The involvement of INSIGs, DGAT1 and APOB in human lipid metabolism

Sergey Krapivner
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Stockholm 2008
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Printed by
REPROPRINT AB
Stockholm 2008
www.reproprint.se
Gärdsvägen 4, 169 70 Solna
ABSTRACT

Atherosclerosis is a complex pathophysiological condition characterized by lipid accumulation in the vascular wall. These lipids are derived from the lipoproteins present in the circulation. Two major plasma lipoprotein fractions, low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs), play significant roles in the atherosclerotic process, whereas the relevance of triglyceride-rich lipoproteins (TRLs) remains less clear. However, recent epidemiological studies demonstrated a high predictive value of the plasma triglyceride (TG) concentration for coronary heart disease (CHD).

The overall aim of this thesis was to identify and characterise major regulatory proteins involved in the control of secretion of TRLs by the liver and the regulation of the plasma TG concentration. To this end, the impact of two critical proteins regulating the synthesis of TGs and other lipids was analysed using a combination of in vitro techniques and population studies. In subsequent studies, the relevance of two additional proteins with putative roles in the regulation of TRL secretion was evaluated.

The insulin-induced genes (INSIG) -1 and -2 code for proteins known to regulate the rate-limiting step in the sterol regulatory element binding protein (SREBP) pathway, a key metabolic pathway regulating TG and cholesterol metabolism. INSIG1 and INSIG2 were screened for genetic variants and functional polymorphisms were identified in the promoters of INSIG1 (-169C>T) and INSIG2 (-102G>A). The associations of these polymorphisms with biochemical and anthropometric traits related to lipid and glucose metabolism were analysed in cohorts of healthy, middle-aged subjects. No relationships between the promoter polymorphisms and plasma lipid levels were observed. However, significant associations were found between the INSIG1 -169C>T polymorphism and parameters related to glucose metabolism and between the INSIG2 -102G>A polymorphism and parameters related to body weight. The unique roles of INSIG1 in hepatic metabolism and of INSIG2 in adipose tissue were corroborated by the results from expression studies in liver and adipocyte cell-lines/tissues.

Factors regulating the secretion of TRLs were studied using as a model system the human hepatoma Huh7 cell-line in combination with the small interfering RNA (siRNA) inhibition technique. The impact of siRNA inhibition of apolipoprotein B (APOB), the major structural protein of TRL particles, was analysed in order to elucidate the physiological significance of variation in APOB mRNA level for the rate of secretion of TRLs. Positive relationships between the APOB mRNA level and the rate of secretion of TRLs were observed under various experimental conditions. A similar positive relationship was observed in human samples between the hepatic APOB mRNA level and plasma LDL concentration, the ultimate product of intravascular remodelling of TRLs secreted by the liver. The data from these in vitro and in vivo studies thus provide further evidence for an important role of transcriptional regulation of APOB for lipid metabolism.

Subsequently, we analyzed the significance of hepatic nuclear factor (HNF) 4A, a transcription factor involved in lipid and glucose homeostasis, for the regulation of secretion of TRLs. It was found that siRNA inhibition of HNF4A leads to a marked decrease in the secretion of TRLs, a phenomenon that is associated with a decreased expression of the diacylglycerol acyltransferase (DGAT) 1 gene. Functional studies substantiated the role of DGAT1 in the secretion of TRLs. These studies thus demonstrated that DGAT1 mediates the effect of HNF4A on hepatic secretion of TRLs.
LIST OF PUBLICATIONS

I.  **S. Krapivner**, E. Chernogubova, M. Ericsson, C. Ahlbeck-Glader, A. Hamsten, F.M. van 't Hooft

II. **S. Krapivner**, S. Popov, E. Chernogubova, M-L. Hellenius, R.M. Fisher, A. Hamsten, F.M. van 't Hooft

III. **S. Krapivner**, S. Popov, E. Chernogubova, A. Silveira, N. Spong, J. Tegnér, J. Bjorkegren, A. Hamsten, F.M. van 't Hooft
Hepatic apolipoprotein B mRNA level determines secretion of triglyceride-rich lipoproteins and plasma LDL concentration in man. Manuscript submitted

IV. **S. Krapivner**, A. Silveira, A. Hamsten, F.M. van 't Hooft
DGAT1 mediates the effect of the MODY 1 gene HNF4A on hepatic secretion of triglyceride-rich lipoproteins. Manuscript submitted
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<tbody>
<tr>
<td>APO</td>
<td>apolipoprotein</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
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<tr>
<td>GK</td>
<td>glucokinase</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A reductase</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatic nuclear factor</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoproteins</td>
</tr>
<tr>
<td>INSIG</td>
<td>insulin-induced gene</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>MAF</td>
<td>minor allele frequency</td>
</tr>
<tr>
<td>MODY</td>
<td>maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>MTTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>PCK2</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PPARα</td>
<td>peroxisome proliferator activator receptor alfa</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------------------------</td>
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<tr>
<td>S1P</td>
<td>Site-1 Protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 Protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP Cleavage Activating Protein</td>
</tr>
<tr>
<td>Sf</td>
<td>Svedberg flotation rate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
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<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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To my father who waited for this for so long

“There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened”
Douglas Adams, 1980
GENERAL INTRODUCTION

Epidemiology of atherogenic dyslipidemia and its relationship to CHD

Cardiovascular disease is a major cause of morbidity and mortality in the industrialised world. CHD is a prevalent manifestation of cardiovascular disease. A major underlying cause of CHD is atherosclerosis, a chronic condition associated with accumulation of lipids in lesions along blood vessels, ultimately leading to obstruction of the blood flow. Although the relative input of individual atherogenic risk factors is still under debate, it is accepted that a combination of lipid-related traits referred to as atherogenic dyslipidemia constitutes one of the strongest predictors of CHD (1).

Atherogenic dyslipidemia is characterised by an elevated plasma triglyceride (TG) concentration, increased plasma concentrations of remnant chylomicrons and small dense LDL, and a decreased HDL concentration. While elevated plasma LDL-cholesterol and decreased HDL-cholesterol concentrations are well-defined risk factors of CHD, the role of elevated plasma TG as an independent predictor of CHD progression and outcome was for a long time controversial. Indeed, a number of prospective studies (2-9) conducted a decade ago argued against an independent role of plasma TG concentration in modulating CHD risk. Detailed analysis of the Multiple Risk Factor Intervention Trial (2, 3), the Lipid Research Clinics Coronary Primary Prevention Trial (4, 5), and the Lipid Research Clinics Prevalence and Mortality Follow-up Study (6, 7) did not uncover an independent association between plasma TG concentration and CHD events (10). Accordingly, it was concluded that measurement of the plasma TG concentration does not add any meaningful information beyond that provided by the measurement of the LDL- and HDL-cholesterol concentrations alone. However, two recent comprehensive meta-analyses (11, 12) demonstrated that elevated fasting TG is a risk factor for cardiovascular disease which is independent of HDL-cholesterol. This conclusion is supported by the results of several recent publications (13-15).
Nonfasting plasma TG concentration and the risk of CHD

The plasma TG concentration is conventionally measured following an overnight fast. Nevertheless, there is compelling in vitro evidence that postprandial TRLs may be more atherogenic than fasting TRLs. This raises the question as to the potential predictive power of non-fasting plasma TG measurement with respect to risk of CHD. Prospective studies conducted in Danish (15) and American (16, 17) cohorts identified non-fasting TG concentration as an independent risk factor of CHD. Moreover, while adjustments for total- and HDL-cholesterol weakened the association of fasting TG with the incidence of CHD, non-fasting TG levels maintained a strong independent relationship with the occurrence of cardiovascular events. This suggests that the measurements of non-fasting TG levels may be more useful in clinical practice than the measurement of fasting plasma TG concentration.

Physico-chemical properties of TRLs

The TG molecule is the most concentrated form of energy available to mammalian tissues. An important physical property of TG is the high hydrophobicity that renders TG insoluble in the aqueous medium of blood plasma. TG transport in the circulation is ensured by the assembly of TG molecules into the core of TRLs: the chylomicrons synthesised in the intestine in response to absorption of dietary fats and the very low-density lipoproteins (VLDLs) produced constitutively in the liver. The encasement of TG with a shell of hydrophilic phospholipids and esterified cholesterol molecules provides stability in an aqueous environment. The structural stability of these particles is also dependent on the presence of the large APOB polypeptide, which appears to bind together the hydrophobic core of the TRL with its hydrophilic shell.

VLDL assembly and secretion

The synthesis of VLDL has been studied extensively in cultured hepatic cell systems (18-21). On the basis of these studies a two-step model of VLDL assembly
Figure 1. VLDL assembly and APOB degradation

Two-step model of VLDL assembly (modified from Hooper AJ. et al., 2005 (22)). The first step (I) involves MTTP-mediated lipid transfer to nascent APOB, which in the course of second step (II) fuses with preformed lipid droplets generating mature VLDL particle. Excess nascent APOB is degraded (III).

has been proposed (22), as outlined in Figure 1. In the first step (I), pre-VLDL is synthesized in the presence of lipids. In this step APOB is cotranslationally lipidated with the assistance of the microsomal triglyceride transfer protein (MTTP) that acts both as a lipid transporter and as a chaperon (23-25). This process takes place in the endoplasmic reticulum (ER). During the second step (II), mature VLDL is formed by bulk lipid addition via fusion of pre-VLDL with large TG droplets. The confinement of the second step is under debate and both Golgi apparatus (26) and smooth ER (27, 28) have been implicated. Finally, in the absence of lipids the nascent APOB is degraded (III).

The two-step model is compatible with the idea that the availability of lipids determines the stability of APOB. In agreement with this hypothesis, it was shown that unlipidated or underlipidated APOB is the subject of presecretory proteasomal degradation (29-31). Moreover, the secretion of APOB can be dramatically increased in human hepatoma cells by stimulation of lipid synthesis with sodium oleate (30, 31). However, it is not clear whether this mechanism is a common mode of regulation in normal human or animal physiology. Indeed, it was shown that both short- and long-term feeding of high-fat diets to rats resulted in increase of the size of the secreted TRLs, whereas there was no effect on APOB secretion (32-34). These results suggest
that in vivo, the rate of TRL and APOB secretion is primarily controlled by particle size rather than by posttranslational degradation of APOB.

**Heterogeneity of TRLs and atherogenic dyslipidemia**

The VLDL particles secreted by the liver pass through a number of remodelling steps in the circulation involving lipolysis and protein transfer, giving rise to intermediate-density lipoproteins (IDLs) and ultimately LDL (Figure 2).

![Figure 2](image)

**Figure 2.** Metabolism of APOB-containing lipoproteins

VLDL2 synthesized by the liver is the subject of two metabolic fates: it can either be secreted into the circulation or undergo an additional lipidation step, generating large, TG-rich VLDL1. Insulin suppresses the additional lipidation step generating VLDL1. In the circulation, the lipids of the VLDL1 and VLDL2 particles are hydrolyzed by lipoprotein lipase (LPL) and hepatic lipase (HL). Ultimately, VLDL2 is converted to normal-sized LDL, while VLDL1 gives rise to small, dense LDL. The intermediate-density lipoproteins (IDL) and LDL are removed from the circulation by the LDL-receptor (LDLR), while VLDL1 and VLDL2 are removed by the LDLR-related protein (LRP) and the VLDL-receptor (VLDLR) (Modified from Taskinen M R., 2003 (35))

Both VLDL and LDL particles are heterogenous with regard to their size, density and composition. Small dense LDLs are more atherogenic than larger LDL particles and their prevalence is linked to increased risk of CHD (36-39). The size of VLDLs secreted by human hepatocytes ranges from 350 Å to 700 Å depending on the
amount of TG in the lipid core (40). There are two secretion-competent subclasses of VLDL particles: large buoyant TG-rich VLDL1 (Sf 60-400) and smaller and denser VLDL2 (Sf 20-60). In normolipidemic subjects over 75 % of the increase of postprandial plasma TG concentration is accounted for by the concentration of VLDL1 particles and nearly 50 % of the variation in LDL size is explained by the plasma TG concentration (40, 41). This suggests that the abundance of plasma VLDL1 is causally related to the concentration of small dense LDL (40, 42). While VLDL1 and VLDL2 are secreted into different metabolic channels within the VLDL-IDL-LDL cascade, the switch to preferential synthesis of VLDL1 leads to the plasma lipoprotein profile typical of atherogenic dyslipidemia. This metabolic switch determines the time course of postprandial TG response under pathophysiological conditions such as insulin resistance and dysregulation of liver fat metabolism. Thus, subjects with type 2 diabetes (T2D) are exposed to high TG concentrations throughout most of their lives (35).

