Genes affecting triglyceride metabolism: from steatosis to lipodystrophy

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Genes affecting triglyceride metabolism

from steatosis to lipodystrophy

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Pictures  front: from left to right, molecular structure of triglyceride, microscopic picture of adipose tissue, steatotic liver section with HE staining and MRI of a human subject with lipodystrophy and excess subcutaneous fat.

Back: Sunset at Cap Nègre, Le Lavandou, France

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Genes affecting triglyceride metabolism
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## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>General introduction</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Gene expression in atherogenesis</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>The apolipoprotein L gene cluster has emerged recently in evolution and is expressed in human vascular tissue.</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Apolipoprotein L2 and L3 differentially modulate intracellular triglyceride storage.</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>Lipase H, a new member of the triglyceride lipase family synthesized by the intestine.</td>
<td>73</td>
</tr>
<tr>
<td>6</td>
<td>Familial partial lipodystrophy phenotype resulting from a single-base mutation in deoxyribonucleic acid-binding domain of peroxisome proliferator-activated receptor-gamma.</td>
<td>85</td>
</tr>
<tr>
<td>7</td>
<td>Inherited lipodystrophies and the metabolic syndrome.</td>
<td>99</td>
</tr>
<tr>
<td>8</td>
<td>Discussion</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Samenvatting</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Dankwoord</td>
<td>133</td>
</tr>
</tbody>
</table>
Introduction
Atherosclerosis

Atherosclerosis is the underlying cause of major cardiovascular diseases such as myocardial infarction, peripheral arterial disease and stroke. Atherosclerosis is a chronic and complex process that involves an inflammatory response in the vessel wall induced by lipid particles such as oxidized Low Density Lipoproteins (ox-LDL). The crosstalk between inflammation and lipid metabolism is the hallmark of this process. Atherosclerotic lesions themselves are the best example of this crosstalk. Fatty streaks, that are prevalent in young adults, are focal lesions in the inner layer of the vascular wall, called neo-intima, and contain lipid-laden macrophages. In time, these apparently innocent fatty streaks evolve to the full-blown atherosclerotic lesions that are composed of debris, foam cells, activated smooth muscle cells and many inflammatory cells such as T-cells, dendritic cells and mast cells (1). To understand the exact pathophysiology of atherosclerosis, we need to define how these two processes are coupled. Figure 1 depicts that inflammation is the basis of atherosclerosis and that lipid metabolism is closely linked to these processes. The role of LDL-cholesterol in atherosclerosis has been extensively studied and has been thoroughly reviewed in a number of perspectives (2-6). The impact of hypertriglyceridemia on atherosclerosis has not been appreciated as much until recently. More and more observational and interventional studies are starting to unravel the fact that high triglyceride (TG) is an independent risk factor for cardiovascular disease (7,8). Here, I will focus on the role of TG metabolism and the genes involved in this process. Activation of nuclear transcription factors such as PPARγ and the subsequent transcription of genes involved in TG metabolism could affect lipid content of tissues as well as plasma lipids. On the other hand, liver steatosis could induce insulin resistance and the subsequent increased Very Low Density Lipoproteins (VLDL) secretion and increased plasma TG levels. In the following paragraphs each of the players in this metabolic triangle will be discussed (figure 1).

Figure 1. Atherosclerosis is an inflammatory disease of the vessel wall. The role of lipid metabolism in inflammation and atherosclerosis is depicted. Nuclear transcription factors control the expression of many genes in lipid metabolism and are involved in pathologies like obesity and lipodystrophies. These metabolic pathways in turn affect the inflammatory response and the chronic process of atherosclerosis.
Apolipoproteins and Lipases

To understand the cross-talk between inflammation and lipid metabolism, it is crucial to understand how lipids are formed, transported and metabolized in the body. Lipids are essential for living organisms, not only as a source of energy but they are also involved in many other processes. In summary: inside the cell (i.e. transcription activation, lipid storage and breakdown), at the level of cell membrane (the membrane itself and anchoring the receptors for signal transduction, endocytosis and exocytosis), outside the cells (hormones and inflammatory agents such as leukotriens). The proteins that are associated with the lipid particles in the plasma are called apolipoproteins. The major apolipoproteins are apolipoprotein A1 on High Density Lipoproteins (HDL) particles and apolipoprotein B on LDL particles.

In the fed state, chylomicrons are formed in the gut and are secreted into the plasma and distribute the lipids to the peripheral tissues. After removal of lipids from the core of chylomicrons, the so-called remnant particles are then taken up by the liver and metabolized. In the fasting state, VLDL particles are produced by the liver to ensure supply of lipids to peripheral tissues (figure 2). These particles are metabolized in the plasma and, as they drop their cargo, become smaller and form LDL particles. There is substantial evidence that high LDL-cholesterol is associated with cardiovascular disease, and the causal role of LDL-cholesterol in the pathogenesis of atherosclerosis has been proven beyond doubt (2-6). The common concept is that LDL particles are modified in the circulation and become oxidized. These ox-LDL particles are then taken up by the monocytes and activate these cells. Lipid-laden macrophages (foam cells) are then formed and can recruit more inflammatory cells. The result is an inflamed vascular wall at risk of rupture and complete occlusion. Therefore, LDL particles are called atherogenic particles.

The small and dense HDL particles are produced by the gut and liver. The nascent HDL particles can obtain cholesterol from the peripheral tissue and transport it to other tissues. The transport of HDL-cholesterol to the liver is called “reverse cholesterol transport” and has been suggested to be very important in the removal of cholesterol from the arterial wall and thereby reducing atherosclerotic burden (9). Therefore, HDL particles are thought to be anti-atherogenic. But in the past decade it has become clear that the anti-atherogenic effect of HDL is not only by promoting reverse cholesterol transport but also by inhibiting oxidation of phospholipids within LDL and downregulating associated inflammatory cytokines and vascular cell-adhesion molecules (10). HDL can lose its protective capacity and even become proinflammatory in the setting of systemic inflammation (11). Conditions that have been associated with proinflammatory or dysfunctional HDL include coronary heart disease (CHD) (12), metabolic syndrome (13), chronic kidney disease (14), obstructive sleep apnea (15), infections (16) and some rheumatologic diseases (17). Apolipoproteins associated with HDL are essential in these processes and are shown to be modified in patients with coronary artery disease (CAD). Vaisar et al. elegantly showed that the composition of HDL, isolated from healthy subjects and subjects with CAD, was substantially different (18). To disentangle
the anti-atherogenic properties of HDL particles, we need to understand the function of each of these proteins.

Other proteins that interact with lipoprotein particles are the enzymes that are located at the inner-surface of vessel walls, i.e. on the endothelial cells. Lipoprotein Lipase (LPL) is one of the best studied proteins but our knowledge of other lipase such as Hepatic Lipase (HL) and Endothelial Lipase (EL) is growing. LPL is involved in hydrolysis of TGs and as a result, free fatty acids (FFAs) are formed. FFAs are then taken up by other cells and are either used in $\beta$-oxidation in peripheral cells (i.e. myocytes) or re-esterified to TGs again in adipocytes. There is a complex fine tuning of this process where LPL could be stimulated by other proteins such as apolipoprotein CII and inhibited by others such as apolipoprotein CIII.

**Figure 2.** The exogenous and endogenous lipoprotein metabolic pathways. The exogenous pathway transports dietary lipids to the periphery and the liver. The endogenous pathway transports hepatic lipids to the periphery. 

**Legend:**
- **LPL**: lipoprotein lipase
- **FFA**: free fatty acid
- **VLDL**: very low density lipoprotein
- **IDL**: intermediate-density lipoprotein
- **LDL**: low-density lipoprotein
- **LDLR**: low-density lipoprotein receptor
It is known that infection and inflammation induce the acute-phase response, leading to multiple alterations in lipid- and lipoprotein metabolism. Plasma TG levels increase due to increased VLDL secretion, as a result of adipose tissue lipolysis, and decreased VLDL clearance, secondary to decreased lipoprotein lipase activity. In human sepsis, plasma TGs are increased, phospholipids are maintained at near normal levels, while total cholesterol is decreased (19). It is not only the lipids that are altered during inflammation but the protein composition of particles is modified as well. During inflammation, HDL drops dramatically mainly because of a decrease in large apo A1-containing HDL particles, an almost total loss of apoC-I, and an increase in apoE-HDL (20).

Whereas inflammation causes a massive change in lipid metabolism, the same holds true for the opposite direction; lipids and lipoproteins do affect inflammatory responses. Apo A1, which is the major component of HDL, was shown to inhibit the lipopolysaccharide (LPS)-induced release of cytokines by monocytes (21). ApoAl was also reported to bind LPS and to protect mice against LPS-induced mortality (22). Not only apolipoproteins, but also LPL, can modulate inflammatory response. For instance, treatment of endothelial cells by exogenous LPL could attenuate the tumor necrosis factor α (TNF-α) induced expression of VCAM1 on endothelial cells (23).

From the abovementioned details on the crosstalk between inflammation and lipid metabolism, it must be clear that inflammatory responsive apolipoprotein are of interest to resolve the remaining peaces of the puzzles on TG metabolism and inflammation.

### Extracellular TG metabolism

Triglyceride is an efficient energy storage molecule, because its hydrophobicity allows tight packing into droplets without rigidity. Consequently, the energy to mass ratio of TG is at least 10 times that of hydrated carbohydrates or proteins (24). Whereas the ability to store intracellular TG is conserved from *Saccharomyces cerevisiae* to humans, the genes involved in this delicate system of energy storage have been modified during the course of evolution to adjust to environmental demands (25). Moreover, the ability of secreting TG-rich particles is a phenomenon specific for multicellular organisms to ensure energy supply for the rest of body that is obviously not an issue for a single cell organism. This is also the reason that the superfamily of large lipid transfer proteins (LLTP) has been identified in most animals, in vertebrates as well as invertebrates but not in single cell eukaryotes. The LLTP protein family includes apolipoprotein B, insect apolipoprotein (apolipophorin- II/I), vitellogenin (Vtg), and microsomal triglyceride transfer protein (MTP) (26). These major LLTP genes arose in the earliest animals, suggesting that the creation of this genetic module coincided with animal multicellularity (27).

The pool of TG-rich particles in human plasma are from two separate sources; the so-called endogenous and exogenous pathway (figure 2). The endogenous pathway refers to the continuous production of TG-rich VLDL particles by the liver. This ensures that there is a constant supply of energy to other tissues. The exogenous pathway is the production of
chylomicrons by the gut after a meal. The TGs from the exogenous pathway are mainly used for storage in adipocytes but are also used, although to a lesser extent, by other tissues. The energy balance is dictated by different organs in the body and by the interrelations among them. Food intake, energy storage and energy expenditure is well orchestrated in normal situation. Many genes are involved in this process that influence appetite and food intake, TG breakdown, β-oxidation and ATP formation or for storage in the adipose tissue. Appetite is the first step in setting this interplay in motion. During the past few years many encouraging findings have been reported about the role of hypothalamus as the control center of food intake. The gastrointestinal tract is the organ responsible not only for food digestion but also transmitting signals to the brain through hormones. The role of these hormones in satiety and glucose homeostasis has been extensively reviewed elsewhere (28;29).

In the intestine, TGs are split into glycerol and FFAs by pancreatic lipase. These lipids are taken-up by the enterocytes and the TGs are rebuilt and packaged together with cholesterol and proteins to form chylomicrons. These large particles are excreted from the cells and, via the lymphatic system, transported directly to the heart where they are mixed into the blood and distributed to other organs. TGs are then hydrolyzed and taken up by cells as a source of energy. Lipoprotein lipase (LPL) is the rate limiting enzyme for hydrolysis of TGs in the circulation and is mainly expressed by myocytes and adipocytes. LPL is then translocated to the endothelial luminal surface where it can hydrolyse TG. This ensures that TG is hydrolyzed exactly where it is needed, i.e. muscles for energy expenditure and adipose tissue for storage in lipid-droplets. Due to western life style the balance in this delicate system, that has evolved to store energy for the time of famine, is distorted. Because of excess energy input compared to energy output, TGs are stored in adipose tissue as well as non-adipose tissue resulting in diseases such as obesity and non-alcoholic fatty liver disease (NAFLD). Obesity can be viewed as an energy balance disorder, arising when energy input exceeds energy output (29).

**Intracellular TG metabolism**

Once synthesized from FFAs, intracellular TG is processed in three different ways depending on the cell type and the nutritional state. It is either stored in lipid droplets in specialized cells (adipocytes) or transported into plasma as lipoprotein particles by hepatocytes and enterocytes or hydrolyzed again into FFAs for β-oxidation. The intermediate products of TG hydrolysis are also used for phospholipids synthesis and membrane assembly. Here, I will try to summarize the currently known genes that are generally recognized to be important in these processes.

**Genes involved in TG synthesis**

Unesterified fatty acids are highly toxic to cells and have been implicated in much of the pathology associated with obesity and diabetes. Thus, efficient fatty acid esterification into TG and packaging of the newly synthesized TG are necessary for viability of cells apart from energy storage (24). The acylation of glycerol-3-phosphate represents the first
and committed step in glycerolipid biosynthesis (figure 3). The reaction is catalyzed by acyl-CoA: glycerol-sn-3-phosphate acyltransferase (GPAT), resulting in the production of 1-acyl-sn-glycerol-3-phosphate (lysophosphatidate). Phosphatidate is synthesized de novo from the acylation of lysophosphatidate in a reaction catalyzed by acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase (AGPAT). The phosphatidate formed by AGPAT occupies a central branch point in lipid biosynthetic pathways (30). Phosphatidate can be converted to phospholipids or to diacylglycerol (DAG), which serves as the precursor for TG (31). Dacylglycerol:acyl-CoA acyltransferase (DGAT) is the enzyme involved in the conversion of DAG into TG.

![Diagram](image)

**Figure 3.** A schematic presentation of intracellular TG synthesis in the ER. The terminal enzymes for triacylglycerol synthesis residing in the ER are: AGPAT, acylglycerol-P acyltransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; G3P, glycerol-3-phosphate; GPAT, glycerol-P acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; TG, triglyceride; VLDL, very low density lipoprotein.

DGAT activity is highest in organs that have high rates of TG synthesis: adipose tissue, liver, lactating mammary gland, small intestinal mucosa, and adrenal gland. TG synthesis takes place at the surface of endoplasmic reticulum (ER), where the above mentioned enzymes are located. Because TG storage is essential to all eukaryotic cells, the DGAT gene family has been preserved in yeast, plants and animals and there is a clear redundancy. There are multiple pathways to the formation of neutral lipids in eukaryotes. The importance of these reactions is further demonstrated by the fact that they are conserved across many billions of years of evolution and have arisen independently (32). This apparent redundancy in neutral lipid synthesis is obviously advantageous and necessary otherwise these genes presumably would have been mutated or silenced.
Genes involved in TG storage

The exact mechanism behind TG packaging into cytosolic droplets is not well understood. However, the vast majority of TG in mammalian cells is in droplets coated with one or more of the Perilipin-Adipophilin-TIP47 (PAT) family of proteins (33). Mammalian genomes have at least five genes that encode regulatory proteins that coat lipid droplets (24). Perilipin is the major lipid droplet PAT protein of differentiated adipocytes (34). Perilipin is a multifunctional protein, capable of lowering basal lipolysis, promoting lipolysis after PKA activation, and controlling lipid droplet fragmentation through mechanisms that are lipase-dependent and -independent (35). It is generally accepted that lipid droplets emerge from the ER that at some point during expansion buds as a droplet into the cytosol. This model is based on two important observations: i) mature lipid droplets are physically associated and in some cases appear to be continuous with the ER as shown by electron microscopy, ii) many genes involved in TG synthesis have been identified and confirmed to reside in the ER (31). It is not clear when these genes have appeared in the evolution but it has been suggested that TIP47 could be the ancestral gene of PAT protein family because it is ubiquitously expressed and is the only PAT gene that is not PPAR regulated. TIP47 is likely duplicated four times and gave rise to the more specialized PAT proteins that encoded by different chromosomes (24).

Genes involved in TG breakdown

During fasting and exercise TGs are the main source of energy. Therefore, lipolysis and FFA formation is under direct control of glucagon and norepinephrine. Both hormones can activate protein kinase A (PKA) and thereby activate Hormone Sensitive Lipase (HSL) (36). HSL is one of the key regulating enzymes in TG lipolysis that is translocated to lipid droplets during PKA activation (37). Interestingly, insulin reduces HSL activity by reducing PKA activity. This means that PKA functions as a second messenger where its activity related to the level of lipolysis (38). Until the discovery of adipose triglyceride lipase (ATGL), HSL was considered to be the main triglyceride lipase inside the cell. ATGL has strong TG hydrolase activity, but no activity against diacylglyceride or monoacylglyceride substrates. ATGL is highly expressed in adipose tissue of mice and humans. It exhibits high substrate specificity for TG and is associated with lipid droplets (39). Both enzymes likely maintain hepatic lipid homeostasis by mobilizing TG from storage pools to fatty acid (FA) oxidation pathways and possibly also by releasing FFA directly into the circulation (40). The fact that overexpression of these enzymes reduces hepatic TG, without increasing hepatic apoB or TG secretion, may make them attractive therapeutic targets in steatosis.

Genes involved in TG secretion

Once synthesized, TGs are packaged into TG-rich particles and secreted into the plasma. As already mentioned, the major enzymes in the conversion of FFAs into TGs reside in the ER. Apolipoprotein B (apoB) is synthesized in the mammalian small intestine and liver, where it has an essential role in the assembly and secretion of TG-rich lipoproteins. Each particle contains just one apoB molecule (41). Full-length apoB, apoB100, is synthesized in the liver as a 4536
amino-acid polypeptide. Mutations that lead to the production of truncated forms of apoB of insufficient length for assembly of fully-lipidated lipoproteins lead to hypolipidemia (42). ApoB is a large hydrophobic protein that exists in plasma as apoB48 or apoB100, associated with intestinally derived chylomicrons or the liver-derived VLDL, respectively. These two proteins are formed from the same mRNA through an RNA editing event that converts the Gln2153 codon to a stop codon, leading to a truncated form (apoB48) containing 48% of the protein from the N-terminus. This post-transcriptional modification is performed by an enzyme called Apolipoprotein B mRNA-Editing Enzyme 1 (APOBEC1) that is only expressed in the human intestine but not in the liver. Therefore, human chylomicrons carry apoB48, whereas VLDL and LDL carry apoB100. The C-terminal part of apoB is involved in binding to LDL-receptor (LDLR) that is lacking in apoB48. Thus, chylomicrons depend upon apolipoprotein E to bind to the LDLR (and to other members of the LDLR family) to mediate their clearance from the circulation (43). Interestingly, avian species lack apoE and also do not edit apoB: i.e. their intestines produce apoB100 (44). Thus, the appearance, during evolution, of a form of apoB unable to bind to the LDLR coincided with the appearance of another LDLR ligand.

A major determinant of apoB maturation into VLDL particles is the microsomal triglyceride transfer protein (MTP), an ER protein with lipid-transfer activity that exists as a heterodimer with protein disulfide isomerase (PDI) (45). MTP is physically associated with apoB and transfers TG into the lumen of ER while apoB is being synthesized. The importance of this chaperone protein in lipid enrichment of apoB is well demonstrated by a loss of function mutation in MTP that causes a syndrome called abetalipoproteinemia (46). These patients have steatosis in the gut epithelium and liver whereas the lipid levels in the plasma are very low. ApoB is not secreted in this disorder and is degraded intracellularly, due to lack of maturation into VLDL particles. MTP was known to transfer phospholipids in many species, but has gained TG transfer activity during evolution. The TG transfer activity was absent in Drosophila but in fish, amphibians, and birds was shown to be 27%, 40% and 100%, respectively, of that observed in mammals (47). One might argue that MTP triglyceride-transfer activity first appeared in fish, matured in birds, and remained conserved in mammals. This has made possible that sufficient TG is packaged into lipoprotein particles for distribution of energy to the rest of the body.

TG storage disorders

TG is stored in adipose tissue and can be mobilized during fasting. In Western societies, due to sedentary life style and increased caloric intake, there is an imbalance between storage and mobilization of TGs leading to obesity. In time there is also ectopic TG deposition in other tissues such as liver. It is now recognized that non-alcoholic fatty liver disease (NAFLD) is a manifestation of the metabolic syndrome (48). Obesity is a tremendous health issue in Western societies and the number of affected subjects in the United States is dramatically increasing (49). The prevalence of obesity is also increasing in European countries (50). There is higher incidence of cardiovascular morbidity and mortality in obese subjects (51,52). Post
mortal analysis of coronary arteries of adults dying of other causes than cardiovascular disease (CVD) showed a clear association between Body Mass Index and the extent of atherosclerosis (53). The association between obesity and CVD could be explained by the metabolic disturbances that go together with obesity as well as by the associated inflammatory burden. Obesity with enlarged fat cells is associated with an increased number of macrophages in the adipose tissue surrounding individual adipocytes (54). TNF-α is an inflammatory cytokine that is mainly produced by macrophages. Adipocytes also express TNF-α and adipose tissue has been shown to be a major source of TNF-α locally and systemically.

