mutation in the *ABCA1* gene leads to reduced export of cellular cholesterol and phospholipids to apolipoproteins, as well as inhibition of HDL biogenesis. In the homozygous state, the carriers are characterised by the absence of HDL-cholesterol (HDL-C), and in heterozygotes, HDL-C levels are around half of the normal individuals, which consequently leads to an increased cholesterol accumulation in all cell types⁴⁹⁻⁵¹.

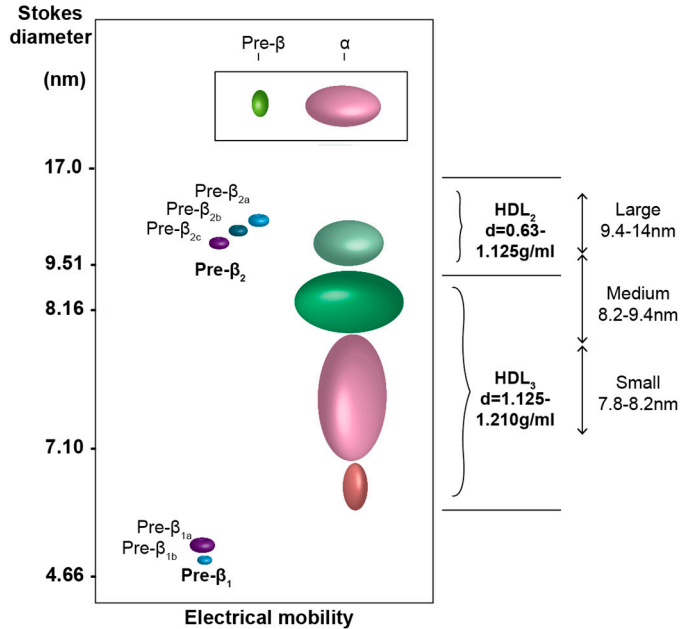


Figure 3. HDL subclasses. Nondenaturing gradient electrophoresis separates HDL into two major subpopulations, HDL₂ and HDL₃. The HDL₃ particles are denser and smaller as compared to HDL₂. HDL are also classified based on their surface charge into particles that migrate to either pre-β -position or α -position during agarose gel electrophoresis. Pre-β -position corresponds to lipid-free (LF) ApoA-I, lipid-poor ApoA-I, and most of the discoidal HDL. Spherical HDL migrate to α -position.

There is a number of plasma factors that regulate the distribution of the HDL subpopulations. One of the most important HDL modulating proteins is **lecithin-cholesterol acyltransferase (LCAT)**, an ApoA-I-activated enzyme⁵². LCAT ensures HDL maturation by the hydrolysis of phospholipid *sn*-2 acyl ester bonds in discoidal HDL. Resulting fatty acyl groups are transferred by LCAT to the 3-hydroxyl group of cholesterol, producing cholesteryl esters (CE) and lysophosphatidylcholine (Figure 4 and Figure 5). Due to the high hydrophobicity of the CE they are enclosed in the core of the lipoprotein particle esters which consequently expand the core of the HDL particles forming **spherical HDL** (Figure 5). The lysophosphatidylcholine (removed from the HDL particle) associates with albumin. The LCAT-driven reaction exhausts discoidal HDL of un-esterified cholesterol, leading to the formation of a concentration gradient where more cholesterol is transferred from other lipoproteins and from cell membranes to the HDL surface. This ensures a constant generation of CE^{53,54}.

Of note, people with LCAT deficiency were reported to have primarily discoidal HDL in their plasma⁵⁵. LCAT activity is proposed to be a rate limiting step in RCT, making it a potential therapeutic target for prevention of atherosclerosis⁵⁶. Structural⁵⁷ and

functional^{34,58} studies strongly suggest that LCAT interacts directly with ApoA-I on HDL, with almost 300-fold increased enzymatic activity in the presence of the protein⁵⁹, which makes ApoA-I the most potent LCAT-activator. Another important HDL-modulating factor is **cholesteryl ester transfer protein (CETP)**, which activity leads to a decrease of plasma HDL cholesterol levels and reduction of HDL particle size. CETP stimulates the bidirectional transfer of CE and TG between lipoproteins (Figure 4). People with CETP deficiency are characterised with larger HDL particles as well as overall higher plasma levels of HDL cholesterol^{54,60}. For this reason, HDL raising therapies using CETP inhibitors were under investigation over the past fifteen years. However, the failure of clinical trials on CETP inhibitors^{61,62} shows the complexity of HDL biology and the need for further research. **Phospholipid transfer protein (PLTP)** ensures the transfer of phospholipids between lipoproteins. PLTP remodels HDL particles by stimulating either particle fusion or dissociation of LF ApoA-I (Figure 4)⁶³. Re-entering of the LF or the lipid-poor ApoA-I into the circulation promotes HDL biogenesis by augmenting cholesterol and phospholipid efflux from cells expressing ABCA1⁶³. **Hepatic lipase's (HL)** activity leads to preferential hydrolysis of HDL-associated TG, but also to smaller extent phospholipids, and generation of small core-lipid depleted lipoproteins followed by dissociation LF or lipid-poor ApoA-I (Figure 4). CETP and VLDLs-mediated enrichment of HDL with TG makes an excellent substrate for HL. **Endothelial lipase (EL)** preferentially hydrolyses HDL phospholipids and to a smaller extent TG. EL's phospholipase activity only slightly decreases HDL size and does not lead to dissociation of LF or lipid-poor ApoA-I⁵⁴. Research shows that in mice overexpression of EL is associated with lower plasma concentration of HDL and ApoA-I⁶⁴. In humans, a loss-of-function mutation in the *EL* gene has been linked to an increase in HDL cholesterol levels⁶⁵. The role of **scavenger receptor B, type I (SR-BI)** in the regulation of plasma HDL cholesterol levels is also of importance. SR-BI mediates selective uptake of CE from HDL to the liver, where they are further metabolised and secreted^{66,54} (Figure 4).

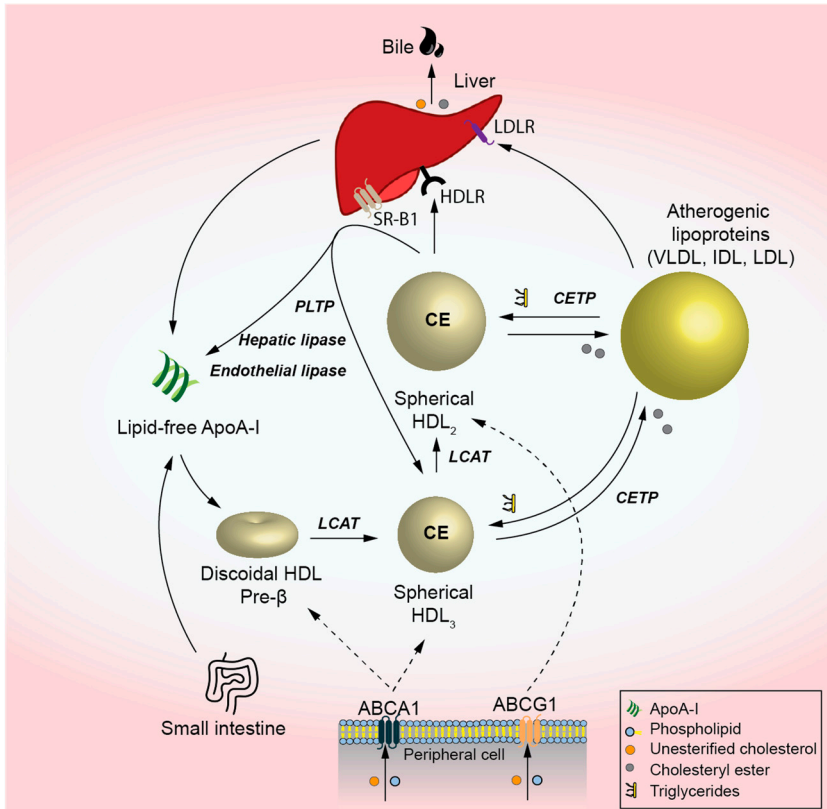


Figure 4. Metabolism of HDL. ApoA-I is synthesized in the liver and small intestine, followed by secretion to blood and mesenteric lymph. Lipid-free (LF) or lipid-poor ApoA-I accepts phospholipids and un-esterified cholesterol from cell membranes that express ATP-binding cassette (ABC) transporter A subfamily (ABCA1), leading to the formation of discoidal HDL. Lecithin-cholesterol acyltransferase (LCAT) binds to the discs and is activated by ApoA-I, resulting in the formation of spherical HDL particles, HDL₂ and HDL₃. LCAT action leads to the esterification of free cholesterol to become cholesteryl esters (CE), which reside in the core of the HDL. HDL₂ and HDL₃ contain two to four molecules of ApoA-I. Of note, spheroidal, large HDL (mainly HDL₂) can efflux cholesterol and phospholipids from peripheral tissues via ATP-binding cassette (ABC) transporter G subfamily (ABCG1) receptor. Unesterified cholesterol and cholesteryl esters are transferred from HDL₂ to the liver by reversibly binding to Scavenger receptor class B type I (SR-B1). Alternatively, the transfer is via cholesteryl ester transfer protein (CETP) to atherogenic lipoproteins (VLDL, IDL, LDL), followed by uptake by the LDL receptor (LDLR). In hepatocytes, CE are oxidized to bile acids and secreted out. HDL are subjected to remodelling catalysed by CETP, hepatic lipase, and plasma phospholipid transfer protein (PLTP). This results in LF ApoA-I being dissociated from the HDL particle which can then interact with ABCA1 in the next lipidation cycle. The activity of endothelial lipase leads to slightly decreased HDL particle size. HDL can also be hydrolysed in the liver via uptake through HDL receptor (HDLR)⁶⁷.

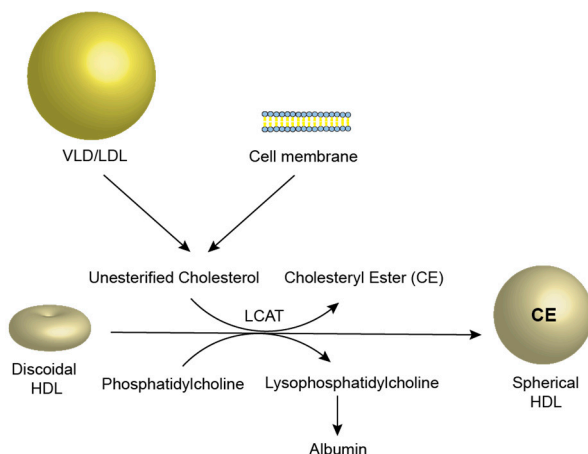


Figure 5. Biogenesis of spherical HDL particles. LCAT hydrolyses the phospholipids in discoidal HDL which leads to the release of fatty acyl groups and lysophosphatidylcholine. The fatty acyl groups are transferred to unesterified cholesterol, leading to the generation of cholesteryl esters (CE) which are integrated into the core of the spherical HDL. The remaining lysophosphatidylcholine binds to albumin. Discoidal HDL, depleted from unesterified cholesterol, is re-filled in the concentration gradient manner with additional cholesterol from VLDL/LDL and cell membranes, which can be hydrolysed by LCAT.

Molecular aspects of cholesterol efflux

Cholesterol catabolism is a very important process as it prevents from cholesterol overloading which, as in the case of macrophages foam cells in the arterial wall gives rise to atherosclerotic plaques⁶⁸. Most cells of the body are not capable of cholesterol catabolism, therefore efficient cholesterol efflux and transport by the HDL particles is crucial for maintaining homeostasis. RCT starts with efflux of the free cholesterol (FC) from the cell plasma membrane to HDL, facilitated by passive and active processes. The passive pathways involve simple diffusion and SR-BI-mediated diffusion. The active pathways involve transmembrane transporters ABCA1 and ATP-binding cassette (ABC) transporter G subfamily (ABCG1). It is noteworthy that, at least in mice, two-thirds of the cholesterol efflux is mediated by active pathways, mostly via ABCA1⁶⁹. Indeed, deficiency of ABCA1 and ABCG1 transporters results in foam cells accumulation and consequent progression of atherosclerosis in mice⁷⁰. LF ApoA-I interacts with ABCA1-expressing macrophages leading to active transport of phosphatidylcholine. Thanks to the phospholipid translocase activity of the ABCA1, this is followed by simultaneous FC and phospholipids efflux and formation of nascent (pre- β) HDL particles⁷¹. The half-life of ABCA1 is very short (1-2 h) and its cellular levels depend on the availability of ApoA-I, as the binding of ApoA-I to ABCA1 protects the transporter from calpain-driven proteolysis⁷². It has been shown that C-terminal α -helix of the ApoA-I determines the efflux of FC, possibly due to the high

hydrophobicity and high lipid affinity of this region⁷³. After ApoA-I binding to the cell surface, phospholipids are transported and solubilized allowing the formation of the nascent HDL, which is a rate-limiting process for the overall FC and phospholipid efflux. It is noteworthy that the catalytic efficiency (B_{max}/K_d) is highest for LF ApoA-I.

ABCG1 transporter is expressed by most of the cells, including arterial macrophages. Unlike ABCA1 mediated transport of FCs and phospholipids, ABCG1 enhances efflux to HDL but not to LF ApoA-I and does not require binding of the lipoprotein to the cell surface⁷⁴. Aqueous diffusion efflux pathway includes FC efflux to HDL particles in a nonprotein-mediated process. Highly hydrophobic cholesterol molecule is desorbed from the donor particle creating a high free energy state. When cholesterol desorbs completely to the aqueous phase it collides with an acceptor particle, leading to rapid absorption. Cholesterol can diffuse in both directions between the donor and acceptor particle in a concentration gradient-dependent process⁷. The second passive pathway of cholesterol transport is via SR-BI. Due to its role in the RCT, SR-BI is mostly expressed by the liver, but as well, by the steroidogenic tissue where it facilitates cholesterol delivery. SR-BI mediates cholesterol uptake into cells in a selective process where only CEs are transferred without degradation of the HDL particle, and additionally, its action has been shown to enhance efflux of cellular cholesterol to HDL⁷⁵. The selective CE uptake into cells involves first HDL binding to the receptor followed by CE transfer from the HDL-bound particle to the plasma membrane. Importantly, the K_d for the HDL binding to the receptor depends on an HDL particle size. Larger particles have lower K_b , meaning enhanced binding of the bigger particles to SR-BI. On the other hand, SR-BI mediated FC efflux depends on the local concentration of HDL. At low HDL concentration, the binding of the HDL is critical for the bidirectional transfer of FC through the hydrophobic tunnel of the SR-BI's extracellular domain. At high HDL concentration, binding to the receptor is saturated, but still, FC efflux to HDL is increased. This is due to FC reorganization in the plasma membrane, introduced by the SR-BI. The reorganized FC is desorbing more easily from the plasma membrane and can then associate with HDL⁷.

ApoA-I in glucose control

The role of HDL and ApoA-I in the prevention of atherosclerosis and cardiovascular diseases (CVD) is well established. More recently, ApoA-I has been shown to play important roles in the regulation of glucose control, providing a compelling link between diabetes and CVD. The positive effect of ApoA-I/HDL on glucose disposal includes stimulation of glucose uptake, increase of glucose-stimulated insulin secretion (GSIS), as well as improvement of cellular insulin sensitivity. These and other aspects of ApoA-I/HDL in glucose control are the subject of the current section and are discussed in detail below.

Diabetes

Diabetes mellitus (DM) is a metabolic disorder which is currently the fastest increasing disease worldwide. In 2015, there were around 415 million people with diabetes and this number is expected to rise up to 643 million by the year 2040⁷⁶. Understanding the disease and finding anti-diabetic solutions are therefore of great importance. The two main types of diabetes are type 1 (T1D) and type 2 (T2D). A main difference between these two types is the presence (in T1D) or absence (in T2D) of autoantibodies against pancreatic islet beta cell autoantigens, which occurrence leads to little or no insulin production in T1D patients. Another difference is the age at diagnosis, with T1D being diagnosed early in life. Of note, around 90% of the diabetes patients are diagnosed with T2D. While this classification is widely used, a recent study distinguishes five clusters of diabetes based on patient characteristics and prevalence of diabetic complications. The refined classification may greatly contribute to future, more precise diagnosis and better treatment of diabetes⁷⁷. The risk factors for developing T1D are mostly genetic predispositions and, to smaller extent, co-existent autoimmunity⁷⁶. Even though it has been over 100 years since the discovery of insulin, it is still the main therapy choice for the T1D patients⁷⁶.