**Metabolic context that favours VLDL1 production and secretion**

Hepatic cholesterol and fatty acid synthesis is regulated by a family of nuclear factors called SREBPs (43). This family of transcription factors comprises three members of trans-membrane proteins anchored in the ER, designated SREBP-1a, -1c and -2. SREBP-1a and -1c are mainly involved in the regulation of fatty acid synthesis, whereas SREBP-2 is a master regulator of cholesterol metabolism. Although the origin and intracellular localisation of the lipid pools from which TGs constituting the core of VLDL particles are derived is still uncertain, it is generally assumed that both de novo lipogenesis governed by SREBP-1c (44-46) as well as flux of free fatty acids (FFA) into the liver (44) contributes to the shift of cellular metabolism from lipid oxidation to lipid storage. While insulin exerts an inhibitory effect on the production rate of TG-rich VLDL1 particles (47-50) it is believed that insulin resistance influences VLDL assembly and secretion. Importantly, both SREBP-1c expression and release of fatty acids from adipose tissue are subjected to tight regulation by insulin. In short, the events driving the shift in VLDL assembly towards generation of atherogenic VLDL1 are: 1) impaired insulin signalling,
enhancing lipid accumulation to nascent APOB, 2) upregulation of SREBP-1c, stimulating de novo lipogenesis, and 3) excess availability of lipids in the liver (35).

**Analysis of factors affecting VLDL synthesis and secretion: focus on candidate genes**

TGs, phospholipids, cholesteryl-esters, and unesterified cholesterol are the principal lipid-components of the VLDL particle. The main VLDL proteins are APOB, APOE and several different APOCs. The rate of VLDL secretion is dependent on the availability of both lipids and APOB. There are two sources of lipid molecules, most importantly TGs, utilised for VLDL assembly: 1) de novo synthesis and 2) uptake, lipolysis and reesterification of exogenous lipids. There is compelling evidence that the second pathway is quantitatively of greater importance for the regulation of VLDL secretion (51-53). This underlines the potential role of esterification enzymes in the regulation of VLDL secretion (51). In short, numerous proteins are involved in VLDL synthesis, and all of these proteins may be regarded as putative “candidates” for the regulation of VLDL secretion.

**Analysis of candidate genes using single nucleotide polymorphisms**

Variation in the DNA sequence has been observed in all species, and it has been estimated that a common polymorphism (usually defined as a nucleotide substitution with a frequency of the minor allele of at least 0.01 in the general population) is present, on average, in one of every 500 base pairs (bp) of the human genome. When a polymorphism is present in a section of the genome that is of critical importance for the structure of a protein (e.g. the exons of the gene), or the regulation of the expression of the gene (for example the promoter of the gene), it can be expected that an association can be found between the polymorphism and a phenotype related to the function of the protein. These so-called functional polymorphisms therefore provide an opportunity to examine the physiological role of a candidate gene and are potential tools to examine the relationship with CHD.
The analysis of single nucleotide polymorphisms (SNPs) as a tool for the study of genetic risk factors underlying CHD is limited by two major factors. First, not all genes harbour functional polymorphisms, and the analysis of SNPs can therefore not be used as a general approach for the study of genetic risk factors for CHD. Second, the human genome contains millions of SNPs, and only a small fraction of these will have physiological significance. It is therefore of importance to examine the functional relevance of a particular SNP prior to large scale testing in human populations.

**Analysis of candidate genes using RNA interference**

Important insights into the roles played by putative candidate genes can be gained from studies in human cell lines using tools to manipulate the transcription of these genes. A new and efficient tool to achieve this objective is RNA interference (RNAi) using siRNAs. This technique makes use of naturally existing antiviral defence mechanisms. This pathway operates in two steps (54-58). In the first step, the foreign RNA is cleaved into small fragments of 19-21 bp. In the second step, the small fragments direct the RNA induced silencing complex (RISC) to the complementary RNA, mediating its degradation. Since the siRNAs can be introduced into cells by transient transfection, it is possible to silence RNA in a highly specific fashion. Thus, the siRNA technique is a unique tool for the specific silencing of in principle every gene. siRNA silencing can therefore be used for the analysis of candidate genes of CHD.

**Cell models used in studies of TRL secretion: pros and cons**

The McA7777 rat hepatoma cell line is a popular model for studies of lipid metabolism. These cells secrete considerable amounts of TRLs, which is obviously very convenient for the study of TRL metabolism. However, a drawback is the etiology of the cell-line, which raises the question of the extent to which results derived from these cells can be extrapolated to human liver cells.
It is theoretically feasible to use primary human hepatocytes for the study of TRL metabolism. Primary human hepatocytes retain most of the functions of the cells contained in the intact liver, for example with regard to drug and xenobiotic metabolism (59). However, primary hepatocytes are expensive, are derived from different donors, require delicate cell-culture conditions and are difficult to manipulate with transfection techniques. Most of the studies on TRL metabolism have therefore been performed using HepG2 cells, a human hepatocellular carcinoma cell line with epithelial morphology that secretes all the major plasma proteins typical for liver (60-63). However, HepG2 cells secrete lipoproteins of abnormal size and composition at very low levels (62). A potential alternative to the HepG2 cell-line is the Huh7 human hepatoma cell-line. Surprisingly, this cell-line has rarely been used in lipoprotein research, although Huh7 cells, like HepG2 cells, can be obtained commercially, only require standard cell-culture conditions and can be easily manipulated using a variety of molecular biological techniques.

The expression of genes involved in drug metabolism was recently compared between human liver biopsies and the human hepatoma HepG2 and Huh7 cell-lines (59). Moderate agreements in mRNA levels were observed, but the agreement was better for Huh7 cells than for HepG2 cells. Unfortunately, little is known regarding possible differences between the HepG2 and Huh7 cells as regards lipid and lipoprotein metabolism. We therefore compared the expression of 80 genes in Huh7 and HepG2 cells to the expression in human liver. As shown in Figure 3 overall comparable expression levels were observed for the HepG2 and Huh7 cell-lines as compared to human liver. However, direct comparison of gene expression between the hepatoma cell-lines demonstrated markedly reduced mRNA levels for INSIG1 and MTTP in HepG2 cells as compared to Huh7 cells, indicating that HepG2 cells may be defective in some critical aspects of lipid metabolism.

Comparative analysis of TRL secretion demonstrated that Huh7 cells secrete TRLs at ~ 2-fold higher rate than HepG2 cells when analyzed under standard cell-culture conditions. Nevertheless, both HepG2 and Huh7 cells show a substantial increase in the rate of secretion of TRLs following oleate treatment. However, both hepatoma cell-lines secrete TRLs with a size that is intermediate when compared with VLDL and LDL.
The mRNA levels of 80 genes in human liver, HepG2 and Huh7 hepatoma cells were measured by real-time PCR using the Low Density Array system. For each gene, the log_{10}-transformed fold difference in mRNA level between human liver and HepG2 (left panel) or Huh7 (right panel) was calculated, using human liver as reference. The frequency distribution was analyzed using the GraphPad Prism 4 program.

There are reports that HepG2 cells are relatively resistant to transcriptional changes induced by lipoprotein deficiency (64). We therefore tested the effects of lipoprotein deficiency on the expression of nine SREBP target-genes in the hepatoma cell-lines.

HepG2 and Huh7 cells were incubated for 24 hours in the presence or absence of fetal bovine serum (FBS) in the cell-culture medium. The mRNA levels of nine SREBP-target genes were subsequently measured by real-time PCR. For each gene, the fold increase in mRNA level induced by the absence of FBS was calculated using as reference the mRNA level in the presence of FBS (control). Values are expressed as mean ± SE for four experiments.*p<0.05, **p<0.01
As shown in Figure 4, lipoprotein deficiency increased the mRNA levels of nearly all SREBP target-genes in the Huh7 cells, while the changes in mRNA levels were small in the HepG2 cells.

In short, human hepatoma HepG2 and Huh7 cells are cheap and reliable cell-lines and are easy to culture and manipulate using various molecular biological techniques. Nevertheless, a detailed comparative analysis of lipid and lipoprotein metabolism revealed a number of potentially important defects of the HepG2 cell-line as compared to the Huh7 cell-line. For this reason, mainly Huh7 hepatoma cells were used in this thesis for the analysis of genes involved in the regulation of secretion of TRLs.
SPECIFIC INTRODUCTION

INSIG1 and INSIG2

Hepatic cholesterol and fatty acid synthesis is regulated to a large extent by a family of nuclear factors called SREBPs (43). As described earlier, this family of transcription factors comprises three members of trans-membrane proteins (designated SREBP-1a, -1c and -2), which are anchored in the ER. When the concentration of cellular sterols is low, SREBPs are transported to the Golgy apparatus where they undergo two sequential proteolytic cleavages (Figure 5).

Figure 5. SREBP pathway
In the presence of cholesterol or oxysterols, SREBP, SCAP and INSIG form a complex which is retained in the ER. In the absence of sterols there is a conformational change of SCAP, leading to the release of INSIG from the SREBP-SCAP complex. The SREBP-SCAP complex is subsequently incorporated into COPII vesicles via interaction with Sec23/24 and Sar1 proteins. Following the transfer from the ER to Golgi, the SREBP undergoes two sequential proteolytic cleavage events mediated by the proteases S1P and S2P. The mature form of SREBP generated in this process is subsequently transported to the nucleus, where it activates the transcription of SREBP-target genes such as HMG-CoA reductase and LDL receptor. SCAP: SREBP cleavage activating protein, S1P: Site-1 protease, S2P: Site-2 protease. (Modified from Espenshade PJ., et al, 2007 (65)).
As a result of this process the transactivation domain of the SREBPs is released and transported to the nucleus where it activates different genes involved in lipid metabolism. The translocation of SREBP to the Golgi apparatus is inhibited when the concentration of cellular sterols is high (66).

There is considerable evidence that the balance between SREBP retention in the ER and trafficking to the Golgi apparatus is rate-limiting in the regulation of the SREBP pathway (67, 68). The retention of the SREBPs in the ER is mediated by two proteins called INSIG1 and INSIG2 (69, 70). The INSIGs are close homologues with 59% amino-acid identity between the two proteins and it is generally assumed that INSIG1 and INSIG2 play similar roles in regulating cholesterol homeostasis (71).

INSIG1 is a hydrophobic protein with multiple membrane-associated domains, located in the ER both in the presence and absence of sterols (72). In the presence of sterols, INSIG1 retains the SREBPs in the ER, which leads to reduced amounts of the mature (nuclear) form of SREBP and decreased levels of expression of SREBP-regulated genes. This dependence on sterols for the retention of SREBP in the ER may be overridden when INSIG1 is abundant. Under these conditions, INSIG1 retains SREBP even in the absence of sterols (72). Moreover INSIG1 is itself a subject of SREBP-mediated transcriptional regulation (73). Thus, SREBP activates the expression of its own negative regulator. INSIG1, but not INSIG2, also imposes another level of feedback regulation of sterol metabolism: in the presence of sterols it binds to the sterol-sensing domain of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) and triggers its proteasomal degradation (74, 75). Conversely, in the absence of sterols INSIG1 is rapidly ubiquitinated and degraded in proteasomes (76).

Unlike INSIG1, INSIG2 is not a transcriptional target of SREBP, and in order to retain SREBP in the ER it is totally dependent on sterols (71). Moreover, its stability is not affected by sterols. In mice, Insig2 produces two distinct mRNAs that differ in their first non-coding exons (77). One mRNA, designated Insig2b, is ubiquitously expressed. The other mRNA, named Insig2a, is liver-specific. However, in our preliminary analysis of human INSIG2 we did not find any indication of the existence of an INSIG2a isoform.
The INSIG proteins differ in their mode of regulation by insulin. In mice, the expression of Insig1 is up-regulated in response to high levels of insulin (78). In contrast, the expression of the insulin-sensitive isoform of Insig2 (Insig2a) is down-regulated by insulin (77). This suggests that INSIG2 may have an as yet unidentified role in the SREBP pathway, distinct from that of INSIG1. Moreover, the fact that the expression of INSIG1 is markedly enhanced by insulin also suggests that INSIG1 may play an as yet unidentified role in glucose metabolism.