The term metabolic syndrome (MetS) has been introduced to describe this cluster of metabolically related cardiovascular risk factors i.e. obesity, insulin resistance, dyslipidemia and hypertension. The most important obstacle in identifying genes involved in MetS is the fact that the definition of MetS is not universal and the population is very heterogeneous. Therefore, monogenic disorders that resemble metabolic syndrome are perfect disease models that could help us understand the pathogenesis of MetS. Lipodystrophies represent a heterogeneous group of diseases characterized by an abnormal, subcutaneous fat distribution that are associated with metabolic abnormalities comparable to the metabolic syndrome. Familial partial lipodystrophy type 3 (FPLD3) is one of these monogenic disorders that is caused by mutations in the gene for the Peroxisome Proliferator-Activated Receptors γ (PPARγ). FPLD3 patients have a reduced expression of PPARγ due to haploinsufficiency of the PPARG gene.

**Nuclear transcription factors**

Nuclear receptors orchestrate the expression profile of cells upon activation. They act as intracellular sensors that become activated by biologically active molecules. Liver X receptor (LXR), Farnesoid X receptor (FXR) and Peroxisome Proliferator-Activated Receptors (PPARs) are nuclear receptors that play a major role in lipid metabolism. LXR and FXR are activated by cholesterol derivatives, whereas PPARs become active by FFAs and eicosanoids. Because transcription factors are at the top of a pathway-specific regulatory hierarchy, they have been the target of drug design. One drug can affect not only one gene but an array of genes that are involved in the same pathway. There are also many drugs available for activation of these nuclear receptors that have been tested in clinical settings. I will discuss the key transcription factors in lipid metabolism with emphasis on TG metabolism.

LXR plays a key role in promoting removal of excess cholesterol from cells and is important in the so-called reverse cholesterol transport (RCT). LXR is activated by endogenous oxysterols and upregulates ATP binding cassette transporter (ABC) A1 and ABCG1, promoting cholesterol efflux to apoAI and HDL, respectively. LXR also promotes fecal excretion of cholesterol by upregulating ABCG5 and ABCG8 in the intestine. Hence, LXR agonists are a potential approach to promote cholesterol efflux and RCT and to reduce atherosclerosis. In rodents, but not in man, LXR activation enhances hepatic cholesterol catabolism partly through increased expression of cholesterol 7-α-hydroxylase gene (CYP7A1), the rate-limiting
enzyme in the classic conversion of cholesterol to bile acids (55). Therefore, rats and mice have the unusual capacity to convert cholesterol to bile acids by LXR-mediated stimulation of CYP7A1 transcription, whereas other species do not respond to cholesterol and develop hypercholesterolemia on a high cholesterol diet. Kotokorpi et al. also showed significant differences in the response to GW3965 (a synthetic LXR agonist) between human and rat hepatocytes (56). These data demonstrate that pathophysiology of lipid metabolism in rodents could not be unequivocally extrapolated into humans. Caution is needed when drugs affecting lipid metabolism tested in rodents are going to be used in humans.

FXR plays a major role in protecting the hepatocyte from the toxic effects of excess bile acids (BA). BAs are the major ligand to activate FXR and consequently downregulate BA synthesis and the import of BAs from the plasma. In addition, BAs upregulate their export into the bile. FXR also downregulates the intestinal reabsorption of BAs (57). Taken together, excess BAs activates FXR and thereby promotes the removal of excess BA. Whether FXR activation is a useful therapeutic target is still not clear. Chenodeoxycholic acid (CDCA) is a bile acid and acts as a natural FXR agonist and reduces plasma TG in man (58). The synthetic FXR agonist GW4064 (cholic acid) significantly reduced plasma TG as well as glucose when used in diabetic mice (59). Therefore, FXR seems to be potential drug target for treating dyslipidemia, mainly hypertriglycerideremia. Whether FXR activation could have a role in atherosclerosis is uncertain because FXR deficiency in mice seems to be anti-atherogenic, but only in females (60). Future investigations are needed to define the therapeutic potential of FXR activation in humans.

PPARs, including the three known isotypes PPARα, PPARγ and PPARδ, are members of the steroid hormone nuclear-receptor superfamily (61). Like other nuclear receptor family members, PPARs contain both a ligand-binding domain, and a DNA-binding domain that mediates binding to specific PPAR response elements in the promoter region (62). In response to ligand binding, PPARs undergo a conformational change that facilitates the formation of a hetero-dimeric complex with another ligand-activated nuclear receptor, retinoid X receptor (RXR). This ligand-induced conformational change also facilitates the binding and release of small accessory molecules that are critical for transcription. Natural ligands of PPARs include FA and eicosanoids. The three isotypes of human PPAR (α, δ and γ) have been characterized, showing distinct tissue distributions, physiological roles and ligand specificity (63).

PPARα is predominantly expressed in the liver and is activated by fibrates. Drugs such as cipofibrate or fenofibrate have been successfully prescribed in patients with hypertriglycerideremia, but their role in PPARα activation was demonstrated long after their introduction as a drug (64). PPARα activation by fibrates initiates FA oxidation and thereby reducing the intracellular TG store. Apparently, this also affects VLDL production due to lack of sufficient TG in hepatocytes. It has been shown that fibrates also enhance the catabolism of VLDL particles by activating LPL (65). Eventually, these changes will lead to reduction of TGs in liver (less steatosis) as well as in plasma. The effect of fenofibrate on steatosis was
more pronounced in a mouse model of non-alcoholic fatty liver disease (NAFLD) than in human subjects (66;67). Although their role in dyslipidemia has been extensively studied, their role in atherosclerosis is still under debate. Gemfibrozil, a PPARα agonist, showed 34% reduction in the incidence of coronary heart disease in hyperlipidemic subjects (68), whereas the role of other fibrates in preventing cardiovascular disease is not clearly established yet. PPARγ is classically characterized by its high expression and functional role in adipose tissue, where it was first identified (69). Considerable evidence has established the importance of PPARγ in adipose tissue, including its high level of expression in adipocytes, the lack of white fat in PPARγ-deficient mice, PPARγ regulation of adipokine expression, and the association of a PPARγ deficiency with lipodystrophy (70). In addition to adipogenesis, PPARγ also regulates genes involved in TG metabolism, including LPL, acyl-coenzyme A synthetase, and glucose control such as the glucose transporter GLUT4 (71). The relationship of PPARγ and inflammation is a two way communication. On the one hand, PPARγ ligands have been shown to attenuate the inflammatory response of macrophages by inhibiting the production of inflammatory cytokines such as TNF-α (72), whereas in T lymphocytes it also limits the production of cytokines such as IFNγ and interleukin-1 (73). On the other hand, inflammatory agents like endotoxin, via the increase in TNF-α release, downregulates PPARγ expression in macrophages (74). TNF-α was discovered not only as a soluble protein that induces the death of tumor cells but also as a molecule (cachectin) that causes hypertriglyceridemia and wasting of muscle and fat tissue (75). TNF-α has been shown to affect hepatic lipogenesis due to reducing the expression and activity of different nuclear hormone receptors such as PPARγ (76;77). Adipose tissue macrophages are suspected to be the major source of inflammatory mediators such as TNF-α and IL-6 that interfere with adipocyte function by inhibiting insulin action (78). Taken together, PPARγ appears to be anti-inflammatory and its activation by specific ligands could be used for treating atherosclerosis.

Whereas PPARα and PPARγ have been studied extensively over the past 20 years, relatively little is known with respect to PPARδ. PPARδ has almost ubiquitous tissue expression which suggests that it might be involved in different metabolic pathways. In fact, by analysis of PPARδ null mice (79), it could be demonstrated that PPARδ-deficiency was associated with multiple developmental and metabolic abnormalities, including aberrant growth, diminished adipose tissue mass, demyelination, impaired wound healing and skin abnormalities, which were apparently due to altered inflammatory responses in the skin (80). Selective PPARδ agonists raise plasma HDL-cholesterol levels in rhesus monkeys as well as in human subjects (81;82). This increase in HDL-cholesterol is at least partly due to enhanced ABCA1 expression. In monkey and in man, but not in mice, PPARδ agonists decrease plasma TGlevels as well (83). Primates differ in their lipid metabolism when compared to rodents with the most prominent difference being lack of CETP in the latter species (84). This is in line with the differences in lipid metabolism of primates compared to other mammalians such as rodents. It has been suggested that PPARδ agonists could be used in the whole spectrum of metabolic syndrome because of their effects on HDL-cholesterol, TG and adipose tissue (85).
Outline of this thesis

In chapter 2, I will go into details on inflammatory response and gene expression of different cell types of the vessel wall involved in this process. Special attention has been made to nuclear transcription factors such as Peroxisome proliferator-activated receptor gamma (PPARγ).

In chapter 3, I will characterize the expression profile of the novel TNF-α-responsive Apolipoprotein L gene cluster. Their expression in different species as well as in human atherosclerotic tissues is demonstrated.

In chapter 4 I will present the function of the intracellular apolipoproteins L2 and L3 in TG metabolism.

Chapter 5 describes the expression pattern of a novel gene, Lipase H (LIPH) that is a member of the triglyceride lipase family.

Chapter 6 describes a lipodystrophic patient with metabolic syndrome due to a genetic mutation in PPARG.

In Chapter 7 all types of lipodystrophy and their associated metabolic abnormalities will be discussed.

Finally, Chapter 8 summarizes the findings in this thesis and the role of genes involved in TG metabolism and fat storage in adipose tissue as well as in non-adipose tissue in relation to the introduced common diseases and syndromes.
Reference List


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Chapter 2

Gene Expression in Atherogenesis

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Summary

It is conceivable that the extent and spatio-temporal expression of dozens or even a few hundred genes are significantly altered during the development and progression of atherosclerosis as compared to normal circumstances. Differential gene expression in vascular cells and in blood cells, due to gene-gene and gene-environment interactions can be considered the molecular basis for this disease. To comprehend the coherence of the complex genetic response to systemic and local atherosclerotic challenges, one needs accessible high through-put technologies to analyze a panel of differentially expressed genes and to describe the interactions between and among their gene products. Fortunately, new technologies have been developed which allow a complete inventory of differential gene expression, i.e. DD/RT-PCR, SAGE and DNA micro-array. The initial data on the application of these technologies in cardiovascular research are now being reported. This review summarizes a number of key observations. Special attention is paid to a few central transcription factors which are differentially expressed in endothelial cells, smooth muscle cells or monocytes/macrophages. Recent data on the role of nuclear factor-κB (NF-κB) and peroxisome proliferation-activating receptors (PPARs) are discussed. Like the PPARs, the NGFI-B subfamily of orphan receptors (TR3, MINOR and NOT) also belongs to the steroid/thyroid hormone receptor superfamily of transcription factors. We report that this subfamily is specifically induced in a sub-population of neointimal smooth muscle cells. Furthermore, intriguing new data implicating the Sp/XKLF family of transcription factors in cell-cell communication and maintenance of the atherogenic phenotype are mentioned. A member of the Sp/XKLF family, the shear stress-regulated lung Krüppel-like factor (LKLF) is speculated to be instrumental for the communication between endothelial cells and smooth muscle cells. Taken together, the expectation is that the fundamental knowledge obtained on atherogenesis and the data that will be acquired during the coming decade with the new, powerful high through-put methodologies will lead to novel modalities to treat patients suffering from cardiovascular disease. In view of the phenotypic changes of vascular and blood-borne cells during atherogenesis, therapeutic interventions likely will focus on reversal of an acquired phenotype by gene therapy approach or by using specific drugs which interfere with aberrant gene expression.
Introduction

Atherosclerosis is a complex, chronic inflammatory disease of the arterial vessel wall which is manifested at predilected locations of the vasculature [reviewed in (1-3)]. Accordingly, the current opinion is that this disease is caused by both local and systemic risk factors. Local risk factors are determined by the geometry of the vessels and the view has emerged that bifurcations, branch points and curvatures intrinsically cause irregularities of the otherwise uniform laminar shear-stress (4-6). Areas of turbulent flow, resulting in abrupt transitions of low and high shear stress, are particularly prone to develop atherosclerotic lesions. The formation of atherosclerotic lesions is considered as the basis for the etiology of most pathological, cardiovascular events such as coronary heart disease (CHD) (7, 8). The systemic risk factors which cause these diseases, have been thoroughly defined by epidemiological studies and traditionally include high plasma cholesterol, smoking, male sex, advanced age, diabetes mellitus, overweight and high blood pressure (9). Novel risk factors have now been introduced and are under investigation such as hyperhomocysteinemia, impaired fibrinolysis, lipoprotein (a), small dense low-density lipoprotein (LDL) and inflammatory markers (10). Ever since the epidemiological studies identified the risk factors for CHD, scientists have been challenged to translate these risk factors into patho-biological pathways and define a molecular basis for atherosclerosis.

Among the patho-biological processes involved in atherosclerosis, the importance of lipoprotein metabolism in initiation and progression of atherosclerosis has been established by human as well as animal studies. The earliest form of atherosclerosis, called “fatty streaks”, could start in infancy and these lesions contain inflammatory cells, such as macrophages and lymphocytes, in addition to accumulated lipids. This has led to the generally accepted hypothesis that atherosclerosis is a chronic inflammatory disease of the vessel wall [reviewed in (1-3)]. According to this hypothesis, endothelial cell dysfunction upon different “injuries”, such as oxidized low-density lipoprotein (ox-LDL), is the first step in atherogenesis. The dysfunction leads to increased permeability and to an increased adhesion of leukocytes and platelets to the endothelium. The interaction between endothelial cells and leukocytes or platelets is mediated by selectins, surface-exposed receptors, that bind to defined ligands on the indicated blood cells (11). Subsequently, leukocytes (e.g. monocytes and T cells) transmigrate through the endothelium by transiently disrupting the gap junctions and the tight junctions between adjacent endothelial cells [reviewed in (12)]. In the sub-endothelial space, monocytes differentiate into macrophages and get engaged in digesting large amounts of (modified) lipoprotein particles. Consequently, these cells turn into lipid-laden macrophages (“foam-cells”). The monocytes/macrophages release cytokines and chemokines which will reach the medial smooth muscle cells and strongly affect their fate. Under normal conditions, smooth muscle cells are specialized to provide elasticity and contractability to the arterial vessel wall. However, due to the monocyte/macrophage-derived factors, smooth muscle cells leave their medial location, traverse the internal elastic lamina and migrate into the intima. Subsequently or simultaneously, smooth muscle cells change from a contractile
(differentiated) phenotype into a non-contractile, proliferative (partially dedifferentiated) phenotype, reminiscent of transitions observed during early fetal development (13-15). In addition, dedifferentiated smooth muscle cells synthesize large amounts of extracellular matrix proteins, ultimately resulting in a fibrous lesion that may partially obstruct the circulation. The susceptibility of the lesion with respect to rupture is dependent on the lipid content of the plaque or, more precisely, on the relative amount of fibrous material versus atheromatous core.

Each of the distinct steps in atherogenesis is driven by a set of genes which is expressed by a variety of cells as outlined above. Although it has been announced that the nucleotide sequence of the entire human genome has now been resolved, it is obvious that only a fraction of the genes and gene products has been characterized. It is conceivable that, among the tremendous number of unidentified genes, a subset will be responsible for atherogenesis. Hence, we are faced with the challenge of identifying these genes and elucidating their function, in order to get a firm understanding of the molecular basis of this multifactorial disease. Fortunately, during the past decade, novel high through-put technologies have been developed which provide unbiased approaches to make an inventory of differentially expressed genes. These technologies have recently been applied in cardiovascular research by our laboratory and by others and will be briefly outlined in the next section.

There is no doubt that genes expressed in the liver and the intestine have an important role in lipid metabolism and in atherogenesis. However, this review focuses on genes expressed by the vascular cells and, consequently, we will refrain from discussing genes expressed in the aforementioned organs. We have chosen to discuss individually the contribution of endothelial cells, smooth muscle cells and monocytes/macrophages to atherogenesis. It is expected that, in the near future, integration of the results obtained for separate cell types will be done e.g. by employing co-culture systems of vascular cells as has been described previously (16, 17). Furthermore, in view of the molecular complexity of the pathophysiology of atherogenesis, we have restricted this review to a discussion of recent data on the contribution of specific (families of) transcription factors which regulate genes which are possibly causative determinants for initiation and progression of atherosclerosis. In particular, recent data on the role of nuclear factor-κB (NF-κB), peroxisome proliferation-activating receptors (PPARs) and the Sp/XKLF family of zinc finger proteins will be discussed. It may be hypothesized that interfering with the action of these transcription factors, or with up-stream or down-stream events related to these factors, may provide opportunities to develop new diagnostic tools and therapeutic agents and modalities either to prevent the formation of lesions or to induce their regression.
High Through-put Techniques to Monitor Differential Gene Expression

Three major techniques have been developed during the last decade which allow high through-put analysis of differential gene expression in complex organisms. These techniques are denoted differential display of randomly-primed mRNA by RT-PCR (DD/RT-PCR) (18, 19), serial analysis of gene expression (SAGE) (20) and DNA micro-array (21). Our laboratory has implemented and validated each of these techniques and we believe that each of them has its specific merits and drawbacks. For example, in principle, DD/RT-PCR reveals differential expression of both high and low expressing genes, but does not provide quantitative insight into the steady-state concentration of mRNAs. Furthermore, DD/RT-PCR is based on the use of anchored- and arbitrary primers and the number of primer combinations determines the actual “coverage” of the collection of different mRNAs (denoted “transcriptome”). For example, usually 144 DD/RT-PCR reactions are performed with 12 anchored- and 12 arbitrary primers, theoretically comprising about 80% of the mRNA profile, meaning that the remaining part of the transcriptome is lacking (19). SAGE does provide quantitative data on the level of (differential) mRNA synthesis, but obtaining a collection of statistically relevant data depends on the availability of large-scale DNA sequencing facilities. Since both techniques rely on amplification of mRNA by RT-PCR, they are both very sensitive. DNA micro-array is based on hybridization of radiolabeled or fluorescently labeled cDNA preparations to either filter- or glass-immobilized single-stranded cDNA or a series of gene-specific and control oligonucleotides. The amplified, labeled cDNA preparation should be a qualitative and quantitative representation of the mRNA profile of a given tissue, cell type or mixture of different cells and, hence, is highly heterogeneous in terms of amount of distinct mRNAs. As a consequence, the sensitivity of hybridization, which is determined among other things by the concentration of the hybridizing counterpart (probe), might be modest for genes that are expressed at a very low mRNA level. On the other hand, DNA micro-array is a genuine high through-put technique which in principle allows a “genome-wide” comparison of, for example, gene expression of a chosen cell type/tissue under different conditions or of two different cell types under the same condition. As an illustration of large-scale gene expression profiling, tumor endothelium has been compared with normal endothelium, using SAGE (22). Undoubtedly, similar studies will be undertaken to compare the transcriptome of vascular cells under atherosclerotic circumstances and that of their healthy counterparts.

We have recently designed and applied a combined protocol of DD/RT-PCR and DNA micro-array. Initially, DD/RT-PCR had been employed to isolate and identify genes differentially expressed in cultured human vascular endothelial cells and smooth muscle cells that were subjected to a strong atherosclerotic stimulus (23,24). In addition, we also performed an extensive SAGE to delineate genes that are specifically induced or repressed upon activation of cultured human smooth muscle cells (25). Together with cDNAs, corresponding to genes differentially expressed upon the transition of cultured human monocytes into macrophages (V. Sier-Ferreira, B.M.M, van den Berg, H. Pannekoek, unpublished data), we composed a
limited, “custom” human vascular DNA micro-array which contains about 350 cDNAs (26). These arrays are currently used to analyze expression of this pre-selected set of genes in specimens collected during major vascular surgery or in vascular material that has been obtained from organ donors (27). Obviously, the bioinformatics required to “read” these custom arrays is relatively straightforward as compared to genome-wide screens which will be available in due time.