When blood glucose increases, pancreatic beta cells secrete insulin, the only hormone of the human body capable of lowering blood sugar level. The pathophysiology of T2D includes development of insulin resistance (IR), a condition characterised by a reduced response to insulin-stimulated glucose uptake in liver, muscle, and adipose tissue. At first, in order to compensate for a greater peripheral tissue demand, insulin production significantly increases. This leads to beta cell exhaustion and may consequently result in beta cell failure⁷⁸ (Figure 6). It is debated whether IR occurs first, leading to hyperinsulinemia or, reversely, if a primary increase in beta cell insulin secretion causes the IR. Many excellent studies are published in support of both notions and are presented in the review by Czech et al⁷⁹. Additionally, abdominal and visceral fat, which is a highly metabolically active tissue, secretes unesterified fatty acids which in turn impair the action of insulin contributing to the development the IR. Around 75-80% people with T2D is or had been obese, therefore obesity is among the most important risk factors for the development of the disease. Also, age, physical inactivity, genetic and epigenetic predispositions play a part in the development of the disease. The diagnosis of T2D is based on the levels of fasting glucose (≥ 7.0 mmol/L) and glycated haemoglobin ($\text{HbA1C} \geq 48$ mmol/mol or 6.5%)⁸⁰. The HbA1C provides information about how much glucose is bound to the blood cells. As the haemoglobin is a very stable protein, the test allows for the determination of prolonged hyperglycemia. The treatment of T2D involves lifestyle changes, such as weight loss, healthy diet and increased physical activity. From the therapeutic point of view, metformin is considered as a first line of treatment. Metformin inhibits liver gluconeogenesis, thus decreasing fasting glucose levels by around 20% and HbA1C by around 1.5%. The main advantages of taking metformin are low grade of side effects and possibility to combine

it with other medications. Among the newly developed drugs are glucagon-like peptide 1 (GLP1) analogues, i.e., incretins that stimulate postprandial insulin release, as well as sodium-glucose cotransporter 2 (SGLT2) inhibitors, which block renal reabsorption of glucose⁸¹. When untreated, prolonged hyperglycemia and dyslipidaemia may lead to a number of complications. Among the most common T2D-complications there are heart failure, cardiovascular diseases (CVD), retinopathy and molecular oedema, nephropathy, and non-alcoholic steatohepatitis (NASH)⁸²(Figure 6). CVD is the most common cause of death among T2D patients, indeed, an estimated 75% of deaths will result from cardiovascular events⁸³.

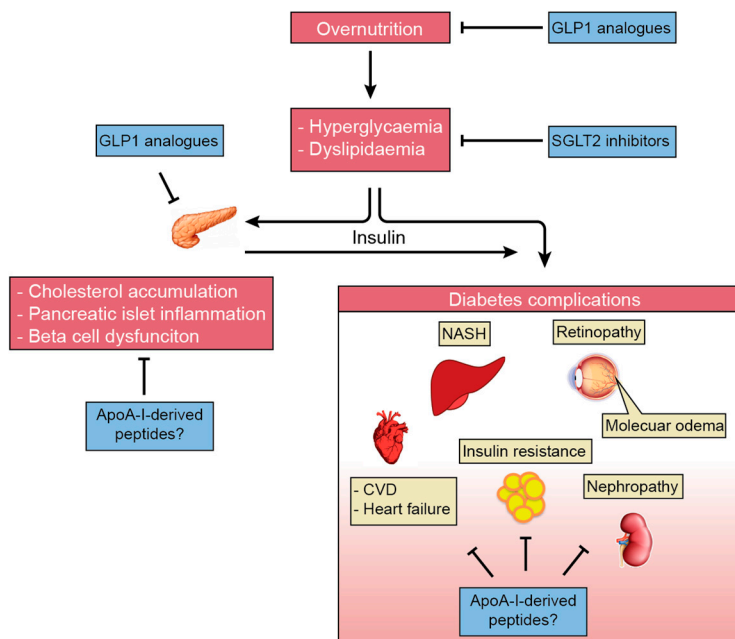


Figure 6. Diabetes complications. Overnutrition and consequent hyperglycaemia and dyslipidaemia result in pancreatic beta cell dysfunction and development of type 2 diabetes (T2D). Prolonged, untreated T2D leads to complications. Among the most common diabetes complications are heart failure, cardiovascular diseases (CVD), retinopathy and molecular oedema, nephropathy, non-alcoholic steatohepatitis (NASH) and insulin resistance (IR). Therapeutics, such as glucagon-like peptide 1 (GLP-1), augment beta cell insulin secretion and promote satiety, or improve glucose clearance via the kidney, as the sodium-glucose cotransporter 2 (SGLT2) inhibitors. ApoA-I was shown to improve beta cell functionality, reduce inflammation and IR, have potent anti-CVD properties, and improve kidney function. These properties make ApoA-I-derived peptides promising future anti-diabetic agents.

HDL-C/ApoA-I levels in glucose control

Metabolic syndrome includes a cluster of conditions such as high blood sugar, excess body fat around waist, high levels of LDL cholesterol and TG, as well as high blood pressure. Together these conditions increase the risk of development of CVD and T2D. The levels of ApoA-I and HDL are potent modulators of the metabolic syndrome and are, therefore, important biomarker of glycaemic control⁸⁴⁻⁸⁷. The link between low HDL and ApoA-I levels and elevated cardiovascular risk is well established⁸⁸. In line with this, high HDL levels were shown to protect from CVD, as shown by the Framingham Heart Study⁸⁹. Conversely, low and dysfunctional HDL leads to development of T2D and are associated with pathologies such as IR, obesity and high plasma TG. Studies show that infusions of reconstituted HDL (rHDL) particles reduces circulating glucose and increases insulin levels in diabetic mice⁹⁰ and in T2D patients⁹¹. Furthermore, in the Chinese population, low ApoA-I levels were found to be independently correlated with occurrence of T2D and the lower ApoA-I levels were proposed to improve T2D risk predictions⁸⁴. Additional, cross-sectional study presented by Feng et al.⁸⁵, showed that people with impaired glucose tolerance exhibit significantly lower ApoA-I levels, thus suggesting a negative correlation between low ApoA-I and development of IR, and proposing low ApoA-I as an independent risk factor for impaired glucose tolerance. In further support of this notion, IR is accompanied by dyslipidaemia, a common hallmark of T2D. This includes hypertriglyceridemia together with low HDL-C levels which lead to the progression of the IR in the early stages of T2D, as supported by multiple studies⁸⁶. Among those, epidemiological investigations, such as the Uppsala Longitudinal Study of the Adult Man (ULSAM), determined that HDL-C is a long-term predictor of insulin sensitivity⁹². Additionally, therapies aimed at lowering TG or increasing HDL levels, resulted in improved insulin sensitivity (as reviewed in⁸⁶). In established T2D, lower levels of HDL-C and ApoA-I were found to be associated with earlier initiation of pharmacological control of glucose⁸⁷. Important knowledge regarding the importance of ApoA-I levels in glucose control comes from genetic mice models. Both ApoA-I deficient and ApoA-I overexpressing were investigated by Lehti et al⁹³. ApoA-I deficient mice were characterised by fasting hyperglycemia and impaired glucose tolerance test as compared to wild type (WT) counterparts. On the other hand, ApoA-I overexpressing mice exhibited decreased fasting glucose, improved glucose tolerance test (GTT), reduced fat mass, when on a normal diet. Of note, mice with genetically increased HDL/ApoA-I did not develop diet-induced hyperglycemia due to increased glucose utilization. However, increased HDL/ApoA-I levels did not protect the mice from high-fat diet related body weight and fat mass gain.

HDL/ApoA-I in peripheral tissue metabolisms and insulin sensitivity

Regulation of glucose uptake

Glucose is the main metabolic fuel for mammalian cells, therefore its transport into the cells is tightly regulated. Glucose transporter isoform 4 (Glut4) is specifically expressed in insulin-sensitive tissues, such as skeletal muscle, heart, and adipose tissue⁹⁴. The action of the Glut4 in transporting glucose is regulated by insulin and the AMP-activated protein kinase (AMPK) contraction-induced pathway⁹⁵. Under basal conditions when insulin levels are low, Glut4 is located intracellularly. When insulin levels rise, Glut4-containing vesicles translocate to the cell surface, where transport of glucose from the extracellular environment into the cell can be ensured. For this reason, Glut4 plays a key role in normal glucose homeostasis as well as during the development of IR in T2D⁹⁴.

ApoA-I/HDL in glucose uptake

As shown by Han et al.⁹⁶, the stimulation of the C2C12 myotubes with ApoA-I led to an increase in phosphorylation of AMPK and acetyl-CoA-carboxylase (ACC), and subsequent stimulation of the glucose uptake. Moreover, the authors proposed that clathrin-dependent ApoA-I internalisation into the cells is a determinant of this positive action. Additionally, ApoA-I knockout mice were shown to have reduced AMPK phosphorylation in liver and skeletal muscles and increase in liver gluconeogenesis. These findings were translated into humans by Drew et al.⁹¹. Muscle biopsies obtained from T2D patients who had been infused with rHDL confirmed activation of AMPK. The proposed mechanisms involved binding of ApoA-I/HDL to cell surface receptors, like ABCA1, leading to recruitment of intracellular Ca²⁺ and activation of calcium/calmodulin-dependent protein kinase (CaMKK), resulting in phosphorylation of AMPK and glucose uptake. The role of ApoA-I in the stimulation of glucose utilization was further investigated. As shown by Dalla-Riva et al.⁹⁷, discoidal HDL is a potent activator of the Glut4 translocation in cultured skeletal muscles. Moreover, discoidal HDL promoted glucose uptake to the levels comparable with insulin stimulation. Of importance, the C-terminal domain of ApoA-I (190-243 peptide) was identified to potently induce glucose uptake on its own, opening doors for future ApoA-I-based peptide formulations⁹⁷. ApoA-I/HDL was furthermore shown to directly improve mitochondrial functionality in C2C12 muscle cells resulting in enhanced glucose utilization⁹³. This functionality of ApoA-I was proposed to be a protective mechanism against the development of high-fat-diet-induced impairment of glucose homeostasis in ApoA-I overexpressing mice⁹³. Additionally, the role of ApoA-I/HDL in reducing plasma glucose levels was shown to include a rise in glycogen synthesis in skeletal muscles. Glycogen serves as a cellular storage form of glucose, which can be used when energy is needed. Its synthesis is facilitated by the action of glycogen synthase, which is in turn negatively regulated by glycogen synthase kinase-3 (GSK3)-

mediated phosphorylation⁹⁸. The effect of long-term infusions of HDL into the T2D mice was reported to induce an increased glycogen deposit in the muscle tissue, due to inhibition GSK3⁹⁰. Importantly, in insulin-resistant diet-induced obese (DIO) mice, a single injection of ApoA-I was found to potently induce glucose uptake by the heart in addition to the previously described skeletal muscles⁹⁹. A transient insulin secretion blockade allowed to conclude that ApoA-I-mediated glucose uptake acts in an insulin-independent manner, involving activation of the AKT (also known as protein kinase B) pathway⁹⁹. These findings were further confirmed by others in the primary human skeletal muscle cells¹⁰⁰. The proposed mechanisms of action involve ApoA-I-stimulated phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), activation of the P13K/AKT/AS160 pathway and consequent translocation of Glut4 to the cell membrane. Of note, ApoA-I's ability to promote insulin-dependent and insulin-independent glucose uptake by skeletal muscle was shown to be mediated by ABCA1 and SR-B1.

HDL/ApoA-I in beta cell function

Pancreas

The pancreas consists of endocrine and exocrine tissue. The endocrine tissue accounts for 1-2% of the entire organ and consists of neuroendocrine cells which form highly vascularized islets of Langerhans. The cells secrete a number of hormones, neurotransmitters and peptides which play a crucial role in the metabolic and physiologic functions of the body¹⁰¹. The islets consist of insulin producing **beta cells** (most abundant), glucagon producing **alpha cells**, somatostatin producing **delta cells**, pancreatic polypeptide producing **gamma cells**, and ghrelin producing **epsilon cells**. The vast majority of the pancreas accounts for the exocrine tissue. It is made out of acinar, centroacinar, and ductal cells which together form the acinus. The islets are distributed across the acinar tissue which ensures interactions between the exocrine and endocrine tissue. This is of importance as insulin and other pancreatic islet peptides were shown to activate exocrine tissue. Also, ductal and acinar cells were shown to regulate the physiology of endocrine tissue by secretion of cytokines and growth factors¹⁰².

Insulin secretion

Insulin is the main glucose-lowering hormone of the human body, therefore its release is tightly regulated. Increasing concentrations of glucose and its cellular metabolism are the triggers for beta cell insulin secretion. Glucose induced insulin secretion begins with glucose entering the beta cells via the glucose transporter-GLUT2, where it is immediately phosphorylated by glucokinase. This results in the formation of glucose-6-phosphate, further metabolised via glycolysis. Formed pyruvate is utilized as a

substrate in the mitochondrial citric acid cycle leading to formation of ATP. Increasing intracellular ATP to ADP ratio causes the closure of the ATP-sensitive potassium (K^+) channels in the beta cell membrane. Consequential beta cell membrane depolarization opens voltage-dependent calcium channels, allowing for influx of calcium ions (Ca^{2+}). As the basal levels of free intracellular Ca^{2+} are approximately 100nM, around 20 000 times lower than free extracellular Ca^{2+} , transient opening of the calcium channels results in an up to 10 times increase of the cellular (Ca^{2+})¹⁰³. The signal is further amplified by 3',5'-cyclic adenosine monophosphate (cAMP) and leads to the fusion of insulin containing granules with the plasma membrane and subsequent release of insulin into the circulation¹⁰⁴. Of note, other nutrients, such as free fatty acids and amino acids can potentiate GSIS¹⁰⁵. Insulin secretion is also regulated by hormones such as glucagon-like peptide 1 (GLP-1), acetylcholine, somatostatin, and adrenaline^{104,106}.

Regulation of insulin synthesis

The regulation of insulin synthesis begins on a transcriptional and translational levels. Among the most important positive transcriptional regulators is pancreatic and duodenal homeobox-1 (Pdx1)¹⁰⁷. This transcription factor plays a central role in beta cell function and survival. The action of Pdx1 is dependent on its nuclear location. It has been shown that deleterious effects of fatty acids on beta cell function are accompanied by sequestration of Pdx1 into the cytoplasm¹⁰⁸. Importantly, positive effect of glucose on insulin transcription was proposed to be a result of Pdx1 nuclear translocation¹⁰⁹. The insulin gene encodes an insulin precursor-preproinsulin, containing a N-terminal signal peptide. Preproinsulin translocates across the rough endoplasmic reticulum (ER) membrane to the ER lumen. There, signal peptide is cleaved by a signal peptidase resulting in the formation of proinsulin. Proinsulin maturation involves folding and formation of three disulfide bonds and translocation from the ER to the Golgi apparatus¹⁰⁶. The proinsulin consists of A chain, B chain and C-peptide. The formation of mature insulin requires action of processing enzymes. Prohormone convertase enzyme PC3 (PC1/3) cleaves at the B chain/C-peptide junctions and PC2 at C-peptide/A chain. This is followed by removal of COOH-terminal basic arginine residues from A and B chain by carboxypeptidase E (CPE)¹¹⁰ (Figure 7). Of note, mice with deficiency in PC1/3 action have severely impaired proinsulin processing¹¹¹. Mature insulin consists of 21 amino acid residues A chain and 30 amino acid B chain bound together by disulfide bridges. Insulin and C-peptide are kept in beta cells as densely packed granules. Insulin is stored in a hexameric form consisting of 6 molecules of insulin peptide, in the form of 3 dimers¹⁰⁶.