The physiological relevance of the INSIG proteins has been analysed in knock-out animals (70). A double knock-out of both Insigs (but not each of them alone) was associated with defects in facial development such as cleft palate and cleft face, and the animals died within one day from birth. Administration of an inhibitor of HMGCR to pregnant mice ameliorated these phenotypes, suggesting that the observed effects of INSIG-deficiency are attributable to abnormal cholesterol metabolism. In addition, the double knock-out mice showed gross accumulation of cholesterol and triglycerides in the liver and a blunted response to cholesterol-feeding (70).

In order to investigate the role of INSIG proteins in a human setting, we screened INSIG1 and INSIG2 for common polymorphisms and analysed their associations with biochemical parameters related to lipid and glucose metabolism.

**APOB**

The role of APOB in the regulation of lipoprotein transport.

APOB is a large amphipathic glycoprotein with a crucial role in mammalian lipid metabolism. APOB ensures an efficient partitioning of the transport process for the lipids of endogenous and exogenous origin, which is reflected by the existence of two isoforms of APOB: APOB100 associated with VLDL and secreted by the liver, and APOB48 associated with chylomicrons and secreted by the gut. APOB100 is a 4536-residue protein and APOB48 corresponds to the 2152 amino-terminal residues of APOB100. The molecular mechanism responsible for the conversion of APOB100 to APOB48 involves site-specific RNA modification and is referred to as C-to-U RNA editing (79-81). APOB100 is synthesised in humans exclusively in the liver and is present in VLDL, IDL, and LDL. APOB48 is expressed in the intestine and is
present in chylomicrons and their remnants (82). In contrast, considerable amounts of both APOB100 and APOB48 are synthesised in the liver of rodents (83, 84).

APOB contains multiple domains involved in lipid binding and protein-protein interactions that ensure the multiplicity of its functions in regulating lipoprotein transport. APOB is a ligand for the LDL-receptor and is therefore important for the clearance of LDL particles from the circulation. Mutations in APOB that interfere with APOB binding to the LDL receptor are the cause of the genetic disorder familial defective APOB100, characterised by hypercholesterolemia (85-88).

APOB is also important for the assembly and secretion of TRLs, whereby the proper folding of the protein determines the generation of secretion-competent lipoprotein particles. Truncation mutations of APOB render the protein unable to adopt a proper folding, leading to defective synthesis and secretion of TRLs with ensuing severe dyslipidemia (89-91).

**Atherogenic potential of APOB**

There are currently three hypotheses explaining the events that initiate atherogenesis. The “response-to-injury” hypothesis (92) emphasises injury to smooth muscle cells and vascular endothelium as an initiating event and considers APOB-containing lipoproteins in this context. The “oxidation hypothesis” (93) stresses the role of oxidative modification of LDL in mediating the inflammatory responses underlying the atherosclerotic process. Finally, the “response-to-retention” hypothesis (94-96) is based on the concept that APOB-containing lipoproteins are retained and accumulate in the artery wall and that this retention is the initiating event in atherogenesis (97). The “response-to-retention” hypothesis emphasises the atherogenic role of the APOB protein and not just the lipid cargo of LDL (98, 99). According to the “response-to-retention” hypothesis, the APOB protein itself accounts for the initial steps of atherogenesis by influencing the plasma concentration of APOB-containing lipoproteins as well as the retention time of LDL in the arterial wall. It has been demonstrated that APOB interacts with collagen, elastin and proteoglycans of the extracellular matrix that are synthesised by smooth muscle cells (99-103). It is proposed that this mechanism increases the retention time of the LDL particle in the subendothelium, extending the exposure to the modification processes of the inflammatory atherogenic response (97). In agreement with the “response-to-
retention” hypothesis, it was found that transgenic mice expressing proteoglycan-binding defective APOB have LDL with decreased proteoglycan binding capacity and greatly reduced atherogenic potential, which provides direct evidence that binding of LDL to the arterial wall is an early step in atherogenesis (104).

**APOB as a predictor of CHD**

Although the conventional approach to assessing the risk of CHD remains the measurement of the LDL-cholesterol concentration, the predictive potential of the APOB concentration with regard to the risk of CHD seems to be considerable (105-108). Indeed, there is evidence that the plasma APOB concentration is superior to the plasma LDL-cholesterol concentration as a predictor of cardiovascular events (109). In particular, the prognostic value of the LDL-cholesterol concentration is confined to young subjects, while the APOB concentration is predictive in all age categories. Moreover, the APOB concentration was predictive of fatal myocardial infarction in both men and women, whereas the LDL-cholesterol concentration is only predictive in men. These data suggest that the measurement of the APOB concentration adds information beyond that provided by LDL-cholesterol measurement. More recently the APOB/APOA1 ratio has come into focus as an alternative to the LDL-cholesterol measurement. It appears that the APOB/APOA1 ratio could serve as an independent predictor of CHD risk under conditions where lipid parameters lost their prognostic value (for example during treatment with lipid-lowering medication) (110).

**Transcriptional versus posttranslational regulation of APOB secretion**

Extensive studies using cell-biological model systems have provided evidence that the secretion of APOB and TRLs by the liver is regulated at both the cotranslational and posttranslational levels (79, 111, 112). These regulatory processes appear to be governed primarily by the availability of lipid substrate. In agreement with this hypothesis, it was shown that the level of APOB mRNA in cell culture shows little variation under different metabolic conditions (79), indicating that transcriptional regulation of APOB does not play a major role in the regulation of the
secretion of APOB and TRLs. However, several recent reports have questioned this conclusion. Studies in hepatoma cell lines expressing recombinant human APOB demonstrated that the APOB mRNA level has an important effect on the secretion of TRLs (113). Moreover, overexpression of APOB in mice (114, 115) and rabbits (116) resulted in increased plasma LDL concentrations, and a positive correlation between transgene copy number and plasma LDL concentration was observed (116). Conversely, siRNA knockdown of Apob in mice resulted in decreased plasma LDL-cholesterol and Apob concentrations (117). These studies indicate that transcriptional variation of APOB can contribute to the regulation of the rate of secretion of TRLs by the liver. However, all of these studies were conducted under conditions that induce profound changes in the expression of APOB. The question was therefore raised whether a moderate change in APOB transcription, presumably representing a more physiological condition, influences the rate of secretion of TRLs by the liver and is associated with the plasma LDL-cholesterol concentration. In this project, we have examined this question by studying the effects of siRNA inhibition of APOB on the secretion of TRLs in human hepatoma cells and by analyzing in a human population the relationship between the APOB mRNA concentration in the liver and the plasma LDL concentration.

**HNF4A**

HNF4A was discovered as a liver-enriched nuclear orphan receptor that regulates the transcription of genes encoding transthyretin and APOC3 proteins (118). The HNF4A protein belongs to the family of steroid-thyroid nuclear receptors and constitutively activates the transcription of target genes. The prominent role of HNF4A in human lipid and glucose homeostasis is illustrated by a recent chromatin immunoprecipitation (ChIP) study (119), which generated an extensive list of putative
HNF4A target genes, including different apolipoproteins, coagulation factors and enzymes involved in lipid, amino acid and glucose metabolism. However, HNF4A does not appear to be a sensor of intracellular fatty acid levels, as was initially proposed (120).

HNF4A is expressed predominantly in the liver, kidney and pancreas (120, 121). Mutations of HNF4A are the cause of maturity-onset diabetes of the young 1 (MODY1) (122). In addition, recent studies indicate that common polymorphisms in HNF4A may contribute to the development of late-onset T2D (123, 124). MODY1 patients are characterised by compromised insulin secretion in the presence of glucose, which has been linked to HNF4A deficiency in pancreatic β-cells (125, 126). Also, it is known that the plasma TG concentration is markedly reduced in subjects with MODY1 and this impairment is due to HNF4A haploinsufficiency and precedes the onset of hyperglycemia (127). This underlines the potential role of hepatic HNF4A in the regulation of the plasma TG concentration, but the target gene involved in this process has not been defined.

HNF4A has recently emerged as a potential target for treatment of hyperlipidemia in humans. It has long been known that peroxisome proliferator activator receptor alpha (PPARα) activators possess hypolipidemic activity (128). However, some PPARα activators influence the plasma lipoprotein profile in rats, but not in humans. This paradox was recently resolved by Hertz et al. (129), who demonstrated that the hypolipidemic potency of different PPARα activators in humans, but not in rats, is dependent on the intracellular esterification of the compound to acyl-CoA. It was shown that this esterification step is necessary to convert the PPARα agonist into a HNF4A antagonist. Those PPAR agonists that retain their PPARα activating capacity but are not convertible into acyl-CoA (and therefore do not act as HNF4A inhibitors) are hypolipidemic agents in rats, but not in humans. These experiments demonstrated the existence of an HNF4A-dependent arm of PPAR action in humans, in contrast to the PPARα-dependent pathway in rats.

The in vivo analysis of the physiological role of hepatic HNF4A was hampered by embryonic lethality of the knock-out mouse model (130). However, conditional liver-specific HNF4A knock-out mice were characterised by decreased plasma cholesterol and TG concentrations (131, 132). These abnormalities were attributed to impaired expression of HNF4A target genes involved in lipid metabolism and transport. However, in this in vivo model it is rather difficult to distinguish
between the primary effect of compromised gene expression and the secondary effect of severe liver dysfunction observed in these animals.

We used siRNA inhibition and a gentle overexpression method to gain insight into the role of HNF4A in the regulation of synthesis and secretion of TRLs by the liver. We demonstrate that variation in HNF4A concentration in human Huh7 hepatoma cells is mirrored by changes in the rate of secretion of TRLs. It was previously proposed that the effect of HNF4A on the plasma TG concentration is accounted for by upregulation of APOC3 (133-135), a known repressor of lipoprotein lipase activity (136-139). However, we found that the decrease in secretion of TRLs by HNF4A siRNA inhibition precedes the reduction of APOC3 expression in Huh7 cells, suggesting that APOC3 does not mediate the effect of HNF4A on the secretion of TRLs. Accordingly, we set out to identify the functional intermediary protein and demonstrated that DGAT1 is responsible for the HNF4A-dependent regulation of secretion of TRLs.
HYPOTHESES

- INSIG1 and INSIG2 play distinct roles in the regulation of the SREBP pathway in man.
- The APOB mRNA level influences the hepatic secretion of TRLs and the plasma LDL concentration.
- The reduced plasma TG concentration in subjects with MODY 1 is due to changes in the regulation of an unknown HNF4A target-gene involved in the secretion of TRLs.
AIMS OF THE THESIS

The overall aim of this thesis was to identify and characterise major regulatory proteins involved in the control of secretion of TRLs by the liver and the regulation of the plasma triglyceride concentration. To this end, the impact of two critical proteins (INSIG1 and INSIG2) regulating the synthesis of TG and other lipids was analysed using a combination of in vitro techniques and population studies. In subsequent studies, the relevance of two additional proteins (APOB and HNF4A) with putative roles in the regulation of secretion of TRLs was evaluated.
MATERIALS AND METHODS

Subjects

A biobank and database comprising a total of 629 healthy 50-year-old men, recruited for biochemical and molecular genetic studies of mechanisms predisposing to atherothrombosis, were used in studies I and II. A cohort of 956 middle-aged male survivors of myocardial infarction and age-matched healthy control subjects was evaluated in study II. Two groups of healthy middle-aged men (n = 181 and n = 291), who had been sampled as control subjects for case-control studies of young survivors of myocardial infarction, were analysed in study I. Gluteal subcutaneous adipose tissue biopsies, obtained from a separate cohort of 40 healthy middle-aged men, were used for the gene expression analysis described in study II. Liver biopsies were obtained from 80 patients undergoing coronary artery by-pass grafting at the Karolinska University Hospital as part of the Stockholm atherosclerosis Gene Expression (STAGE) study. All control subjects were population-based and selected at random from a registry containing all permanent residents of Stockholm County. The Ethics Committee of the Karolinska Hospital had approved the protocols of the different projects, and all subjects had given informed consent to their participation.