Vascular Cells

The non-atherosclerotic (“healthy”) vessel wall contains only two different cell types, endothelial cells and smooth muscle cells, each of which has a well-defined role. Under these conditions, the monocellular endothelial layer forms a border that permits transport of nutrients to the sub-endothelial space. The endothelial cells synthesize products that are important to maintain the contractile function of the underlying smooth muscle cells. This communicative function is attributed in particular to small molecules like prostanoids, endothelins and nitric oxide (NO). Indications are at hand, however, suggesting that additional pathways are also involved in the communication between endothelial cells and smooth muscle cells. Hence, it is foreseen that future studies will focus on the identification of these molecules and the elucidation of their function. In this respect, the seminal studies on the interplay between cardiac microvascular endothelial cells and cardiomyocytes may serve as an excellent example (28-31). Here, it was demonstrated that differential (regional) vascular-bed specific expression of von Willebrand was mediated by signal transduction initiated by way of hetero-dimeric platelet-derived growth factor AB (PDGF AB). Remarkably, endothelial cells and cardiomyocytes both contribute to the generation of PDGF AB. In the absence of cardiomyocytes, cardiac microvascular endothelial cells exclusively synthesize PDGF A. Co-culturing of these two different cell types results in the induction by cardiomyocytes of endothelial PDGF B synthesis, allowing the subsequent generation of heterodimeric PDGF AB. PDGF AB is a ligand for the so-called α-receptor and the interaction between ligand and receptor ultimately leads to von Willebrand factor synthesis. This example elegantly illustrates the importance of cell-cell communication and provides a mechanistic explanation for regional-specific gene expression. In the following paragraph, we will present a similar, though less well understood, example of communication between the endothelial cells and smooth muscle cells.

The atherosclerotic vessel wall harbors inflammatory cells, notably monocytes and T cells, in addition to endothelial cells and smooth muscle cells. As indicated before, these cells transmigrate through the endothelial cell layer by interaction with the proteins that constitute the gap junctions and the tight junctions between adjacent endothelial cells (reviewed in [12]). Communication between monocytes, which differentiate in the vessel wall into macrophages, and smooth muscle cells, is mediated by cytokines and chemokines which are not synthesized at these locations under non-atherosclerotic circumstances (reviewed in [1-3]). Hence, the accumulation of lipid-laden macrophages in the vessel wall constitutes a hallmark
of this disease and conceivably forms a decisive factor in the development of a rupture-prone plaque. Taken together, the picture is emerging that dysregulation of gene expression of different cell types and, consequently, altered communication between these cells is the molecular basis for this disease. However, since alterations of cell-cell communication in atherogenesis are still poorly understood, we will discuss a number of aspects of differential gene expression of “isolated” vascular cells.

Endothelial Cells

The endothelium covers the luminal side of the entire cardiovascular system and has been considered as a distinct organ. It covers approximately 700 m², weighs 1.5 kg and regulates many processes (32, 33). It forms a non-thrombogenic, non-adhesive layer and regulates vascular tone. It is now generally accepted that endothelial dysfunction is the first step in atherosclerosis [reviewed in (1-3)]. Endothelial dysfunction refers to impaired biological processes in endothelial cells, leading to increased adhesiveness to monocytes, increased permeability, procoagulant properties and changes in vascular tone. However, in clinical settings endothelial dysfunction has been used to describe the impaired nitric oxide (NO) derived vasodilatation. Endothelial cells are exposed to a variety of biochemical and biomechanical stimuli, such as circulating LDL, inflammatory cytokines, fluid shear stress and cyclic stretch (4-6,34). Since it is generally assumed that atherosclerosis is due to an interplay between genes and environmental factors, individuals should react differently to these stimuli. Accordingly, experiments with mice of different genetic backgrounds illustrate a different sensitivity towards atherogenic stimuli and differences in the gene expression pattern of their endothelial cells (35). By using two strains of mice, one resistant (C3H/HeJ) and the other susceptible (C57BL/6J) to diet-induced atherosclerosis, it was shown that cultured endothelial cells isolated from these strains of mice reacted differently to stimuli. Minimally-modified LDL induced higher levels of inflammatory genes in cultured endothelial cells derived from the C57BL/6J mice than in those derived from C3H/HeJ mice. This study not only explains, at least partly, the differences in susceptibility of these mice, but also illustrates that factors acting in the vessel wall could protect against diet-induced atherosclerosis. Finally, these observations imply that altering the expression profile of vascular cells by, for instance, gene therapy or local drug delivery, might be a useful way to treat atherosclerosis.

Since atherosclerosis is considered a chronic inflammatory disease of the vessel wall, it is obvious that special attention should be given to the transcription factor, nuclear factor κB (NF-κB). NF-κB is a heterodimeric, DNA-binding protein, being one of the key regulators of gene expression in the vessel wall and plays a coordinating role in inflammation. Its role in atherosclerosis is emphasized by the notion that the activated form is exclusively encountered in vascular lesions and not in healthy tissue (36). The mode of activation of NF-κB has been elucidated in detail. It involves the activation of a dimeric IκB kinase complex which phosphorylates the NF-κB inhibitor κBα, leading to its subsequent ubiquitination and degradation and, ultimately, release of NF-κB (37). Free NF-κB dimers then translocate to the
nucleus where the assembled transcription factor regulates the mRNA synthesis of target genes. Recently, it was shown in a pig model that a high cholesterol diet resulted in higher expression of the activated NF-κB in the coronary vasculature (38). In view of the mediating role of activated NF-κB in inflammation, it is conceivable that high cholesterol levels cause inflammation of the vessel wall, as evidenced by the presence of activated NF-κB. Similarly, tumor necrosis factor alpha (TNF-α) is a potent inflammatory cytokine which also activates the NF-κB signaling pathway.

Although the role of the NF-κB pathway in atherogenesis is undisputed, it is obvious that many other known and unknown genes play a crucial role in initiation and progression of this disease. To identify novel and known genes expressed by endothelial cells and involved in atherosclerosis, differential display of gene expression by randomly primed mRNA, using RT-PCR was employed (DD/RT-PCR; 18, 19). The mRNA profile of quiescent human umbilical vein endothelial cells (HUVEC) was compared with that of TNF-α-stimulated HUVEC, resembling the pro-atherogenic, inflammatory phenotype (23). A total of 106 differentially expressed gene fragments were identified and these included 22 known genes (Table 1).

Many of these known genes have previously been implicated in atherosclerosis, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8), showing the validity of this approach. On the other hand, as noted before, DD/RT-PCR analysis does not cover the entire transcriptome since a number of anticipated, differentially expressed mRNAs were not encountered. Notably, we did not detect a differential display (DD) fragment corresponding to transcripts of e.g. NF-κB nor to plasminogen activator inhibitor 1 (PAI-1). The latter transcript was, however, encountered in a rather limited SAGE of resting HUVEC versus cells activated with TNF-α-containing conditioned medium of ox-LDL-activated cultured monocytes (39). This observation demonstrates that it is worthwhile to simultaneously explore different (complementary) high through-put technologies to make an inventory of genes which are differentially expressed. Some of the remaining unknown endothelial cell DD gene fragments (37 out of 84) are present in public databases (dbEST), indicating that these genes are indeed expressed and represent genuine transcripts. A subset of these ESTs is currently under investigation to explore their role in vascular disease. Finally, it should be noted from the catalogue of differentially expressed genes (Table 1) that activation of endothelial cells with a “strong” atherosclerotic stimulus, i.e. TNF-α or TNF-α-containing conditioned medium of ox-LDL-treated monocytes, results in alterations of an impressive number of diverse processes. These include haemostasis, leukocyte trafficking, regulation of transcription, protection against oxidation, cell shape and cell cycle, signal transduction and apoptosis.

Despite the importance of systemic factors for its initiation and progression, atherosclerosis develops at preselected sites in the arterial tree, such as the outer edges of vessel bifurcations, branch points and curvatures (4-6, 34). In these susceptible areas, laminar blood flow is disturbed resulting in an altered shear stress. It has been postulated that the gene expression pattern of the endothelial cells is modulated at the transition of high to low shear stress, leading to a focal atherogenic phenotype of these cells (40-42). Indeed, in vitro studies have shown that the mRNA profile of cultured endothelial cells changes upon applying
shear stress. For instance, endothelial NO-synthase (eNOS), an enzyme that catalyses the production of the vasodilator NO, is increased in vitro by fluid shear stress (43). En-face examination of endothelial-cell surfaces in human thoracic aortas revealed a pro-atherogenic phenotype in areas with low shear stress (accumulation of sub-endothelial macrophages and lymphocytes, irregular endothelial morphology with denuded regions) (44). Recently, evidence has been acquired that the effects of low shear stress are also transmitted through the NF-κB pathway (45). These investigators showed that areas with disturbed flow (high probability) in mice aorta display a higher expression level of NF-κB as compared to areas with high shear stress (low probability region). Upon feeding an atherogenic diet to these mice,
NF-κB was predominantly activated in those high probability areas, leading to the conclusion that these areas are primed for activation by various stimuli, whereas the low probability regions with high shear stress are apparently resistant to diet-induced atherosclerosis. These studies show that high shear stress indeed induces a quiescent, anti-atherogenic phenotype of the endothelial cells and it is speculated that this is accomplished by interfering with inflammatory pathways.

How do the endothelial cells “sense” these biomechanical forces and how do they change their expression profile? Unfortunately, neither the receptor(s) for shear stress nor the down-stream signaling pathway(s) have yet been clarified. Recently, however, we found by using commercial DNA macro-arrays that, among other genes, lung Krüppel-like factor (LKLF) was up-regulated by shear stress (R. J. Dekker, H. Pannekoek and A. J. G. Horrevoets, unpublished data). LKLF is a zinc finger transcription factor that belongs to the Sp/XKLF family (reviewed in ref 46). Most of these proteins have been reported to promote transcription, but some members of this family exert an inhibitory effect. LKLF was previously shown to be expressed in naïve T cells and was rapidly repressed after T cell activation (47). In that study, it was concluded that LKLF is required to program the quiescent state of T cells and to maintain their viability in the peripheral lymphoid organs. LKLF is also highly expressed in vascular endothelial cells between E9.5 and E12.5 of mouse embryogenesis, a critical time for vessel wall stabilization (48). It turned out that LKLF-deficient mice die in utero due to haemorrhages that are caused by severe defects in vessel wall integrity. Apparently, LKLF−/− mice are unable to form an organized tunica media. However, we found by in situ hybridization that LKLF is exclusively expressed in endothelial cells of the human aorta and it seems to be confined to areas with high shear stress. No expression was observed in the media, presumably since smooth muscle cells do not synthesize LKLF (R. J. Dekker, H. Pannekoek and A. J. G. Horrevoets, unpublished data). Consequently, we propose that these observations can be reconciled by assuming that secreted endothelial factors synthesized down-stream of LKLF may interact with the smooth muscle cells and are involved in the organization of the media. Interestingly, LKLF was down-regulated by cultured HUVECs which were treated with TNF-α, suggesting that the NF-κB signaling pathway counteracts gene regulation by LKLF.

Smooth Muscle Cells

Smooth muscle cells do not terminally differentiate and do exhibit different phenotypes, dependent on the presence of specific growth factors and cytokines, secreted by endothelial cells and surrounding macrophages. Contractile (differentiated) smooth muscle cells are quiescent and regulate the vascular tone (13). In contrast, under atherosclerotic conditions, smooth muscle cells respond to growth factors and cytokines and dedifferentiate into a “synthetic”, proliferative phenotype. These cells are able to migrate from the media into the intima and to produce large amounts of extracellular matrix constituents (13, 14). Consequently, a substantial part of the atherosclerotic, neointimal, lesion is formed by smooth
muscle cells and their matrix proteins. Conversely, apoptosis of smooth muscle cells would decrease the extent of lesion formation and promotion of this process may thus constitute a promising strategy to relieve obstruction of the circulation.

The alterations in gene expression, resulting from the transition of quiescent to activated smooth muscle cells, are largely unknown. At present, only a few genes have been identified that are specifically induced in cultured smooth muscle cells upon applying an atherosclerotic stimulus (indicated below). To create an unbiased inventory of differential gene expression of resting versus activated cultured, human smooth muscle cells, we performed both DD/RT-PCR (24) and SAGE (25). Here, the results obtained with DD/RT-PCR will be briefly summarized (Table 2). By applying DD/RT-PCR, using 12 different anchored primers and 12 different arbitrary primers, we identified 40 genes that are induced in smooth muscle cells upon activation with the conditioned medium of ox-LDL-treated cultured human monocytes. Ten differentially expressed genes have a known function, whereas 30 are novel genes of unknown function of which only a few are encountered in dbEST. Among the 10 known genes, some have been reported to be involved in atherogenesis, notably IL-8, intercellular adhesion molecule-1 (ICAM-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Interestingly, a comparison of the differential gene expression profile of cultured endothelial cells and smooth muscle cells, exposed to the same atherogenic stimulus, reveals that these cell types respond via a virtually unique inRNA repertoire. Except for a few genes [IL-8, GM-CSF and the human inhibitor of apoptosis protein-1 (hIAP-1)] both the inRNAs of differentially expressed known genes and the ESTs are apparently specific for each of these cell types.

As in previous sections, we will focus here on differential expression of transcription factors in smooth muscle cells. Interestingly, we found that the central transcription factor NF-κB is

<table>
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up-regulated in activated cultured smooth muscle cells and encountered in the DD/RT-PCR analysis. As mentioned above, this observation with cultured smooth muscle cells coincides with a study showing that activated NF-κB is present in human atheromas, but not in apparently healthy arteries (36). The data are also in agreement with the finding that the products of many genes which are induced by NF-κB are detected in the plaque (49). NF-κB is also a crucial player in the delicate balance that determines apoptosis or protection against programmed cell death. These processes are of prime importance for the generation and regression of fully developed plaques. The properties of NF-κB to both promote apoptosis and to counteract this process are illustrated by the following findings. Activation of smooth muscle cells by a cocktail of cytokines (containing e.g. TNF-α) causes synthesis of NF-κB, as revealed by our DD/RT-PCR experiment (Table 2). As a consequence of the activation of the NF-κB pathway, down-stream genes are induced which are known to inhibit apoptosis, notably inhibitor of apoptosis protein-1 (hIAP-1) and FLICE-like inhibitory protein (FLIP) (50-54).

Next to the induction of NF-κB and its subsequent down-stream genes, it is also of interest to discuss the induction of the peroxisome proliferator-activated receptors (PPARs) in these cells and the probable mechanistic relation between the PPARs and NF-κB (55). The PPARs are transcription factors and members of the family of the nuclear steroid/thyroid hormone receptors. PPARα stimulates the β-oxidative degradation of fatty acids, whereas PPARγ regulates adipocyte differentiation and glucose homeostasis. PPARα has been linked to the NF-κB signal transduction pathway, since it antagonizes the nuclear translocation of NF-κB (56). It is conceivable that inhibition is mediated by the formation of inactive complexes between NF-κB and PPARα. These observations are consistent with the reported anti-inflammatory activities exerted by PPARα ligands (57, 58). Similarly, it was demonstrated that PPARγ ligands inhibit the development of atherosclerosis in LDL-receptor deficient mice (59). However, the explanation of this beneficial effect does not imply interference in the NF-κB pathway. Rather, it has been suggested that PPARγ ligands would reduce the expression of MCP-1 in macrophages and vascular-cell adhesion molecule-1 (VCAM-1) in endothelial cells (60-62), although some investigators did not observe these effects (59). Recently, different laboratories have reported exciting new data on the effect of activated PPARγ on lipid influx and efflux of macrophages and these results will be discussed in the following paragraph.

Interestingly, a distinct subfamily of the nuclear steroid/thyroid hormone receptor superfamily was found in our DD/RT-PCR of resting versus activated smooth muscle cells. This nerve growth factor inducible gene B (NGFI-B) subfamily consists of three genes which encode orphan nuclear receptors and are denoted TR3, mitogen-induced orphan receptor (MINOR) and nuclear orphan receptor of T cells (NOT) [reviewed in (63)]. All three members are induced in cultured smooth muscle cells, treated with an atherosclerotic stimulus. The NGFI-B family members have been implicated in different cellular processes and are also expressed by other vascular cells in atherosclerosis (E. K. Arkenbout, H. Pannekoek, C. J. M. de Vries,
unpublished observations). Presently, no data are available on the function of these receptors in atherogenesis.

Finally, we will mention new data which may serve as an example for the intricate regulation of gene expression which determines the phenotype of smooth muscle cells (15). In addition, it may be instructive to illustrate the potentially crucial role of the Sp/XKLF family of zinc finger transcription factors in atherogenesis. As stated before, dedifferentiation of quiescent, contractile smooth muscle cells into proliferative cells is characterized by down-regulation of contractile proteins, such as SM-actin and SM22α (13). Conversely, maintenance of the quiescent, contractile state is strongly promoted by transforming growth factor β (TGF-β) (1,2). This effect of TGF-β is mediated by a specific TGF-β control element (TCE), identified in the promoter of SM-actin and SM22α (15, 64). It was shown that members of the Sp/XKLF family of transcription factors interact with the TCE. Gut-enriched Krüppel-like factor (GKLF) was found to bind to the TCE and to repress TGF-β-promoted transcription of SM22α. Accordingly, expression of GKLF in proliferative smooth muscle cells is high and is specifically down-regulated by TGF-β when the cells become quiescent. In contrast to the repressing effect of GKLF, overexpression of another XKLF family member (BTEB2) augmented the TGF-β-dependent increase in SM22α mRNA synthesis: these findings are specific for BTEB2 since LKLF did not affect SM22α expression (15). In conclusion, the often ubiquitously expressed members of the Sp/XKLF family are structurally similar proteins that bind to the socalled GC/GT boxes in transcription regulatory elements. Nevertheless, they exhibit a high degree of specificity which is explained by a competitive mechanism in which differences between DNA binding affinity to a given GC/GT box and the relative concentration of the XKLFs would determine the prevalence of a particular family member (46). In aggregate, these studies together with the discussed effect of endothelial LKLF on the organization of smooth muscle cells during mouse embryogenesis, and possibly also post-natally, emphasize the importance of the Sp/XKLF family in the maintenance of the integrity of the vessel wall.

Monocytes/Macrophages
The attachment of monocytes to activated endothelial cells, followed by extravasation into the intima, are crucial steps in the development of atherosclerotic lesions. Monocytes differentiate in the vessel wall into macrophages and generate lipid-laden “foam cells”. To define the molecular mechanisms of gene expression during the process of macrophage differentiation, Hashimoto and colleagues employed SAGE to delineate differential gene expression upon the transition of monocytes into macrophages, induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) (65). Interestingly, in view of our specific identification of the involvement of the Sp/XKLF family of transcription factors in atherogenesis, these investigators detected down-regulation of a member of the XKLF family, although they did not unveil its explicit identity. Remarkably, none of the PPAR family members were identified in this screen. This result is unexpected since it has been shown that treatment of monocytes with ox-LDL causes differentiation into macrophages and induction of PPARγ expression (66,
The ultimate influence of PPARγ expression and activation on atherosclerosis has been puzzling, although recent observations have shed light on this enigmatic issue (see below). First, it has been shown that PPARγ-specific ligands inhibit the expression of inflammatory genes [e.g. TNF-α, interleukin-1β (IL-1β)] (68, 69). Hence, these results would indicate that PPARγ and its specific ligands act as anti-inflammatory and anti-atherogenic agents. Second, it has been demonstrated that activated PPARγ up-regulates the synthesis of CD36, a major scavenger receptor on macrophages which mediates uptake of ox-LDL and, consequently, promotes removal of its own inducer (i.e. ox-LDL) (66). It should be noted, however, that up-regulation of CD36 would be expected to be pro-atherogenic, since CD36 deficiency in mice susceptible to diet-induced atherosclerosis due to apoE deficiency (CD36−/−-apoE−/−) protects against atherosclerosis (70). An apparently contradictory observation is that the PPARγ-specific agonists, rosiglitazone and GW7845, strongly inhibit the development of atherosclerosis in LDL receptor-deficient (LDL-R−/−) mice (59). These conflicting data might be reconciled by yet another effect of activated PPARγ. Notably, PPARγ ligands promote apoptosis by inhibiting the anti-apoptotic NF-κB signalling pathway (71). Possibly, programmed death and subsequent removal of macrophages may restrict the size of the lesion. Finally, recently, three “back-to-back” papers provided a rationale for the puzzling effect of PPARγ activation on atherosclerosis (72-74). It was demonstrated that the induction of CD36 by PPARγ is actually counteracted by a reduced expression of another scavenger receptor (SR-A). In addition, a nuclear receptor of the steroid/thyroid family LXR-α is up-regulated by PPARγ and induces the expression of the reverse cholesterol transporter ABCA1. Hence, the net result of PPARγ activation is anti-atherogenic in foam cells and now provides a rational explanation for the beneficial effects of PPARγ specific agonists.
Gene Expression in Atherogenesis

Reference


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The Apolipoprotein L Gene Cluster Has Emerged Recently in Evolution and Is Expressed in Human Vascular Tissue

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ABSTRACT

We previously isolated Apol3 (CG12-1) cDNA and now describe the isolation of Apol1 and Apol2 cDNA from an activated endothelial cell cDNA library and show their endothelial-specific expression in human vascular tissue. Apol1-Apol4 are clustered on human chromosome 22q13.1, as a result of tandem gene duplication, and were detected only in primates (humans and African green monkeys) and not in dogs, pigs, or rodents, showing that this gene cluster has arisen recently in evolution. The specific tissue distribution and gene organization suggest that these genes have diverged rapidly after duplication. This has resulted in the emergence of an additional signal peptide encoding exon that ensures secretion of the plasma high-density lipoprotein-associated Apol1. Our results show that the Apol1-Apol4 cluster might contribute to the substantial differences in the lipid metabolism of humans and mice, as dictated by the variable expression of genes involved in this process.
INTRODUCTION

Atherosclerosis is the major cause of mortality and morbidity in the Western societies due to the development of clinical events such as myocardial infarction and stroke. The role of lipoprotein particles in coronary heart disease (CHD) is well established. Elevated plasma low-density lipoprotein (LDL)-cholesterol levels are associated with CHD [1], whereas there is a strong inverse relationship between high-density lipoprotein (HDL)-cholesterol levels and the incidence of CHD [2]. Endothelial cells are the first cells of the vessel wall to come in contact with lipoprotein particles in blood and there is growing evidence that these cells have a major effect on lipid metabolism [3]. Endothelial cells not only express a variety of lipoprotein receptors, which suggests a direct interaction with lipoprotein particles, but also can bind many lipases expressed in other tissues at the cell surface that have the ability to hydrolyze lipoprotein triglycerides and phospholipids [3].