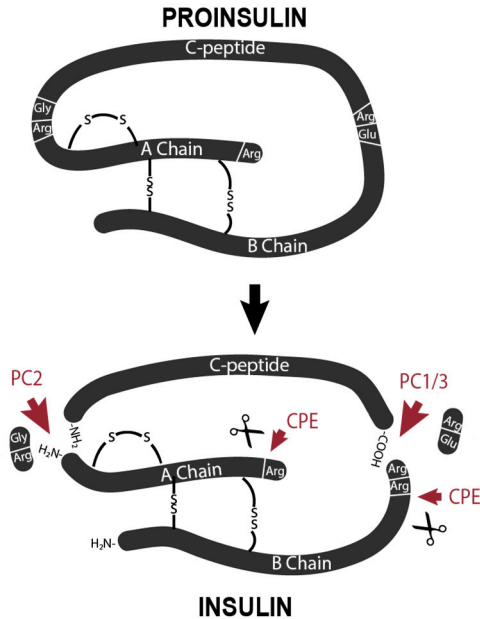


Figure 7. Processing of proinsulin. The proinsulin consists of A chain, B chain and C-peptide. The formation of mature insulin, consisting of A chain and B chain, requires action of processing enzymes. Prohormone convertase enzyme PC3 (PC1/3) cleaves at the B chain/C-peptide junctions and PC2 at C-peptide/A chain. This is followed by removal of COOH-terminal basic arginine residues from A and B chain by carboxypeptidase E (CPE).

ApoA-I/HDL and insulin secretion

One of the main hallmarks of T2D is beta cell exhaustion, resulting from an increase of insulin secretion to compensate for the progressing IR. Deterioration of the beta cell function and reduction in beta cell mass is accompanied by apoptosis and loss of beta cell identity^{112,113}. Studies show that ApoA-I restores beta cell functionality. Firstly, HDL-raising agents, such as CETP inhibitors were found to enhance postprandial insulin secretion in humans and potentiate *ex vivo* beta cell GSIS¹¹⁴. Furthermore, in obese and insulin-resistant mice a single injection of ApoA-I, followed by 3h incubation, led to improved glucose tolerance and insulin secretion. As an explanation of this, it was proposed that in addition to direct stimulation, ApoA-I primes the beta cells for improved GSIS¹¹⁵. In a follow-up study, ApoA-I's ability to improve beta cell function was proposed to involve the amplification of insulin release after glucose challenge⁹⁹. As the main function of ApoA-I is the transport of lipids, another study investigated whether long-term ApoA-I injections into mice with beta-cell specific ABCA1 and ABCG1-deletions, characterised with high islet cholesterol content and impaired GSIS¹¹⁶, would improve their beta cell function. The treatment with ApoA-I significantly increased GSIS in these mice, however, islet cholesterol levels were not changed. This led to the conclusion that ApoA-I improves beta cell function

independent of ABCA1 and ABCG1¹¹⁷. Valuable information regarding ApoA-I's role in improving beta-cell functionality also comes from *in vitro* studies that utilize clonal beta cell lines. In mouse-derived Min6 cells, incubation with either HDL isolated from human plasma, rHDL, or LF ApoA-I, led to an increased beta cell insulin secretion in basal glucose as well as in stimulatory glucose environment. Importantly, cholesterol accumulation in beta cells was proposed to deteriorate beta cell functionality in T2D^{118,119}. To explore whether incubation with ApoA-I can reverse this deleterious effect, cholesterol-enriched Min6 were incubated with LF ApoA-I. Surprisingly, while insulin secretion was potentiated, the intracellular cholesterol levels were not changed, suggesting that ApoA-I positive effect on insulin secretion is independent of cholesterol efflux¹²⁰. In another cellular model of beta cell, rat derived INS-1E, incubation with ApoA-I was found to directly increase glucose-stimulated, and basal insulin release¹²¹. Of note, prolonged incubation with ApoA-I resulted in a significantly higher transcription of the insulin coding genes *Ins1* and *Ins2* as well as *Pdx1*. The authors proposed a mechanism explaining the positive effect of ApoA-I on beta cell functionality, which involves the protein interaction with the cell surface expressed ABCA1. The direct interaction of ApoA-I with ABCA1 was proposed to increase GSIS and initiate transcription of *Ins1* and *Ins2* by activation of the G-protein subunit. This was followed by the increase of intracellular cAMP and activation of protein kinase A (PKA), which in turn phosphorylates and excludes the transcription factor forkhead box protein O1, the repressor of insulin gene transcription, from the INS-1E nucleus¹²¹. There are apparent inconsistencies in the proposed explanation of ApoA-I's positive action on improving beta cell functionality. As described above, ApoA-I significantly increases GSIS in beta cell-specific ABCA1 knockout mice¹¹⁷, therefore ApoA-I's interaction with ABCA1 does not seem to be a determinant of this phenomenon. The activation of the G-protein subunit may be achieved by alternative pathways, which remains to be investigated. To better understand the mechanisms of ApoA-I-mediated improvement of beta cell function, a study was performed (**paper I**) and is presented in detail in the following chapters¹²².

ApoA-I as a potential therapeutic

The HDL-C-raising therapies, including CETP inhibitors and rHDL infusions, exhibited anti-diabetic properties. This includes protection against diabetes development, attenuation of diabetes progression, and improvement of glycaemic control in individuals with established disease^{91,123,124}. However, the negative outcome of the clinical trials employing these agents as an anti-CVD solution decreases the likelihood of reusing them for anti-diabetic purposes. Another way to raise the HDL levels is by lifestyle interventions. After one year of calorie restriction diet and increased physical activity, the T2D subjects were shown to have improved glucose control, reduced intake of anti-diabetic therapeutics and increased HDL levels¹²⁵. Other

promising candidates for anti-diabetic HDL-targeted therapies are ApoA-I mimetic peptides. There are multiple advantages of employing therapeutic peptides over full-length proteins or antibodies. They are smaller and therefore easier to synthesize, they have high activity and specificity, and are less likely to accumulate in organs which minimises the possibility of toxic side effects. Peptides are also less immunogenic than recombinant proteins or antibodies¹²⁶. The administration of the L-4F ApoA-I-mimetic peptide into obese mice was shown to reduce visceral fat content and improve glucose tolerance and insulin sensitivity. The proposed mechanism involved a reduction of systemic inflammation, phosphorylation of AMPK and insulin receptor, and increase in plasma adiponectin levels¹²⁷. A recent study deriving from our group proposes a novel anti-diabetic ApoA-I-derived peptide, the RG54 peptide, which exhibits added anti-CVD benefits. DIO and diabetic *Lep^r^{db}* mice injected with RG54 were shown to have significantly improved glucose clearance in a GTT. Supported by additional *in vitro* studies, this positive effect was proposed to be via dual mechanisms. The first includes direct stimulation of glucose uptake in skeletal muscles and the other priming of the beta cell for improved GSIS. This is agreement with conclusion derived from our previous study for full-length ApoA-I⁹⁹. The potential role of the RG54 in the treatment of cardiometabolic disease was presented in the atherosclerotic *ApoE*^{-/-} mice model. The treatment with RG54 reduced the number of atherosclerotic plaques as well as the aortic plaque area in these animals. In support of this, RG54 more efficiently than full-length ApoA-I, catalysed cholesterol efflux from cultured macrophages¹²⁸. Altogether, HDL-raising therapies and ApoA-I-mimetic peptides provide a very promising treatment avenue. This is of particular interest for diabetic and pre-diabetic individuals with increased risk of CVD.

Glycation of ApoA-I

Chronic hyperglycemia in T2D is a causative factor for the development and progression of the disease and diabetic complications. Additionally, prolonged exposure to high glucose environment modifies many biomolecules, including ApoA-I, leading to the deterioration of their functionality.

Advanced glycation end-products (AGEs)

Biomolecules are modified by sugars in the process called Maillard reaction, leading to the formation of advanced glycation end-products (AGEs). Reactive reducing sugars, such as glucose, are capable of non-enzymatic reaction with amino groups in proteins, lipids and nucleic acids, forming precursors of AGEs called Schiff bases and Amadori products (Figure 8). Glycation is often accompanied by oxidation, leading to the

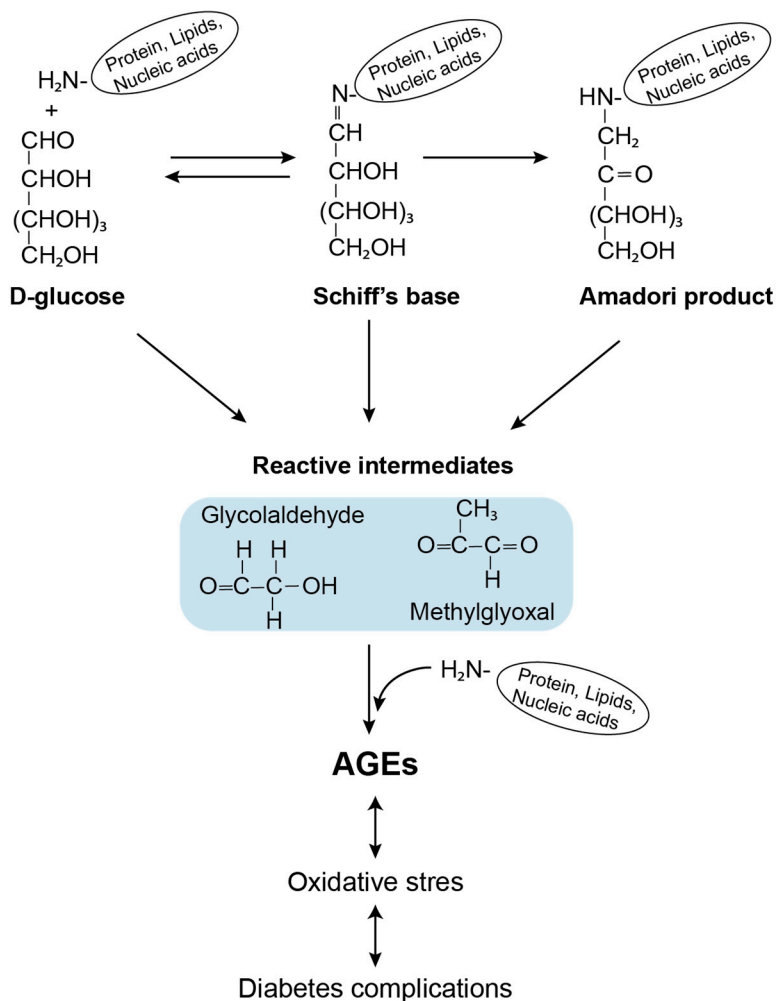


Figure 8. Formation of advanced glycation end products (AGEs). In the Maillard reaction, an aldehyde group deriving from reactive reducing sugars, such as glucose, reacts with an amino group of proteins, lipids or nucleic acids. Formed Schiff's base undergoes rearrangement to form more stable Amadori product. A spontaneous cleavage of glucose, Schiff's base or Amadori products leads to the formation of reactive intermediates, which can as well form AGEs by interacting with amino groups. Accumulation of AGEs leads to oxidative stress and diabetes complications¹²⁹.

formation of so-called glycoxidation products¹³⁰. Advanced glycation occurs over longer periods, hence affecting long-lived proteins such as haemoglobin. As the Maillard reaction is enhanced in diabetes^{131,132}, the rate of glycated haemoglobin (HbA1C) is a common biomarker of hyperglycemia events monitored over past 6 to 12 weeks¹³³ and used for diagnosis of diabetes. Notably, Maillard reaction is accompanied by the formation of reactive intermediate products called alpha-dicarbonyls, including

methylglyoxal (MG) and glycolaldehyde (GA), which are capable of creating AGEs. The build-up of reactive dicarbonyls, as well as glycooxidation and lipoxidation products, leads to the development of carbonyl stress. It has been proposed that carbonyl stress is a common hallmark of diabetes and uraemia leading to an acceleration of vascular damage¹³⁴. Furthermore, AGEs have been associated with a number of conditions such as renal, retinal, neural complications in diabetes^{135,136} as well as ageing¹³⁷.

Functional aspects of glycated ApoA-I

ApoA-I, together with many other plasma proteins, is subjected to non-enzymatic glycation in both type 1 and type 2 diabetes¹³⁸. HDL isolated from diabetic patients were found to have an 400% increase in glycation events as compared to control subjects¹³⁹. Multiple studies have therefore addressed the functional consequences of ApoA-I glycation. Firstly, modification of ApoA-I/HDL with MG was shown to affect the conformation of ApoA-I in the region important for its interaction with LCAT and lipid binding. This was manifested by a decreased size of HDL as the LCAT-mediated cholesterol esterification was reduced when ApoA-I was glycated¹⁴⁰. Furthermore, glycation of ApoA-I was demonstrated to impair its anti-inflammatory properties. Infusions of glycated ApoA-I into rabbits with induced vascular inflammation less effectively counteracted the inflammation as compared to non-modified ApoA-I/HDL. As supported by *in vitro* studies, the authors proposed a mechanism involving the diminished ability of the glycated ApoA-I/HDL to inhibit activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) protein complex and increased formation of reactive oxygen species (ROS). It is suggested that structural and conformational changes, which occur as a result of ApoA-I glycation, are important contributing factors to this phenomenon¹⁴¹. On that note, modification of ApoA-I with reactive carbonyls such as malondialdehyde (MDA), was proposed to mainly target lysine residues (Lys) in the C-terminal of ApoA-I. The resulting MDA-Lys are proposed to be responsible for impairment of cholesterol efflux through the ABCA1 pathway¹⁴². Since a main function of ApoA-I/HDL is the reverse transport of lipids and cholesterol, which ensures its cardioprotective and antiatherogenic properties, the capability of glycated ApoA-I to mediate cholesterol efflux has been thoroughly investigated¹⁴³⁻¹⁴⁶. The studies, however, provided conflicting results. Firstly, HDL isolated from plasma of healthy individuals and thereafter glycated *in vitro* were shown to mediate cholesterol efflux from cultured macrophages to the same extent as the non-modified HDL¹⁴³. This was further supported by another study, where the authors used ApoA-I isolated from plasma and subjected to a similar analysis. No change was observed for the ability of either LF glycated ApoA-I or glycated rHDL to mediate cholesterol efflux from cultured macrophages. Notably, a negative effect of glycation on the ability of ApoA-I to mediate cholesterol efflux has also been described¹⁴⁴⁻¹⁴⁶. This was shown in THP-1 macrophages, in isolated human monocytes and in ABCA1-transfected HeLa cells, which exhibited more than 70% reduction in the ability of AGE- modified ApoA-I to

promote cholesterol mobilisation. This deleterious effect was reversed by the prevention of AGE formation¹⁴⁴. Additionally, another report stated that metformin, a commonly used anti-diabetic therapeutic, can protect from glycation-induced impairment of HDL to accept cholesterol¹⁴⁵. With the aim to bring clarity to these contradictory findings, we performed additional experiments, and the emerging findings are included in this thesis in **paper II**¹⁴⁶.

ApoA-I is a potent positive regulator of glucose control. Given its antiatherogenic properties, therapeutics based on the ApoA-I biology may provide a promising therapeutic solution for people with metabolic syndrome. As summarized above, a lot of research has been done in order to unravel the molecular mechanisms behind ApoA-I's positive effect on glucose control. The research shows that ApoA-I, due to its versatile functional benefits, exerts potential for the treatment as well as the prevention of the disease. There is still a lot to be understood, especially in terms of its action on beta cells. Additional research and cohort studies will hopefully bring a renaissance to clinical trials of ApoA-I/HDL-based therapies.

ApoA-I related amyloidosis

The previous section was devoted to the beneficial functions of ApoA-I but there is a darker side to this protein. This following chapter is centred around the deleterious propensities of natural, but rarely occurring, ApoA-I variants to become misfolded and to form amyloids in vital organs.