Polymorphism detection and genotyping

The ABI 3100 capillary sequencer was used for direct sequencing of \textit{INSIG1} and \textit{INSIG2}. All exons, 50-200 bp of flanking introns, approximately 600 bp of the proximal promoter and 900-1300 bp of the 3’-untranslated region were analyzed. Sections with a size ranging from 750 bp (study I) or 900 bp (study II) were amplified and purified using the QIAquick PCR purification kit (Qiagen). These sections were used as templates for sequencing using nested primers.

The -237T>C \textit{INSIG1} promoter polymorphism, and the 805-362 A>G, -337 T>G, -324T>C, -319C>T, -318A>G, and -212T>C polymorphisms in intron 5 of \textit{INSIG1} were analyzed using the sequencing technique employed for polymorphism detection. The -425A>G, 537+45C>T and 805-119C>A polymorphisms of \textit{INSIG1} and the -102G>A polymorphism of \textit{INSIG2} were genotyped using PCR amplification.
followed by digestion with specific restriction enzymes. The rs12986752 polymorphism of INSIG2 was genotyped using PCR amplification followed by pyrosequencing analysis of the amplified fragments. The -633G>C and -169C>T polymorphisms of INSIG1 and the rs7566605, rs7589375, rs13393332 and rs1559509 polymorphisms of INSIG2 were genotyped using TaqMan assays (Applied Biosystems).

**Cell culture and transfection procedures**

Human hepatoma Huh7 and HepG2 cells were cultured in DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin. For transfection studies, subconfluent hepatoma cells were preincubated with FBS-free medium for 30 minutes. The cells were subsequently transfected with 50 nM gene-specific siRNA or negative control siRNA or with recombinant plasmid construct using Lipofectamin 2000 (Invitrogen) as a transfection agent. The transfection medium was removed after a 5-hours incubation, and the cells were cultured for another 24 to 48 hours under standard cell culture conditions. In the overexpression studies, cells were incubated for 36 hours with Lipofectamin 2000 and 2 µg of HNF4A expression plasmid or 1 µg DGAT1 promoter construct, followed by 24 hour incubation under standard cell culture conditions.

Mouse 3T3-L1 preadipocytes were grown in high glucose DMEM (Gibco), supplemented with 10% FBS (Gibco), 110 mg/L pyruvate, 50 units/ml penicillin and 50 µg/ml streptomycin (all from Gibco). A adipocyte differentiation was induced in 2-day post-confluent cells by addition of 1.7 µM insulin, 0.5 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma) to the medium.

**siRNA probes and DNA constructs**

Gene-specific oligonucleotides were designed using the siRNA Target Finder program (www.Ambion.com) and supplied by a commercial supplier (Proligo) or purchased from Ambion as pre-designed probes. A siRNA targeting the pGL2 plasmid vector was used as negative control in all experiments. Double-stranded 21-bp oligonucleotides, comprising the sequence around the normal or the mutated
HNF4A binding site in the DGAT1 promoter, were annealed into the pGL3-promoter vector according to the manufacturer’s instructions (Promega). An expression plasmid encoding functional HNF4A protein was a generous gift from Dr. Paolo Parini, Department of Clinical Chemistry, Karolinska University Hospital, Huddinge, Sweden. pSV-β-glactosidase plasmid (Promega) was cotransfected with reporter constructs and used for normalisation of their activities.

**Real-time quantitative PCR**

Total cellular RNA was isolated using the RNeasy mini kit (Qiagen). Total cDNA was synthesised from 0.5 μg RNA in a polymerase reaction using oligoT primer (2.5 pmol/μl - final concentration), dNTPs (0.5 mM - final concentration) and SuperScript™II RNAse H⁻ reverse transcriptase (Invitrogen). cDNA from triplicate experiments were pooled and the specific gene expression was analyzed by real-time quantitative PCR, using 18s as invariant control. All assays and reagents were obtained from Applied Biosystems. Single gene assays were performed using the ABI PRISM 7700 sequence detection system and the Delta Rn analysis method. Multiple gene assays were conducted with the Low Density Array method using the 7900HT Fast Real Time PCR system. The relative difference in expression was calculated using the C_T method. All samples were analyzed in duplicate and all genes were analyzed in 4 or more independent experiments.

**Western blot analysis**

Cells were lysed on ice with RIPA buffer (150 mM NaCL, 10 mM Tris-HCL pH 7.2, 0.1 % SDS, 1 % Triton X-100, 1 % deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 10 mM benzamidine and 2 μg/ml leupeptin). The extracted proteins (25 μg) were subjected to SDS-PAGE and electrophoretically transferred onto a Hybond P PVDF membrane (Amersham Biosciences). Protein-specific primary antibodies (all from Santa Cruz Biotechnology) at different dilutions were used to detect specific protein bands. Horseradish peroxidase conjugated immunoglobulins (Santa Cruz Biotechnology) were used as the secondary antibodies.
TG and APOB assays

TG secretion was measured following a 24-hour incubation of the cells with C\textsuperscript{14}-glycerol (Perkin Elmer Life Sciences) at a final concentration of 2.85 mCi/ml. Lipids were extracted from the media using the methanol:chloroform:sodium-dihydrogen phosphate (0.2 M) extraction method (1:2:3 vol/vol). The lipids were separated by thin layer chromatography using Kieselgel 60 F\textsubscript{254} plates (Merck) and the hexane:diethyl-ether:acetic acid (80:20:1 vol/vol) elution method. Spots corresponding to TG were visualized using a Fujifilm BAS 2500 phosphorimager and excised. The radioactivity was then counted using a Beckman LA-1501 counter.

APOB in the cell medium was quantified by ELISA (ALerCHEK Inc). For chromatographic separation of (lipo-) proteins, 1.0 ml of cell culture medium was applied to a column (1.0 x 50 cm) of Sephacryl S-300 HR (GE lifesciences) equilibrated with PBS, containing 0.01 % azide. The fractions eluted from the column were analyzed for protein content (absorbance at 280 nm), APOB concentration and C\textsuperscript{14}-radioactivity associated with TG.

DGAT1 activity measurements

DGAT activity was measured under apparent V\textsubscript{max} conditions in microsomal membranes from Huh7 cells isolated as described for 3T3 cells (140). The assay was modified for the specific measurement of DGAT1 activity as outlined by Cases et al. (141). In brief, the incorporation of C\textsuperscript{14}-palmitoyl-CoA (Perkin Elmer Life Sciences) into TG was measured in the presence of 100 mM MgCl\textsubscript{2} in a 5-min assay using 0.25 mM 1,2-dioleoyl-sn-glycerol and 25 \textmu M palmitoyl-CoA (Sigma) as substrates.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from Huh7 cells were prepared according to Alksnis et al. (142). All buffers were freshly supplemented with leupeptin (0.7 \mu g/mL), aprotinin (16.6 \mu g/mL), phenylmethylsulfonyl fluoride (0.2 mmol/L) and 2-mercaptoethanol (0.33 \mu L/mL). Incubation for EMSA was conducted as described (143). The HNF4A and INSIG2 consensus gelshift oligonucleotides and the supershift HNF4A antibodies
were purchased from Santa Cruz Biotechnology. Non-radioactive competitor DNAs, identical or of the opposite allelic variant or of non-specific origin, were added in 100-fold excess of the labelled DNA.

**Statistical methods**

Distribution of continuous variables in groups was expressed as mean ± SD or mean ± SEM. Coefficients of skewness were calculated to test deviations from a normal distribution. Logarithmic transformation was performed on all skewed variables to obtain a normal distribution before statistical computations and significance testing was undertaken. Differences in continuous variables between groups were tested by Student’s unpaired two-tailed t test. Differences in biochemical and anthropometric measurements according to genotype were assessed using one-way analysis of variance. The ConSite program was used for human-mouse sequence comparison. The Associate program was used for LD calculations.
RESULTS

Functional polymorphisms affecting body mass index (BMI) and plasma glucose

Experimental design

The nucleotide sequences of INSIG1 and INSIG2 were screened for common polymorphisms using DNA samples from 18 healthy subjects. The potential relationships between these polymorphisms and quantitative traits related to lipid and glucose metabolism were then analysed in a cohort of apparently healthy, 50-year-old men. Replication studies were performed in other cohorts of apparently healthy subjects. The functional significance of a limited number of polymorphisms was analyzed in more detail using in vitro techniques, among others transient transfection and EMSA analysis.

Association of the -169 C>T polymorphism in the INSIG1 promoter with fasting and postload plasma glucose concentration

The promoter, exons and intron-exon borders of INSIG1 were screened for common polymorphisms. Fourteen SNPs were found: ten polymorphisms in the introns and four polymorphisms in the proximal promoter of INSIG1 (Figure 6).

Figure 6. Organisation of the human INSIG1 gene and delineation of SNPs
The boxes indicate the locations and approximate sizes of the six exons of INSIG1. The closed and open boxes represent the transcribed and untranscribed sections, respectively.
No polymorphisms were discovered in the exons or sections close to the intron-exon borders. Pair-wise linkage disequilibrium (LD) was analyzed for 12 polymorphisms in 80 subjects, and significant LD was observed for all polymorphisms.

The pattern of evolutionary conservation of the INSIG1 promoter was evaluated in order to establish whether any of the promoter polymorphisms is located in a putatively regulatory segment of the promoter. Human-mouse alignment revealed considerable degree of conservation for the first 600 bp of the INSIG1 promoter, with a very high conservation for a section surrounding the -169C>T polymorphism.

Four polymorphisms were analysed in the cohort of 50-year-old men for possible associations with biochemical traits related to human lipid and glucose metabolism. A significant association between the -169C>T polymorphism and fasting plasma glucose was observed (4.9 ± 0.7 mmol/l for C/C versus 4.8 ± 0.5 mmol/l for C/T+T/T, p<0.01), while the other three SNPs showed borderline-significant associations with the same parameter. This relationship was confirmed in a replication study of a cohort of 472 healthy, middle-aged men (5.2 ± 0.9 mmol/l for C/C versus 4.8 ± 0.7 mmol/l for C/T+T/T, p < 0.02). The effect of the -169C>T polymorphism on glucose tolerance was analysed in a subgroup (n = 181) of the replication population. Significantly lower plasma glucose levels were found at time points 30, 45 and 60 minutes after the glucose load in subjects carrying the -169T allele as compared with subjects homozygous for the -169C allele (Figure 7).

Figure 7. Impact of -169C>T polymorphism on oral glucose tolerance
Plasma glucose concentrations were analysed according to INSIG1 -169C>T genotype. Subjects heterozygous or homozygous for the -169T allele (n=18) (open squares) had faster glucose clearance than subjects homozygous for the -169C allele (n=163) (filled triangles). *p<0.05, **p<0.01
Overall, the genotype-phenotype association studies indicate that the -169T allele is associated with a significantly lower fasting plasma glucose concentration and enhanced clearance of glucose as compared with the -169C allele.

The downstream effects of siRNA inhibition of \textit{INSIG1} in Huh7 hepatoma cells were subsequently analyzed in order to dissect the possible mechanism(s) by which \textit{INSIG1} influences glucose homeostasis. The impact of \textit{INSIG1} siRNA inhibition on the expression of more than 40 different genes was analyzed. As expected, all SREBP target-genes evaluated in this study showed >1.5-fold increase in expression in response to an approximately 80 % decrease in \textit{INSIG1} mRNA concentration. In addition, the expressions of two genes, glucokinase (\textit{GK}) and phosphoenolpyruvate carboxykinase (\textit{PCK2}) were evaluated. These genes are involved in opposing metabolic pathways: \textit{GK} is a major enzyme of glycolysis, whereas \textit{PCK2} is an enzyme involved in gluconeogenesis. \textit{GK} was expressed at extremely low levels in Huh7 cells, and it was therefore not possible to evaluate the effect of \textit{INSIG1} siRNA inhibition on \textit{GK} expression. However, a significant (p = 0.02) reduction in expression of \textit{PCK2} was observed in \textit{INSIG1} siRNA treated cells. It is proposed that the observed effect of the -169C>T polymorphism in the \textit{INSIG1} promoter on fasting and postload plasma glucose concentrations is the consequence of decreased expression of the -169T allele as compared to the -169C allele, which is in turn associated with reduced expression of \textit{PCK2}, ultimately leading to lower glucose secretion by the liver.