Previously, we isolated a panel of novel genes from human vascular endothelial cells by differential display of gene expression [4]. One of these genes, ApoL3 (CG12-1), was highly homologous to ApoL1, sharing 50% identity at the amino acid level. ApoL1 was isolated from plasma of fasting normolipidemic human subjects and was reported to be expressed by the pancreas [5]. Recently, two different groups described the genomic organization of an ApoL gene family on chromosome 22, consisting of ApoL1-ApoL6 [6,7]. Here, we show that the ApoL1-ApoL4 cluster is the result of tandem gene duplication, whereas ApoL5 and ApoL6 have a different origin. ApoL1-ApoL3 are expressed in various organs, and in human vascular tissue specifically by the endothelial cells. ApoL1-ApoL3 were detected only in primates (human and green African monkeys) and not in other placental mammals (dogs, rabbits, pigs, rats and mice). The expression of these genes in human vascular endothelial cells indicates a novel species-specific role in lipid metabolism and vascular biology.

MATERIAL AND METHODS

Sequencing of clones isolated from an endothelial cell cDNA library.

The cDNA library was constructed and screened using a radioactive probe, representing the sequence 1687-2298 of ApoL3 (CG12-1; GenBank acc. no. AF070675), as described [4]. Sequencing of the clones was performed on purified plasmid DNA using the AutoRead Sequencing-kit and Cy5-labeled T7- or SP6-oligonucleotides and analyzed on the ALF-express automatic sequencer (materials and protocol: Pharmacia, Uppsala, Sweden). Exon 4 of the ApoL1 transcript was amplified from the Marathon-ready placenta cDNA library (Clontech, Palo Alto, CA). An intron-spanning set of primers was designed containing the forward sequence from exon 2b (5’-CCTCGGTGACTGGGCTGCTGGC-3’) and the reverse sequence from exon 4b (5’-GATATCGCCACTGCACTCCAGCCTG-3’). The PCR product was cloned and sequenced. Sequence files from the ALF-express were exported in GCG format and analyzed and stored using the GCG program (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI). Sequence homology was confirmed by BLAST searches at the National Center...
RNA isolation and northern blot analysis.

Total RNA was extracted from unstimulated (0 hour) human umbilical vein endothelial cells (HUVEC) and HUVEC exposed to TNF-α (for 1.5, 3, 6, or 20 hours) and blotted to Hybond-N nylon membranes (Amersham, Buckinghamshire, UK) as described [4]. Purified DNA fragments, corresponding to the following sequences, were used as probes: ApoL1, 141-1324 (GenBank acc. no. AF305428); ApoL2, 698-1194 (GenBank acc. no. AF305429); ApoL3, 703-1362 (CG12-1; GenBank acc. no. AF070675); and commercially available β-actin cDNA probe (Clontech). Filters were hybridized, washed as described, and analyzed by autoradiography [4]. Radioactivity was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The human multiple tissue northern blot (Clontech) was probed according to the manufacturer’s protocol with the same fragments as described above.

In situ hybridization.

Human vascular tissue specimens, displaying various stages of atherosclerosis, were collected during organ transplantation from multi-organ donors who did not have a prior history of vascular disease (approved by the AMC Medical Ethical Committee 95/146). All specimens were fixed in formalin within 5 minutes after resection and subsequently paraffin-embedded. Paraffin sections (5 μm) were mounted on 3-aminopropyl-triethoxysilane-coated slides. The following sequences were used to synthesize various riboprobes: ApoL1, 647-1041 (GenBank acc. no. AF305428); ApoL2, 709-1194 (GenBank acc. no. AF305429); ApoL3, 703-1362 (CG12-1; GenBank acc. no. AF070675); and human vWF, 8239-8442. Riboprobes were synthesized by in vitro transcription of cDNA fragments cloned in various pGEM vectors (Promega, Madison, WI), containing T7 and SP6 RNA polymerase transcription initiation sites [4]. In situ hybridization was performed as described [4].

Multispecies Southern blot.

Tissues from different species were obtained from the animal facility (GDIA, AMC, Amsterdam). High molecular weight DNA from rabbit, rat, mouse and pig was isolated by incubating approximately 200 mg of minced liver tissue in a buffer containing 100 mM Tris-HCl (pH 8), 5 mM EDTA, 200 mM NaCl, 0.1% (w/v) SDS, and 0.3 mg/ml proteinaseK (Sigma) during 16 hours at 55°C under continuous rotation. Insoluble material was pelleted and the supernatant was incubated for 15 minutes at room temperature with 1 mg/ml RNase A. The supernatant was then extracted three times with equal volumes of phenol, once with a phenol chloroform isoamyl alcohol mixture (25:24:1, v:v:v), and once with a chloroform isoamyl alcohol mixture (24:1, v:v). The DNA was then precipitated from the aqueous phase and picked up using a glass rod. After washing in 70% (v/v) ethanol and brief drying, the DNA was solubilized in 10 mM Tris-HCL (pH 7.6), 1 mM EDTA. High molecular weight DNA from insect, dog, Chinese hamster, human and African green monkey was isolated from respectively Sf9, MDCK, CHO-I,
RESULTS

Isolation of ApoL1-ApoL3 cDNAs

Screening of the activated HUVEC cDNA library with a probe from the 3' end of ApoL3 led to the identification of two additional homologous cDNAs. One of these contained the complete coding sequence of ApoL1 with an open reading frame of 398 amino acids (Fig.

\[\text{FIG. 1. Alignment of the amino acid sequences of APOL1–APOL3 proteins. Amino acid sequences were deduced from cDNA sequences and aligned by ClustalW (European Bioinformatics Institute at http://www.ebi.ac.uk/). When all three proteins were aligned, 42% of the amino acids were identical (*)}.\]
The first 27 amino acids were found to be a secretory signal peptide when analyzed by GCG SPScan. This signal peptide is 15 residues longer than that previously published, due to an additional methionine (start codon) in-frame and upstream of the previously suggested initiating methionine [5]. The amino-terminal sequence of the predicted mature protein, after cleavage of this putative signal peptide, matches exactly the sequence of the secreted ApoL1 that was determined by direct N-terminal microsequencing [5]. Analysis of the chromosomal sequence of ApoL1 revealed that all intron-exon boundaries conform to the GT/AG rule (Table 1), except for the sequence of 0.7 kb that lacks from the last exon of our 2.2 kb cDNA of ApoL1. As the size of the ApoL1 mRNA by northern blot analysis is approximately 3 kb, this sequence probably does not represent a genuine intron, but might have been spliced in our HUVEC cDNA because of the specific genotype of the donor. This apparent polymorphism, leading to a smaller cDNA, will not affect the encoded protein because it lies within the 3' UTR. The composite sequence of ApoL1, now being 2.9 kb, is very close to the actual size of the mRNA as observed by northern blot analysis.

The second clone constituted a partial cDNA with an incomplete open reading frame lacking the 5' sequences. Searching dbEST we found an IMAGE consortium cDNA clone (GenBank acc. no. AA531428) [8] containing the 5' portion of the cDNA and the complete coding sequence. Although this clone lacked part of the 3' end due to internal priming, it shared an 878-bp overlap with our incomplete clone. Combining these two sequences revealed the full-length sequence of ApoL2 cDNA, which was 2403 bp, encoding a novel protein of 337 amino acids (Fig. 1). The 3' UTR of ApoL2 contained two polyadenylation signals (AATAAA) at positions 1971 and 2383, which may lead to two different transcripts. Indeed, both types of transcripts are present in dbEST. ApoL1 shows an N-terminal extension compared with the other sequences due to the presence of an upstream start codon, which is in-frame with

<table>
<thead>
<tr>
<th>Intron</th>
<th>Gene</th>
<th>Intron-exon boundaries</th>
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<tbody>
<tr>
<td>1</td>
<td>Apo1</td>
<td>ATTCCTTGGtaagtg...tgctcaagGAGGAGGC</td>
</tr>
<tr>
<td>2</td>
<td>Apo1</td>
<td>TGCATCTGtgaagctt ccacacagGATGAGTG</td>
</tr>
<tr>
<td>X</td>
<td>Apo1</td>
<td>GGACCTTGGtaagtta ccctcacagGACAAGAG</td>
</tr>
<tr>
<td>2a</td>
<td>Apo1</td>
<td>GGAGCGAGgtaggtgt tctcaagGATGCAAC</td>
</tr>
<tr>
<td>2b</td>
<td>Apo1</td>
<td>GGACCCAGtgaggctt ccactagAGAGCAGT</td>
</tr>
<tr>
<td>3</td>
<td>Apo1</td>
<td>GTGCCCAAGttaagctc tgtgcagGATGAGG</td>
</tr>
<tr>
<td>4a</td>
<td>Apo1</td>
<td>TCGCCAAGttggagt ctgggagGTGAGCT</td>
</tr>
</tbody>
</table>

Capital letters represent exons and small letters are introns. All intron–exon boundaries conform to the gt/ag rule except for the last part of APOL1.
that used by ApoL2 and ApoL3 (Fig. 1). This part contains the signal peptide and apparently ensures the secretion of ApoL1. Neither ApoL2 nor ApoL3 contained a consensus signal peptide.

**Genomic Organization**

All three genes are clustered on chromosome 22q13.1 on a region of 130 kb, between nucleotide 33,178,000 and 33,308,000, as can be viewed at the Genome browser (August 2001 freeze; http://www.genome.ucsc.edu/; Fig. 2). There is an additional highly homologous region of 15.7 kb between ApoL2 and ApoL3, corresponding to ApoL4. Dot-plot analysis

![Genomic organization of the Apol1-Apol4 gene cluster on chromosome 22q13.1. "Percent identity plot" or pip files were produced by MultiPipMaker (http://bio.cse.psu.edu/pipmaker/). The sequences of the Apol gene cluster were obtained from the genome browser (http://www.genome.ucsc.edu/; position 33,178,000-33,308,000). Repetitive elements were masked by the RepeatMasker program (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker/). Self-comparison of this 130-kb region of chromosome 22 shows an additional homologous region, designated Apol4. Regions with CpG/GpC > 60% are shown by small empty boxes and CpG/GpC > 70% by small gray boxes. Large gray boxes are noncoding exons and large black boxes are coding exons. Arrows depict the direction of the transcription. The overall genomic organization of the exons and introns of the Apol1-Apol3 transcripts is almost identical, except the additional exon X of Apol1. This exon contains a start codon that is in-frame with the initiating methionine of the other two transcripts, encoding the larger, signal peptide containing N-terminal part (Fig. 1).
of the genomic sequences of the members of this gene cluster revealed a high homology throughout the genomic sequences including the intronic sequences, implying genomic duplication (Fig. 3A). The previously reported ApoL5 and ApoL6 are only distantly related to the ApoL1-ApoL4 cluster, as homology is restricted to the cDNA level with no homology in the intronic regions (Fig. 3B). ApoL3 spans approximately 21 kb of the genomic DNA, whereas ApoL2 and ApoL1 span 14 and 15 kb, respectively. This difference is mainly caused by the length of the first intron, which is 11, 6, and 4 kb in ApoL3, ApoL2, and ApoL1, respectively. The first intron of ApoL3 is also the only non-homologous region of the genomic sequence, when compared with ApoL1 or ApoL2, and contains seven Alu repeats.
Expression Profile of the ApoL Gene Family

Because of the high homology among the ApoL genes, specific probes with low homology were chosen and found by Southern blot analysis to yield less than 1% cross hybridization (data not shown). Northern blot analysis showed ApoL1, ApoL2, and ApoL3 mRNA to be upregulated in HUVEC after prolonged tumor necrosis factor (TNFα) stimulation (Fig. 4). Northern blot analysis with a probe derived from a putative exon from ApoL4 did not give a detectable signal in cultured endothelial cells. When normalized for β-actin expression, ApoL1 was more than 10-fold upregulated after 48 hours and ApoL2 was upregulated more than sixfold after 6 hours. As previously reported, ApoL3 was also upregulated by 10-fold after 6 hours.

To assess the tissue specificity of these genes, we carried out multiple tissue northern blot analysis with the same probes. For all three genes, a single hybridizing band of the expected size was observed (Fig. 5A), which demonstrates both the specificity of these probes and the existence of a single prominent splice form. An additional band (2.7 kb) observed for ApoL2 in brain only is in agreement with the presence of two polyadenylation sites in the transcript. Again, a similar experiment with an exon from ApoL4 failed to produce a detectable signal, indicating that no genuine mRNA is transcribed in any of the tissues examined. We quantified the hybridization signals after normalization for the amount of poly(A)+ RNA loaded in each lane (Figs. 5A and 5B). The expression of ApoL1 mRNA (3 kb) was high in placenta, lung, and liver, and low in the kidney and heart, but there was virtually no expression in pancreas. The tissue distribution of ApoL3 mRNA (2.3 kb) expression was similar to that of ApoL1. In contrast, ApoL2 mRNA (2.4 kb) was mostly expressed in brain and to a lesser amount in heart, with very low expression levels in other tissues (Fig. 5B).

In situ hybridization on human vascular tissue was carried out to evaluate the cell specificity of the expression of the various ApoL1 transcripts in vivo. As a positive control, von Willebrand factor (vWF) was used, which is exclusively expressed by the endothelial cells in the vessel wall.

![Multiple tissue northern blot analysis.](image)

**Fig. 5.** Multiple tissue northern blot analysis. A single hybridizing band corresponding to the sizes of the isolated cDNAs was observed for Apol1-Apol3 in all tissues, but an additional band for ApoL2 was detected in brain only (A). The expression of ApoL1-Apol3 in each of the lanes represented in (A) was quantified and normalized for the amount of poly(A)+ RNA to correct for loading differences (B). ApoL1 and ApoL3 are widely expressed, with high expression in the liver, lung, placenta, and heart. ApoL1 has a more restricted expression pattern, being highly represented in the heart and brain.
ApoL expression across species

Presently, several model organisms are used to study the effects of aberrant plasma lipid profiles on cardiovascular disease, most notably the mouse and rabbit. We searched the NCBI EST databases for expressed ApoL homologs in other species, but were unable to find any. Therefore, we determined the genomic presence of this gene family in different species using specific probes for Southern blot analysis, as shown in Fig. 7. As expected, the evolutionary well-conserved α-tubulin gene was detected in all species tested under the low stringency hybridization conditions used. ApoL1, ApoL2, and ApoL3 each show specific bands in humans corresponding to the sizes of the predicted restriction fragments. Unlike

FIG. 6. In situ hybridization of the Apol gene family in human abdominal aorta. (A and B) An antisense ribo-probe to vWF was used to demonstrate the identity and integrity of the endothelial cell lining and the quality of the mRNA in situ. Apol1 (C and D) was expressed in endothelial cells and some isolated neointimal cells (arrows). Apol2 (E and F) and Apol3 (G and H) were expressed exclusively by the endothelial cells. The neointimal scattered silver grain for Apol2 and Apol3 represents background noise, which can be distinguished from the signal because it lies outside the cytoplasm of the cells.
the α-tubulin gene, however, the ApoL gene family is only detected in humans and monkeys under identical low-stringency hybridization conditions. When less unique sequences from Apol1-Apol3 were used, each probe detected its paralogs in primates, but still no specific bands were detected in other species (data not shown).

DISCUSSION

Although endothelial cells are the first cells of the vessel wall to come in contact with lipoprotein particles in blood, their role in local lipid metabolism is not fully understood. It is generally accepted that endothelial injury caused by various agents, such as oxidized LDL, is the first step in the chronic inflammatory process in the vessel wall leading to atherosclerotic plaques. The ApoL1 protein was first isolated from HDL particles and was reported to be expressed specifically by the pancreas [5]. We have described here the isolation of the complete cDNA encoding ApoL1 by conventional cDNA cloning and shown expression of ApoL1 mRNA in liver, heart, lung, and placenta, but virtually no expression in pancreas. In vascular tissue, ApoL1 was specifically expressed by endothelial cells. Due to the positive correlation between plasma levels of ApoL1 and plasma triglyceride levels, it was suggested that ApoL1 might have a role in lipolysis of triglyceride-rich particles [9]. The site of ApoL1 expression is consistent with its possible role in lipolysis because this process takes place at the luminal surface of endothelial cells [3]. Furthermore, the expression of all three members of the ApoL gene family was upregulated by TNF-α, which is a potent proinflammatory cytokine. During infection and inflammation, plasma lipid profiles show characteristic proatherogenic
changes such as increased triglyceride levels, which are mostly due to decreased lipolysis [10]. Therefore, the effect of TNF-α on the expression of the ApoL gene family could be of importance in regulating the lipid profile in vivo during inflammation.

Two different groups recently reported the PCR cloning of ApoL gene family members and their mapping to human chromosome 22q13.1 [6,7]. Using a conventional cloning procedure, we isolated ApoL1-ApoL3 from an endothelial cell library and show expression of these genes in vivo. We did not detect ApoL4 expression in the tissues tested here. However, using RT-PCR, ApoL4 expression has been reported by others [6,7]. Hence, the importance of ApoL4 expression in vivo remains to be established. Although splice variants of ApoL1-ApoL4 have been reported [7], we did not detect these, indicating that in vivo the reported splice variants for the ApoL gene family are apparently expressed at a very low level, beyond the detection limit of northern blot analysis. One of the ApoL3 splice variants (ApoL3c), although not detected (Fig. 5A), was also found in our endothelial cell cDNA library. Using an in vitro transcription-translation assay, protein expression was only detected for ApoL3 and not for ApoL3c (data not shown). These observations cause uncertainties on the biological role of the reported splice variants, which might originate from the recent emergence of this gene cluster.

The ApoL gene family consists of the ApoL1-ApoL4 cluster and the ApoL5-ApoL6 cluster [6,7]. There is a 400 kb intergenic region between these two clusters that contains non-related genes (genome browser at http://www.genome.ucsc.edu/). Four observations indicate that the ApoL1-ApoL4 cluster is the result of recent tandem duplication. First, dot-plot analysis of the genomic sequences of the ApoL1-ApoL4 cluster revealed a sustained high homology among these genes, including the intronic regions (Fig. 3A), whereas ApoL5 and ApoL6 show homology to this cluster mainly at the protein level. The only non-homologous region in this cluster was the first intron of ApoL3, which also contained many additional Alu repeats. Nishio et al. used these repetitive DNA elements to analyze genomic expansion of the albumin gene family on human chromosome 4 [11]. In their case, the vitamin-D-binding protein gene was the earliest gene in the line of evolution and had the highest number of repetitive elements, indicating that such differences accumulate after duplication. Given that ApoL3 is both the least homologous of the three family members and has a much higher number of repetitive elements, ApoL3 may be the ancestor gene in this gene cluster. Second, the overall gene structure (that is, intron-exon boundary structure) has remained almost identical, indicating a recent duplication in the human genome. Third, the original analysis of human chromosome 22 suggested that it contains a high degree of segmental duplications [12], amounting to as much as 10.8% of the finished sequence [13]. Most of these segmental duplications are recent in evolution and even partly human-specific, and have contributed to primate genome evolution. The most recently duplicated sequence lies most proximal to the centromere, whereas more “ancient” duplications lie more distal. Fourth, in accordance with these observations, we indeed could only detect the ApoL1-ApoL4 cluster in humans and African green monkey. The ApoL1-ApoL4 gene cluster is located more distal at chromosome 22q13.1 (position 33 Mb) and must have arisen before the divergence.
of African green monkeys. Finished sequences of different species are needed for a more accurate phylogenetic analysis.