Protein misfolding and diseases

Proteins constitutes the building blocks of the living cell and are, therefore, vital for life-maintenance. Numerous disorders can arise from the acquisition of proteins dysfunctional conformations. These are termed conformational or protein misfolding diseases, and lead to the formation of protein aggregates and fibrils, also termed amyloids, that result in a loss of function and in an acquisition of toxic properties. There are approximately 50 different proteins or peptides known to assemble into amyloid fibrils causing human disease¹⁴⁷. Some proteins are more prone to become misfolded and the risk of forming fibrils increases with age and protein concentration. Among these, there are intrinsically disordered β -Amyloid peptide (A β), responsible for the development of Alzheimer's disease, α -synuclein of Parkinson's disease, and islet amyloid peptide of the pancreatic islets of Langerhans. These "natively unfolded" proteins are in their soluble form characterised by high flexibility and lack of secondary structure¹⁴⁸. There are many endogenous and exogenous factors which can increase the propensity of proteins to change their conformation and become misfolded. Among the most common are disturbed interactions with endogenous factors such as membranes, chaperones, and extracellular matrix components, as well as point mutations, exposures to toxins, impaired posttranslational modifications, and oxidative stress. Misfolded proteins transition from their soluble state into amyloid fibrils, which are stable, highly organized, filamentous aggregates¹⁴⁹. Amyloids can deposit in various organs and tissues causing multiple disorders, collectively termed amyloidoses. Even though amyloids of different proteins largely vary in size, they share several characteristics. They are of varied length, consist of two to six unbranched protofilaments of each 2-5 nm diameter that together make rope-like like fibrils that are 7-12 nm thick¹⁵⁰, and display core cross- β -sheet structure with polypeptide chain organized in β -sheets parallel to the fibril axis¹⁵¹. Native proteins exist in dynamic equilibrium with their disordered states. The fibril formation starts when one of these states assemble into oligomeric species. The further assembly of these pathogenic oligomers leads to the formation of higher-order oligomers containing fibril nucleus, which recruits other monomers, in the lag time of assembly. The next phase, termed elongation phase, includes exponential growth by conversion of the monomers into fibrils and recruitment of aggregation-prone species. These fibrils can then associate

with one another or with other proteins, leading to the development of amyloid plaques and intracellular inclusions¹⁴⁷.

The similarity of amyloidogenic fibrils originating from structurally diverse proteins implies that a major structural rearrangement must take place. Well-folded globular proteins are not susceptible to such rearrangements thanks to the presence of tertiary structure constrains. The amyloidogenic properties of these proteins were proposed to be due to the destabilization of their native structure by occurring mutations, changes in pH, or temperature^{149,152}. This is the case of ApoA-I and its amyloidogenic N-terminal fragment as discussed below.

Table 1. The mutations occurring in ApoA-I sequence related to amyloidogenic propensities.

Mutation	Affected organ or tissue	Ethnic origin
Internal mutations		
G26R ¹⁵³	Kidney, liver, peripheral nerves, GI tract	British, Scandinavian
E34K ¹⁵⁴	Kidney, liver	Polish
S36A ¹⁵⁴	Asymptomatic	British
G35V	Asymptomatic	Caucasian
W50R ¹⁵⁵	Kidney, liver, GI tract	Jewish
L60R ¹⁵⁶	Kidney, liver, testes, heart	British
L64P ¹⁵⁷	Kidney,liver	Italian
Δ60-71+VT ins ¹⁵⁸	Liver	Spanish
Δ70-72 ¹⁵⁹	Kidney,liver,choroid	German
F71Y ¹⁵⁴	Liver,palate	British
N74K frameshift ¹⁵⁹	Kidney, uterus, ovaries, pelvic lymph nodes, GI track	German
L75P ¹⁶⁰	Kidney, liver, testes	Italian, German, other
L90P ¹⁶¹	Skin, heart, larynx	French, American
External mutations		
ΔL107 ¹⁶²	Circulatory system	Scandinavian
A154 frameshift ¹⁵⁹	Kidney	German
H155M frameshift ¹⁵⁴	Kidney	British
L170P ¹⁵⁹	Asymptomatic	German
R173P ¹⁶¹	Kidney, skin, heart, larynx	American, British
L174S ¹⁶³	Skin, testes, heart, larynx	Italian, Dutch
A175P ¹⁵⁴	Larynx, testes	British
L178H ¹⁶⁴	Larynx, skin, heart, nerves	French
L178P ¹⁶⁴	Heart, larynx	Dutch

ApoA-I and amyloidosis

There are more than 50 naturally occurring human ApoA-I variants and most of them are associated with low plasma levels of ApoA-I/HDL. While some of them are beneficial, such as the ApoA-I_{MILANO} (Leu173Cys) variant that exhibits protective role against atherosclerosis¹⁶⁵, the majority have deleterious effects as in the case of amyloidogenic variants. There are two forms of human ApoA-I amyloidosis, acquired and familial. Acquired amyloidosis, independent of genetic predisposition, is the most common and it involves the deposition of full-length, wild-type ApoA-I as fibres in aortic plaques¹⁶⁶. This process is proposed to be proatherogenic as it is shown to increase the vulnerability of the plaques, activate macrophages, and exert toxic properties^{167,168}. Based on the *in vitro* studies, WT ApoA-I forms amyloids upon methionine oxidation, which may also be the mechanism of ApoA-I fibrillogenesis in the oxidative environment of atherosclerotic plaques¹⁶⁹.

Of importance, more than twenty ApoA-I variants lead to the development of familial ApoA-I amyloidosis (AApoA-I). These variants differ from the WT protein for point substitutions, frame shifts, and deletions^{33,154}(Table 1), mainly concentrated to two regions of the ApoA-I protein (residues 26-107 and 154-178). AApoA-I is a late-onset, autosomal, dominant condition, where N-terminal (9-11 kDa, amino acids 80-100 amino acid residues long) of the mutant ApoA-I accumulate in various vital organs (kidney, liver, skin testes, heart), leading to their damage^{33,170}. The mutations occurring in the N-terminal domain (amino acid residues 26-107), termed “internal” mutations, result in the preferential deposition of the amyloidogenic fibres in liver, kidneys and testes. Instead, mutations in the C-terminal segment (amino acid residues 154-178), termed “external mutations”, result in a preferential amyloid accumulation in heart, skin and larynx (Figure 9). To date, the only available treatment is end-stage organ transplant¹⁷¹.

There are many unknowns regarding the development and mechanisms of this disease. Firstly, different AApoA-I mutations form N-terminal fibrillogenic fragments, which are sometimes distant from the actual mutation site. Secondly, the same mutation can result in different severity of symptoms, as in case of the carriers of Leu75Pro mutation^{33,154,172}. Thirdly, and most strikingly, even though AApoA-I patients have reduced by 30 to 50% plasma levels of HDL/ApoA-I^{172,173}, they are not at higher risk of developing atherosclerosis^{174,175}. This suggests that AApoA-I is a complex disease where a lot still remains to be understood. Due to the rare nature of the disease and limited material, only few research groups are working towards a better understanding of this complex condition. Nevertheless, a number of functional and structural methods have been applied, providing important new knowledge on the subject. These advancements are summarised in the following section.

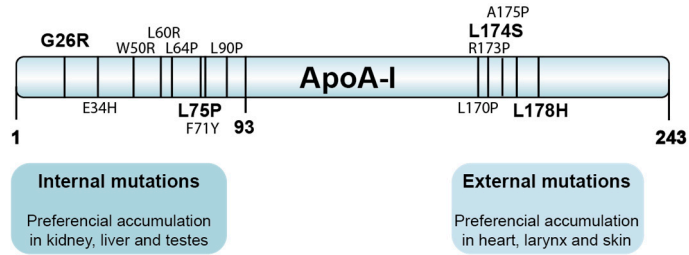


Figure 9. A map of ApoA-I single point amyloidogenic mutations. The mutations occurring in the N-terminal domain of the ApoA-I protein are termed “internal” mutations, whereas mutations occurring in the C-terminal domain are termed “external” mutations. The location of the mutation in the *APOA1* gene determines the preferential organ accumulation of the ApoA-I variant fibrils.

Clinical phenotype of the AApoA-I patients

Clinical description of AApoA-I patients based on small case studies. One example is the evaluation of 253 carriers of Leu75Pro ApoA-I amyloidogenic variants, who belong to 50 families from a region in the north of Italy¹⁷². The disease penetrance was shown to be age-dependent and clinical phenotype emerged with rising age. The kidney was identified as a predominant target organ for amyloid deposition and, to a smaller extent, liver and testes. Another case study of two patients with Leu174Ser and Leu178Pro AApoA-I variants identified amyloids deposits in the larynx¹⁶⁴. Severe lesion progression was found after a 4-year follow-up in these patients. Of note, although AApoA-I carriers are heterozygous for the mutated gene, only the variant isoforms is detected in the amyloid deposits. Interestingly, in patients with amyloidogenic mutations WT ApoA-I is expressed at higher level as compared to the variant protein, at least in the Leu174Ser and Leu75Pro carriers^{176,177}. There is a challenge with the diagnosis of AApoA-I as it often requires examination of the affected organs by biopsy. Limited data describing the clinical manifestation of this complex condition provide difficulty in the characterisation of the disease and establishment of the treatment avenues. Together, these observations suggest that AApoA-I is an underdiagnosed condition.

Structural and functional features of the ApoA-I fibrillogenic peptide

Identification of the structural and environmental factors driving the fibrillogenic process is key to a better understanding of the pathology and to the discovery of novel strategies to prevent it. ApoA-I fragment extracted from amyloid deposits of a patient affected by ApoA-I-related amyloidosis was characterised as natively unfolded at neutral pH and consisting of N-terminal 1-93 residues. The exposure of the isolated fragment to acidic environment induced a complex fibrillogenic pathway, which led to a shift from a random coil structure to a transient helical conformation, and final aggregation into a β -sheet polymeric structure¹⁷⁷. It is proposed that accumulation of the N-terminal fragments as amyloid fibrils is a result of the structural destabilization, induced by mutations, and consequent proteolysis, that generates a high concentration of the amyloid precursors^{33,178}. This is supported by mass spectroscopic analysis of several “internal” variants, where only the fragments of the mutant protein were identified in fibrils¹⁵⁴. Of note, for “external” variants, N-terminal deposited fragments were mutation-free^{33,178}.

A breakthrough in understanding the structure-function relation of AApoA-I came with the crystallization of the C-terminal deletion mutant of ApoA-I³⁶, which contains all sites of known amyloidogenic mutations. It allowed for the identification of the two amyloidogenic “hot-spot” mutation sites included in the *APOA1* gene, N-terminal residues 26-107 and C-terminal 154-178¹⁷⁸. According to the model, LF ApoA-I forms a dimer made of two antiparallel, and largely α -helical ApoA-I molecules, stabilized by two four-segment bundles at its ends. The perturbations of the bundle structure in the N-terminal domain by mutations are proposed to be a causative factor for the development of the ApoA-I amyloidosis. The destabilization of ApoA-I interaction with lipids leads to dissociation of LF ApoA-I from HDL. Additionally, the presence of mutations destabilizes the four-helix bundle of the protein which changes its conformation. The increased mobility of the protein exposes the helical repeats 3 and 4 (H3-H4, residues 80-120) to the solvent, making that region more susceptible to proteolysis. Moreover, the misfolding of the N-terminal fragment from native α -helical structure to β -sheet results in fibril formation^{178,179}. The studies on crystal structure, as well as other low resolution studies¹⁸⁰, also revealed that the residue segment 44-55 adopts high β -sheet propensity, which plays an important role in the α -helix to β -sheet conversion. Of note, the presence of these residues was proposed to be a prerequisite for fibril formation¹⁸¹ (Figure 10).

The observation that ApoA-I amyloidogenic variants are characterised by a destabilized structure were confirmed by a hydrogen-deuterium exchange (HDX) – mass spectrometry (MS) analysis¹⁸². This method allows for the monitoring of high structural resolution local changes in the dynamics of a protein. The authors compared WT ApoA-I with amyloidogenic G26R (also referred as ApoA-I_{Iowa}). In agreement with previous studies^{33,178}, major structural differences between the proteins occurred in the

N-terminal fragment, which is largely destabilized in the variant. Moreover, the authors found that the disordering in G26R leads to a loss of 30 helical residues translating to around 12% total alpha-helix reduction, as estimated by circular dichroism (CD) spectroscopy¹⁸². A major advancement in understanding the structure-function relationship of the amyloidogenic ApoA-I variants comes from the studies performed by our group, included in the presented thesis in **paper III and paper IV**¹⁸³.

In summary, AApoA-I is a very complex condition affecting multiple vital organs and the late-onset clinical phenotype manifestation provides a challenge for its characterisation and treatment. Understanding how structural rearrangements and destabilizations lead to ApoA-I amyloidosis is likely key in the development of new therapeutics.

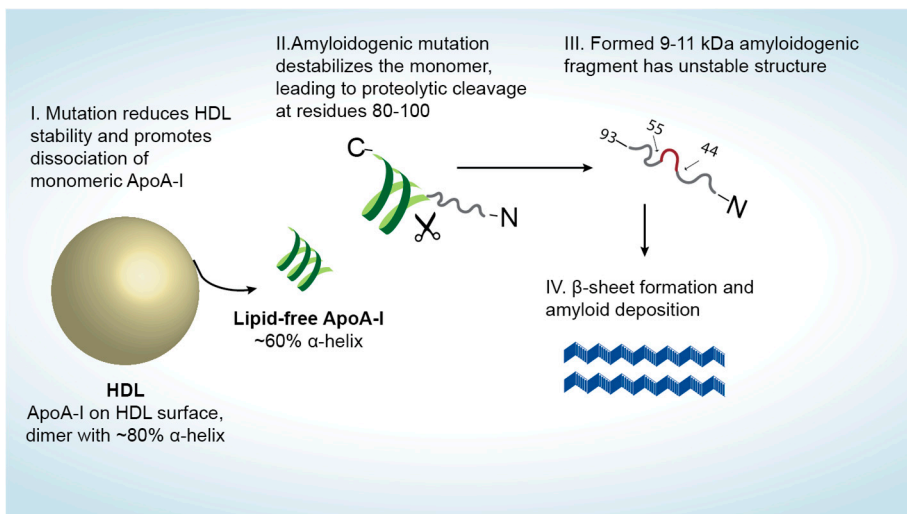


Figure 10. Proposed model of molecular mechanism leading to ApoA-I misfolding in hereditary amyloidosis. Variant ApoA-I is susceptible to dissociation from HDL. Free, variant ApoA-I undergoes a proteolytic cleavage and formation of N-terminal amyloidogenic fragment. The misfolding of the fragment leads to the transition from the native α-helical structure to parallel β-sheet and amyloid deposition.

Aims

Overall aim

The aim of my PhD was the in-depth investigation of the structural and functional properties of ApoA-I.

Specific aims

The goals were to determine

- the mechanisms of ApoA-I-mediated increase of glucose stimulated insulin secretion from pancreatic beta cell.
- the structural and functional consequences of ApoA-I glycation in hyperglycemia.
- the function-to-structure connection of ApoA-I amyloidogenic variants that cause improved capabilities to mediate cholesterol efflux.

Methods

Glucose stimulated insulin secretion (GSIS)

GSIS is a widely used laboratory technique which measures the sensitivity of the beta cells to secrete insulin in response to stimulatory glucose levels. It provides valuable information regarding the beta cell functionality. In the GSIS experiments included in papers II and III, we developed “the priming model”, as shown below, to assess the role of ApoA-I/HDL on the biological processes.

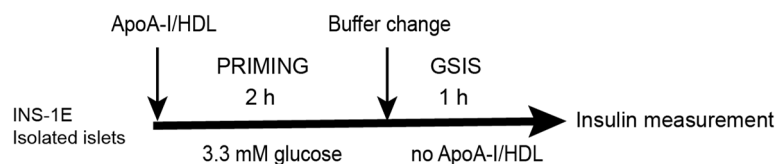


Figure 11. “The priming” experimental model. INS-1E or isolated mouse islets were incubated for 2 h in the absence (Control) or in the presence of ApoA-I or rHDL in low glucose environment (priming). Buffer was replaced to one containing either low or stimulatory glucose, in the absence of the protein. Insulin secretion was measured after 1 h glucose challenge.