Association of the -102G>A polymorphism in the \textit{INSIG2} promoter with BMI

The promoter, exons and intron-exon borders of \textit{INSIG2} were analyzed in search for the functional polymorphism responsible for the relationship between the rs7566605 polymorphism located 10 kb upstream of the transcription start site of \textit{INSIG2} gene and BMI (144). No polymorphisms were discovered in the exons or in sections close to the intron-exon borders. Eight SNPs were found: three were located in the introns (rs13393332, rs1559509 and rs13003121), three in the proximal promoter (rs7589375, rs12986752, -102G/A) and two (+136delA and +893C>T) in the 3'-UTR of \textit{INSIG2} (Figure 8)
Five SNPs with minor allele frequencies (MAFs) >10% were evaluated in association studies to investigate possible relationships with biochemical and anthropometric traits. This analysis also included the previously described rs7566605 polymorphism associated with BMI (144). Significant relationships were observed between the -102G>A polymorphism and BMI and plasma leptin concentration in two cohorts of middle-aged men. No relationships were found with biochemical parameters related to lipid or glucose metabolism.

The functional significance of the -102G>A polymorphism was evaluated using EMSA. Two synthetic oligonucleotides containing the -102G and -102A alleles were incubated with increasing concentrations of 3T3-L1 nuclear extract, followed by separation of the incubation products by 7% polyacrylamide gel electrophoresis. Quantification of a specific DNA-protein complex revealed stronger binding of nuclear factors to the -102G allele as compared to the -102A allele. The specificity of the DNA-protein complex was demonstrated in competition experiments. The corollary of these studies is that the -102G>A polymorphism influences the binding of transcription factor(s) to the INSIG2 promoter, presumably leading to allele-specific differences in the rate of transcription of INSIG2. Accordingly, INSIG2 mRNA concentration was measured in subcutaneous fat biopsies obtained from healthy middle-aged men to test this hypothesis. INSIG2 mRNA concentrations tended to be lower in subjects heterozygous for the -102A allele (27.7 ± 7.5 arbitrary units, n = 29) as compared to individuals homozygous for the -102G allele (33.5 ± 9.0 arbitrary units, n = 11), but this difference did not reach the conventional level of statistical significance (p = 0.07).
Differential expression of INSIG1 and INSIG2 in human tissues

The mRNA expression levels of INSIG1 and INSIG2 were analysed in different human tissues. INSIG1 was highly expressed in the liver, whereas relatively low expression levels were observed in extrahepatic tissues. In contrast, INSIG2 showed a more ubiquitous expression pattern. The INSIG1/INSIG2 mRNA ratio in the liver was 3.51 ± 0.43. The INSIG1/INSIG2 mRNA ratio for all extrahepatic tissues combined was 0.57 ± 0.1, and none of the extrahepatic tissues had an INSIG1/INSIG2 mRNA ratio exceeding 1.

Effects of INSIG siRNA inhibition on the expression of SREBP-target genes in human hepatoma cells

SiRNA inhibition experiments were performed in human hepatoma cells to delineate the relative importance of INSIG1 and INSIG2 for hepatic lipid metabolism. The effects of INSIG1 and/or INSIG2 siRNA inhibition were monitored by expression analysis of eight SREBP target-genes (ACLY, ACSS2, FASN, FDPS, HMGCR, HMGSC1, LDLR and PCSK9) in Huh7 hepatoma cells. INSIG1 siRNA silencing led to 74 % reduction of INSIG1 mRNA and was associated with significant increases in the expression of all eight SREBP-target genes. The increased expression was paralleled by an increased concentration of mature SREBP1 protein, as demonstrated by Western Blot analysis. In contrast, siRNA inhibition of the INSIG2 gene by 76 % did not result in significant changes in the expression of the eight SREBP-target genes.

The INSIG1/INSIG2 mRNA ratio for Huh7 cells was 2.84 ± 0.53. As discussed above, a comparable INSIG1/INSIG2 mRNA ratio was found for human liver tissue. This suggests that the INSIG1 protein concentration is higher compared to the INSIG2 protein concentration in Huh7 cells. The relatively low INSIG2 expression raises questions as regards the interpretation of the INSIG2 siRNA inhibition experiments in Huh7 cells. Additional siRNA experiments were therefore performed in HepG2 cells, another human hepatoma cell line, characterized by an extremely low INSIG1 expression level as compared to INSIG2. Again, no effect of INSIG2 siRNA silencing on the expression of SREBP target-genes was observed. On
the basis of these experiments it is proposed that INSIG1 plays a critical role in the regulation of the SREBP pathway in human hepatocytes.

Increase in Insig2 expression during differentiation of 3T3-L1 cells

The differentiation of mouse 3T3-L1 preadipocytes into adipocytes is characterised by morphological changes such as lipid droplet accumulation and an increase in the concentration of intracellular TG. The putative roles of Insig1 and Insig2 in adipocyte differentiation of 3T3-L1 preadipocytes were analysed. The expected changes in morphology (accumulation of lipid droplets), cellular TG concentration, and expression of marker genes for adipocyte differentiation (Figure 9)

![Figure 9](image)

**Figure 9. Expression of Insig1 and Insig2 during 3T3-L1 differentiation**
The expression of Pparg (top-left panel), Fabp4 (top-right panel), Insig1 (bottom-left panel), and Insig2 (bottom-right panel) during differentiation of 3T3-L1 cells was analysed using real-time PCR. The changes in expression were calculated relative to 2-days post-confluent levels, i.e. start of adipocyte differentiation. All values are mean ± SEM of four to six independent experiments.

(Pparg and Fabp4, shown in Figure 9; Cebpa, Dlk1, Fasn, not shown) were observed after induction of differentiation. As shown in Figure 9, a marked increase in expression of Insig2 and a modest increase in expression of Insig1 were observed during adipocyte differentiation. The Insig1/Insig2 mRNA ratio at the end of the 10-day experiment was 0.84 ± 0.17, which is comparable to the INSIG1/INSIG2 mRNA
ratio of $0.73 \pm 0.16$ in human adipose tissue. These observations underline the significance of Insig2 in adipogenesis.

**Genes affecting secretion of TRLs by the liver**

Experimental design

The roles of APOB and HNF4A in the regulation of secretion of TRLs were analyzed using the Huh7 and HepG2 human hepatoma cell-lines. A robust system for the measurement of the rate of secretion of TRLs in the cell-culture medium was developed. SiRNA inhibition techniques were subsequently employed to study the effect of gene-specific reductions in mRNA levels on TRL secretion. Gene expression studies were conducted to uncover potential intermediary proteins responsible for the observed effects of siRNA inhibition on TRL secretion. Additional in vitro experiments were performed to define in greater detail the physiological role of DGAT1, the intermediary protein responsible for the effect of HNF4A on TRL secretion. Finally, expression studies in human liver samples were conducted to substantiate the roles of APOB and HNF4A/DGAT1 in TG metabolism.

TRL secretion assay

The rate of secretion of TRLs by the hepatoma cells was estimated by the quantification of TG and APOB secreted in the cell-culture medium. TG secretion was measured by the analysis of $C^{14}$-labelled TG present in the medium following incubation of the cells with $C^{14}$-glycerol. APOB secreted in the medium was quantified by an ELISA for human APOB. As reported in study III, the rates of secretion of TG and APOB were linear during the 24-hour experiment. Molecular sieve chromatography experiments were conducted to determine the size of the complexes containing the $C^{14}$-labelled TG and APOB secreted in the cell-medium. $C^{14}$-labelled TG and APOB were recovered in the VLDL-LDL size range, indicating that the two molecules are associated with lipoprotein particles secreted by the Huh7 cells.

The question was raised to what extent the rate of accumulation of TG and APOB in the cell-medium was influenced by re-uptake of secreted lipoprotein particles and/or extra-cellular modifications of the secreted lipids.
**Figure 10.** Experimental design for the analysis of TRL re-uptake by Huh7 cells
Huh7 cells were incubated for 24 hours in cell-culture medium supplemented with C\(^{14}\)-glycerol (left upper box), followed by a 24 hour incubation with regular culture medium (CM) (left lower box). The conditioned medium, containing C\(^{14}\)-labelled TRLs secreted by Huh7 cells, was subsequently transferred to Huh7 cells incubated with gene-specific or control siRNA probes (right upper box). The change in TG-associated radioactivity was measured at different time-points during the subsequent 24-hour incubation period (lower right box).

The experimental procedure outlined in Figure 10 was used to address these questions. In these experiments, conditioned medium was generated by incubation of Huh7 cells for 24 hours with C\(^{14}\)-glycerol, followed by a 24-hour incubation of the cells with fresh medium, thus allowing accumulation of C\(^{14}\)-TG in the medium in the absence of C\(^{14}\)-glycerol. The conditioned medium was subsequently transferred to fresh Huh7 cells and the removal of radioactivity from the medium was monitored over a 24-hour period. However, no evidence was found for significant removal of the TRLs from the cell-culture medium. Nevertheless, it remained possible that the C\(^{14}\)-labelled lipids were modified (for example hydrolyzed) following secretion by the Huh7 cells. The relative quantities of C\(^{14}\)-label associated with TG and diglycerides (DG) were therefore analyzed to test this hypothesis. However, the overall proportions of C\(^{14}\)-TG and C\(^{14}\)-DG remained unchanged during the 24-hour incubation period. Taken together, the various control experiments indicate that quantification of the 24-hour accumulation of C\(^{14}\)-TG and APOB in the medium provides a reliable estimation of the rate of secretion of TRLs by Huh7 cells.
Relationship between APOB mRNA level and secretion of TRLs by human hepatoma cells

APOB siRNA silencing experiments were performed in order to evaluate the role of transcriptional regulation of APOB on the secretion of TRLs. APOB siRNA inhibition reduced the APOB mRNA level by 82 ± 2 % and was associated with a 83 ± 4 % reduction in APOB protein and 71 ± 4 % reduction in TG secreted by Huh7 cells. Analysis of the cell-medium by molecular sieve chromatography confirmed that the reduced secretion of APOB and TG was due to decreased secretion of TRLs in the VLDL-LDL size range. Similar results were obtained for APOB siRNA silencing experiments in HepG2 cells. Moreover, this effect was specific for APOB, while the expressions of 37 other genes involved in lipid and lipoprotein metabolism were not affected by APOB siRNA inhibition.

The studies described above demonstrated that a marked reduction in APOB mRNA level is associated with a substantial decrease in secretion of TRLs. The question was then raised to what extent more moderate changes in APOB mRNA level influence the secretion of TRLs. To this end, three different APOB siRNA probes were analyzed with different inhibition potencies, leading to 82 ± 4 %, 71 ± 9 %, and 59 ± 12 % (mean ± SD) reductions in APOB mRNA level, respectively. A positive relationship was observed between the APOB mRNA level and the secretion of TRLs was observed ($r^2 = 0.56$ for APOB and $r^2 = 0.67$ for TG secretion), indicating that also moderate changes in APOB mRNA level affect the secretion of TRLs. This conclusion was confirmed in a series of experiments analyzing the effect of in total eight different APOB siRNA probes. As shown in Figure 11 strong correlations were observed between the APOB mRNA level and TG secretion (Figure 11, left panel) and between the APOB mRNA level and APOB secretion (Figure 11, right panel).
Figure 11. Relationships between the APOB mRNA level and secretion of TG (left panel) and APOB (right panel) by Huh7 cells.

The APOB mRNA levels and the TG and APOB concentrations were measured 24 hours after APOB siRNA inhibition using 8 different APOB-specific probes. Each probe was used in 2-5 experiments, in total 28 experiments. All values are expressed as percent of control experiment.

Several studies have shown that HepG2 cells incubated in the presence of oleic acid have an increased secretion of TRLs. It was tested whether addition of oleic acid has a similar effect in Huh7 cells. Indeed, 24-hour incubation of Huh7 cells with 0.8 mM oleic acid was associated with a 4.4 ± 0.8 fold increase in TG secretion and a 2.1 ± 0.3 fold increase in APOB secretion. Also, significant relationships were observed between the APOB mRNA level and the secretion of TG ($r^2 = 0.91$, $p<0.0001$) from Huh7 cells incubated with oleic acid. Taken together, these experiments indicate that regulation of APOB mRNA level plays a significant role in determining the secretion of TRLs by human hepatoma cells.