Most duplicated genes experience a brief period of relaxed selection early in their history [14]. The vast majority of gene duplicates are silenced within a few million years, unless they have evolved effectively and survive the selection. In this regard, it is conceivable that ApoL4 is in the process of silencing and, although expressed at low levels, has no in vivo relevance [15]. It has been proposed that a multi-gene system provides an efficient mechanism for generating genetic diversity (that is, generating new alleles that are useful for the organism) [16]. The DNA sequence of the coding regions of the ApoL1-ApoL4 gene cluster members was more than 80% homologous, whereas the amino acid identity was only 42%, indicating that these proteins have evolved diversely after duplication. The most striking difference among the protein-encoding parts of ApoL1-ApoL4 was the additional exon in ApoL1 (exon X), which contained an upstream start codon, in-frame with the one used by the other transcripts. This gives rise to a larger signal peptide encoding N-terminal part. Thus, the introduction of this new exon has directly resulted in the generation of a secreted protein, ApoL1, which has been shown to be specifically associated with plasma HDL particles. The diversification is not only in the protein-encoding regions but also in the regulatory elements, leading to the tissue-specific expression of ApoL1-ApoL3, with ApoL2 being the only member highly expressed in the brain.

Although a large proportion of the mouse genome has been sequenced, there is no evidence for the existence of the ApoL1-ApoL4 gene cluster in mice. Completion of genomic sequencing of different species would be important in the final phylogenetic analysis of this gene cluster. The mouse is a widely accepted animal model for the study of lipoprotein metabolism and atherosclerosis and indeed it has been proven to be useful in several studies [17]. Still, the value of the mouse as a human lipoprotein disease model is limited by the differences in lipid metabolism between these two species [18]. First, a specific ortholog, ApoLipoprotein E, is more important in lipoprotein distribution in mice than it is in humans. Second, there is different tissue distribution of a specific ortholog; ApoLipoprotein B mRNA editing enzyme (APOBEC1) is expressed only in the intestine of human, whereas it is expressed in both intestine and liver of mice, leading to APOB48 expression in both organs. As a result APOB48 accounts for a major proportion of plasma APOB in mice [19,20]. Third, cholesteryl ester transfer protein (CETP), which is an important enzyme in human lipid metabolism, does not exist in mice and rats [21]. CETP is a key enzyme in the process of reverse cholesterol transport and its role in atherosclerosis has been established [22]. Another example of a primate-specific gene product involved in lipid metabolism is ApoLipoprotein (a) (LPA). This component of lipoprotein (a), a major inherited risk factor for atherosclerosis, resulted from the genomic duplication of the plasminogen gene that has only occurred in primates [23]. We have shown that the ApoL1-ApoL4 gene cluster has emerged only in primates and might as such contribute to the differences in the lipid metabolism among species.
REFERENCES

Chapter 4

Apolipoprotein L2 and L3 differentially modulate intracellular triglyceride storage

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ABSTRACT

ApoL gene cluster, consisting of 4 homologous genes on human chromosome 22, is the resultant of a recent genome duplication and only exists in primates. It has been shown that apoL1 is an HDL-associated protein that lyses trypanosomes by pore formation in their membrane. There is a positive correlation between the plasma levels of apoL1 and triglyceride levels that suggests a role in lipid metabolism. The role of apoL2 and apoL3 in lipid metabolism is unknown. Here, we show that apoL2 and apoL3 are both intracellular proteins that have a different subcellular localization: apoL2 co-localized to the endoplasmatic reticulum, whereas apoL3 resides in the Golgi. *In vitro* as well as *in vivo* overexpression studies using adenoviruses containing either apoL2 or apoL3 revealed that they have the opposite effect on intracellular triglyceride levels with no effect on cholesterol levels. ApoL2 induced triglyceride accumulation only in cells capable of storage such as hepatocytes and adipocytes, whereas apoL3 reduced triglyceride levels in endothelial cells and hepatocytes but has no effect in adipocytes. During the differentiation of monocytes to lipid-laden macrophages, the endogenous expression of ApoL2 mRNA increases parallel to PPARγ expression, which is a known transcription factor involved in adipogenesis. On the other hand, ApoL3 mRNA expression shows exactly the opposite and is completely repressed during the foam-cell formation. Therefore, we conclude that although these two genes are highly homologous as the result of tandem duplication, they have evolved and gained different subcellular localization and function.
INTRODUCTION

The Apolipoprotein-L gene cluster is a primate-specific gene family that has arisen recently in the evolution (1). This gene cluster on human chromosome 22 consists of 4 separate genes (ApoL1-4) that are highly homologous and are the result of tandem gene duplication (1-3). We previously reported, based on detailed genome analysis, that ApoL3 might be the ancestral gene that has been duplicated rapidly (1). However, the rationale for the evolutionary creation of this gene cluster into the human genome remains elusive.

The ApoL1-3 genes are expressed in human endothelial cells (ECs), not only in vitro but also in human vascular tissues (1,4). ApoL1 was the first protein of the ApoL gene cluster to be discovered. This protein of approximately 42 kD was found to be mainly associated with HDL particles (5). Because of its specific binding to HDL particles, and the observation that apoL1 was not found circulating in unbound form in plasma, it was suggested that it might have a role in lipid metabolism and was thus designated as apolipoprotein. In later studies, it was shown that the plasma levels of apoL1 were positively correlated with hypertriglyceridemia and hyperglycemia (6,7). Recently, however, Vanhamme et al. unraveled a quite unexpected role for apoL1, being in fact the long sought Trypanosoma lytic factor (8).

There is a high homology among the members of the ApoL gene cluster not only at the DNA level but also at the protein level. The most significant difference between apoL1 and other members of the gene family is the specific signal peptide at the N-terminal part of apoL1, due to the presence of an additional protein encoding exon (1). Therefore, apoL1 is secreted and becomes associated with HDL particles. ApoL2 and apoL3 are most probably intracellular proteins as they lack the signal peptide encoding exon. We could not detect any expression of apoL4 in human tissues and assume that the ApoL4 locus is a pseudogene.

Although apoL1-3 are designated as apolipoproteins, the function of apoL2 and apoL3 in lipid metabolism has not been investigated. To study the function of these proteins, we designed adenoviruses containing either full-length ApoL2 or ApoL3 cDNA to analyze their function in vitro as well as in vivo. As already mentioned, this gene cluster is primate-specific and, accordingly, the murine genome does not harbor any of these genes in their genomes. Therefore, the mouse is a suitable animal model because it mimics a naturally occurring ApoL knock out model and is adequate for studying the role of ApoLs in vivo. Our results show that apoL2 and apoL3 are both intracellular proteins that are involved in triglyceride metabolism, but have opposite effects.

MATERIAL AND METHODS

Cell Culture

Human umbilical vein ECs (HUVECs) were isolated and cultured in the same way as the endothelial cell line (EC-RF24) (9, 10). The culture medium was composed of Medium 199 (Invitrogen, Paisley, U.K.), supplemented with 20% (vol/vol) fetal bovine serum, 50 µg/ml
heparin (Sigma, St. Louis, MO), 12.5 µg/ml endothelial cell growth supplement (Sigma), and 100 U/ml penicillin/streptomycin (Invitrogen). Preadipocyte SW 872 (SW) cells, derived from human liposarcoma (American Type Culture Collection product number HTB-92), were cultured in DMEM/F12 mixture at a ratio of 3:1, supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS). Human peripheral blood monocytes were isolated and cultured in RPMI 1640 (BioWhittaker, Viviers, Belgium) supplemented with 10% (vol/vol) human serum as described (11). Differentiation of monocytes into activated macrophages spontaneously occurs upon prolonged cell culture.

Construction of recombinant adenoviruses
Cloning of full-length ApoL2 and ApoL3 cDNAs was described previously (1,4). The ApoL2 coding sequence was excised from the cloning vector and inserted into pAdCMV shuttle vector to produce Ad-ApoL2 (12). The same procedure was performed for Ad-ApoL3. Both constructs were sequenced and contain the complete coding sequence of ApoL2 and ApoL3 genes. Recombinant second-generation adenovirus was generated as previously described (12). Briefly, the plasmid was co-transfected into 293 cells along with adenoviral DNA and cells were overlaid with agar and incubated for 15 days at 32°C. Plaques were picked and screened by PCR. Those positive for the ApoL2 and ApoL3 cDNA were subjected to a second round of plaque purification. After confirmation of the presence of apoL2 and apoL3 cDNA and the absence of wild-type adenovirus, the recombinant adenovirus was expanded in 293 cells at 32°C. Cell lysates were used to infect HeLa cells for confirmation of the expression of human ApoL2 and ApoL3 mRNA by Northern blotting. The recombinant adenovirus, designated as Ad-ApoL2 and Ad-ApoL3 were further expanded in 293 cells and purified by using cesium-chloride gradient ultracentrifugation. The empty virus (Ad-null) and a Green Fluorescent Protein (GFP)-containing adenovirus (Ad-GFP) were also subjected to plaque purification and purified as described above. The purified viruses were stored in 10% (vol/vol) glycerol/PBS at -80°C.

Construction and expression of apoL2 and apoL3 GFP fusion proteins.
To detect subcellular localization of apoL2 and apoL3, we constructed C-terminal GFP fusion protein by inserting the respective open reading frames, devoid of stopcodons, into pEGFP-N vector (Clontech, Palo Alto, CA). Transfections of various cells with GFP-constructs were performed using Superfect (Qiagen), according to the manufacturer’s instructions. Coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA) and images were recorded, using a Bio-Rad MRC 1024 confocal laser-scanning microscope (CLSM).

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
Semi-quantitative RT-PCR was performed on total RNA isolated by using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA) as described (13). Gene-specific primers for ApoL2, ApoL3, PPARγ and hypoxanthine phosphoribosyltransferase (HPRT) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_
www.cgi). The following primers were used for ApoL2 (GGTGTTAGAGTAGGGAAAGAG and CCACCTGCTGTGCTGGTGC) and ApoL3 (ATGGACTCAGAAAGAAACGC and CGTCCTCAATACTGCATATG), respectively. After correction for HPRT, ApoL2 and ApoL3 mRNA levels were expressed as ratios compared to the control cultures in time.

**Antibodies**

Two separate, non-homologous synthetic peptides of the human ApoL2 (KIHEMLQPGQDQ) and ApoL3 (MDSEKKRFTEEAT) were used to raise antisera in rabbits by the Eurogentec Double-X program (Eurogentec, Seraing, Belgium). The following antibodies were used for colocalization experiments; Anti-giantin (kind gift of dr. H.P. Hauri, University of Basel, Switzerland.), anti-PDI (product number SPA-890, Stressgen, Victoria, Canada), anti Phalloidin-TRITC (Sigma-Aldrich, Zwijndrecht, The Netherlands), anti-tubulin (Cedarlane Laboratories, Burlington, Ontario, Canada) and anti-vimentin (polyclonal ab8545, Abcam, Cambridge, U.K.).

**Animal studies**

Six to eight weeks old, female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and fed a regular Chow diet (Diet 5010, PMI Nutrition International, Richmond, IN). Mice were bled from the retro-orbital plexus after a 4 h fast using heparinized capillary tubes. Blood was drawn into tubes containing 2 mM EDTA, 0.2% NaN₃ (w/vol), and 1 mM benzamidine and placed immediately on ice. Aliquots of plasma were stored at -20º C until analysis. Mice were injected via the tail vein with 1x10¹¹ particles of purified recombinant adenovirus in a volume of 0.1 ml. At the indicated times, blood was collected from the retro-orbital venous plexus into heparinized capillary tubes. Mice were anesthetized, and the vasculature was perfused with cold phosphate-buffered saline (PBS). Liver was removed and stored at -20°C until lipid extraction. All animal experiments were performed according to institutional guidelines of the University of Pennsylvania, School of Medicine.

**Lipid measurements**

Cholesterol and triglyceride were measured using commercially available kits (Infinity Triglyceride and Cholesterol Kit, Thermo Electron Corp.). Hepatic lipids were measured following solubilization of the homogenized tissue with deoxycholate (14). Briefly, livers were homogenized in PBS using 3 ml PBS/g liver. The homogenate was quick frozen on dry ice and kept at -80° C till assayed. Homogenates were quickly thawed at 37° C and then diluted 5-fold with PBS, resulting in a homogenate with 50 mg protein/ml. Solubilization was performed in a 96-well plate with 20 µl of homogenate and 20 µl of 1% (w/vol) deoxycholate and incubated at for 5 min 37° C. For triglyceride measurements, 200 µl of reagent (Infinity TG Reagent, Sigma, St. Louis) was added and incubated for 15 min at 37° C. For cholesterol measurements, 200 µl of reagent (Infinity Cholesterol Reagent, Sigma) was added and incubated for 5 min at 37° C. Samples were agitated during incubations and the optical density read at 500 nm. The triglyceride content was determined using a standard
curve generated with Lipid Lin-Trol prediluted standard set (Sigma). Protein was measured using the BioRad Protein Assay (BioRad, Hercules, CA) with serum albumin as standard. Liver biopsies were stained with oil-red O.

Statistical Analysis
Values are presented as mean±SEM. Results were analyzed by either one-way ANOVA or Student’s t test, where appropriate, with the use of SPSS. P-values of less than 0.05 were considered significant.

RESULTS
ApoL2 and apoL3 are intracellular proteins
To study the function of ApoL gene family members, we generated adenoviruses to express the full-length proteins in HUVECs. The infection efficiency was about 95% as measured by the number of GFP-containing cells. The expression level of apoL2 and apoL3 was confirmed by Western blotting (Fig. 1 A). The molecular weight of both proteins was in agreement with their calculated size: apoL2 is approximately 37 kD, whereas apoL3 is 31 kD. As these proteins have no signal-peptide, we anticipate that they will be intracellular proteins. Indeed, both apoLs were only detected in the cell lysates and not in the culture media.

At this point, we could not establish the subcellular localization of apoL2 and apoL3 with the rabbit antisera, either due to the low endogenous occurrence of these proteins or due to lack of exposure of the specific epitope in tertiary structure of the apoL proteins after cell fixation with various agents. To establish the exact intracellular localization of these two proteins, we

![Fig. 1: Western blotting after apoL2-3 overexpression in HUVEC. ECs were infected with adenoviruses containing full-length cDNA of GFP, ApoL2 or ApoL3. In cell lysates, anti-apoL2 antibody recognized a specific band of 37 kD (lane 2) and anti-apoL3 a protein of 31 kD (lane 3). No cross-reactivity between antibodies was observed (A). Subcellular localization of apoL2-GFP and apoL3-GFP in HUVEC (B-I). ApoL2-GFP fusion protein had a peri-nuclear expression pattern (B) and partly co-localized to PDI (C). There was no co-localization of apoL2 to actin filaments (D), intermediary filaments (E), microtubules (F). Co-localization is observed between apoL3 and the Golgi complex stained for giantin (G-H). ApoL2 did not co-localize with the Golgi apparatus (I). (for colour picture, see back cover)
transfected HUVECs with ApoL2-GFP and ApoL3-GFP fusion constructs. ApoL2-GFP fusion protein was detected in the cytoplasm of ECs and its expression pattern was peri-nuclear, but also appeared to be associated with the cytoskeleton (Fig. 1B). We hypothesized that apoL2 is associated with the endoplasmatic reticulum (ER) and to explore this hypothesis we used an antibody against Protein Disulfide Isomerase (PDI), which is a known ER marker. Indeed, apoL2 partly co-localized with PDI (Fig. 1C). To investigate the association of apoL2 with the cytoskeleton, specific antibodies to actin filaments (anti-Phalloidin antibody), intermediate filaments (anti-Vimentin antibody) and microtubules (anti-tubuline antibody) were used. However, none of them revealed co-localization with apoL2-GFP (Fig. 1D-F). On the other hand, apoL3-GFP specifically co-localized to the Golgi complex as shown by anti-Giantin antibodies (Fig. 1G-H). In contrast, apoL2-GFP did not show any detectable co-localization with the Golgi apparatus (Fig. 1I).

**Triglyceride content of ECs is significantly reduced by Apolipoprotein L3**

As an effect of both apoL family members on lipid homoeostasis could be expected, we analysed the adeno viral-infected cells for various lipid species content, being cholesterol, triglycerides and phospholipid. Although, the presence of apoL3 had no effect on intracellular cholesterol, triglyceride levels were significantly reduced by 30% upon apoL3 overexpression (Fig. 2 A, B). Overexpression of apoL2 in ECs had neither effect on cholesterol nor on triglyceride levels.

**ApoL3 reduces triglyceride levels in vivo with no effect on cholesterol levels**

To evaluate the effect of apoL3 on triglyceride levels in vivo, C57BL/6 mice were injected with either empty virus (Ad-null) or ApoL3-containing adenoviruses (Ad-ApoL3). At day 3, the mice were sacrificed and the lipid profile as well as the lipid content of the liver was measured.

*Fig. 2: Effects of apol2-3 overexpression on HUVEC lipid profile*

The lipid content of adenoviral infected ECs was measured. Neither Ad-ApoL2 nor Ad-ApoL3 affected the intracellular cholesterol levels (A), whereas the triglyceride levels were significantly reduced by overexpression of ApoL3 (B). *p<0.05.*
ApoL3 overexpression had no effect on plasma cholesterol, whereas plasma triglyceride was reduced by 25% (Fig. 3B). Plasma HDL-cholesterol and phospholipids were also unaffected (data not shown). Like in cultured ECs, apoL3 again clearly reduced the liver intracellular triglyceride level by 20%, although this effect did not reach statistical significance (Fig. 3A).

ApoL2 increases triglyceride levels in vivo with no effect on cholesterol levels
To detect the effect of apoL2 in vivo, we performed the same experiment in C57BL/6 mice with either Ad-ApoL2 or the control empty virus (Ad-null). ApoL2 had no effect on plasma lipids (Fig. 4A). On the other hand, the livers of ApoL2-injected mice were extremely steatotic as could be visually observed after sacrifice. After measuring the lipids, it was clear that the steatosis was due to increased triglyceride levels (Fig. 4B). Cholesterol as well as phospholipid
levels were not affected by apoL2, whereas the triglyceride level was almost doubled. Oil-red O staining of liver sections revealed that steatosis was diffuse and almost all cells revealed large, intracellular fat droplets (Fig. 5).

**ApoL2 increases triglycerides in adipocytes**

An apparent discrepancy has been reported in the previous paragraphs, while studying different cell types. Whereas a massive effect of apoL2 on intracellular triglyceride was detected in murine hepatocytes, no such effect had been detected in human cultured ECs. Therefore, we addressed the question as to what the role of apoL2 would be in human cells that are specialized in storing fat. Human adipocytes were infected either with Ad-GFP as control, Ad-ApoL2 or Ad-ApoL3. The cell lysates were analyzed for expression of apoL2 and apoL3 and the intracellular lipids were measured. As shown in Fig. 6, the intracellular triglyceride levels were indeed significantly increased by apoL2 overexpression, whereas no

**Fig. 5: ApoL2 overexpression induces murine liver steatosis**

Oil-red O staining of the mice livers, sacrificed after 3 days of apoL overexpression. ApoL2 overexpression clearly induces large fat droplets in the cells, whereas ApoL3-infected livers are devoid of fat.

(for colour picture, see back cover)

**Fig. 6: Effects of apol2-3 overexpression on human adipocyte lipid profiles**

Overexpression of apoL2 in adipocytes had no effect on cholesterol levels (A) whereas it increases intracellular triglyceride content (B). ApoL3 overexpression has no effect on either of the lipids in these fat storing cells. *:p<0.05.
effect on cholesterol was detected. In contrast, apoL3 overexpression did not affect levels of either lipid in this cell type.

**Endogenous expression of apoLs during foam-cell formation**

We then set out to analyze the role of the endogenously expressed apoL2-3 in fat storage in an *ex vivo* human model, relevant for vascular disease. Therefore, human primary monocytes were collected from blood of healthy volunteers and were kept in culture media for 3 weeks. During this period, they differentiate into lipid-laden foam cells, due to accumulation of both cholesterol and triglyceride (15). Peroxisome Proliferator-Activated Receptor γ (PPARγ) is a transcription factor that is mainly expressed in adipose tissue and regulates fat-cell differentiation and fatty-acid uptake and storage. It is well established that PPARγ expression is induced during foam-cell formation of human macrophages. At different time points, mRNA expression of ApoL2, ApoL3 and PPARγ was measured by RT-PCR. ApoL2 as well as PPARγ mRNA expression were both increased in a time-dependent manner, whereas apoL3 mRNA expression was completely repressed during foam-cell formation (Fig. 7). These data indeed reveal a discordant expression of the two pro- and anti-lipid storage apoLs during human foam-cell formation and triglyceride storage, in accordance with the findings reported on apoL2 and apoL3 in the previous paragraphs.

![Fig. 7: Endogenous apoL2-3 expression during human foam cell formation](image)

**DISCUSSION**

The primary findings of this paper are: 1) ApoL2 and apoL3 are both intracellular proteins, but have different subcellular localizations 2) they both change the intracellular triglyceride levels, but in opposite direction and 3) during foam-cell formation the endogenous mRNA expression of both genes are coordinately affected in opposite direction.