Cells – INS-1E

Cellular models are valuable tools in understanding the molecular mechanisms of biological processes. The experiments can be performed in controlled, reproducible environments, which is the main advantage of the cell culture studies over the *in vivo* experiments. Compared to animal models, they are also cheaper, faster to complete, and do not require ethical permit. The main disadvantage is that the cells are not no longer exposed to naturally occurring interactions, which raises the question of translatability of the results obtained in cells to physiological environment. Nevertheless, cellular models are needed to elucidate the biological processes.

Rat insulinoma-derived clonal beta-cell line INS-1E¹⁸⁴ was used in experiments included in **paper I** and **paper II**. Cell studies included GSIS preceded by pre-incubation with ApoA-I/HDL or glycated ApoA-I/HDL (**paper II**). Additionally, in

paper I, INS-1E were used for immunological and microscopy analysis. Cell studies with INS-1E largely contributed to uncovering the mechanisms behind ApoA-I-mediated increase in GSIS (**paper I**) as well as verification of the observed *in vivo* effect (**paper II**), confirming the deleterious effect of the glycation on ApoA-I's function.

Isolated mouse islets

To more closely represent the *in vivo* processes, we used isolated mouse islets to verify findings emerged from the *in vitro* studies in **paper I**. Pancreas from sacrificed male C57BL/6 mice were digested by intraductal injection of collagenase and the islets were handpicked using pipette and light microscope. After overnight recovery, cells were stimulated with ApoA-I/HDL and glucose followed by insulin determination according to the “priming model” presented above, and transmission electron microscopy was performed.

The use of isolated mouse islets includes the effects of the neighbouring endocrine cells, such as glucagon secreting alpha cells. As the treatment is performed *ex vivo*, it allows for precise dosing, although it does not fully represent the natural environment. Even though mouse islets share a lot of structural and functional similarities with the human islets, there are still some differences. As an example, as oppose to human islets, mouse islets have alpha cells concentrated in the outer ring of the islet¹⁸⁵. This can lead to functional differences, which should be verified in human pancreatic islets in future experiments.

Glucose tolerance test (GTT)

In **paper II**, we used a mouse model to study the impact of the ApoA-I glycation on ApoA-I's ability to improve glucose tolerance. Using mice is a well-regarded approach to study biological processes in the *in vivo* setting. Mice are inexpensive, small, and easy to house, which allows for quick acquisition of reliable results. Ethical considerations are an important aspect when working with mice. Good experimental planning and proper handling are crucial for obtaining reliable results with using minimal number of animals.

Prior to the experiment, male C57BL/6 mice were fed a high-fat diet (60% fat content) for two weeks, which leads to the development of IR in these animals, as shown before¹¹⁵. After overnight (12 h) fasting, mice were intraperitoneally injected with ApoA-I (glycated or not). After 3 h, glucose was administered followed by a collection of serum samples (for determination of insulin) and measurement of blood glucose levels.

ApoA-I amyloidogenic variants

Production of recombinant variants

ApoA-I amyloidogenic variants, as well as the WT protein were produced in a bacterial expression system. The *Escherichia coli* strain BL21 (DE3) containing ApoA-I expressing pEXE-5 plasmid was used, as we previously described^{186,187} with modification included in the paper IV¹⁸³. ApoA-I variants were purified from bacteria lysate using two-step immobilized metal affinity chromatography system (His-Trap-Nickel-chelating columns, Äkta, GE Healthcare).

Using a recombinant system for ApoA-I production is very advantageous. It provides a fast, efficient and inexpensive way of producing the protein without being limited by the availability of plasma material. Moreover, ApoA-I does not undergo major post-translational modifications, as it lacks glycosylation sites and disulfide bonds³². These properties of ApoA-I justify the use of recombinant protein in studying naturally occurring processes. The possible disadvantages include the risk of bacterial-derived endotoxin accumulation and lack of exposure to plasma modifying agents.

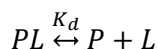
Clinical samples

Serum samples from carriers of ApoA-I amyloidogenic variants and from unrelated healthy controls were obtained from Dr Laura Obici (Amyloidosis Research & Treatment Centre, Pavia, Italy). The samples included 11 controls, 11 L75P carriers and 4 L174S carriers. The subjects were between 37 to 77 years of age, both males and females. The HDL species distribution and functional properties of these clinical samples were studied in **paper III**. To research the functional properties of the HDL contained in plasma of ApoA-I amyloidosis patients, we performed cholesterol efflux experiments. Prior to the analysis, samples were depleted of Apolipoprotein B, which could potentially serve as additional cholesterol acceptor¹⁸⁸. HDL subspecies distribution was studied by Western Blot from denaturant PAGE and native PAGE.

The use of clinical samples for the experiments shown in **paper III** reinforced the findings obtained with the study on the recombinant proteins (in **paper IV**), and further strengthened the physiological relevance of drawn conclusions on higher efficiency of the amyloidogenic variants to catalyze efflux cholesterol.

Cholesterol efflux

Cholesterol efflux assay was performed in **paper II** and **papers III, IV** included in this thesis. The assay is designed to measure the ability of ApoA-I/HDL (WT, variant, glycosylated) to accept cholesterol released from macrophages. First, radioactively labelled cholesterol was added into mouse-derived J774 macrophages, followed by 24 h incubation. Next, the medium was changed to one containing low serum concentration to promote equilibration of the labelled cholesterol among the intracellular cholesterol reservoir. In order to unify results, chemical agents were added to inhibit cholesterol esterification and induce ABCA1 expression. Finally, ApoA-I was added to the macrophage cultures and cholesterol efflux to the media was measured by the scintillation counter and presented as the percentage of the total ^3H -cholesterol. Cholesterol efflux follows the Michaelis-Menten kinetics allowing for the determination of efflux capacity- B_{\max} and efflux efficiency- K_d . While commonly used Michaelis constant (K_m) is a measure enzyme kinetics, here we apply the dissociation constant- K_d . It provides an information about the affinity of the ligand (L) for the binding site of the protein (P), as determined by the thermodynamic preferences.



The higher the affinity of the ligand to the protein binding side the lower the K_d value. In our case, the ligand corresponds to ABCA1 delivered cholesterol and K_d determines its affinity to bind cholesterol acceptor.

The main difference between both constants originates from their definition. K_m is a kinetic parameter and K_d is a thermodynamic parameter.

Another parameter used in the analysis of cholesterol efflux is B_{\max} , which informs about acceptor capacity¹⁸⁹. The higher the acceptor capacity- B_{\max} the greater the efflux capacity of the protein.

HDL particle preparation

Reconstituted HDL (rHDL) were prepared in two different ways, depending on the nature of experimental analysis. Lyophilized DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) was used for the assessment of the kinetics of solubilisation of phospholipid multilamellar vesicles (MLV) to form discoidal ApoA-I-PL complexes, as shown in the lipid clearance assay presented in **papers III** and **IV**. The decrease in absorbance (at 325 nm) is proportional to the rate of phospholipid incorporation (or phospholipid solution clearance) into the protein-lipid complexes, as it was described in detail before¹⁸⁶. Even though the lipid-binding of apolipoproteins is a complex,

multistep process, this assay is a well-accepted method in the field providing an approximate view on the lipid-binding capabilities of an apolipoprotein.

The cholate dialysis method (method described in detail in **paper IV**) was used in order to prepare HDL particles of defined size for structural and functional analysis, included in **paper I**, **paper III**, and **paper IV**. This method provides more physiological-like environment of the prepared particle preparation; however, it requires incubation with detergent, which makes it unsuitable for measurements of dynamic processes such as the lipid clearance assay. Lyophilized POPC (1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine), FC and protein were mixed in two different molar ratios resulting in the formation of defined size particles, either 9.6 or 8.4 nm. To ensure homogeneity of the preparations, size-exclusion chromatography was applied and peak fractions collected.

Circular dichroism (CD)

Circular dichroism (CD) spectroscopy is a widely used spectroscopic technique to study obtain protein low-resolution structural information using a small amount of sample. It allows for determination of conformational changes of protein in their secondary structure as well as monitoring of structural changes under a range of conditions. A good example is thermal stability assay where structural rearrangements are followed during temperature-induced folding and unfolding of proteins (assay presented in **paper II**, **paper III**, **paper IV**). The principles of this method utilize differences in absorption of polarized light by left- and right-circularly polarized light by chiral molecules in the far-UV wavelength region (190-250 nm). Amplitude at 222 nm is used to estimate the helix content¹⁹⁰.

In case of the bench-top, conventional CD the source of light is usually a xenon arc lamp. Important limitations of the conventional CD instruments in performing measurements on ApoA-I/HDL are firstly the presence of large lipid particles which may cause light scattering, and secondly, common buffer components such sodium chloride may have high absorption in the far-UV range. These factors largely increase the noise to signal ratio and thus negatively impact the quality of the obtained spectra, affecting the correct estimation of the structural content distribution of alpha helix, beta strand-turn, and random coil^{191,192}.

Synchrotron radiation circular dichroism (SRCD)

In order to overcome the aforementioned limitations, synchrotron radiation circular dichroism (SRCD) was performed. This method utilizes light produced by a synchrotron source thus allowing for a higher photon flux into the sample across the

UV range. This brings many important advantages for the measurement of lipid-containing samples, such as HDL^{191,192}. The advantages of a high, focused photon flux include (i) an increased signal-to-noise ratio, (ii) minimization of the amount of the material required, (iii) possibility to perform high-throughput screening, (iv) enabled comparison of proteins with subtle differences, (v) extension of the UV region for data collection, of particular importance for determination of unordered structure, which has a major CD peak at wavelengths <200 nm¹⁹³. The SRCD measurements presented in this thesis were performed at the Diamond Light Source, United Kingdom¹⁹⁴ and analysed with the use of in-house developed software CD Apps. By applying a suitable algorithm¹⁹⁵, the data was deconvoluted based on reference spectra allowing for determination of the relative contribution of the different secondary structure elements in the sample, which is a major advantage of this methodology.

Hydrogen-deuterium exchange mass spectrometry (HDX MS)

HDX MS is a powerful technique for investigating protein-ligand interactions, protein folding, and protein dynamics. The conformational changes of proteins in solution are monitored through the rate of exchange of the protein backbone amide hydrogens with deuterium in solution. Hydrogens, which are involved in weak interactions or are located at the surface of the protein can easily undergo exchange with deuterium. On the other hand, hydrogens located in the interior of the protein or involved in the formation of stabilizing hydrogen bonds, are exchanged slower. The monitoring of deuterium incorporations as a function of time is thus informative of conformational dynamics of the protein at the equilibrium. Next, upon sample incubation in deuterated buffer, the pH is dramatically decreased to stop the exchange process and protein enzymatic digestion (usually by pepsin) is performed¹⁹⁶. MS is then applied to reveal localized exchange information in the produced peptides. The comparison of these overlapping peptides provides an overview of protein conformational changes in the defined regions. The results are represented as “heat maps” and are included in **paper III**, where we monitored local changes in the dynamics of ApoA-I amyloidogenic variants. The main advantages of HDX MS-based investigation of conformational dynamics of proteins are (i) the possibility to performing measurements under physiological conditions, (ii) small sample amount requirement, (iii) changes can be linked to protein’s amino acid sequence (region), and (iv) capability to analyse large proteins¹⁹⁷.

Other relevant techniques

Microscopy

Confocal microscopy

Confocal microscopy is a widely used laboratory technique which allows for imaging of cellular processes in the cross sections of cells and tissues. The technique uses lasers and fluorescence to generate an image of the sample. It provides possibility to control depth of field and eliminate or reduce background signal, which are the main advantages of the confocal microscopy over the fluorescence microscopy.

Proteins can be imaged either by the direct coupling with fluorescent label to their amine group or by using fluorescent antibodies. Both approaches were undertaken in **paper I**. ApoA-I was labelled with fluorescein isothiocyanate (FITC) to study its localization in the cell. To monitor other proteins, such as Pdx1 and insulin, we used immunostaining, which first includes incubation with specific primary antibody followed by incubation with fluorescently-labelled secondary antibody. Confocal microscopy is an easy and fast method to visualize proteins in the cell. Challenges with this method involve the development of well-functioning staining and protein-labelling protocols.

Transmission electron microscopy (TEM)

TEM is a powerful imaging technique enabling high-resolution studies. Among the multiple applications of this method there is ultrastructural examination of cell morphology and its organelles, which was applied in **paper I**. The principle of the method involves the use of an electron beam which passes through ultrathin (less than 100 nm) osmium-fixed tissue section. The image is created by the differentiated transmission of the electrons resulting from their interaction with the specimen, captured on a fluorescent screen¹⁹⁸. In the studies presented in **paper I**, TEM was used to visualize insulin granules in the beta cells and in the islet beta cells. Insulin granules can be easily detected by TEM as spherical organelles with a dense-core separated from the surrounding halo. Their size varies between 100-800 nm in mouse beta cells¹⁹⁹. The high-resolution of the images allowed for performing statistically significant quantitative studies of insulin granule distribution with the use of in-house developed software MatLab.

Immunological analysis

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a very convenient and fast method to detect and quantify multiple substances including peptides, proteins, and hormones in a small-sized sample material. It is a plate-based assay, where a capture antibody, upon addition of the sample, immobilizes the antigen of interest. The incubation with the detection antibody conjugated to biotin or streptavidin-HRP allows for visualization of this specific antibody-antigen interaction. The resulting colorimetric reaction allows for signal quantification with the use of spectrophotometric methods. ELISA was used for detection and quantification of insulin in experiments performed in **papers I and II**. An important consideration when using this method is the specificity of detection.

Western blot

Western blot (WB) is an important technique which enables identification of specific proteins from a mixture of proteins existing in, for example, cell extracts. In the studies included in this thesis, the initial electrophoretic separation of the samples was performed either under denaturant or native conditions, followed by blotting and incubation with appropriate antibodies. The native conditions, meaning without the addition of reducing agents and SDS, allow for examination of profiles of HDL species in the sample, whereas the denaturant environment informs about levels of the protein of interest in the sample. Native page is also a good tool to follow HDL formation, as presented in **papers II and IV**. WB is a well-regarded, reliable and fast technique for protein detection in the sample. As compared to ELISA, WB requires more sample and it is more time-consuming. In both techniques, the antibody specificity is an important factor for successful protein detection. A major advantage of WB is a possibility of protein separation under native conditions, which is a key factor for analyses included in this thesis.

Objectives and key findings

Paper I

Objective: Dissect the molecular mechanism behind ApoA-I-induced increase in beta cell insulin secretion.

Key findings: ApoA-I primes beta cell to increase glucose stimulated insulin secretion *via* its internalization into beta cells, promoting Pdx1 nuclear translocation, and stimulating expression of proinsulin processing enzymes. Altogether, these processes lead to an increased number of insulin granules.

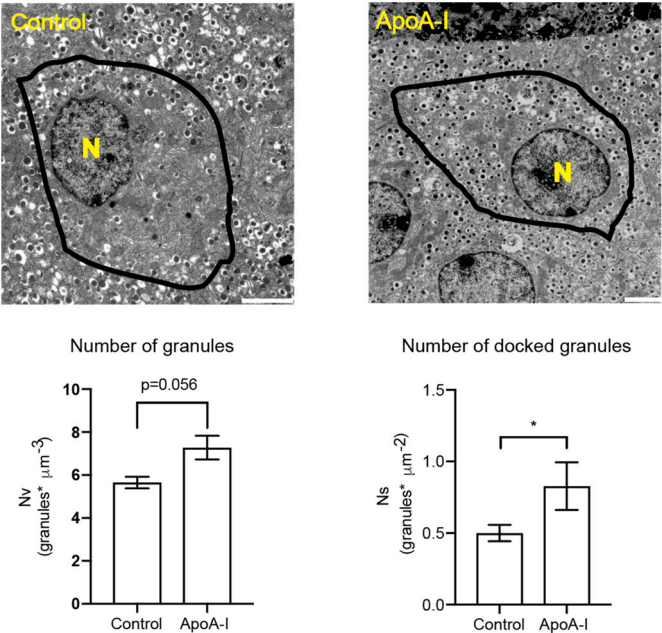


Figure 12. Electron micrographs of single beta cell within a mouse islet after 2 h incubation in low glucose in the absence (Control) or presence of ApoA-I. Below, bar graphs representing an increase in the normalized total number of granules and docked granules, resulting from exposure to ApoA-I. Modified Figure 5 b,c,d in **paper I**.