Relationship between hepatic APOB mRNA level and plasma LDL concentration

The impact of inter-individual variation in hepatic APOB mRNA levels on the plasma LDL-cholesterol concentrations was analyzed in 78 patients with manifest CHD. The APOB mRNA level was assayed for each liver sample by real-time quantitative PCR using a highly standardized protocol, involving eight independent measurements on all samples. The APOB mRNA level was expressed in relative units and expressed in relation to the levels of three housekeeping genes (B2M, GAPDH and GUSB).
Figure 12. Relationship between hepatic APOB mRNA level and plasma LDL-cholesterol concentration

Normalised APOB mRNA levels were calculated from measurements by real-time PCR in liver biopsies, obtained from patients undergoing coronary artery bypass grafting. The mean values of three housekeeping genes (B2M, GAPDH and GUSB) were used as invariant control. The LDL-cholesterol concentration was determined in plasma samples obtained from the same subjects.

As shown in Figure 12 significant relationships were observed between the APOB mRNA level and the plasma LDL-cholesterol concentrations. Thus, in agreement with the in vitro studies in Huh7 cells, these in vivo studies demonstrated that inter-individual variation in hepatic APOB mRNA level is associated with the plasma concentration of LDL, the ultimate metabolic product of the VLDL particles secreted by the liver.

Effect of HNF4A siRNA inhibition on the secretion of TRLs

HNF4A siRNA inhibition experiments were performed in Huh7 hepatoma cells to test the hypothesis that the reduced plasma TG concentration observed in MODY1 subjects is a consequence of decreased expression of genes involved in hepatic TG synthesis. Huh7 cells were transfected with HNF4A-specific siRNA, leading to $77 \pm 2\%$ and $73 \pm 3\%$ reductions in HNF4A expression as measured 24 and 48 hours after transfection, respectively. Western blot analysis confirmed that the reduction in HNF4A mRNA concentration was associated with a marked decrease in
HNF4A protein concentration. The reduced expression of HNF4A was associated with a significant decrease in the secretion of TG and APOB (Figure 13).

![Graph showing effect of HNF4A silencing on the secretion of TG and APOB](image)

**Figure 13** Effect of HNF4A silencing on the secretion of TG and APOB. The HNF4A mRNA level and the secretion of TG and APOB were measured 24 and 48 hours after transfection of Huh7 cells with HNF4A-specific siRNA. The values are expressed as percentage of the control experiments (indicated with dotted line) and represent means ± SEM of five independent experiments. **p<0.01, ***p<0.001

Analysis of the cell-medium by molecular sieve chromatography demonstrated that the TGs and APOB secreted by the cells were recovered in the VLDL-LDL size range, indicating that both components are secreted from the cells as part of TRL particles.

Identification of the HNF4A target-gene responsible for the observed effect on TRL secretion

The Taqman Low Density Array system was used to monitor the expression of 37 genes involved in the regulation of lipid and glucose homeostasis in order to identify the target-gene responsible for the observed effect of HNF4A inhibition on the secretion of TRLs.
Figure 14. Effect of HNF4A silencing on the expression of genes involved in the secretion of TRLs. mRNA levels were measured 24 and 48 hours after transfection with HNF4A-specific siRNA. The values are expressed as percentage of the control experiments (indicated with dotted line) and represent means ± SEM of 4-5 independent experiments. *p<0.05, **p<0.01, ***p<0.001

As shown in Figure 14 substantial reductions in expression were observed for SOAT2 and DGAT1, genes encoding enzymes esterifying cholesterol and diacylglycerides, respectively.

Overexpression studies were conducted to substantiate the regulatory role of HNF4A on the rate of secretion of TRLs. It was found that gentle overexpression of HNF4A (confirmed by Western blot analysis) increases the secretion of TG and APOB and enhances the expressions of DGAT1 and SOAT2. Thus, the results from HNF4A knock-down and overexpression experiments underlined the involvement of HNF4A in the secretion of TRLs and suggested that either DGAT1 or SOAT2 mediates this effect.

Additional experiments were then performed to distinguish the putative roles of DGAT1 and SOAT2 in the secretion of TRLs. Individual silencing of DGAT1 and SOAT2 expression by siRNA inhibition was associated with a significant reduction in the secretion of TRLs by the DGAT1-deficient Huh7 cells, while no measurable effect was observed for the SOAT2-deficient cells. Moreover, a significant decrease in DGAT1 activity was observed in the HNF4A siRNA-treated cells. DGAT1 knock-down alone lead to similar reductions in the secretion of TRLs as double knock-down of HNF4A and DGAT1, indicating that DGAT1 accounts for the observed effect of
HNF4A on the secretion of TRLs. Taken together, these experiments demonstrated that DGAT1 mediates the effect of HNF4A on the rate of secretion of TRLs.

Putative HNF4A binding site in the DGAT1 gene promoter

The DGAT1 promoter was scanned for a putative HNF4A binding site using the MatInspector program. A section with high similarity to the HNF4A consensus-binding site was identified in the -695 to -675 region of the promoter. EMSA studies were performed in order to establish the functional relevance of this section of the DGAT1 promoter. Synthetic oligonucleotides corresponding to the natural or the mutated HNF4A binding sites were incubated with Huh7 nuclear extract in the presence or absence of HNF4A antibodies. A specific DNA-protein complex was detected when the oligonucleotide with the natural HNF4A binding site was analysed, while this complex was barely detectable when the oligonucleotide with the mutated HNF4A binding site was evaluated (Figure 15).

![Figure 15](image)

**Figure 15** Characterisation of the HNF4A binding site in the DGAT1 promoter

EMSA studies using 19-bp DNA fragments containing the HNF4A binding site in the DGAT1 promoter and the HNF4A consensus binding site as probes. The effects of increasing nuclear extract (NE) concentrations were evaluated in the left panel, while the results of supershift analysis using HNF4A antibody (ab) are presented in the right panels. The arrows indicate the protein-DNA complex generated by addition of the antibody.

The complex showed a clear ‘supershift’ when incubated with the HNF4A antibody, indicating that HNF4A protein is binding to the putative HNF4A binding site. Transient transfection studies were subsequently conducted in Huh7 cells with recombinant plasmid constructs containing the natural HNF4A binding site and the
mutated HNF4A binding site. It was found that the mutation reduced the expression of the construct containing the putative HNF4A binding site in the DGAT1 promoter. Moreover, variation in the cellular HNF4A concentration only influenced the expression of the construct containing the natural HNF4A binding site, while no effects were observed on the expression of the construct containing the mutated HNF4A binding site. Taken together, these results suggest that DGAT1 mediates the effect of HNF4A on TRL secretion via direct interaction of HNF4A with the HNF4A binding site in the DGAT1 promoter.
GENERAL CONCLUSIONS

- INSIG1 is critical for hepatic lipid and glucose homeostasis, while INSIG2 is involved in the regulation of the SREBP pathway in extra-hepatic tissues, influencing adipose tissue metabolism.
- Variation in APOB mRNA expression plays a significant role in determining the secretion of TRLs by the liver and is important for the regulation of the plasma LDL concentration in man.
- The reduced plasma TG concentration observed in patients with MODY1 is due to a decrease in HNF4A-mediated DGAT1 expression.
GENERAL DISCUSSION

Association of INSIG1 and INSIG2 promoter polymorphisms with liver and adipose tissue-related phenotypes

The INSIG1 and INSIG2 proteins are close homologues, which are thought to play complementary roles in the regulation of the SREBP pathway (70). Mice with Cre-mediated disruption of Insig1 or germ-line disruptions of Insig2 appear completely healthy and do not show a specific phenotype. However, double Insig ‘knockout’ mice show markedly increased expression of SREBP target-genes and accumulation of lipids in the liver. Thus, only the combined downregulation of Insig1 and Insig2 in the liver leads to a SREBP-related phenotype, underlining the complementary nature of the two Insigs. It was therefore surprising that the functional promoter polymorphisms in INSIG1 and INSIG2 showed liver and adipose-tissue-related phenotypes, as reported in papers I and II, respectively. This suggests that in humans, in contrast to rodents, the INSIGs do not fulfil complementary roles. This hypothesis was explored in greater detail in study II. It was found that siRNA inhibition of INSIG1, but not INSIG2, was associated with a significant upregulation of the expression of SREBP target-genes in the human hepatoma Huh7 cell-line. However, no evidence was found in these experiments for a compensatory increase in expression of the complementary INSIG gene. Moreover, the effects of INSIG1 siRNA inhibition were already evident after approximately 75 % reduction of INSIG1 mRNA concentration, while the phenotype in the mouse model was only observed following near complete suppression of Insig1 and Insig2 expressions. Taken together, the data suggests that in human tissues, in contrast to mouse cells, the INSIGs do not play complementary roles, but instead, each of the INSIG proteins influences a unique physiological pathway.

The tissue expression studies reported in study II suggest that the INSIG2 concentration is low in human liver as compared to the INSIG1 concentration, indicating that only INSIG1 is of physiological relevance in liver tissue. In contrast, non-hepatic tissues express predominantly INSIG2, albeit with substantial additional expression of INSIG1. These tissue expression studies indicate that the proposed divergent down-stream pathways for INSIG1 and INSIG2 are predominantly of
relevance in extra-hepatic tissues. Unfortunately, little is known regarding the effects of the INSIGs in extra-hepatic tissues. Indeed, no systematic analysis of the SREBP target-genes has thus far been performed in extra-hepatic tissues and it is therefore not clear to what extent the effects of the INSIGs are mediated by the SREBP pathway. Data from adipocyte-specific Srebp1 transgenic mice are of relevance in this respect. Overexpression of constitutively active Srebp1a in mouse adipose tissue resulted in adipocyte hypertrophy (145), increased fatty acid secretion and fatty liver, while overexpression of the nuclear form of Srebp1c brought about the opposite phenotype: lipodystrophy. Although Srebp1c and Srebp1a isoproteins have been shown to activate expression of the same genes in the liver (66), differences might exist regarding their target-genes in adipose tissue. These differences may explain the disparity in observed phenotypes. It is therefore tempting to speculate that the INSIGs exhibit isoform-specific association with the SREBPs, possibly mediated by tissue-specific cofactors, accounting for the differential effects of the INSIGs in adipose tissue. Clearly, further studies are required to resolve this issue.

**INSIG1 and glucose metabolism**

INSIG1 was originally discovered as a hepatic gene that was markedly upregulated following exposure to insulin, indicating a role of INSIG1 in glucose metabolism. It is therefore hardly surprising that the -169C>T polymorphism in the INSIG1 promoter is associated with fasting plasma glucose concentration (study I). No relationship with the plasma insulin concentration was found, suggesting that INSIG1 is involved in the regulation of gluconeogenesis. More detailed analysis revealed that INSIG1 siRNA inhibition leads to decreased expression of PCK2, a rate-limiting enzyme of gluconeogenesis regulated by SREBPs (146). Based on these observations, it is proposed that the -169C>T polymorphism influences the transcription of INSIG1, thereby regulating the concentration of the INSIG1 protein, in turn leading to changes in expression and protein concentration of PCK2. Finally, PCK2 influences de novo glucose production and secretion by the liver. In agreement with this hypothesis, it was recently reported that the minor alleles of three polymorphisms in SREBP1 are associated with increased risk of T2D, with raised
plasma glucose concentration in both the diabetic and control groups and with elevated plasma insulin concentration in persons with T2D (147).

**INSIG2 and body mass**

The previously reported association of the rs7566605 polymorphism upstream of the INSIG2 gene with increased BMI in obese people and that of the -102G>A SNP in the INSIG2 promoter described by us suggests some role for INSIG2 in human adipocyte metabolism. One possible explanation for an effect of the INSIG2 promoter polymorphism on BMI is that the -102G>A polymorphism interferes with INSIG2 transcription, which seems to be of considerable importance in the process of preadipocyte differentiation into adipocytes. As shown in study II, Insig2 expression is dramatically enhanced in the course of mouse preadipocyte 3T3-L1 differentiation and it is only paralleled by a modest increase in the expression of Insig1. However, it is not known whether Insig2 plays a significant functional role in the process of differentiation or is just a differentiation marker.

As discussed above, it is possible that the SREBP1a and SREBP1c isoforms have specific modes of transcriptional activation of target-genes in adipose-tissue and it is conceivable that INSIG1 and INSIG2 may be involved in these processes. It is, for example, possible that INSIG2 is primarily involved in the action of the SREBP1c isoform in adipose tissue. In this scenario it can be expected that reduced transcriptional activity of INSIG2 will decrease INSIG2 protein concentration, leading to reduced activation of SREBP target-genes, ultimately leading to a reduction in BMI. The data presented in study II are compatible with this hypothesis. Nevertheless, identification of the SREBP1a and SREBP1c target-genes in adipose tissue will help to test this hypothesis.