The function of apoL1 has been extensively studied and it was found to be the Trypanosoma Lytic factor. Interestingly, Vanhollebeke *et al.* described a patient who was infected
with *Trypanosoma evansi*, whereas normally humans are resistant to infection with this parasite. It turned out that this patient had a complete lack of plasma apoL1 (16), due to mutations in both ApoL1 alleles. Whether apoL1 plays a role in lipid metabolism, beside its trypanolytic activity, remains unanswered. The fact that its level was clearly associated with high triglyceride levels, however, does suggests a certain role in lipid metabolism (6,7). The function of apolipoprotein L2-4 had not been addressed as yet. Due to the lack of ApoL4 mRNA expression as detected by multiple-tissue Northern blot analysis, we argued that ApoL4 might be a pseudogene that has lost its expression during the evolution (1). Therefore, the current experiments were performed to solely address questions regarding the role of apoL2 and apoL3 in lipid metabolism, using both cultured human cells and a murine model.

ApoL2 overexpression significantly increased the intracellular triglyceride levels, but only in cells that are capable of triglyceride storage. Indeed, ECs are not equipped for triglyceride storage and there was no increase detected in these cells. There was also no effect on plasma lipid levels, when expressed in murine liver, but a clear steatosis was observed. Primary human monocytes can differentiate *in vitro* into foam cells (11). Interestingly, during this process the endogenous expression of apoL2 was also increased in the same fashion as PPARγ. The highest levels of PPARγ are expressed in adipose tissue and its expression and activation is sufficient to induce adipogenesis (17-19). The essential role of PPARγ in adipogenesis has been clearly demonstrated in functional and genetic knockdown experiments (20). ApoL2 expression, along with PPARγ, is increased during adipogenesis and in monocytes during foam-cell formation, and apoL2 overexpression increased intracellular triglyceride in adipocytes.

ApoL3 overexpression in human ECs reduced intracellular triglyceride content significantly whereas it had no effect on triglyceride content in adipocytes. In murine liver, apoL3 overexpression reduced the intracellular triglyceride content by 20% and plasma triglyceride by 25%, respectively. Apparently, in cells that are specialized to store triglyceride (adipocytes), apoL3 overexpression does not reduce triglyceride content, whereas it does so in non-adipocytes. In human macrophages, its endogenous expression was completely repressed during foam-cell formation. All these data are in line with the conclusion that apoL3 has an opposite effect on triglyceride storage as compared to apoL2.

How to explain a opposite function of two highly homologous proteins? One option might be that even though the differences between apoL2 and apoL3 are minor, due to the novel protein sequences, the effect on the structure and function of these proteins might be significant. For instance, apoE2-4 isoforms differ by only one amino acid and yet their function is substantially altered. Alternatively, the change of subcellular location might induce novel functions. To define the intracellular localization of apoL2-3, we generated antibodies that can specifically detect either apoL2 or apoL3. In cells infected with adenoviruses,
containing these two genes, we could only detect the protein in the cell lysate and not in the
culture media, showing that these proteins remain intracellular and are not secreted to any
detectable level. This is in agreement with our previous hypothesis based on the lack of signal
peptide in these proteins. The apoL2-GFP fusion protein displayed peri-nuclear appearance
and partly co-localized to the ER, whereas apoL3-GFP co-localized with Giantin, which is
an established Golgi marker (21). The potential large impact of subcellular localization of
proteins in intracellular lipid metabolism is well documented, i.e in the case of Microsomal
Triglyceride transfer Protein (MTP). MTP is an essential protein in the assembly of VLDL in
hepatocytes and of chylomicrons in enterocytes. In these cells MTP is located in the ER.
Interestingly, Mohler et al. reported on a splice variant of MTP that was mainly expressed in
adipocytes and co-localized to the Golgi. The Golgi apparatus is involved in lipid packaging
in lipid droplets as well as in lipoprotein particles. It seems that, changing the location of a
splice variant of the same protein in adipocytes, causes lipid storage instead of secretion (22).
Apparently, the switch in intracellular location of apoLs introduces a novel function, possibly
by changing their interaction partners. However, the exact mechanism by which apoL2 and
apoL3 modify the intracellular triglyceride is yet to be determined.

ApoL3 is the ancestral gene in this gene cluster that has duplicated rapidly in the course of
evolution. It has been postulated that, depending on the selection pressure on duplicated
genes, they could either undergo diversification or concerted evolution (23). In the case of
apoL family members, clearly diversification has occurred, which is also in accord with the
fact that their respective amino-acid sequences differ more then their DNA sequences (1).
ApoL2 and apoL1 have both evolved in different fashions. ApoL1 has become a secreted
plasma protein and apoL2 has adopted a different intracellular localization and has gained
a novel function. One might argue that the environmental pressure, that once may have
caused the increase in gene dosage by duplication, has disappeared in the course of evolution
leading to novel functions of duplicated genes. Most species lack the ApoL2-3 genes, but
their triglyceride metabolism is obviously adequate. Therefore, it is reasonable to assume
that the ApoL genes have a moderate effect on triglyceride metabolism and are involved in
fine-tuning of lipid storage levels specifically for primates. It is conceivable, however, that in
certain diseases where lipid storage is disturbed, such as during liver steatosis, this presumed
fine-tuning property of apoL2-3 may appear essential. Future experiments on the exact role
of these proteins in health and disease should clarify these issues.

In summary, we have shown for the first time that apoL2 and apoL3 are indeed intracellular
proteins but reside on different organelles. They have opposite effects on intracellular
triglyceride levels that might have emerged merely due to this change of subcellular location.
Obesity and non-alcoholic fatty liver disease and the resulting non-alcoholic steatohepatitis
are ever increasing health problems in the Western societies. Understanding the molecular
basis of intracellular triglyceride storage and specially the suppressive effects of apoL3, might
introduce novel strategies for the treatment of these pathologies.
REFERENCES


Chapter 5

Lipase H, a New Member of the Triglyceride Lipase Family Synthesized by the Intestine

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ABSTRACT

We report here the molecular cloning of a novel member of the triglyceride lipase family, a 2.4-kb cDNA encoding human lipase H (LIPH) and the mouse ortholog (Liph). The human LIPH cDNA encodes a 451-amino-acid protein with a lipase domain. Mouse Liph shows 85% amino acid identity and 75% nucleotide identity to human LIPH. Human LIPH exhibits 47% identity with phosphatidylinerine-specific phospholipase A1 (PS-PLA1) and 46% identity with endothelial lipase (LIPG) and lipoprotein lipase (LPL). LIPH is localized on human chromosome 3q27-q28. Northern blot analysis revealed specific expression of LIPH mRNA in intestine, lung, and pancreas. Lipase H protein was also detected in human intestine. Lipase H is a secreted protein with an apparent molecular weight of 63 kDa. Although several lipid substrates were tested, the lipid substrate of LIPG was not identified. Like the other members of this gene family, LIPH may be involved in lipid and energy metabolism.
INTRODUCTION

Members of the triglyceride lipase gene family [1] encode proteins that hydrolyze triglycerides and phospholipids, generating fatty acids to facilitate intestinal absorption or serve for energy production or for storage. This gene family encodes pancreatic lipase (PNLIP), pancreatic lipase-related protein-1 (PNLIPRP1), pancreatic lipase-related protein-2 (PNLIPRP2), lipoprotein lipase (LPL), hepatic lipase (LIPC), endothelial lipase (LIPG), and phosphatidylserine-specific phospholipase A1 (PS-PLA1). PNLIP and PNLIPRP2 [1] are made in the pancreas; they have triglyceridase and galactolipase activities and are believed to be involved in dietary lipid absorption. LPL [2] has a well-established role in energy and lipoprotein metabolism and is responsible for hydrolyzing chylomicron and very low density lipoprotein (VLDL) triglycerides in muscle and adipose tissues. LIPC [3] has a role in lipoprotein remnant and high density lipoprotein (HDL) metabolism by hydrolyzing both triglycerides and phospholipids. The physiological role of LIPG [4] is uncertain, but it hydrolyzes HDL phospholipids ex vivo [5] and reduces HDL cholesterol levels when overexpressed in mice [6]. The physiological roles of PNLIPRP1 and PS-PLA1 [7] are unknown.

Comparisons of amino acid sequences predicted from the cDNAs encoding the various lipases suggest that this lipase family evolved from a common ancestral hydrolase [1]. Members of this gene family share a conserved domain, the lipase domain. We reasoned that there are likely to be other members of this gene family. While searching for homology to the lipase domain of endothelial lipase, we discovered lipase H (LIPH) and found that it has substantial homology with members of this gene family. Here, we describe the cloning, genomic organization, tissue distribution, and protein expression of both the human and mouse forms of this novel lipase.

MATERIALS AND METHODS

Materials

Mouse anti-Flag antibody was purchased from Sigma. We used the cell lines A549, HeLa, HepG-2, 4 HT-29, MCF-7, NCI-H727, SW480, U937, HPAC, and HEK 293 (all obtained from ATCC).

Cloning

tBLASTn analysis of the EST database at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) with the protein sequence of human LIPG revealed numerous ESTs containing a lipase domain. The EST clones (IMAGE catalog number 97000) were obtained from Research Genetics (Huntsville, AL) and sequenced.

A partial mouse Liph cDNA sequence was obtained by searching the EST database for homology to human LIPH cDNA sequence. The complete ORF was cloned by RT-PCR using the following primers: 5'-GATCTCTTTAGGTCC-CTATGCTG-3' and 5'-AGCTACATCTGCTGAGAGC-3', with mouse placenta total RNA as template. The
PCR product was sequenced, digested with BamHI and XhoI, and the insert was cloned into pCR2.1 (Invitrogen, Carlsbad, CA).

**PCR and 5'-RACE**
The transcriptional initiation site of human LIPH was determined by 5'-RACE (LTI, Rockville, MD). RT-PCR was performed using GeneAmp RNA PCR Kit and Core Kit (ABI, Foster City, CA) using the following primers: 5’-AGGCTGAGCTTTCACAGTGCAG-3’ and 5’-TCTCCAA-CA ACCC AG AT ATGTGG-3’.

**Northern blot analysis**
Total RNA was isolated from human tissues, which were obtained from Cooperative Human Tissue Network (Hospital of University of Pennsylvania, PA), and from adult C57BL/6 mouse tissues, using Trizol (Invitrogen, Carlsbad, CA). For northern blot analysis, 50 µg total RNA was separated on 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes. These membranes were then hybridized with 1.35-kb human LIPH cDNA or a 1.35-kb mouse cDNA probe. The probes were labeled with [32P]dCTP by random priming (Stratagene, La Jolla, CA) and hybridized at 68°C for 1 hour in ExpressHyb Hybridization solution (Clontech, Palo Alto, CA). After hybridization, the membranes were washed three times for 15 minutes with 2X SSC buffer with 0.1% SDS at room temperature, followed by two washes for 15 minutes in 0.1X SSC with 0.1% SDS at 50°C. Visualization was achieved by exposure to Kodak Biomax MS film (Eastman Kodak Co., Rochester, NY).

**In situ hybridization**
In situ hybridization slides were prepared [15] from paraformaldehyde-fixed, paraffin-embedded mouse embryos or tissues according to established methodology. A full-length 1.35-kb mouse Liph cDNA was used for in vitro RNA probe synthesis. Both antisense and sense cRNAs were used for hybridization.

**Construction of recombinant adenovirus and generation of polyclonal antibody**
A recombinant adenovirus encoding human LIPH (Adhuman LIPH) was made as described [6]. A rabbit polyclonal antibody to human lipase H was raised using the adenovirus encoding human LIPH as follows: 5 X 1012 viral particles were injected into a New Zealand White rabbit (Hare-Marland) via the ear vein. Sera were obtained at various intervals for analysis of antibody to human lipase H (anti-human LIPH). Three months after injection, the rabbit was anesthetized and exsanguinated. Serum was prepared and stored at -80°C. The control serum was generated using the injection of an “empty” recombinant adenovirus that contains no transgene.

**Western blot analysis**
Culture medium and 4X sample loading buffer (Invitrogen, Carlsbad, CA) were mixed 3:1 (vol/vol) and heated at 70°C for 10 minutes. The samples were size-fractionated using SDS-PAGE.
(precast 10% polyacrylamide gels; Novex) and transferred to Hybond-P (PVDF) membrane (Amersham Pharmacia Biotech). The membrane was incubated and washed using standard procedures. Detection was carried out by the ECL protocol (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Chemiluminescence signals were detected on x-ray films.

**Plasmids and transfection**
The EST clone containing human LIPH was digested with HindIII and BamH1 and subcloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA); this vector was designated pcDNA3.1 human LIPH. The human LIPH with an in-frame double Flag tag at the C terminus, designated pcDNA3.1 human LIPH-Flag, was constructed by PCR. Primers used were 5'-ATAGGATCCACCATGTTGAGATTCTACTTATTCATCAG-3' and 5'-AGGAATCCTCACTTTGTCGTCGTCGTCCTTGTAGTCCTTGTCGTCGTCGTCCTTGTAGTCC-3', and the PCR product was inserted into pcDNA3.1(+). The fidelity of the insert was confirmed by DNA sequencing. All transfections were performed in HEK293 cells using Lipofectamine (Invitrogen, Carlsbad, CA). Conditioned media were collected 24-48 hours post-transfection.

**Computer analysis**
Phylogenetic trees were generated using Vector NTI 6.0 Suite (Informax, North Bethesda, MD). MacVector (Accelrys, Princeton, NJ) was used for primer design and alignment.

**RESULTS**

**Cloning and Sequence Analysis of LIPH**
The human LIPH EST clone AA149682 has a 2426-bp insert containing a complete open reading frame (ORF) of 1356 nucleotides. Mouse Liph cloned by RT-PCR from mouse placenta has an ORF the same size as that in human. Therefore, both human and mouse Lipase H cDNAs encode proteins of 451 amino acids. The mouse Liph and the human LIPH cDNAs share 75.3% identity, and the mouse and the human lipase H proteins share 85.4% identity.

The human LIPH protein sequence is similar to other members of the mammalian triglyceride lipase gene family (Fig. 1A), including 47% amino acid identity to PS-PLA1 and 46% amino acid identity to LPL and LIPG. LIPH is predicted to have the two-domain structure proposed for this lipase family, including an amino-terminal domain (lipase domain) and a carboxy-terminal domain. LIPH shares a high degree of identity with other lipases in its lipase domain.

The phylogenetic tree for this gene family shows that LIPH and PS-PLA1 are in the same subgroup (Fig. 1B).

LIPH commences with an 18-amino-acid hydrophobic sequence characteristic of a secretory signal peptide (Fig. 1A), consistent with other lipases in this family. Alignment with the human PS-PLA1, PNLIP, LIPG, LPL, and LIPC amino acid sequences reveals conservation of the catalytic residues serine (Ser-144), aspartic acid (Asp-178), and histidine (His-249), as well as of the cysteine residues involved in disulfide bridge formation. The sequence contains the
typical "GXSXG" lipase motif. Both human and mouse lipase H proteins have a 12-residue lid region. By analogy with the three-dimensional crystal structure of PNLIP, the LIPH lid probably covers a catalytic pocket and serves to confer substrate specificity [1]. Because the LIPH lid region has minimal sequence homology with the PS-PLA1, LIPG, LPL, PNLIP, and LIPC lids, the LIPH substrate may be different from those of other mammalian lipases. The regions immediately bordering the lid are almost identical among lipases of this family. Similarly, two

FIG. 1. The triglyceride lipase family. (A) Amino acid sequence alignment of the lipase family. The deduced amino acid sequence of human LIPH is provided on the top line and is compared with the other major members of the triglyceride lipase family: PS-PLA1, LIPG, LPL, LIPC, and PNLIP. LIPH residues identical to those in at least one other member of the family as well as the corresponding residue in the other family member are shown in bold or shaded. Gaps introduced for maximal alignment are indicated by dashes. The predicted site of signal peptide cleavage in LIPH is marked with a large arrow between amino acid residues. Asterisks and bold lines indicate the GXSXG lipase motif, and conserved cysteines are marked by dots. A dashed line indicates the lid region. The amino acids of the catalytic triad are marked with filled arrowheads. Potential N-linked glycosylation sites are marked with open arrowheads. (B) The phylogenetic tree of the triglyceride lipase family.
stretches of hydrophobic amino acids (142-163 and 254-262) that are adjacent to the catalytic serine and histidine, respectively, and that presumably are important for the interaction with lipid substrate are also highly conserved. Finally, four potential N-linked glycosylation sites are predicted by the presence of universal acceptor sequence N-X-S/T at positions 50, 58, 66, and 357; the site at position 66 is conserved in LPL, LIPC, and LIPG.

**LIPH Genomic Structure**
A database search of the Human Genome Project revealed two genomic BAC clones (Human Genome Project acc. no. AC069420 and AC068379) that contain most of the LIPH cDNA sequence. These BAC clones map to chromosome 3q27-q28. Comparison between the LIPH cDNA and the BAC genomic sequence revealed the genomic organization of LIPH (Table 1). Human LIPH is composed of 10 exons separated by 9 introns in an approximately 45-kb region. All exon-intron boundaries comply with the GT/AG rule. The 5'-untranslated region of 82 bases was obtained using 5'-RACE. The gene has a 1:30 ratio between the coding and non-coding sequence, and exons vary in length from 90 to 368 bases. The translation initiation codon of LIPH is in exon 1 and the last exon contains 82 bp of coding sequence. A comparison of the exon/intron organization with that of the protein deduced from the cDNA sequence reveals that there is no obvious correlation between the exon/intron structure and the modular organization of the protein. For LIP, the lipase motif is located in exon 3 and the lid region is divided between exons 5 and 6.

**TABLE 1:** Exon-intron organization of human LIPH

<table>
<thead>
<tr>
<th>Exon</th>
<th>bp</th>
<th>5’ Boundary site</th>
<th>3’ Boundary site</th>
<th>Intron</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>131</td>
<td>cactgtgac/AAAATCCCAC</td>
<td>TCAAGATCAG/gtaggttatt</td>
<td>1</td>
<td>18225</td>
</tr>
<tr>
<td>2</td>
<td>368</td>
<td>tatttttcag/ACGCAGAAGA</td>
<td>CCAGATGTTG/gtaagagaat</td>
<td>2</td>
<td>1085</td>
</tr>
<tr>
<td>3</td>
<td>109</td>
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<td>AGAATTACAG/gtaagcttc</td>
<td>3</td>
<td>5608</td>
</tr>
<tr>
<td>4</td>
<td>102</td>
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<td>3301</td>
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<td>4782</td>
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<td>aagtttaaggt/CCCCCTGGCCA</td>
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</tr>
</tbody>
</table>

**RNA Expression**
RT-PCR and northern blot analysis were used to investigate the tissue-specific pattern of human LIPH and mouse Liph expression. The same amounts of total RNA from different human organs were used for RT-PCR. The PCR primers are located in two different exons of LIPH genomic DNA. The specificity of PCR products was verified by DNA sequencing. Results in human tissues (Fig. 2A) demonstrated that testis, colon, pancreas, and lung gave strong
positive signals, whereas small intestine, mammary gland, ovary, brain, and varicose vein gave weak signals. Results in mouse tissues were qualitatively similar (data not shown).

Northern blots (Fig. 2B) of selected human and mouse tissues were hybridized with a 1.35-kb human LIPH DNA or 1.35-kb mouse LIPH probe. We detected two specific bands of 4.3 kb and 3.6 kb in human tissues; three forms of 4.0 kb, 3.8 kb, and 3.2 kb were present in mouse tissues. Expression of human LIPH mRNA was detected in colon, lung, and pancreas. In the mouse, strong expression of Liph mRNA was detected in placenta and colon, and weak expression was detected in small intestine. We also detected human LIPH mRNA in various cultured cell lines using northern blot analysis. Cell lines SW840 and HT-29, derived from human colon, and cell line A549, derived from human lung, showed expression of the two transcripts of LIPH. Human LIPH mRNA was also detected in Caco-2 cells, which are derived from human colorectal adenocarcinoma (data not shown). In situ hybridization (Fig. 3) of an 18.5-day mouse embryo showed that mouse Liph mRNA was detectable in the enterocytes of intestine.

EST database analysis showed that most ESTs (27 of 31) containing LIPH cDNA were derived from the colon. There was one each from skin and prostate, and the other two were...
from pancreas. These results indicate that this new member of the lipase gene family is predominantly expressed in colon, most likely by enterocytes.

**FIG. 3.** In situ hybridization of mouse embryos. 35S-Labeled cRNA sense and antisense probes of mouse Liph were hybridized to sections of mouse embryos. A positive signal is shown by white silver grains. Sections were photographed with brightfield (A, C) and darkfield (B, D) illumination. (A and B) Low-power view of the intestine of mouse embryo at 18.5 days of development. (C and D) High-power view of the intestine reveals a punctate staining pattern that appears to be over enterocytes. Magnification: A and B, X10; C and D, X20.