Paper II

Objective: Investigate the impact of ApoA-I glycation on protein structure and function in lipid and glucose metabolism.

Key findings: Glycation of ApoA-I alters protein structure, worsens its lipid-binding function and its ability to catalyse cholesterol efflux from macrophages. Glycated ApoA-I does not exhibit beneficial effects in the regulation of glucose disposal to the same extent as non-modified ApoA-I (WT).

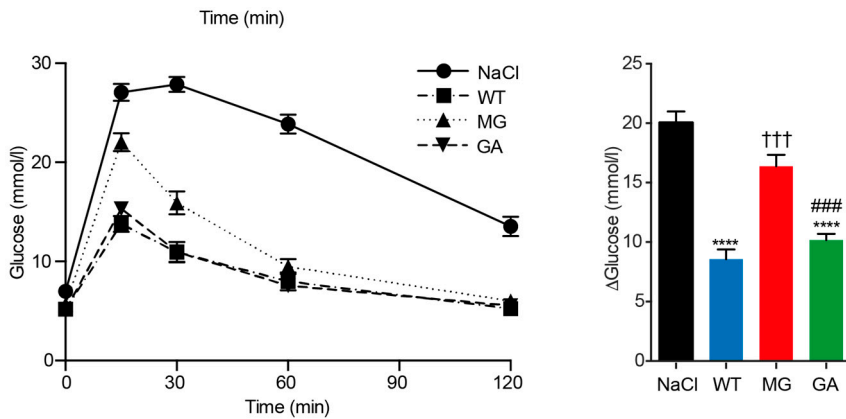


Figure 13. Effects of ApoA-I glycation on glucose disposal. High fat diet-fed mice were treated for 3 h with a single injection of ApoA-I (non-modified-WT or glycated with methylglyoxal-MG, or glycolaldehyde-GA) or saline (NaCl). Mice received an i.p. glucose load followed by determination of blood glucose levels at the indicated time points. The changes in glucose are represented on the right, showing deterioration of glucose-clearance capabilities of glycated ApoA-I. Figure 5 a,c in paper II.

Paper III

Objective: To investigate the structural basis of the improved catalytic function of the ApoA-I variants to mediate cholesterol efflux and to study the clinical samples from the ApoA-I amyloidosis patients in terms of HDL particle distribution and functionality.

Key findings: The reduced HDL-levels of carriers of ApoA-I amyloidogenic variants are compensated by a higher abundance of small, dense particles, which possess a greater capacity to catalyse cholesterol efflux from macrophages. This improved functionality is due to altered, region-specific changes in protein structure dynamics.

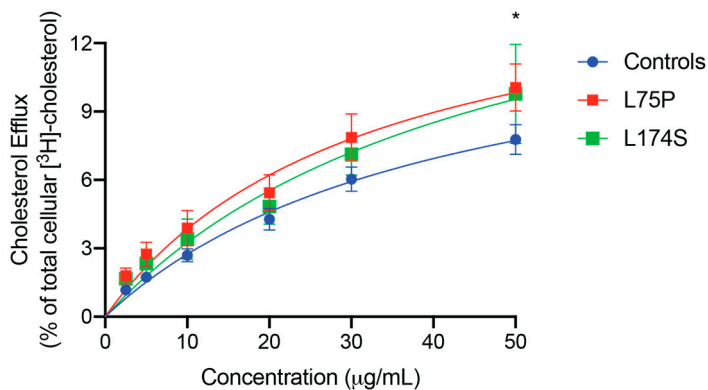


Figure 14. HDL in serum samples from carriers of ApoA-I amyloidogenic variants, L75P and L174S, more efficiently catalyse Cholesterol efflux from cultured macrophages as compared do matched controls, in dose-dependent experiments. Figure 1c in **paper III**.

Paper IV

Objective: Understanding the clinical phenotype by study the connection between the alterations in the protein structure of the ApoA-I amyloidogenic variants and their functionality.

Key findings: ApoA-I amyloidogenic variants are characterised by a decreased lipid affinity and enhanced proteolytic susceptibility, which explains low ApoA-I/HDL plasma levels observed in the carriers of ApoA-I amyloidogenic variants. The reduced levels of ApoA-I/HDL are compensated by a higher efficiency of the amyloidogenic variants to efflux cholesterol from macrophages. Altogether, these findings clarify why the carriers of ApoA-I amyloidogenic variants do not show a higher CVD risk despite the lower ApoA-I/HDL plasma levels.

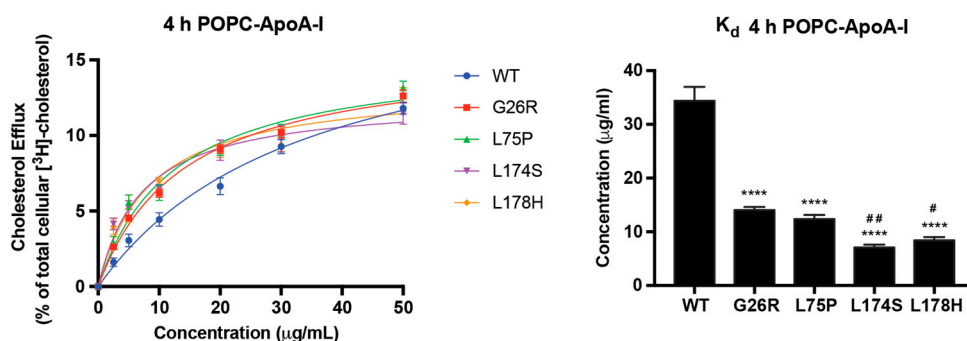


Figure 15. Cholesterol efflux ability of ApoA-I variants associated with POPC lipids-POPC-ApoA-I. The dissociation constant (K_d) was calculated according to Michaelis-Menten equation allowing for estimation of cholesterol efflux efficiency, which is significantly improved for the variants. Modified Figure 8 b,c in **paper IV**.

Results and discussion

The focus of the presented thesis is on ApoA-I and two of its many different functions. The first involves the role of ApoA-I in glucose control and the consequences of ApoA-I glycation on its functionality. The second part focuses on the deleterious effects of mutations occurring in the *APOA1* gene, which lead to the acquisition by ApoA-I of amyloidogenic properties. The results from studying these two different aspects of ApoA-I are included in papers I-IV and will be discussed separately in the following chapter.

ApoA-I in glucose controls

T2D patients are at high risk of developing of CVD⁸³. These two conditions are closely linked and finding a biological compound capable of battling both diseases is of great interest. ApoA-I/HDL has a well-established role in the prevention of atherosclerosis and has been recently shown to exhibit potent blood-glucose-lowering properties^{200,201}. In **paper I**, we show a previously unexplored role of ApoA-I to prime beta cells to increase insulin secretion in response to high glucose load. Moreover, we propose a molecular mechanism which can explain this phenomenon. In **paper II**, we investigated the structural and functional consequences of ApoA-I glycation in the hyperglycaemic environment.

ApoA-I as an insulin secretagogues

It was previously described that short-term incubation with ApoA-I directly leads to an increase in insulin release in low and high glucose environment from clonal beta cells^{120,121}. Additionally, prolonged incubation of beta cells with ApoA-I was shown to increase insulin levels *via* transcriptional activation¹²¹. Importantly, a single injection of ApoA-I to insulin resistant mice improved glucose disposal by an increase in circulating insulin levels^{99,115}. To further explore the function of ApoA-I as an insulin secretagogue, in **paper I** we developed a “priming model” (Figure 11) which allowed us to study the effect of short-term pre-incubation of beta cells with ApoA-I on their insulin-secreting properties.

ApoA-I in either LF or lipid-bound (HDL) form, was added on the clonal beta cells INS-1E for 2 h in low glucose environment. Buffer was then changed to one containing either low glucose or stimulatory glucose with no protein addition. Insulin secreted to media was measured showing significantly higher levels after a glucose challenge (2- to 3-fold increase) when ApoA-I or HDL was added to the pre-stimulatory phase. The same beneficial stimulatory effect of ApoA-I/HDL pre-incubation was observed in the *ex vivo*- treated islets isolated from C57BL/6 mice. In order to understand whether this positive effect is independent of glucose metabolism we employed different known secretagogues and investigated whether the ApoA-I's effect remains. We used tolbutamide and KCl, which trigger insulin release by direct depolarization of the plasma membrane. Triggering insulin secretion with these secretagogues led to a significantly larger ApoA-I mediated amplification of insulin secretion, comparable to that of glucose. Based on a presumption that there may be an increased number of granules present at the close proximity of the cell membrane resulting from ApoA-I's action, we performed microscopy analyses. Initial experiments using confocal microscopy, allowed us to visualize an increased cellular signal from insulin after 2 h incubation with ApoA-I as compared to control. In order to confirm these observations in a high-resolution setting, we used TEM. This method allows for detection of insulin granules as dense-core organelles surrounded by a halo. TEM analysis was performed for both INS-1E cells and isolated mouse islets. In both cases, incubation with ApoA-I in low glucose environment resulted in a significantly higher number of granules in close proximity of the plasma membrane. These granules, so-called docked granules, are more likely to be released upon stimulation¹⁰⁴, which may explain the effects observed with KCl- and tolbutamide-mediated direct cell membrane depolarization.

ApoA-I internalization is of importance for its role as an insulin secretagogue

Chemical blockers monodansylcadaverine (MDC) and Cytochalasin D (CytD) were used to block ApoA-I internalization into INS-1E beta cells. MDC blocks clathrin-dependent endocytosis whereas CytD blocks actin-dependent endocytosis. In both cases, partial inhibition of ApoA-I internalization resulted in a complete loss of ApoA-I's positive effect on GSIS. In order to understand this finding, it is worth reviewing published reports. By interacting with ABCA1 at the plasma membrane, ApoA-I accepts cellular cholesterol and lipids and, consequently, forms nascent HDL⁷. Importantly, ABCA1 beta cell-specific mouse knock out models were created showing an increased beta cell cholesterol levels^{116,119} and accumulation of beta cell cholesterol is a common hallmark of T2D²⁰². It would be plausible to assume that ApoA-I improves beta cell functionality by removing cellular cholesterol, however, published reports do not support this hypothesis. Incubation of unesterified cholesterol-enriched clonal beta cells Min6 with ApoA-I did not show any significant changes in the levels of unesterified cholesterol, CE, or total cholesterol¹²⁰. One possible explanation to this

involves the activation of endogenous protective mechanisms. It has been reported that chemically-induced removal of plasma cholesterol from mouse beta cells leads to migration of SNAP-25 and reduction of docked insulin granules²⁰³. This suggests that insulin secretion is sensitive to changes in plasma membrane cholesterol levels, which could at least in part explain why incubation with ApoA-I does not lead to cholesterol depletion from beta cells.

It remains to be understood how the internalization of ApoA-I relates to signalling and changes in cellular cholesterol levels.

ApoA-I promotes Pdx1 nuclear localization and expression of insulin processing enzymes

Pre-treatment of INS-1E cells with ApoA-I resulted in a 7-fold increase in expression of PC1/3 and moderate increase of CPE, key regulators of proinsulin processing. Notably, PC1/3 action was shown to be more important for the production of active insulin as compared do CPE^{204,205}. Pdx1 is a transcription factor vital for beta cell identity and functionality, and its nuclear exclusion was linked to beta-cell specific dysfunction in islets from T2D patients^{206,207}. We show in **paper I** that incubation with ApoA-I leads to shuttling of Pdx1 from cytoplasm to nucleus (Figure 16). Importantly, expression of PC1/3 and CPE is regulated by Pdx1 and downregulation of Pdx1 in INS-1E was reported to reduce PC1/3 levels by 80-90% leading to severe impairment of proinsulin processing²⁰⁸. To further support a vital role of Pdx1 in glucose control, studies show that lower expression of Pdx1 is linked to chronic hyperglycemia in rodents^{209,210} and T2D patients²⁰⁶, and beta cell-specific deletion of *Pdx1* in mice leads to the development of hyperglycemia within days²¹¹. As shown by others, prolonged incubation of INS-1E with ApoA-I (16 h) significantly increases *Pdx1* mRNA levels¹²¹. Altogether, our observations indicate that internalized ApoA-I leads to nuclear localization of Pdx1 and consequent increase in expression of PC1/3.

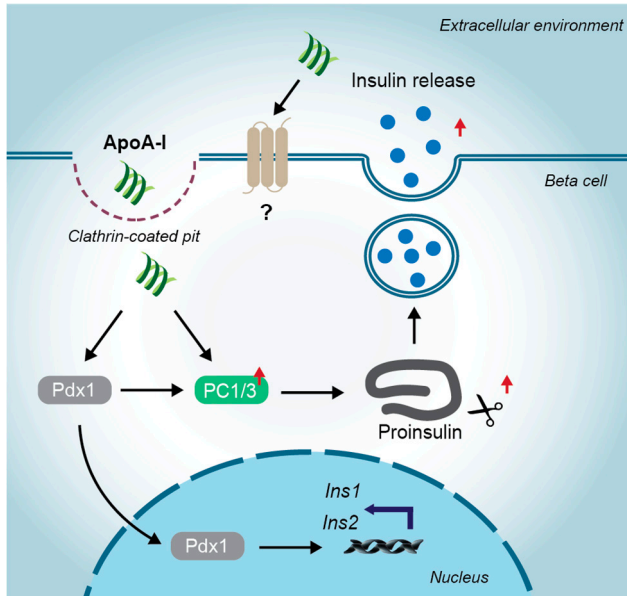


Figure 16. Proposed model of the mechanism behind ApoA-I-dependent increases in GSIS. ApoA-I enters the beta cell via clathrin-mediated endocytosis. Once internalized, ApoA-I promotes translocation of the Pdx1 to the nucleus where it can stimulate insulin expression. Pdx1 and/or ApoA-I promote an increase of expression of proinsulin processing enzyme – PC1/3. As a result, proinsulin processing is increased, leading to the formation of more insulin granules. More insulin in the beta cell translates into more insulin secretion upon stimulation. It remains to be explained which of the beta cell receptors are of importance for the interaction with ApoA-I.

ApoA-I is glycated in hyperglycemia

Chronic hyperglycemia is a common hallmark of T2D and the main cause of long-term complications such as CVD²¹²⁻²¹⁴. Prolonged hyperglycemia leads to the glycation of many biomolecules, including ApoA-I²¹⁵. In the Maillard reaction, glucose and reactive carbonyls chemically modify proteins and lipids to become AGEs. In **paper II**, we addressed the effect of glycation on ApoA-I's structure and functionality. It has been shown that, *in vivo*, ApoA-I is mainly modified by the two major reactive glucose metabolites, methylglyoxal (MG) and glycolaldehyde (GA), which target two different amino acid side chains of the ApoA-I protein¹⁴². Of importance, diabetic patients exhibit a 50 to 70% increase in MG-modified HDL^{216,217} and glycated fraction can account for 50% of the total ApoA-I in circulation²¹⁸. Undoubtedly, given a vital role of ApoA-I/HDL in the RCT and glucose control, understanding the consequences of this abundant modification is of great importance.