**INSIGs do not regulate plasma lipoprotein concentrations in man**

The SREBP pathway is of critical importance for the regulation of the expression of genes involved in lipid and glucose metabolism. Several SREBP target-genes are directly or indirectly involved in processes regulating plasma lipoprotein
concentrations, and it has therefore been generally assumed that perturbations of the SREBP pathway will promote dyslipoproteinemia. Accordingly, variations in the expression of INSG1 and INSIG2, major regulators of the SREBP pathway, would be expected to influence the plasma lipoprotein concentrations in man. However, as shown in study I and study II, no evidence was found for associations between the functional promoter polymorphisms in INSIG1 and INSIG2 and plasma lipoprotein concentrations. Needless to say, this apparently surprising observation could be due to insufficient power to detect a small impact of these polymorphisms on the plasma lipoprotein concentrations. Alternatively, it is conceivable that the SREBP pathway primarily influences intracellular lipid metabolism. The extensive data from knockout and transgenic mice appears to be in line with this hypothesis. For example, mice with Cre-mediated disruption of Insig1 and germ-line disruptions of Insig2 overaccumulated cholesterol and triglycerides in the liver, but exhibited no significant changes in plasma lipoprotein pattern. Nevertheless, the expression of genes involved in TG and cholesterol synthesis were markedly increased in the double knock-out mice (70). Comparable changes in the expression of SREBP target-genes were also observed in other mouse models, like Srebp1a (148), Srebp2 (149) and Insig1 (150) transgenic mice and Srebp1 knockout mice (151), again without consistent alterations in the plasma lipoprotein concentrations. However, liver specific Cre Slp (152) and Scap (153) knockout mice showed substantial reductions in plasma cholesterol and triglyceride concentrations, consistent with a significant role of the SREBP pathway in the regulation of the plasma lipoprotein concentrations.

It was proposed by Horton et al. (66) that the apparent lack of the effect of Srebp gene overexpression on plasma TG and cholesterol concentrations is due to a simultaneous increase in the expression of the LDL receptor, which leads to augmented intracellular degradation of pre-formed VLDL and LDL uptake. This view is supported by the observation that transgenic overexpression of Srebp1a in the Ldrl-deficient mice leads to an almost ten-fold increase in plasma cholesterol and TG concentrations (154). Thus, the proposed increased secretion of TRLs induced by Srebp1a overexpression is counteracted by enhanced degradation, ultimately leading to only marginal changes in plasma cholesterol and TG concentrations. It noteworthy in this context that population studies indicate that polymorphisms in SREBP1 and SREBP2 are only associated with plasma lipid concentrations in subjects with familial hypercholesterolemia or polygenic hypercholesterolemia (155-158). However, it
should be emphasized that the results of multiple knockout/transgene mouse studies must be interpreted with caution. Indeed, preliminary studies of INSIG1 siRNA inhibition in Huh7 cells provided no evidence for an increased rate of secretion of TRLs (Krapivner, unpublished observation). In summary, results from our own studies and the data in the literature indicate that the variations in the expression of INSIGs will not have a major impact on plasma lipoprotein concentrations in man.

**In vitro model system to study TRL secretion**

The secretion of TRLs by hepatocytes or hepatoma cells is usually quantified using two parameters: 1) TG, representing the predominant lipid component of TRLs, and 2) APOB, the major structural protein of the TRLs. The conventional way of measuring the rate of TG secretion is by addition of radioactive fatty acyl-CoA (typically oleoyl- or palmitoyl-acyl-CoA) to the cell medium and subsequent monitoring of the accumulation of the tracer in the TGs in the medium. Unfortunately, fatty acyl-CoAs are utilized by cells for the synthesis of a large array of products, each with their own metabolism, a phenomenon which complicates the interpretation of fatty acyl-CoA tracer data. One way to simplify the interpretation of fatty acyl-CoA tracer data is by introducing a pulse-chase procedure, thereby exposing the cells for a limited period of time to the radioactive fatty acyl-CoA. However, there are several caveats with this approach, including the increased complexity of the procedure and the need for careful timing of the different steps of the experiment.

The rate of secretion of APOB by hepatoma cells is conventionally monitored by an APOB immunoprecipitation procedure, followed by Western blot analysis. This method is time-consuming and tedious given the size and hydrophobicity of APOB. However, the major advantage of this procedure is that the two APOB isoproteins, APOB100 and APOB48, can be quantified separately. This requirement is essential for the analysis of secretion of TRLs by rodent hepatocytes, since these cells secrete both APOB100 and APOB48 TRLs. In contrast, human hepatocytes and hepatoma cells only secrete APOB100 TRLs, which makes the Western blot method somewhat redundant. Nevertheless, the same procedure can be used for the analysis of intracellular APOB synthesis and TRL assembly, which explains the popularity of this
method for the analysis TRL synthesis in HepG2 cells. Unfortunately, we were not able to establish a similar Western blot method in Huh7 cells.

In this thesis a simple and robust in vitro system for the analysis of secretion of TRLs was devised, using a combination of three important components. First, human hepatoma cell-lines were employed, ensuring that only APOB100 containing TRLs were secreted by the cells. Second, radioactive glycerol instead of fatty acyl-CoA was used as lipid tracer. Glycerol enters the cell at a very slow rate and is incorporated almost exclusively in TGs. These features ensure a linear increase in C\textsuperscript{14}-glycerol labeled TGs secreted by the Huh7 cells over an extended time-period. Thirdly, conditions for the APOB ELISA assay were optimized to measure specifically human APOB secreted by the Huh7 cells in the presence of bovine APOB that is present in the FBS added to the cell-culture medium. Under the conditions employed in our studies, a linear increase in C\textsuperscript{14}-TG and human APOB protein was observed over a 24-hour period. Thus, all of these modifications combined made it possible to study the rate of secretion of TRLs under various experimental conditions using a simple experimental procedure.

Three critical features of the TRL secretion system employed in this thesis were analyzed in greater detail. First, the question was raised to what extent the APOB and the labeled TG are components of TRLs. Using molecular sieve chromatography it was found that all APOB and labeled TG secreted by the cells was associated with molecular complexes in the VLDL-LDL size range, presumably representing TRLs. Second, the issue of possible re-uptake of TRLs secreted by the Huh7 cells was addressed. An ingenious experimental design, outlined in Figure 11, was devised to measure re-uptake. However, no significant re-uptake of TRLs was detected under various experimental conditions. Thirdly, the question was raised to what extent the C\textsuperscript{14}-glycerol labeled lipids secreted by the Huh7 cells are hydrolyzed (or in any other way modified) during the subsequent sojourn in the cell-medium. It is, for example, possible that the lipases secreted by the Huh7 cells convert the labeled TG to mono- or di-glycerides, leading to an underestimation of the rate of secretion of TG by the cells. However, in long-term incubation experiments, no evidence was found for significant changes in the composition of the labeled TRLs secreted by the cells.
Role of transcriptional regulation of APOB for the regulation of plasma lipoprotein metabolism

In study III the question was addressed to what extent inter-individual variation in APOB transcription influences secretion of TRLs and the plasma LDL-cholesterol concentration. Older studies, conducted in African green monkeys (34), rabbits (159) and baboons (160) suggested that the hepatic APOB mRNA concentration is refractory to nutritional challenges and does not correlate with the rate of secretion of TRLs. These studies argued against a regulatory role of APOB transcription in the secretion of TRLs. However, more recent studies (161, 162) provide opposite results.

In this thesis the effects of variation in hepatic APOB transcription on lipoprotein metabolism was analysed using two complementary approaches. First, the relationship between the inter-individual variation in APOB transcription in human liver samples and the plasma LDL concentration was analyzed. A reliable real-time Taqman procedure was developed to measure accurately small variation in APOB mRNA concentrations. A significant relationship between hepatic APOB mRNA concentration and the plasma LDL concentration was observed.

In the second approach, siRNA-mediated silencing of APOB was used as an in vitro model for the analysis of the effect of variation in APOB mRNA concentration on the secretion of TRLs. Eight APOB siRNAs, with silencing capacities ranges from <20 % to >80 %, were used to introduce variable reductions in APOB mRNA level in human hepatoma Huh7 cells. A positive relationship between the APOB mRNA concentration and the secretion of TRLs was observed. In summary, two different human model systems demonstrated that the hepatic APOB mRNA level influences the secretion of TRLs by the liver and shows positive relationships with the plasma LDL-cholesterol concentrations.

The data presented in study III appears to contradict the paradigm that TG availability in the endoplasmic reticulum (ER) dictates the amount of APOB that escapes proteosomal degradation. This conventional model postulates that APOB is synthesized in excess compared to the TG available for incorporation in TRLs. In this scenario, variation in APOB mRNA level is not expected to affect the rate of TRL secretion. However, several recent studies have provided evidence that marked changes in APOB expression are associated with variation in secretion of TRLs and/or
are related to changes in the plasma LDL-cholesterol concentration (113-117). It can be argued that these profound changes in APOB mRNA level create unphysiological conditions under which the availability of APOB becomes rate-limiting in the secretion of TRLs. However, study III demonstrates that TRL secretion is sensitive to moderate changes in APOB mRNA level, underlying the physiological nature of this regulatory mechanism. Moreover, variations in the secretion of APOB were mirrored by variations in the secretion of TG. These observations suggest the existence of a regulatory mechanism that coordinates the incorporation of APOB and lipids (principally TG) in nascent TRL particles secreted by the liver, as outlined in figure 16.

**Figure 16** Hypothetical site of coordination of APOB and TG synthesis

TG-translocation involves the transfer of cytosolic TG to the incipient VLDL. It is proposed that the site of TG-translocation is co-localised with the site where APOB is associated with the inner leaflet of the ER membrane. The hypothetical regulatory system coordinating APOB and TG synthesis presumably acts at or near this junction of APOB translation and TG translocation.

The data presented in study III indicates that the availability of APOB plays an important role in this process. Nevertheless, the data from oleate-stimulated cells in study III and the analysis of the effect of DGAT1 on the secretion of TRLs reported in study IV indicate that the availability of lipids is of equal importance in this regulatory process. Apparently, this regulatory mechanism directs and coordinates the
interaction between the supplies of APOB and lipids for the synthesis of TRL particles.

Numerous studies have demonstrated that addition of oleic acid to liver cells increases the rate of secretion of TRLs. It has generally been assumed that the increased availability of fatty acids is the principal driving force for this action of oleic acid. However, it was recently shown that oleic acid decreases the activity of the MEK-ERK pathway, thereby increasing the activities of the DGAT enzymes and ultimately leading to enhanced TG synthesis (163). It is therefore possible that the MEK-ERK pathway is primarily responsible for the observed effect of oleic acid on TG synthesis. In addition, it was found in study III that treatment of Huh7 cells with oleic acid does not override the sensitivity of TRL secretion to variation in APOB transcription. This indicates that the availability of TG is not the primary regulatory signal for TRL secretion and suggests that the action of oleic acid does not interfere directly with the hypothetical regulatory mechanism coordinating APOB and TG synthesis.