**Protein Expression**

To study lipase H protein expression, we generated an anti-lipase H polyclonal antibody by immunizing a rabbit with recombinant adenovirus encoding human LIPH. This antibody (Fig. 4A) detected a 63-kDa immunoreactive band in the culture medium of HEK 293 cells transfected with pcDNA3.1 human LIPH, but not in medium from cells transfected with a control plasmid (pcDNA3.1 GFP). It did not cross-react with other lipases (LIPG, LPL, LIPC) by western blot (data not shown). In addition, a band of slightly larger size (Fig. 4A) was detected in medium from cells transfected with a Flag-tagged LIPH (pcDNA3.1 human LIPH-Flag) using either the anti-human LIPH antibody or the anti-Flag antibody, consistent with the additional mass conferred by the Flag tag. These results all demonstrate that LIPH is a secreted protein of 63 kDa. The band detected by western blot is larger than that predicted by computer analysis of the protein sequence, which suggests lipase H is post-translationally modified. LIPH can bind to heparin-Sepharose beads (data not shown), indicating that it is a heparin binding protein similar to other members of this family. Expression of lipase H in human intestine and colon was detected by anti-lipase H antibody, but not by control antibody (Fig. 4B).
DISCUSSION

We have reported the discovery of lipase H (LIPH), a new member of the triglyceride lipase gene family. It is most closely related to PS-PLA1, and its sequence homology with other lipases of this family suggests that LIPH is an α/β-hydrolase-fold enzyme. LIPH has an identical catalytic triad, “GXSXG” lipase motif, and conserved cysteines. LIPH has a short lid domain of 12 amino acids, similar to PS-PLA1 (11 amino acids), and shorter than that of PNLIIP (23 amino acids), LPL (22 amino acids), LIPC (22 amino acids), and LIPG (19 amino acids). The conservation of the positively charged residues in the N-terminal domain as well as the ability of LIPH to bind heparin suggests that LIPH might associate with cell surface heparan sulfate proteoglycans, similar to other members of this gene family.

Two forms of LIPH transcripts were detected in human tissues and three forms were detected in mouse tissues. Only a single PCR product was amplified using the 5'-RACE method in human colon, suggesting that these transcripts are derived from the same gene and might be generated by alternative splicing and polyadenylation. Because the EST clone contains a shorter sequence of human LIPH cDNA than those transcripts detected by northern blot, we tried to obtain the full-length 3'-UTR using 3'-RACE, but we were unable to obtain additional sequence. The boundary of divergence and the significance of those splicing forms are uncertain. This phenomenon has also been demonstrated for other lipases in this family: there are two transcripts (3.6 and 3.2 kb) of human LPL [8] and two transcripts (4.4 and 3.8 kb) of human LIPG [6]. Another isoform [9] of the LIPC transcript has also been reported.

LIPH mRNA and protein were found in human intestine, both small intestine as well as colon. The small intestine is well known for its role in lipid absorption and metabolism. The colon is less involved in fat absorption, but it is involved in the absorption of vitamins and short chain fatty acids released by colonic microflora on dietary fiber. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes [10] and have been used as a model for intestinal lipoprotein synthesis and secretion [11], as well as for surfactant-like particle secretion [12]. Expression of this new lipase in Caco-2 cells will allow further study of its role.
in lipid absorption and lipoprotein metabolism. Other tissues involved in lipid metabolism also express lipase H. Expression of LIPH mRNA in pancreas suggests that, like pancreatic lipase, it might be secreted by the pancreas into the digestive tract. LIPH mRNA is also detected in lung and a human alveolar type II cell line, suggesting that lipase H might also be involved in lung lipid metabolism.

The substrate for lipase H is unclear. We used several lipids to try to identify substrates hydrolyzed by lipase H, including Triton X-100 or bile acid stabilized micelles of a variety of phospholipids, as well as a glycerol-stabilized triolein substrate [13] and tributyrin [14]. Two cofactors, apoC-II and colipase, that are known to influence the lipase activity of other lipases were also examined. However, we were not able to detect significant lipolytic activity of this lipase under any of these conditions (unpublished data).

We have reported a new member of the triglyceride lipase gene family. The unique structure and expression pattern of lipase H indicate that it may have a distinct role in lipid metabolism. Understanding the physiologic function and pathophysiologic roles of LIPH will require further studies.
REFERENCES

Chapter 6

Familial partial lipodystrophy phenotype resulting from a single-base mutation in DNA binding domain of peroxisome proliferator-activated receptor gamma

Houshang Monajemi, Lin Zhang, Gang Li, Ellen H. Jeninga, Henian Cao, Mario Maas, C.B. Brouwer, Eric Kalkhoven, Erik Stroes, Robert A. Hegele, Todd Leff

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ABSTRACT

**Context:** Familial partial lipodystrophy (FPLD) results from coding sequence mutations either in LMNA, encoding nuclear lamin A/C, or in PPARG, encoding peroxisome proliferator-activated receptor gamma (PPARγ). The LMNA form is called FPLD2 (MIM 151660) and the PPARG form is called FPLD3 (MIM 604367).

**Objective:** To investigate whether the clinical phenotype of this proband is due to mutation(s) in PPARγ.

**Design:** Case report

**Setting:** Academic medical center

**Patient:** A 31-yr-old female with the clinical phenotype of FPLD3; i.e., lipodystrophy and early childhood diabetes with extreme insulin resistance and hypertriglyceridemia leading to recurrent pancreatitis.

**Results:** The proband was heterozygous for a novel C>T mutation in PPARG gene that led to the substitution of arginine 194 in PPARγ2 isoform, a conserved residue located in the zinc finger structure involved in DNA binding, by tryptophan (R194W). The mutation was absent from the genomes of 100 healthy Caucasians. In vitro analysis of the mutated protein showed that R194W (and R166W in PPARγ1 isoform) could not bind to DNA and had no transcriptional activity. Furthermore, R194W had no dominant negative activity.

**Conclusions:** The R194W mutation in PPARG disrupts its DNA binding activity and through haploinsufficiency leads to clinical manifestation of FPLD3 and the associated metabolic disturbances.
INTRODUCTION

Dunnigan-type familial partial lipodystrophy results from rare coding sequence mutations either in LMNA, encoding nuclear lamina A/C, or in PPARG, encoding peroxisome proliferator-activated receptor gamma (PPARγ) (1;2). The LMNA and PPARG forms are called FPLD2 (MIM 151660) and FPLD3 (MIM 604367), respectively. These mutations underlie profound redistribution of fat stores, characterized by lipoatrophy of the extremities and gluteal region in combination with lipohypertrophy in face, neck, trunk and central adipose stores. This redistribution can be accompanied by a variety of clinical characteristics, including severe insulin resistance, often with acanthosis nigricans, and hypertriglyceridemia, sometimes associated with pancreatitis and eruptive xanthomata (3). The core clinical phenotype is fat loss with subsequent development of secondary metabolic disturbances that are characteristic of the insulin resistance syndrome.

The presence of lipodystrophy in subjects with dysfunctional PPARG missense mutations, such as R425C, F388L, E138fsΔAATG, V290M, P467L and Y355X (4-9) and in PPARγ-deficient murine models (10;11) has confirmed the central role of PPARγ in adipogenesis. PPARγ interacts with retinoid X receptor (RXR), binds DNA as a heterodimer and subsequently regulates transcription of PPARγ-responsive genes. Heterozygous loss of function or haploinsufficiency is clinically important when gene dosage is strictly regulated. Here, we show that a heterozygous mutation of a conserved arginine residue into tryptophan in the PPARγ (referred as R166W in PPARγ1 and R194W in PPARγ2 isoform) zinc finger II region disrupts DNA binding and transcriptional activity and thus underlies FPLD3.

METHODS

Study subject

The study was approved by the University of Western Ontario Ethics Review Panel (protocol 07920E) and the subject gave informed consent to participate.

Magnetic Resonance Imaging

MRI was performed using a 1.5 Tesla scanner (Signa, GE Medical Systems, Milwauk ee, WI, USA) with a neck coil and body coil. Axial and sagittal T1 weighted images of the c-Spine, Axial T1 weighted images of the abdomen and the lower leg were acquired according to the procedure described earlier.

DNA sequence analysis

After DNA sequencing showed no mutation in LMNA, we amplified and sequenced the 6 exons of PPARG plus >100 bp at intron-exon boundaries and ~700 bp of the promoter (5;7). The R194W mutation was genotyped by scoring the electropherogram tracing of exon 4 sequences from the Applied Biosystems 3730 Automated DNA Sequence Analyser (ABI, Mississauga, ON). Genomic DNA from 100 healthy Caucasian subjects was studied,
permitting 70% power to exclude a mutation with frequency \(>2\%\) in the healthy population (two-tailed alpha<0.05).

**PPAR gamma clones**

A cDNA encoding full length human PPAR\(\gamma\)1 was cloned into the pTRE-shuttle2 eukaryotic expression vector (Clontech, Palo Alto, CA). A double-FLAG epitope tag (MDYKDHDGDYKDHD) was added to the N-terminus of the clone. The pCDNA3-PPAR\(\gamma\)1 and pCDNA3-PPAR\(\gamma\)2 constructs were kind gifts from Dr. V.K.K. Chatterjee. The R194W mutation was introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by sequencing.

**Electrophoretic mobility shift assays (EMSA)**

EMSA experiments were performed as described (12). In short, a radiolabelled double-stranded DNA oligomer, containing the PPRE from the rat acyltransferase-coenzyme A oxidase promoter, was incubated with *in vitro* translated PPAR\(\gamma\) (wild type or mutants) and/or *in vitro* translated RXR\(\alpha\) proteins. For supershift experiments 1 \(\mu\)g of \(\alpha\)-RXR (sc553; Santa Cruz Biotechnologies), \(\alpha\)-PPAR\(\gamma\) (sc7273) or \(\alpha\)-Gal4 (sc510) antibodies were added. Receptor-DNA complexes were separated from unbound DNA on native gels and visualized by autoradiography. At least three independent experiments were performed. The complete probe sequences used for binding and competition analysis were as follows: PPRE-wild-type, 5’-CCG GGG ACC AGG ACA AAG GTC ACG AAG CT-3’ and PPRE-mutant, 5’-CCG GGG GAC CAG CAC AAA GCA CAC GAA GCT-3’. Western blot analyses of the different *in vitro* translated PPAR\(\gamma\) proteins was performed as described (13). \(\alpha\)-PPAR\(\gamma\) antibody (sc7196) was used to probe for PPAR\(\gamma\) protein and ECL (Amersham Biosciences) was used for detection.

**Cell culture, reporter assays and dominant negative assays**

NIH 3T3 mouse fibroblasts and human U2OS osteosarcoma cells were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), 100 \(\mu\)g of penicillin/ml and 100 \(\mu\)g streptomycin/ml (Gibco Life Technologies). NIH 3T3 mouse fibroblasts were grown in 24-well plates (1.0\(\times\)10\(^5\) cells/well) in DMEM+10% fetal calf serum. Cells were transfected with 25 ng WT or R194W expression plasmid, 6 ng of pTET-off, 25 ng of pRXR, 2 ng of a \(\beta\)-galactosidase control plasmid and 200 ng of the PPAR-dependent luciferase reporter pFATP-Luc (i.e. three copies of the mouse FATP gene PPRE inserted upstream of the minimal thymidine kinase promoter). Cells were transfected for 4 h with Lipofectamine-plus and then treated with DMSO or increasing doses of rosiglitazone for 16 h. Transfections were performed in triplicate. Mixing experiments examining dominant negative activity (Fig. 4E) were conducted as described above except with the amounts of PPAR\(\gamma\) plasmids: NIH 3T3 cells that were transfected with the combination of 5 ng of WT PPAR\(\gamma\) and increasing amounts of WT, the R194W mutant, or the dominant-negative mutant P467L (5, 10, or 20 ng; indicated in the figure as 1:1, 1:2 and 1:4 respectively), in the presence or absence of rosiglitazone (Fig. 4E and 4F).
U2OS were also seeded in 24-wells plates and transiently transfected using the calcium-phosphate precipitation method. Each well was cotransfected with 1 μg reporter construct, 10 ng pCDNA-PPARγ expression constructs and 2 ng pCMV-Renilla (Promega). After washing, cells were maintained in medium in presence or absence of rosiglitazone (1 μM) for 24 h. Activities of luciferase plus β-galactosidase (NIH-3T3) or luciferase plus renilla (U2OS) were measured with the Dual-light assay system (ABI, Foster City, CA) or Dual-Luciferase Reporter Assay System (Promega), respectively, using a 96-well luminometer (Berthold Technologies, Bad Wildbad, Germany).

RESULTS

Patient medical history and clinical evaluation
The proband was a 31-yr-old Turkish female living in the Netherlands. Menarche occurred at age 11, followed by regular menstrual cycles. At age 15, she was diagnosed with diabetes with severe insulin resistance. Despite insulin therapy, she developed severe hypertriglyceridemia, with plasma concentration > 50 mmol/L, leading to eruptive xanthomas on her trunk and extremities. At age 17, her menstrual cycle became irregular and her extremities and face developed excessive hair.

Figure 1 Clinical aspects of the proband. (A and B) showing the masculine appearance with a clear trunk-sparing lipodystrophy; (C-E), acanthosis nigricans on her neck, axilla and feet; (F), eruptive xanthoma.
growth, leading to the diagnosis of polycystic ovarian syndrome (PCOS). At age 19, she became pregnant after in vitro fertilization and gave birth to a healthy son. Subsequently, she was hospitalized twice more for pancreatitis at ages 20 and 22. During outpatient follow-up, her insulin dose was increased >300 U per day. At the end of 2005, she was referred to the Academic Medical Center, Amsterdam for management of refractory hypertriglyceridemia despite fibrate and insulin treatment. On examination, she was mildly obese (weight 68 kg; height 167 cm and body mass index [BMI], 25 kg/m²). Her resting blood pressure was 130/70 mm Hg. She had excess subcutaneous (sc) fat on the face, neck, trunk, and abdomen, with lack of sc fat on the gluteal region and extremities (Figure 1). This was confirmed with magnetic resonance imaging (Figure 2), which showed excessive and relatively symmetrical deposition of sc fat on the face, neck, and upper trunk, with disproportionate depletion of sc fat in the lower body. Furthermore, she had acanthosis nigricans on her feet, axillae and neck. She was also hirsute. Measurements from fasting plasma: glucose 8.8 mmol/L; HbA1c 8.2%; insulin 1074 pmol/L (reference 34-172); C-peptide 950 pmol/L (reference 176-664); total cholesterol 9.42 mmol/L; HDL cholesterol 1.33 mmol/L; and triglyceride 35.0 mmol/L. APOE genotype was E3/E3. Lipoprotein lipase (LPL) activity was normal and no genomic DNA sequence changes were seen in the LPL gene (data not shown). The free androgen index was 134 (normal ratio 0-8). At the time of these measurements, she was being treated with multiple daily insulin injections totaling 300 U/day, ciprofibrate 100 mg and cyproterone 50 mg daily.

Figure 2 Magnetic resonance imaging (MRI) scans. T1 weighted images were obtained: (A and B), scans of the neck showing a layer of sc fat measuring 2.52 cm; (C), cross section at the abdomen, showing a symmetrical layer of sc fat measuring 1.09 cm; (D), cross section at the gluteal region, showing sc fat measuring 0.80 cm; (E), cross section at the level of upper leg region, showing a dorsal layer of sc fat measuring 0.64 cm; (F) cross section at the level of lower leg region, showing a dorsal layer of sc fat measuring 0.56 cm;
Her 53 year-old father had a history of type 2 diabetes and dyslipidemia, but was not lipodystrophic clinically. Her mother died at age 20 from meningitis. The proband’s sister, half-sister and two half-brothers were healthy; none had criteria for metabolic syndrome diagnosis. None of the family members were willing to participate in additional clinical or genetic testing.

**DNA sequence analysis**

In the genome of the proband, we found a heterozygous nucleotide substitution C>T at position 1762 in the PPARγ isoform 4 (Figure 3). All other regions analysed were free of DNA sequence changes. This mutation was absent from the genomes of 100 normal Caucasian controls. This mutation causes an amino acid substitution R194W in PPARγ isoform 2 (R166W in PPARγ isoform 1).

*R194W mutant PPARγ does not bind DNA and is transcriptionally inactive*

The location of the mutation within the DNA binding domain of PPARγ suggested that it might influence DNA binding. To investigate this possibility, the binding of the R166W as well as R194W mutant to a standard PPRE sequence was assessed using an electrophoretic mobility shift assay (EMSA). While PPARγ wild type in the presence of RXRα was capable of binding to PPARγ response element, the R166W mutant had no detectable DNA binding.

![Diagram of PPARγ isoforms and mutations](image)

**Figure 3** Reported *PPARG* mutations in FPLD3 and genomic DNA sequence electropherograms of heterozygous R194W mutation. Schematic genomic map of *PPARG*, showing the positions of known mutations, and the disease mechanism indicated. Coding exons are shown with Arabic numerals, while non-coding exons are designated alphabetically. The start of transcription for γ1, γ2, γ3 and γ4 isoforms is shown. The electropherogram tracing shows both alleles from proband (R194W) compared to corresponding genomic DNA sequence from a healthy subject. The position of the mutation is indicated by the arrow. Normal nucleotide and amino acid sequence is shown above the WT electropherogram tracing. Note that the mutation numbers refer to gamma 1 isoform in a few and to gamma 2 isoform in other subjects.
activity (Figure 4A). As expected, lack of DNA binding was also observed in the PPARγ2 isoform (Figure 4B).

The transcriptional activity of the R194W mutant PPARγ was assessed by transient transfection of PPARγ expression plasmids into NIH 3T3 cells and analysis of luciferase activity from a PPAR responsive reporter. The R194W mutant receptor was inactive at all doses of the ligand rosiglitazone (Figure 4C). In addition, U2OS cells were transfected with PPARγ1 (WT and R166W) or PPARγ2 (WT and R194W). Whereas both WT isoforms had a slight basal expression level that was highly induced by rosiglitazone, both mutant isoforms displayed no transcriptional activity in the absence or presence of exogenous ligand (Figure 4D).

**R194W mutant PPARγ displays no dominant-negative activity**

To investigate if the R194W receptor had dominant-negative activity against WT PPARγ, a mixing experiment was performed in which an increasing amount of mutant or wild-type receptor were mixed with a fixed amount of WT PPARγ (Figure 4E). While simply increasing the amount of the WT receptor caused a significant increase in transcriptional activity (WT+WT), the addition of increasing amounts of R194W PPARγ to a fixed amount of WT receptor resulted in no change in total PPARγ transcriptional activity (WT+R194W). For comparison, the same experiment was conducted with the P467L that has dominant-negative activity(4). Increasing amounts of P467L PPARγ caused a dose-dependent decrease in WT PPARγ
transcriptional activity (WT+P467L). When the cells were treated with high concentration of rosiglitazone (Figure 4F), the dominant negative activity of P467L was abolished as described earlier(4). Together, these findings indicate that the R194W mutant does not possess any dominant-negative activity against the WT PPARγ receptor.

DISCUSSION

The principal findings of this study are: 1) association of a novel heterozygous PPARγ missense mutation, R194W (R166W in gamma1 isoform), with FPLD3, including fat redistribution, severe insulin resistance, hypertriglyceridemia, hirsutism and acanthosis nigricans; and 2) functional analysis showing that the R194W mutant is transcriptionally inactive, independent of PPARγ isoform (γ1 and γ2) and cell type (NIH-3T3 and U2OS).

The substitution of a hydrophilic arginine to a hydrophobic tryptophan within an α-helix would predict disrupted structure and decreased DNA binding, as was seen with EMSA. The importance of the conserved arginine residue is underscored by natural mutations in other nuclear receptors causing hormone resistance. For instance, a R614H mutation and deletion of this amino acid (Δ614) in the androgen receptor (AR) have been reported in two patients with complete androgen insensitivity (14). Furthermore, mutation of the analogous residue (R477H) in the glucocorticoid receptor (GR) was detected in a patient with primary cortisol resistance (15). In addition, mutation of this conserved arginine residue in the photoreceptor-specific nuclear receptor PNR into tryptophan (R104W) (16) or glutamine (R104Q) (17) were found in patients with enhanced S-cone syndrome. DNA binding of the AR Δ614 and R614H mutants and the GR R477H mutant was impaired (14;15) analogous to the PPARγ R194W mutant, emphasizing the importance of this conserved arginine residue in nuclear receptor signaling.

R194W brings the number of reported PPARG mutations associated with clinical phenotypes to fourteen. Only the PPARγ2 P115Q mutation was not associated with FPLD3 (18). Two PPARγ missense mutations (P467L and V290M), along with the recently published subjects by Agostini et al (C114R, C131Y, C162W, F5315X and R357X) act via a dominant negative mechanism (19), while five (-14A>G, F388L, E138fsΔAATG, Y355X and R194W) caused FPLD3 through haploinsufficiency (5;7;20) (Figure 3). The R425C mutation (9) also lacks dominant negative activity (E.H. Jeninga et al., submitted). All patients with PPARG haploinsufficiency mutations were ascertained based upon a diagnosis of FPLD; almost every patient with a PPARG mutation had partial lipodystrophy as a core phenotype. FPLD3 has proven to be a useful and appropriate clinical designation; the term acknowledges the centrality of lipodystrophy, while concurrently distinguishing FPLD3 from phenotypically similar but molecularly distinct forms of lipodystrophy, such as FPLD2 due to LMNA mutations.