Glycation alters ApoA-I's protein structure

ApoA-I was chemically modified by MG or GA to mimic the process that occurs in circulation. The modified protein was subjected to mass-spectroscopic analyses revealing, in agreement with other studies²¹⁹, that MG preferentially reacted with arginine side residues of the protein while GA preferentially modified lysine residues. Importantly, and in addition to published reports, we showed that ApoA-I is glycosylated to the same extent in LF and HDL-bound state and we identified specific arginine and lysine residues modified by these reactive carbonyls. This observation is of high importance since ApoA-I is a very dynamic protein that undergoes interchanges between LF and lipid-bound states²²⁰. The changes in the protein secondary structure upon glycation were monitored using CD for LF proteins and SRCD for lipid-bound proteins. Despite the different pattern of modification by MG and GA, the secondary structure distribution in the LF proteins were altered in a similar way, showing an increase in alpha helical content as compared to the non-modified ApoA-I (44% for WT and 51-52% for MG/GA). To a similar extent, MG- and GA-modified proteins had reduced ability to bind lipids, as revealed by lipid clearance assay and native gel, after incubation of the proteins with MLV or mouse serum. To overcome the background noise from the lipids, SRCD was chosen to monitor secondary structure changes in lipid-bound protein. There were no major changes with respect to secondary structure composition between the lipid-bound proteins (WT vs MG, WT vs GA, MG vs GA), however, the thermal melting of the glycosylated HDL revealed higher thermal stabilities suggesting more compact structures. The formation of more rigid protein structures characterized by reduced flexibility may explain the reduced capability of the modified protein to accommodate lipids. Another important factor to consider is the change in the net protein charge upon modification of lysine and arginine residues which may affect the interaction of the protein between phospholipid bilayer and aqueous phase and lipid binding capabilities²²¹.

The positive effect of ApoA-I on glucose and lipid metabolism are hampered by glycation

The ability of ApoA-I to mediate cholesterol efflux from macrophages plays a pivotal role in the prevention of atherosclerosis. We, therefore, investigated the ability of the glycosylated ApoA-I to efflux cholesterol from cultured, cholesterol-loaded macrophages. We found this process to be negatively affected by glycation, especially after introducing MG modifications to arginine residues. Lipid binding efficiency and lipid binding capacity in cholesterol efflux were negatively affected when protein was glycosylated with MG, in both LF and lipid-bound state. Surprisingly, GA modification did not significantly affect the ApoA-I's ability to catalyse cholesterol efflux (being similar to WT), despite earlier discussed decreased ability of the GA-modified ApoA-I to bind lipids in lipid clearance assay and HDL formation. This suggests that the modified

arginine residues may be involved in the interaction with cellular receptors at the macrophage surface, such as ABCA1 and ABCG1. In fact, the importance of polar residues in ApoA-I-ABCA1 interaction has been proposed by others^{183,222}. Notably, an involvement of arginine residues shown here to be modified by MG was proposed to be critical for ApoA-I's interaction with LCAT²²³. This suggests that this region is of importance in protein-receptor interaction and lipoprotein maturation. Moreover, human carriers of ApoA-I Arg149Ser mutation have been described to have lower levels of ApoA-I/HDL in circulation and the mutation is negatively associated with cholesterol efflux and LCAT activation. This mutation-dependent charge neutralization and consequent functional deterioration further confirms an important role of the region around R149 for ApoA-I interaction and particle formation.

ApoA-I's positive action on glucose control has been shown to involve positive regulation of beta cell insulin secretion^{115,120-122} and direct stimulation of glucose uptake by skeletal and cardiac muscles^{99,100,224,225}. As we present in **paper II**, these favourable properties are hampered in the hyperglycemic state. Our results show that MG- or GA-glycated ApoA-I in HDL negatively affects HDL-driven glucose uptake in cultured skeletal muscle myotubes. On the same note, ApoA-I's glycation had a negative effect on the ability of ApoA-I to potentiate GSIS (the mechanisms of this phenomenon are described in **paper I**).

To summarize this part of the thesis, we demonstrate a positive action of ApoA-I to potentiate GSIS and we propose ApoA-I as a promising biological compound for the treatment of the metabolic syndrome. At the same time, we present that prolonged hyperglycemic state, which is associated with diabetes, leads to modification of ApoA-I which removes these beneficial properties and may contribute to etiology of diabetes and associated CVD. Therefore, finding therapeutic solutions targeting the prevention of ApoA-I's glycation and the reduction of hyperglycemia are of great importance.

ApoA-I in ApoA-I related amyloidosis

Familial ApoA-I-related amyloidosis is a rare, autosomal dominant disorder where a mutation in the *APOA1* gene leads to the deposition of the N-terminal fragments of variant ApoA-I as fibrils in multiple vital organs. In **paper III** and **paper IV** we investigated four recombinant ApoA-I amyloidogenic variants, two of which preferentially accumulate in the liver and kidney (G26R and L75P) and the other two in heart and skin (L174S and L178H), and WT protein for comparison^{36,178}. Additionally, in the **paper III**, we studied clinical samples obtained from AApoA-I patients carrying L75P and L174S variants.

ApoA-I amyloidogenic variants have higher efficiency at catalysing cholesterol efflux from macrophages

Carriers of ApoA-I amyloidogenic variants exhibit significantly lower serum ApoA-I and HDL levels as compared to healthy relatives^{163,172,173}. Despite this unfavourable lipid profile, they do not show a higher risk of developing cardiovascular diseases, and it has been suggested that the low HDL levels observed in the affected subjects must be balanced by an unknown “protective” mechanism¹⁷⁵.

In **paper IV**, we sought to shed light on these subject by performing functional assays employing recombinant ApoA-I amyloidogenic variants (G26R, L75P, L174S, L178H) and WT protein. As the main function of ApoA-I is to efflux cholesterol from macrophages at the vascular wall and then transport to the liver where the cholesterol is catabolised, we researched the ability of the variants to catalyse cholesterol efflux from J774 macrophages. Amyloidogenic variants were found to have higher efficiency (lower K_d) to efflux cholesterol as compared to the WT protein. This observation was true for both LF and lipid-associated (9.6 nm particles containing either DMPC- or POPC) proteins. In **paper III**, we performed cholesterol efflux experiments using serum samples patients carrying either L75P or L174S amyloidogenic variants and matched controls, as acceptors of cholesterol. In agreement with the findings emerged from in **paper IV**, we found that HDL from the carriers of mutated ApoA-I show improved ability to promote cholesterol mobilization. Importantly, we found that serum containing amyloidogenic variants exhibit a unique pattern in pre-beta HDL particle distribution with a higher abundance of small 8.4 nm particles, as compared to controls which were characterized by a higher abundance of the larger 9.6 nm particles. It has been shown that HDL particle size is a determinant of ABCA1-mediated cholesterol efflux, with small dense particles being the most potent mediators of this process^{226,227}. We therefore investigated in **paper III**, the relative contribution of HDL particles of defined size (8.4 nm or 9.6 nm POPC) to the improved cholesterol mobilization. We

found that cholesterol efflux, in the case of 9.6 nm rHDL particles, was higher for both L75P and L174S variants as compared to WT. This observation directly indicates an important effect of the mutations at residue 75 or 174 on protein/rHDL functionality. In the case of 8.4 nm rHDL particles, only L75P showed improved cholesterol efflux capacity, implying different, size-dependent, molecular mechanisms being targeted by L75P and L174S mutations. The fact that ApoA-I was shown to adopt specific conformations depending on HDL particle sizes²²⁸, supports this conclusion. Moreover, as described in the **paper III** and **paper IV** and determined by SRCD, amyloidogenic variants in rHDL, in particular L75P in 8.4 nm rHDL, are characterised by a higher proportion of the alpha helical content. Since the organization of the primary structure into amphipathic alpha helices is crucial for the lipid binding process of ApoA-I, this highly helical organization may lead to an improved readiness of the small amyloidogenic particles to accept cholesterol in cholesterol efflux process.

To sum up, we propose that the improved ability of HDL from carriers of amyloidogenic ApoA-I variants to efflux cholesterol is due to higher levels of small, dense HDL particles with improved catalytic function. This can explain why ApoA-I amyloidosis carriers do not have a higher risk of developing CVD despite significantly lower HDL plasma levels.

ApoA-I variants have decreased affinity for lipids

In **paper IV**, we analysed the particle size of DMPC-rHDL variants and WT by native electrophoresis and dynamic light scattering (DLS). In agreement with particle size distribution represented by serum from AApoA-I patients in **paper III**, the variants tended to form smaller-size HDL particles. Moreover, in **paper IV**, amyloidogenic variants were shown, in a lipid binding assay, to have significantly decreased lipid binding efficiencies. LF variants were also found to have lower efficiency to form rHDL in *ex vivo* mouse serum, as compared to the WT protein. Using SRCD, we monitored the conformational changes in the secondary structure of the proteins upon the HDL formation. We found that structural rearrangements are very rapid and can occur as soon as the protein is mixed with the lipids (for WT and L178H) or after 1 h incubation (for G26R, L75P, L174S), reaching alpha helical values similar to that of the fully formed rHDL. Furthermore, in **paper III**, 8.4 nm rHDL particles containing amyloidogenic variants or the native protein were subjected to thermal unfolding, followed by electrophoretic analyses under non-denaturing conditions, revealing that amyloidogenic LF ApoA-I dissociates from the particles at lower temperatures as compared to the WT protein in the native particle. Additionally, thermal stability of the variants in 8.4 nm rHDL was investigated by monitoring changes in the CD signal at 222 nm. The thermal denaturation follows a biphasic unfolding process, characterized by two transition temperatures (T_m). The T_{m1} reflects protein structural rearrangements whereas the T_{m2} informs about dissociation of protein from lipids. We

did not observe any changes in Tm1 between the variants and the native protein, however, the Tm2 was significantly lower for the variants, implying higher protein flexibility and lower affinity for lipids.

ApoA-I variants display higher structural flexibility

Our initial experiments in **paper IV**, showed a higher susceptibility to proteolysis of the LF amyloidogenic variants as compared to the native protein, as determined by limited proteolysis experiment. This agrees with previously reported data^{180,187,229}. The higher predisposition to proteolytic cleavage observed around the mutation site indicates an increase in the local flexibility resulting from single point mutations^{178,230}. Of importance, the decreased lipid affinity together with increased proteolytic susceptibility of the ApoA-I amyloidogenic variants may explain the low ApoA-I and HDL plasma levels observed in the AApoA-I patients.

Structural features of the variants were further investigated in **paper III**, where we employed HDX MS methodology to closely monitor local structural rearrangements in ApoA-I proteins (L75P, L174S and WT) in 8.4 nm rHDL particle resulting from amyloidogenic mutations (L75P, L174S and WT). Overall, the particles containing either of the variants exhibited more dynamic protein-lipid interaction. Moreover, we found a specific increase in backbone flexibility in the regions close to the aminoacidic substitution site, i.e. 55 to 89 region for the L75P variant and the 170 to 178 region for the L174S variant. This local structure flexibility resulting from point mutation may be a determinant of the improved cholesterol efflux capacity of the amyloidogenic variants.

There is a great need for a better understanding of mechanisms driving cholesterol efflux beyond explaining the variant-specific differences. Impaired cholesterol efflux resulting from low²³¹ and dysfunctional²³²⁻²³⁵ HDL-C was linked to immune-activation^{234,235} and consequent cardiovascular disease in chronic kidney disease^{236,237}. Of importance, abnormalities in HDL subpopulation distribution and particle size were shown to contribute to a higher risk of developing coronary heart disease in diabetes patients²³⁷. Clearly, finding solutions to improve HDL functionality is of a great interest. Based on the findings emerging from **paper III** and **paper IV**, a promising possibility is the use of destabilizing factors which, by introducing greater region-specific flexibility in the ApoA-I structure, improve its functionality without displaying a risk of amyloidogenic properties.

Future perspectives

There is still a lot to understand about the mechanisms driving the positive and deleterious effects of ApoA-I. In this thesis, we described an important structure-function connection for the observed ApoA-I properties. In future experiments, we plan to further explore and explain the different mechanisms of action of this versatile protein, in both health and disease.

Firstly, we would like to research whether the positive action of ApoA-I to potentiate GSIS from cultured beta cells and isolated mouse islets (presented in **paper I**) can be translated to humans. For this reason, functional and microscopic (TEM) experiments with the use of human islets are planned. The islets will be obtained from Human Tissue Lab at Lund University Diabetes Centre. Furthermore, we would like to identify beta cell receptors which are important for the interaction between the beta cell and ApoA-I. There are conflicting results published with respect to the involvement of the ABCA1 receptor^{117,121} in mediating ApoA-I's positive role in potentiating GSIS. With the aim to shed light on this subject, we plan to apply the TRICEPS method²³⁸, a fast and sensitive method which allows for receptor identification in the near-physiological conditions as well as its quantification (coupled to quantitative MS). Once the key receptors are identified, we plan to use siRNA for selective knock down of the receptor and investigate whether the ApoA-I beneficial effects on increasing GSIS remains.

As for the second part of my thesis, focused on the role of ApoA-I in amyloidosis, we would like to investigate the mechanism behind the preferential accumulation of the ApoA-I fibrils in the specific organs²³⁰. It is very intriguing and unique for ApoA-I that the location of the mutation in the protein sequence drives its preferential accumulation into a specific tissue. Since different tissues are enriched with different extracellular matrix (ECM) components, we plan to investigate the effects of the ECM environment on the fibrillogenic propensity of the ApoA-I amyloidogenic variants. We plan to perform biochemical and biophysical analyses of the variants in the presence of the selected, tissue specific ECM components. This knowledge may contribute to the development of precision medicines which could inhibit this deleterious interaction between ECM component and the ApoA-I variant thus prevent its accumulation.

As we have shown in **paper III** and **paper IV**, ApoA-I amyloidogenic variants are characterised by a local destabilization resulting from the mutation and overall higher flexibility as compared to the WT protein. This structural difference results in a greater

capacity of the variants to efflux cholesterol from macrophages. In our future experiments, we would like to identify an agent capable of introducing a similar local destabilization to the structure of the WT protein. The aim of this approach is to improve the catalytic function of the WT protein without carrying a risk of becoming amyloidogenic.

In conclusion, ApoA-I has great therapeutic potential, especially in the treatment of T2D and its most common complication-CVD. More research is needed to fully understand the processes driving its function and to formulate effective ApoA-I-based therapies.

Science for everyone

Apolipoprotein A-I – a good player with a dark side

The presented doctoral thesis describes Apolipoprotein A-I (shortly named ApoA-I), the main protein of HDL also known as “the good” cholesterol. Based on the experiments performed in the scientific papers included in this thesis, we propose that ApoA-I can be used as a promising treatment for diabetes and cardiovascular diseases. However, some people with genetic predispositions are born with mutated ApoA-I. This not properly functioning ApoA-I, accumulates in the vital organs leading to their failure. Both aspects are in the focus of this thesis and are explained below.

I am sure that most of you have heard of the “good” and the “bad” cholesterol and often were wondering which one it is while looking at the blood test results. I hope that after reading this thesis (or just this chapter), there will be no longer any confusion. Let’s start from the beginning. Our diet is highly enriched in fat, also called lipids. Fat is not soluble in water-based environments, such as blood, and must be therefore transported in the bloodstream with the help of specialised proteins, like ApoA-I. The lipid-protein complexes are called lipoproteins. There is a variety of different lipoproteins which ensure that lipids are transported to cells, where they are used for energy, or to fat tissue, where they can be stored, or to the liver, where the lipids are broken down and removed from the body. When too much fat is consumed, the human organism cannot take care of it properly and this leads to accumulation of lipids in different cells of organs including blood vessels. The build-up of the lipids in the arteries leads to atherosclerosis. The blockade of the blood supply, which provides vital oxygen to the organs, by an atherosclerotic plaque or a blood clots, leads to serious consequences such as heart attack or stroke. The lipoprotein which specialises in the removal of this deadly cholesterol from cells and transport to the liver where it can be eliminated is called HDL, or the “good” cholesterol. On the other hand, the lipoprotein which brings additional cholesterol to the cells, causing its accumulation, is called LDL, or the “bad cholesterol”. People who have high levels of LDL-cholesterol are at a high risk to develop cardiovascular diseases, whereas those with high HDL-cholesterol levels are protected from these diseases. Thus, it is not bad at all to have high cholesterol as long as we are referring to the HDL-cholesterol.