**Role of DGAT1 in the regulation of TRL secretion**

In study IV, reciprocal effects of transient HNF4A knock-down and overexpression were observed on the secretion of TRLs by human hepatoma Huh7 cells. Gene expression profiling revealed that several genes previously reported as HNF4A targets (119, 132) were downregulated by HNF4A siRNA knock-down. The most prominent decreases in mRNA levels were observed for SOAT2 and DGAT1, genes encoding two enzymes involved in lipid metabolism. SOAT2- and DGAT1-specific siRNA knock-down experiments demonstrated that DGAT1 mediated the effect of HNF4A on secretion of TRLs. Analysis of DGAT1 identified a sequence closely resembling the consensus HNF4A binding site. Functional tests, such as EMSA and transient transfection studies, provided further support for the conclusion that the sequence identified in the DGAT1 promoter is, indeed, an HNF4A binding site. Furthermore, it was shown that the enzyme activity of DGAT1 was markedly reduced in response to HNF4A siRNA treatment of Huh7 cells. Taken together, these findings suggest that DGAT1 expression is regulated by HNF4A via a direct interaction with the HNF4A-binding site in the DGAT1 promoter.
Whether DGAT1 is involved in the regulation of TRL secretion remains controversial. Dgat1-knockout mice have normal plasma triglyceride concentrations on low-fat as well as high-fat diets (164), (165) indicating that Dgat1 is not involved in the regulation of the hepatic secretion of TRLs. Nevertheless, Dgat1-knockout mice exhibit reduced hepatic triglyceride content (164, 165) and disturbed chylomicron secretion (166). Moreover, overexpression of human DGAT1 in McA-Rh7777 rat hepatoma cells (167) and long-term overexpression of Dgat1 in mice (168, 169) increases the secretion of TRLs. We therefore evaluated the putative roles of HNF4A and DGAT1 in triglyceride metabolism by genetic association studies in human cohorts (Krapivner, unpublished observation). It was found that the rs2144908 polymorphism, located in the vicinity of the P2 promoter of HNF4A (approximately 40 kb upstream from the P1 promoter), had a significant relationship with BMI and a related parameter, waist circumference, as was previously reported (124). Moreover a trend toward decreased plasma TG concentrations was observed in carriers of the minor rs2144908 allele, but this effect did not reach the conventional level of statistical significance. In summary, our data support a role for DGAT1 in the regulation of the plasma triglyceride concentration in man, suggesting that modulation of DGAT1 activity may be beneficial for the treatment of dyslipidaemia (166).
ACKNOWLEDGEMENTS

This work is dedicated to my family; but I must be forgiven for having my own superstitions about public declarations of love to nearest and dearest.

I was born in between the east and the west. Then I graduated and dropped in between basic science and medicine. Some think that the most interesting things happen on this in-between-territory. This section is dedicated to all the people who made me believe so.

To my supervisors, co-authors and senior researchers at the Atherosclerosis Research Unit:

To Ferdinand M. van’t Hooft: My father’s acknowledgements to the main supervisor of his PhD thesis read: “Since I have never seen you, but always felt your presence in everything around me, your part in my thesis might be called truly divine”. During my first days in the lab I was glad to realize that I am in no way writing something like that about you. Your physical and spiritual presence in all my projects is undeniable and perhaps difficult to hide even if I wanted to. From the beginning you have been my dedicated coach (a Dutch coach is not a joke, as Russian football players say) who helped me to get a sense of reality in this new world where I found myself. From the beginning, you proved to be a decent man and I always enjoyed your support.

To Anders Hamsten: Without your support I, like all other PhD students from the Unit, would be in great trouble. Thanks to you my thesis took its final shape. Your immense scientific scope and skills as a reviewer helped us a lot in writing manuscripts. Like the US in the Second World War you joined the main action (my thesis) in the final stage, but unlike the Americans your participation was decisive and you have not claimed all the credits for the victory (the completion of my PhD). Thank you for placing some hopes in me and for enduring my presence in the lab for so long.

To Per Eriksson: We are both exceptional guys. Me, because I’m one of very few PhD students in the Atherosclerosis Research Unit who did not have you as an official co-supervisor. You, because you are a prominent scientist and a charming person, which is a rare combination. All these years it was so comforting to realize that I was
working with a man of immense and diverse scientific competence who also has a magic touch solving administrative problems.

To Ewa Ehrenborg You were the first person I contacted at KI. You kindly endured my bad English and decided that I was worthy to be introduced to the other GV members. That was quite a while ago and ever since then you have proved to be a generous collaborator always ready to share your knowledge and reagents. Good luck to you and your PhD students.

To Rachel Fisher: During my time in the group we had many occasions to talk both life and science. In English intelligent means just smart, in Russian, intelligent is a title, comprising a whole complex of qualities. For me the most important of them is the sincerity and ability to listen and hear. In this respect you are a Russian intelligent. Thank you for revising my papers, for all your support and your love of good literature.

To Angela Silveira Champion of the world in organizing is how you positioned yourself once upon a time. I don’t know about world championships, but the way you have organized and run our scientific meetings has been very stimulating for my brain. Moreover without you I would have spent so much more time and energy trying to get around some practical biochemical issues that you solved so easily.

To Mai-Lis Hellenius You have been our generous and charming collaborator who also helped me with some of my personal problems. Thank you very much for that.

To the power plant of biochemistry and molecular biology Alexandro Bertorello Your presence in the lab has been an inspiration to me. Your enthusiasm for science is contagious and you willingness to give thought to scientific matters sometimes very remote from your own field of interest is admirable. Thank you for sharing with me your expertise and for taking on as a PhD student my friend and namesake Sergey Popov.

To those who helped me keep my integrity in Sweden:

Nonpolar molecules in aqueous solution create hydrophobic interactions, the same happens with countrymen when they meet far away from home. The foreign environment is a strong driving force bringing them together. This section is dedicated to my Russian and Russian-speaking friends:

To my dear friend Sergey Antipov: If the saying is true and we have the kind of friends that we deserve, than I am proud of myself. There is nothing new that I can
say to you, you know everything. If you, big guy, did not exist someone should have made you up. Keep growing but take it easy, I think sometimes you try to push up such a weight that even your mighty back might snap. Thank you for not forgetting about my parents in Moscow, God bless you and you dearest and nearest.

To Alexey Shemyakin, one of the last St. Petersburg aristocrats: Although our long insane intercourses have been hard on our brains and livers, every time one of us got too intoxicated by this intense spiritual exchange, friends were around to take care of our bodies. Let our wishes coincide with our desires (Alexey Shemyakin unpublished) and God speed with your personal developments, medical or scientific.

To Igor Bazov, the chairman of the club of extraordinary gentlemen: You are a guy in charge of a parallel reality so it is hard for you to feel solid ground under your feet in this world. As a member of your club I wish you to find your way on the map, whichever map you choose. The show must go on.

To Sergey Popov soon Shigi Tamae- my Russian/Swedish/Japanese buddy: You are back to square one my young friend and I am glad. After the successful completion of your PhD you will return to the East full of happy memories and with you liver much bigger than it used to be. Friends will be friends.

To Valeria Golozubova: When I came to Sweden, my thirst for meeting new people and learning from them was for quite a while satisfied by you and Krissi alone. You are intelligent, strong and funny. I know that whatever happens you will always retain two of these qualities but keep up your strength as well. God bless you and your polylingual son Vladimir.

To Dr. Krissi, Krismundur Sigmundsson: Your spirit has been touched by the magic of Iceland. I’ve been there, it is too much beauty for a man to take. We have not seen each other for quite a while but your Egil’s saga still lives within me. I have always been inspired by your dedication to science and your dissertation party was the wildest and the funniest I’ve been to. I wish you to find the right turn at the crossroad.

To a devoted apologist of Great Latvia my Australian friend doctor Aigars Rubulis: Your brutal laughter, your sergeant jokes, your big heart, bright head and light spirit are missed here. I sincerely hope that in Australia all of it is as appreciated as it was here. In the end a guy who is a double doctor and lion/tiger by horoscope can make his way everywhere.
Working in a pretty multinational environment of Karolinska Institutet I also made friends with some of my foreign colleagues. This friendship is precious for me and I want to express my thanks to:

**Justo Sierra Jonson**, a Mexican in a suit: Some people get born rich, some poor. You and me were born rich because we have friends all over the world. Wherever you are I will remember all the fun that we had, your lessons in tequila drinking and will try not to shame my teacher. You were one of very few people with whom I talked about most important things in English. It was too cold for you in this northern country so you went over the ocean, but things seem to have worked out so far. So they should henceforth. I am not saying goodbye.

**Vincent Fontain**: Paris or London, Milan or Madrid, the life of a postdoc is a gypsy’s life, but your Russian friend will remember you. Hopefully he belongs to the good part of the memories that you take away from Stockholm because you definitely belong to the good part of his. Apart from memories I hope not to lose contact with you wherever we are.

**Valentina Paloschi**: Three guys, whose presence in Stockholm on October the 24th would please me so much, were snatched away by the cities of Indianapolis, Melbourne and Paris. But there is a consolation: someone from Italy, who we missed a lot, has made up her mind to start a PhD here. This girl is coming right in time to support me on that very important day. It is great that you are back.

**Massimiliano Ria**: Some time ago we started as fellow PhD students with the same supervisor. Since then many things have happened, but we always stayed friends, who had a lot to share with each other, talking life and science, past and future. Maybe soon we are going to work at the same institution again? All the best to Ilaria and Linnea.

**Maria Jesus Iglesias**: In its sleep my computer still gets delusional that it is Rocky Balboa. Nevertheless I have been happy to share the desk space with you and to be occasionally involved in your Spanish-speaking club and all the fun that comes with it. You are an amazing person, gorgeous woman and a cordial friend. God bless you and little Santiago.

**Karl Gertow** who is coming back: I owe my first and very bright and positive impressions about Sweden to the beauty of Stockholm and to some Swedes I met during my early days here. You, my friend, are one of them. Your sense of humour and our beer clubs is something that I have missed ever since you left for Italy.
Many of my colleagues contributed to this thesis in different ways and I owe my thanks to:

**Dick Wågsäter**: Thank you for our joint social activities and drinks and talks about very important things.

**Josefin Skogsberg**: You helped me so many times that I do not know if I can ever repay you. Your smile and your kindness made my days in the lab so full of sun even despite the winter darkness. Thank you for helping me to love Sweden.

**Maria Kolak**: Thank you for the comfort of working with you in the same lab. You have been one of my most warm-hearted and helpful colleagues and I always feel like speaking Russian to you, I don’t know why. Soon you will be finishing your PhD so be strong.

**Maria Nastase Mannila**: Thank you for being such an angel all the time that we shared doktorandrummet at GV and for helping me with Linkage Analysis. Good luck with your own projects for science and medicine.

**Alexander Kovacs** a guy who left the shallows of science for the deep ocean of investment business: It is difficult to explain, but I recognized you as my own kind quite soon after we made acquaintance. We never worked side by side, but had many chances to discuss issues that require mutual trust. Beware of the sharks in your deep waters and good luck with everything.

**Per Sjögren**: It was so nice to have a beer and to talk to you from time to time and even carrying freezers together from one floor to the other was fun. Good luck with your research in Uppsala.

**Kristina Eneling** and **Karin Stenström**: You have been the driving force of many theatrical activities in the Unit. The time any foreigner spends doing their PhD can be estimated by how good he/she becomes in singing at our yearly Lucia celebrations. Thank God I will not become a real pro. A part from always being friendly and fun to talk to, you also shared with me your expertise in Western blots. Thank you for that and all the best to you and your little ones.

**Petra Thulin, Anders Målarstig, Anna Aminoff**: I have been glad to have you as my room mates and collaborators to discuss ups and downs of academic life. Good luck with your own projects.

**Kerstin Lundberg**: Thank you for being so knowledgeable, modest and friendly, your advices on some aspects of molecular cloning were very helpful.
Barbro Burt: I fail to imagine just how many things would have gone wrong in the lab if you had not been around. There is so much that we take for granted, but in fact there are people who make it work. Thank you so much for helping me with my lab work and for being friendly even when I occasionally messed things up

Karin Husman, Karin Danell-Toverud, Birgitta Söderholm, Fariba Forough.
Thank you for doing so excellently your job preparing sample sets, keeping in order the reagents, creating databases and everything that makes efficient lab work possible, thank you for always being ready to help.

Peri Noori: It was great to have our Russian-speaking tea clubs. It was great to work close to you. Thank you for the superb quality of the samples you prepared for us.

Shohreh Maleki: A stout fighter and a wise woman who honoured me sharing her thoughts, her hopes, frustrations and doubts and also her political views. It influenced my way of thinking and I am grateful for that.

To those who ensure the functioning of the Atherosclerosis Research Unit:
To Ami Björkholm: Thank you for being professional, doing your job that is so essential for the whole of the Atherosclerosis Research Unit. You easily solve issues that otherwise would become problems for me and the others
To Camilla Berg: Thank you for your competence and kindness. A part from being most efficient at taking care of the logistics of PhD education, you are such a charming person to talk to.

Magnus Mossfeldt: Thank you for quickly solving my IT issues.

Administrative staff: Caroline Hamilton, Malin Toverud, Karin Blomberg, Helena Öhgren, Christina Hadders-Medin, Annetty Jansson. Thank you for being good and reliable professionals.
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