Now, that difference between the “bad” and the “good” cholesterol is clear, I would like to direct your focus on ApoA-I, the main protein of HDL. ApoA-I has a very important function in the removal of the cholesterol from the cells thus preventing the development of atherosclerosis. It interacts with special receptors on the surface of cholesterol-enriched cells and transfers it to the HDL lipoprotein. Another, beneficial function of ApoA-I involves the regulation of blood glucose levels, as described in **paper I** and **paper II** included in this thesis.

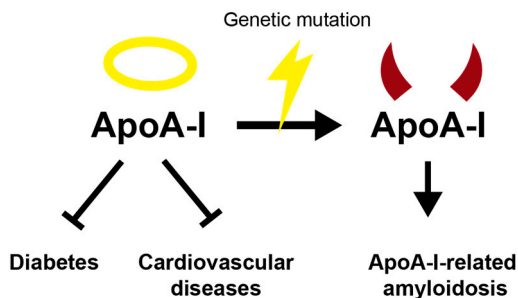
Insulin is a hormone produced by specialised cells, called beta cells located in islets of Langerhans of the pancreas. It is produced after a meal when glucose levels in the blood are high. Glucose is the main energy source for cells and proper regulation of its levels is very important. Insulin interacts with cells of the human body to let them know that they can take up blood glucose and use it for energy. In people with diabetes, these cells do not respond to the signal given by insulin, therefore blood glucose levels stay high. This is called insulin resistance and its development leads to the progression of the disease. To compensate for the high glucose, beta cells produce even more insulin, leading to their exhaustion and dysfunction. Diabetes can be therefore described as a combination of insulin resistance, beta cell dysfunction and consequently high blood glucose levels. Prolonged and untreated diabetes leads to many complications, among which cardiovascular diseases are the most common.

In **paper I**, we found that ApoA-I can improve the beta cell function to make more insulin in response to high glucose. We used beta cells grown in the lab as well as isolated from mice, to understand how this is happening. We used microscopes to see ApoA-I interaction with the beta cells. We found that ApoA-I is mostly inside the beta cells and that the incubation with ApoA-I leads to more insulin being present close to the cell's edge. This insulin is ready to be released as soon as there is a need. In the light of our previous experiments, which showed that ApoA-I increases the sensitivity of cells to insulin, we propose that ApoA-I can be used as a future medicine to treat diabetes and cardiovascular diseases, which often go hand-in-hand.

One of the most common ways to diagnose diabetes is the measurement of so-called glycated haemoglobin. High blood glucose levels, also referred to as hyperglycemia, existing for a long time leads to the glycation modification, of many proteins including haemoglobin of red blood cells. Haemoglobin is a remarkably stable protein and the levels of its glycation allow to estimate how severe was the hyperglycemia during past up to 12 weeks.

ApoA-I, similarly to haemoglobin, is glycated in hyperglycemia and in **paper II** we investigated the consequences of this incidence. We created a glycated ApoA-I and researched its functionality. We found that the glycated ApoA-I does not bind to lipids as efficiently as the not modified protein. Additionally, its function in removing cholesterol from cells and lowering glucose levels in mice was not as efficient as

compared to not glycosylated ApoA-I. We concluded that regulation of blood glucose is very important to ensure fully functional ApoA-I.



The good and the dark side of ApoA-I. ApoA-I has mainly beneficial functions as it protects from cardiovascular diseases and, as recently shown, from diabetes. In rare cases, a genetic mutation leads to formation of mutated ApoA-I, which aggregates in the vital organs causing their damage. This disease is called ApoA-I related amyloidosis and the only treatment available so far is organ transplant.

The second part of this thesis, discussed in **paper III** and **paper IV**, is focused on a different aspect of ApoA-I. Some people are born with a genetic mutation located in the gene encoding ApoA-I. These people, often belonging to the same family, have ApoA-I that has different properties. This mutated ApoA-I can associate with one another and form amyloids, which are dysfunctional protein aggregates that can be accumulated in various organs leading to their damage. This condition is called ApoA-I related amyloidosis. Amyloidoses are a group of diseases which are caused by a build-up of different protein amyloids in various organs. Most likely you have heard of Alzheimer's or Parkinson's diseases, amyloidoses that cause protein amyloid accumulation in the brain.

In case of ApoA-I related amyloidosis, the mutated ApoA-I accumulates in different organs depending on where the mutation occurred in the *APOA1* gene. Among the most commonly affected organs are kidney, liver, larynx, and heart. Since this disease is very rare and shows symptoms late in life, it is very difficult to diagnose. As for today, the only available treatment is organ transplant. People with this disease have much lower amounts of HDL in their blood, but surprisingly, they are not in a higher risk to develop cardiovascular diseases. In **paper IV**, we made and tested four of the mutated ApoA-I and we found that they are more effectively taking cholesterol from cells as compared to normal ApoA-I. This could explain why, despite lower levels of HDL, people with mutated ApoA-I do not have higher risk to develop cardiovascular diseases. In **paper III**, we used plasma from people who are affected by ApoA-I related amyloidosis for our experiments. We tested how functional are the HDL from their plasma and we came to the same conclusion as before. Their HDL were more effective in removing cholesterol from cells as compared to people who do not have mutated ApoA-I. We wanted to understand what happens to the protein when it has a mutation.

We made mutated ApoA-I and performed special analysis, which allowed us to see if the protein's structure is changing. We concluded that the mutation makes the protein more flexible. It means that the protein can adapt better to bind more cholesterol from cells.

Maybe we could make normal ApoA-I more flexible and therefore more efficient in taking care of the deadly cellular cholesterol? We will see in the future experiments.

Populärvetenskaplig sammanfattning

Apolipoprotein A-I – en bra spelare med en mörk sida

Doktorsavhandlingen behandlar Apolipoprotein A-I (ApoA-I), det huvudsakliga proteinet i HDL, även känt som ”det goda” kolesterolet. Baserat på de experiment som gjorts i de vetenskapliga artiklar som ingår i denna avhandling föreslår vi att funktionen hos ApoA-I kan ligga till grund för nya behandlingar av diabetes och hjärt-kärlsjukdomar. Vissa personer med genetiska predispositioner föds emellertid med ett muterat ApoA-I. Deras ApoA-I proteiner ansamlas i vitala organ vilket leder till organsvikt. Båda dessa aspekter är i fokus för denna avhandling och förklaras nedan.

Jag är säker på att de flesta av er har hört talas om det ”bra” och det ”dåliga” kolesterolet och ofta undrat vilket som är vilket när man ser på sina blodprovresultat. Jag hoppas att det inte längre kommer finnas något tvivel efter att ha läst denna avhandling (eller bara detta kapitel). Låt oss börja från början. Vår mat innehåller mycket fett, vilka även kallas lipider. Fett är inte lösligt i vattenbaserade miljöer, såsom blod, och måste därför transporteras i blodomloppet med hjälp av specialiserade proteiner, som ApoA-I proteinet. Lipid-proteinkomplexen, som till exempel kan ApoA-I som binder lipider till sig, kallas lipoproteiner. Det finns en mängd olika lipoproteiner som säkerställer att lipider transporteras till celler där de används för energi, till fettvävnad där de kan förvaras eller till levern där lipiderna bryts ned och tas bort från kroppen. När för mycket fett konsumeras kan den våra kroppar inte ta hand om det ordentligt vilket leder till ansamling av lipider i celler i olika organ, inklusive i blodkärlen. Uppbyggnaden av lipiderna i artärerna leder till åderförkalkning. Blockering av blodtillförseln, som tillhandahåller livsviktigt syre till kroppens olika delar, av en aterosklerotiskt plack eller en blodpropp leder till allvarliga konsekvenser såsom hjärtattack eller stroke. HDL (Hög-densitetslipoprotein), det ”goda kolesterolet”, är det lipoprotein i våra kroppar som är specialiserat på att avlägsna överflödigt, och därmed skadligt kolesterol från celler och transporterar det till levern där det kan elimineras. LDL (Låg-densitetslipoprotein), å andra sidan, är det lipoprotein som ger ytterligare kolesterol till cellerna, vilket orsakar dess ansamling. Människor som har höga nivåer av LDL-kolesterol har en förhöjd risk att utveckla hjärt-kärlsjukdomar, medan de med höga HDL-nivåer är skyddade från dessa sjukdomar.

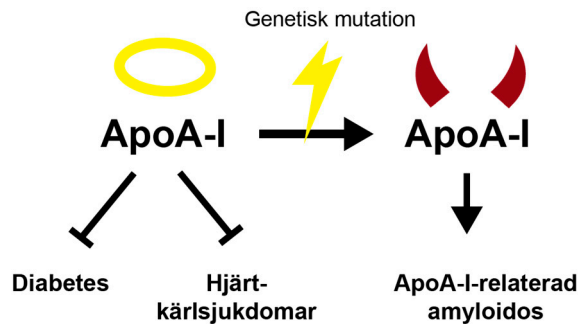
Nu när skillnaden mellan det "dåliga" och det "goda" kolesterolet är tydligare, skulle jag vilja rikta fokus på ApoA-I, huvudproteinet i HDL. ApoA-I har en mycket viktig funktion i avlägsnandet av kolesterolet från kärlväggens celler, vilket förhindrar utvecklingen av åderförkalkning. ApoA-I proteinet interagerar med speciella receptorer på ytan cellerna som innehåller höga nivåer av kolesterol och överför det till det växande HDL-lipoproteinet. En annan fördelaktig funktion hos ApoA-I involverar regleringen av blodsockernivåer; detta beskrivs i två av de vetenskapliga artiklar (paper I och paper II) som ingår i denna avhandling.

Insulin är ett hormon som produceras av specialiserade celler, så kallade betaceller, som finns i Langerhanska öar i bukspottkörteln. Det utsöndras efter måltider när glukosnivån i blodet är hög. Glukos är den viktigaste energikällan för celler och korrekt reglering av dess nivåer är mycket viktigt. Insulin interagerar med celler i människokroppen för att låta dem veta att de kan ta upp blodglukos och använda det som energi. Hos personer med diabetes svarar dessa celler dock inte på signalen som ges av insulin och därför förblir blodsockernivåerna höga. Detta kallas insulinresistens och till utvecklingen av diabetes-sjukdomen. För att kompensera för den höga glukosnivån producerar beta-cellerna ännu mer insulin, vilket leder till utmattning av cellerna och dysfunktion. Diabetes kan därför beskrivas som en kombination av insulinresistens och beta-celldysfunktion, och därpå följande höga blodsockernivåer. Långvarig och obehandlad diabetes leder till många komplikationer, bland vilka hjärt-kärlsjukdomar är de vanligaste.

I paper I fann vi att ApoA-I kan förbättra betacellfunktionens förmåga att utsöndra mer insulin som svar på hög glukosnivå. I våra studier så använde vi betaceller som odlades i labbet och Langerhanska öar som isolerades från möss för att förstå hur detta händer. Vi använde mikroskopi för att se ApoA-I-interaktion med betacellerna. Vi fann att ApoA-I tas upp av betacellerna och att detta leder till att mer insulinet, som förvaras i cellerna i så kallade granuler, mobiliseras och förs närmare cellens yttre skal. Detta gör att insulinet kan utsöndras snabbt och effektivt till cellens omgivning och till blodomloppet så snart behov finns dvs i samband med en måltid. Detta tillsammans med våra tidigare studier på ApoA-I proteins funktion i kroppens kontroll av glukosnivåer, vilka visade att ApoA-I ökar musklers upptag av glukos, föreslår att ApoA-I proteins mekanismer kan användas för att utveckla framtida läkemedel för att behandla diabetes och hjärt-kärlsjukdomar.

Ett av de vanligaste sätten att diagnostisera diabetes är mätningen av så kallat glykerat hemoglobin, eller HbA1c. Höga blodsockernivåer, eller hyperglykemi, under en längre tid kan leda till glykeringsmodifiering av många proteiner inklusive hemoglobin i röda blodkroppar. Hemoglobin är ett anmärkningsvärt stabilt protein som stannar länge i blodomloppet innan det bryts ner. Detta gör att nivåerna av glykerat hemoglobin kan användas för att uppskatta hur allvarlig hyperglykemin har varit under upp till de senaste 12 veckorna.

ApoA-I, på liknande sätt som hemoglobin, glykeras i hyperglykemi och i paper II undersökte vi konsekvenserna av denna förändring av ApoA-I proteinet. Vi skapade ett glykerat ApoA-I och undersökte dess funktionalitet. Vi fann att den glykerade ApoA-I inte binder till lipider lika effektivt som det icke-modifierade proteinet. Dessutom var dess funktion i att avlägsna kolesterol från celler och att sänka glukosnivån hos möss inte lika effektiv jämfört med icke-glykerad ApoA-I. Vi drog slutsatsen att reglering av blodglukos är mycket viktigt för att säkerställa fullt funktionell ApoA-I.



Den goda och den mörka sidan av ApoA-I. ApoA-I har huvudsakligen fördelaktiga funktioner eftersom det förhindrar hjärt-kärlsjukdomar och, som nyligen visats, även har en glukosreglerande funktion med relevans för diabetes. I sällsynta fall leder en genetisk mutation till bildning av muterat ApoA-I, som aggregeras i vitala organen och kan leda till organsvikt. Denna sjukdom kallas ApoA-I-relaterad amyloidos och den enda behandling som hittills är tillgänglig är organtransplantation.

Den andra delen av denna avhandling (paper III och IV) är inriktad på en annan aspekt av ApoA-I proteinet. Vissa människor föds med en genetisk mutation som finns i genen som kodar för ApoA-I. Dessa genetiska mutationer leder till förändrade egenskaper hos ApoA-I proteinet. En viktig förändring är att de muterade ApoA-I proteinerna kan associeras med varandra och bilda amyloider, som är dysfunktionella proteinaggregat som kan ackumuleras i olika organ där de gör skada. Detta tillstånd kallas ApoA-I-relaterad amyloidos. Amyloidoser är en grupp sjukdomar som orsakas av en uppbyggnad av proteinamyloider i olika organ. Troligtvis har du hört talas om Alzheimers eller Parkinsons sjukdomar, amyloidoser som orsakar proteinamyloidansamling i hjärnan. I fallet med ApoA-I-relaterad amyloidos ackumuleras de muterade ApoA-I proteinerna i olika organ; vilket organ som påverkas beror på var mutationen sitter i APOA1-genen. Bland de mest drabbade organen är njurar, lever, struphuvud och hjärta. Eftersom denna sjukdom är mycket sällsynt och visar symptom sent i livet är det mycket svårt att diagnostisera. I dag är den enda tillgängliga behandlingen organtransplantation.

Människor med denna sjukdom har mycket lägre mängder HDL i blodet, men förvånansvärt så har de inte förhöjd risk att utveckla hjärt-kärlsjukdomar. I paper IV tillverkade och testade vi fyra av de muterade ApoA-I och vi fann att de mer effektivt tar kolesterol från celler jämfört med normalt ApoA-I. Detta kan förklara varför

personer med muterad ApoA-I, trots de lägre nivåerna av HDL, inte har högre risk att utveckla hjärt-kärlsjukdomar. I paper III använde vi plasma från personer som drabbades av ApoA-I-relaterad amyloidos i våra experiment. Vi testade funktionen hos HDL från deras plasma och vi kom till samma slutsats som tidigare. Deras HDL var mer effektivt på att ta bort kolesterol från celler jämfört med personer som inte har muterat ApoA-I. Vi ville därför förstå vad som händer med proteinet när det har en mutation. Vi producerade muterat ApoA-I och utförde en speciell analys som gjorde det möjligt för oss att se om och i så fall hur proteinets struktur förändras. Vi drog slutsatsen att mutationen gör proteinet mer flexibelt. Det betyder att proteinet kan anpassas bättre för att binda mer kolesterol från celler.

Kanske kan vi göra normalt ApoA-I mer flexibelt och därför effektivare när det gäller att ta hand om det skadliga cellkolesterolet? Förhoppningsvis kan vi utvärdera detta i framtida experiment.

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And “diabolical” if you are a pro.

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