Mutations can lead to disease through either i) loss of function; ii) gain of function; or iii) dominant negative activity. According to the “classical” dominant negative hypothesis, the mutant allele eliminates the WT function by direct interference. For instance, in the case of nuclear receptors the mutant receptor competes with the WT for binding DNA. However,
there is some evidence that nuclear receptors can also have indirect dominant negative activity by affecting the bioavailability of other components of the transcriptional machinery, such as coactivators, and hence could interfere with the WT allele. We have shown that R194W has neither direct (figure 4 A) nor indirect (Figure 4E) dominant negative activity under our experimental conditions. With haploinsufficiency, 50% reduced gene expression results from one nonfunctional allele, whereas dominant negative mutations induce even greater reduction in gene expression. How do these two mechanisms underlie the same phenotype? One possibility is that subjects with either mutation type might have slightly different clinical phenotypes that are not easily discerned using current methods. For instance, hypertension in human subjects with dominant negative mutations seems to be more severe than in subjects with haploinsufficiency mutations (21). Additional pedigrees with PPARG would allow for better comparisons of these two mechanisms in vivo.

Since the first publication on familial partial lipodystrophy by Dunnigan and Kobberling (22), awareness of this condition by clinicians has increased. Several mutations both in LMNA and PPARG have been described. Yet, many such patients are probably overlooked, because of clinical similarities with the common obesity-related metabolic syndrome that currently is endemic to westernized societies, largely due to lifestyle changes. Careful physical examination of patients with insulin resistance and hypertriglyceridemia could help identify partial lipodystrophy. In summary, in a proband with FPLD3 we found a novel PPARG mutation that fails to bind DNA and is transcriptionally inactive. Human PPARG mutations will improve our understanding of mechanisms involved in lipodystrophy and insulin resistance.
REFERENCE LIST


FPLD phenotype resulting from a single-base mutation in DNA binding domain of PPARγ


Inherited lipodystrophies and the metabolic syndrome

Houshang Monajemi, Erik Stroes, Robert A. Hegele, Eric Fliers

SUMMARY

Lipodystrophies represent a heterogeneous group of diseases characterized by an abnormal subcutaneous fat distribution, the extent of which can vary from localized, partial to generalized lipoatrophy. Whereas partial and generalized lipodystrophies are each associated with metabolic abnormalities, the localized form is not. These metabolic changes include insulin resistance with type 2 diabetes, acanthosis nigricans, dyslipidemia predominantly consisting of hypertriglyceridemia (associated with the onset of pancreatitis) and depressed HDL cholesterol, liver steatosis and hypertension. Affected women are often hirsute and this can be associated with the presence of polycystic ovarian syndrome (PCOS). Most of these clinical features are present to some extent in patients with the common metabolic syndrome. Since the prevalence of metabolic syndrome far outweighs that of lipodystrophy, the diagnosis of this rare disorder may often be overlooked and the affected patient diagnosed as merely being ‘yet’ another case of metabolic syndrome. In this article, we draw attention to the importance of recognizing patients with lipodystrophy, who present with metabolic abnormalities since both the diagnostic as well as the therapeutic approach of these patients differ profoundly from patients with the metabolic syndrome.
INTRODUCTION

Since the first description of lipodystrophy associated with metabolic abnormalities by Berardinelli (1), major advances have unraveled the molecular genetic basis of these disorders. Several classifications have been proposed during the last decade. Whereas the initial classifications were based predominantly on clinical phenotype (generalized vs. partial), the mode of inheritance entered later classifications (dominant vs. recessive). More recently, with discovery of the genetic basis for several types and subtypes of lipodystrophies, diagnosis and classification is based upon the causal molecular genetic defect.

For a physician the clinical phenotype is the most important and expedient diagnostic tool; thus we will discuss the clinical classification (i.e. generalized vs. partial, summarized in table 1), based on the OMIM database (Online Mendelian Inheritance in Man http://www.ncbi.nlm.nih.gov/entrez), taking into account recently published articles on lipodystrophy. Acquired lipodystrophies will not be discussed, since this has been the subject of an excellent recent review (2).

GENERALIZED LIPODYSTROPHY

Congenital Generalized Lipodystrophy (CGL)

CGL was first described in 1954 by Berardinelli and was further characterized by Seip, hence the eponym Berardinelli-Seip congenital lipodystrophy (BSCL)(1;3). Because the characteristic features of these patients are present at birth, CGL is usually diagnosed by neonatologists and pediatricians. Males and females are affected in equal proportions. Due to lack of subcutaneous fat, affected individuals have a masculinized appearance and soon develop acromegaloid features. Despite an excessive appetite, they usually remain lean, but frequently develop fatty liver disease that can progress to fibrosis. Early in childhood, they develop pronounced insulin resistance, often leading to diabetes with concomitant dyslipidemia. The invariably high triglyceride levels can result in recurrent episodes of pancreatitis. ~300 patients with CGL have been reported to date (2;4).

In 1999, by using a genome wide linkage analysis of 17 pedigrees with CGL, a locus on chromosome 9q34 called BSCL1 was identified (5). A few years later, AGPAT2 (1-acylglycerol-3-phosphate O-acyltransferase 2) was identified as the causative gene among the positional candidate genes at this locus (6). AGPATs are members of acyltransferase family of enzymes that are involved in glycerolphospholipids and triacylglycerols. Therefore, it has been suggested that mutations in AGPAT2 could cause lipodystrophy through a direct effect on lipid biosynthesis (7).

In 2001, Magre et al, discovered a new locus for CGL on chromosome 11q13. They analyzed 29 families from different geographical regions and in 11 families found linkage with markers from this new locus that they called BSCL2; they showed that causative gene within this region was GNG3L1 encoding a novel protein coined “seipin” (8). The function of seipin
is still under investigation. In a joint effort both groups analyzed 45 families with CGL (9): 26 had a mutation in \textit{APGAT2} while 11 had a mutation in \textit{BSCL2} (seipin), so that the conditions were renamed CGL1 and CGL2, respectively. Because 8 families with CGL had no underlying genetic defect, it became clear that there must be additional genes underlying this phenotype. Before the detection of the genetic basis of these disorders, CGL1 and CGL2 were not considered to be distinguishable. But with molecular classification, a slight difference in clinical presentation was found: CGL2 patients appeared to have increased prevalence of mental retardation and cardiomyopathy, an observation that was subsequently confirmed by another group (10). This clearly demonstrates the importance of collecting more CGL families for genetic analyses that could potentially lead to discovery of new causative genes.

**Generalized lipodystrophies associated with dysmorphic features**

Mandibulo-acral dysplasia (MAD) was first described in 1971 and is a rare autosomal recessive disorder characterized by mandibular hypoplasia, acroosteolysis, stiff joints, cutaneous atrophy (11). Other clinical features include postnatal growth retardation, progeroid features
and lipodystrophy with metabolic abnormalities. Because of the rarity of this disorder, little is known about the natural course or history of this disease. Following extensive clinical and biochemical analysis as well as MRI fat measurements in 4 patients with MAD, Simha et al found that 3 of the patients had a partial lipodystrophy characterized mainly by the loss of subcutaneous fat in the extremities, which they referred to as MAD type A (12). The fourth patient, in contrast, had a generalized lipodystrophy that they called MAD type B; this patient was subsequently found to have compound heterozygote mutations in a zinc metalloproteinase called ZMPSTE24 (13). This enzyme plays a critical role in proteolytic cleavage of prolamin A to lamin A. Novelli et al investigated 9 individuals from 5 different consanguineous families with MAD and found homozygous mutations in LMNA, the gene encoding lamin A/C (14). All MAD patients with LMNA mutations had the type A pattern of lipodystrophy. The LMNA gene encodes several nuclear lamin isoforms that play a key structural role in the nuclear envelope. Various mutations in this gene underlie a very wide range of clinical phenotypes that have been called “laminopathies”, which have been the subject of recent comprehensive reviews (15,16).

Lipodystrophies as a component of progeroid syndromes
Hutchinson-Gilford progeria syndrome (HGPS) is a rare disorder characterized by accelerated aging. These patients are normal at birth, but age quickly, with an estimated 10 years of biological aging per one calendar year. The average life span of these patients is approximately 13 years and they often die of cardiovascular disease. One of the characteristic features of these patients is profound atrophy of subcutaneous fat stores. There are more than 100 case reports and the incidence has been estimated to be around 1: 4-8 million (17). Although HGPS was first described in 1886, the underlying genetic defect was found in 2003 to be a spontaneous mutation in LMNA affecting splicing (18). Neonatal Progeria Syndrome (also called Wiedemann-Rautenstrauch syndrome) was first described by Rautenstrauch in 1977 (19) and there are more than 20 case reports in the literature (20). It is an autosomal recessive syndrome that is characterized by aged appearance at birth with triangular face, natal teeth, hypotrichosis of the scalp hair, eyebrows, and eyelashes, relative macrocephaly, micrognathia and generalized lipodystrophy. All these patients died at young age. One patient had hyperinsulinemia at age 16 months but no diabetes was reported. These syndromes are not associated with metabolic abnormalities, presumably because the patients do not survive long enough to develop the features of metabolic syndrome.

Partial lipodystrophy
In 1974, Dunnigan reported a dominantly inherited partial lipodystrophy with metabolic abnormalities (21). One year later, Kobberling described another type of partial lipodystrophy that was limited to the extremities (22). The first classification of familial partial lipodystrophy (FPLD) was proposed (23). Diabetes mellitus, hyperlipoproteinaemia, and acanthosis nigricans are present to a variable degree in patients with FPLD, and the abnormal distribution of
subcutaneous fat is the essential hallmark of the syndrome. Most clinical detected FPLD cases were female patients and therefore, Kobberling and Dunnigan proposed an X linked dominant mode of transmission. Ever since, numerous case reports have been described and it has become clear now that, whereas female patients are most readily recognized clinically, males are also affected.

**Kobberling type of lipodystrophy, FPLD1**

In type 1 familial lipodystrophy, loss of subcutaneous fat is confined to the limbs, sparing the face and trunk (figure 1). To date, no genetic mutation has been found that underlies this disease. It is generally agreed that there are more FPLD1 phenotypes than are observed. Herbst et al elegantly demonstrated this by specifically looking for FPLD1 phenotype (24). They were able to identify 13 female subjects with FPLD1 within tertiary care outpatient clinics over the course of a few months by skinfold measurement of fat. This suggests that this syndrome is indeed more common than previously thought. No male subjects were identified as having FPLD1. A similar finding was done for FPLD2 until more detailed cross-sectional imaging of fat was performed (25). These data imply that both men and

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**Figure 1.** Anatomic features of FPLD1 in an obese subject. A: Gluteal view demonstrating central adiposity and the ledge of fat above the gluteal area (arrow). B: Upper and lower arm demonstrating ledge of fat over triceps area (arrow). C: Lower extremities demonstrating prominent musculature and veins. D: Face and neck demonstrating cushingoid appearance of face, with moon facies and fat under chin. Copyright © 2003 American Diabetes Association. From Diabetes Care®, Vol. 26, 2003; 1819-1824. Reprinted with permission from The American Diabetes Association.
women with FPLD1 are often misdiagnosed. A combination of careful clinical examination with additional imaging is required for definite recognition of these syndromes.

**Dunnigan type of lipodystrophy, FPLD2**

In type 2 familial lipodystrophy, the trunk is also affected with the exception of the vulva, giving an appearance of labial hypertrophy (figure 2). There is usually fat hypertrophy in the neck and head region, giving the patient a cushingoid appearance (26). Often patients have been evaluated for Cushing’s disease before lipodystrophy is considered. In 1998 Peters et al carried out a genome-wide scan with a set of highly polymorphic short tandem-repeats (STR) in individuals from five well-characterized pedigrees and mapped the FPLD2 locus to chromosome 1q21-22 (27). Two years later, after genetic analysis of 5 FPLD2 probands, the causative gene was found to be *LMNA* (28), again implicating nuclear lamin A in adipose metabolism.

**Dunnigan type of lipodystrophy, FPLD3**

In 1999, by analysis of 85 unrelated subjects with severe insulin resistance, all coding exons of PPARγ1 and PPARγ2 were studied and new missense mutations in the ligand-binding domain of PPARγ were found in only two subjects (29). The lipodystrophic features of
those individuals were not reported at that time. In 2002, the direct link between FPLD and PPARγ was reported by two groups (30;31). In 2003, a close evaluation of patients with the first PPARγ mutations revealed the presence of a partial lipodystrophy phenotype (32), demonstrating how lipodystrophy can be missed during routine physical examination. Additional techniques for fat measurements, e.g. MRI, are needed to improve diagnostic accuracy, especially in men. Since the association of PPARG with lipodystrophy, this syndrome is annotated as FPLD3. To date fourteen patients have been reported to have mutations in PPARG gene causing FPLD3 (33). Interestingly, it was recently shown that use of MRI measurements and quantification of lower extremity subcutaneous fat depots in patients with FPLD2 and FPLD3 could distinguish between these two forms (34). This corroborated

![Figure 3. Anatomic features of FPLD3.](image)

Although these patients have also a masculine appearance, the subcutaneous fat loss in the extremities is less obvious compared to FPLD2 subjects. Furthermore, the trunk is not affected, hence the name trunk-sparing lipodystrophy. Note the “Cushingoid” appearance.
the impression that there is a difference between these two and showed that subcutaneous fat loss in the FPLD2 subject was greater than in the FPLD3 individual (figure 3).

**Other FPLDs**

Recently, by screening genomic DNA from 104 unrelated subjects with severe insulin resistance for mutations in genes that are implicated in insulin signaling, George et al identified a missense mutation in the serine/threonine kinase gene AKT2 in one Caucasian proband (35). Analysis of the proband’s body composition revealed a –35% difference in total body fat compared to that predicted for her height and weight. This finding is consistent with the recent finding that AKT2 knockout mice develop severe diabetes and age dependent loss of subcutaneous fat (36). Unfortunately, the authors have not yet reported the localization of lipodystrophy in the subject with Akt2 mutation.

**Future directions**

Generalized lipodystrophies are easily detected clinically and are usually diagnosed by pediatricians because of the characteristic features from birth onwards. Partial lipodystrophies, on the other hand, are more difficult to recognize, only causing metabolic abnormalities later in life. Since many of the metabolic features of partial lipodystrophy resemble those of the metabolic syndrome and/or type 2 diabetes mellitus, patients with lipodystrophies are often misdiagnosed. The only clinical signs that are unique to lipodystrophies are lipoatrophy and the onset of severe metabolic abnormalities, comprising recurrent episodes of pancreatitis related to hypertriglyceridemia and severe insulin resistance often combined with only modestly increased BMI. Due to the marked difference in natural courses of the metabolic derangements in lipodystrophic patients versus metabolic syndrome subjects, more attention should be paid in order to improve correct identification of lipodystrophic subjects. Using standardized MRI protocols quantifying subcutaneous fat, lipodystrophic patients can be identified accurately. With respect to therapeutic strategies, current options are largely limited to managing metabolic abnormalities with drugs for treatment of hypertension, diabetes and dyslipidemia. However, targeted interventions are emerging. First, administration of specific adipokines, such as leptin, in lipodystrophic patients was shown to induce sustained improvements in glycaemia, dyslipidemia, and hepatic steatosis (37;38). Long term effect of this treatment modality is currently being addressed in several centers. A second option could be transplantation of subcutaneous fat. In fact, white adipose tissue transplantation has been shown to be effective in leptin deficient mice (39;40). These mice were prevented to become obese and had normal insulin levels. It is too soon to determine whether adipose transplantation will be effective in lipodystrophic patients. Finally, an attractive option for treating lipodystrophic patients might be the use of gene therapy. However, this therapeutic area remains unexplored at this time.
REFERENCE LIST


Discussion
The focus of this thesis is on genes affecting triglyceride (TG) metabolism. Fredrickson and his colleagues were one of the first pioneers in the field of human lipid metabolism who started to investigate the hyperlipidemias by using techniques available at the time (1). By performing paper electrophoresis, they were able to separate different lipoprotein particles according to their size and electric charge. By doing so they defined two major lipoprotein particles, which were designated as α- and β-mobility particles corresponding to high density lipoprotein (HDL) and low density lipoprotein (LDL) particles. Accordingly the main protein constituents of these particles are designated as apolipoprotein A and B, respectively. During the past decades our knowledge of lipoprotein metabolism has grown substantially as the alphabet of lipoproteins. The latest characterized apolipoprotein is apolipoprotein M. Many of these proteins have also several isoforms showing the diversity of proteins on lipoprotein particles. Beside the analysis of plasma lipoproteins, the evolution of techniques in genetic field has been the ground for a tremendous leap forward in understanding the lipid metabolism. Knockouts as well as transgenic animal models have been used to study the effect of a single gene in this complex pathway. Many genes have been sequenced and characterized in de past years and the number of players are increasing. Recently, Gue et al elegantly showed that about 1,5 % of all genes of Drosophila are involved in lipid-droplet formation, demonstrating not only the importance of this organelle inside the cells but also the complexity of intracellular lipid metabolism (2). It is reasonable to assume, knowing the complexity of human lipid metabolism intra- as well as extracellularly, that even more genes are involved in human lipid metabolism.

Phenotypic as well genotypic characterization of human subjects with lipid abnormalities is essential to gain insight in this process, especially because of the difference in lipid metabolism among species. This necessitates an integrative approach to discern these lipid abnormalities where, both clinicians and fundamental research scientists should be involved. Translation of basic research results into daily clinic (from bench to beside) is as important as the opposite direction (from beside to bench). For instance, observing lipid abnormalities in well defined pedigrees has made it possible to pinpoint the locus of the mutated gene. Maybe the best example of this kind of research is the finding of ABC-transporter A1 mutation in Tangier disease (3). Understanding the exact role of each gene involved in lipid metabolism, will eventually lead to novel therapeutic strategies.

The first part of my thesis is about unraveling the function of apoL genes and translating it to human diseases (from bench to beside), whereas in the second part, I described a clinical phenotype and defined the mutation in the PPARγ gene, causing the disease (from beside to bench). The necessity of bidirectional flow of information is exemplified in chapter 5 where a novel gene, Lipase H (LIPH) is described. We show that this gene, which is at the protein level homologous to triglyceride lipase family, is mainly expressed in intestine, lung, and pancreas (4). LIPH is a secreted protein with an apparent molecular weight of 63 kDa. Because of its structural resemblance to that of other triglyceride lipases, we were almost sure that it should have an effect on lipid metabolism. Although we thoroughly searched...
for the lipolytic activity of this protein, we were unable to identify its substrate. Recently, two separate groups found mutations in LIPH in families who show an inherited form of hair loss (5;6). Hereditary hypotrichosis is a rare autosomal recessive disorder characterized by sparse hair on scalp and rest of the body of affected individuals. Both groups suggest that LIPH is involved in hair growth and showed also expression in hair follicles of normal subjects. Although, these results are very convincing in the role of LIPH in hair growth, the exact function of this secreted protein remains unresolved. We found high expression of LIPH in the colon, suggesting that this protein must have other functions than merely hair growth. It would be interesting to characterize the phenotype of these patients further in detail, especially the lipid metabolism, to find a clue on the exact function of LIPH. This example shows clearly that fundamental research and clinical research can mutually affect our understanding of biological processes.

**Evolutionary changes in lipid metabolism**

Although mice have been used extensively to analyze lipid metabolism, the extrapolation of the data to human lipid metabolism has been very difficult. Genetic differences among species are becoming more obvious due to of high throughput sequencing of species. Phylogenetic analysis of specific genes can help us understand how the lipid metabolism has changed during the evolution and has adapted to the environmental demands. One of the essential differences in the lipid metabolism between mice and humans is the fact that mice lack Cholesterol Ester Transfer Protein (CETP) (7). In humans, CETP is a major determinant of plasma HDL-cholesterol levels. TG is transferred from TG-rich lipoprotein particles such as VLDL to cholesteryl ester-rich lipoproteins such as HDL with cholesteryl ester moving in the opposite direction. In doing so, CETP effectively lowers HDL-cholesterol levels. There are many other differences in lipid metabolism between men and mice, such as the evolution of apoB and its editing enzyme as well as microsomal triglyceride transfer protein (MTP) (see for details chapter one).

The Apolipoprotein L gene cluster is a gene family, arisen recently during evolution, that only exists in primates. Apolipoprotein L1 is the only secreted protein in this family and in plasma it is associated with HDL particles and has trypanosome lytic activity (8;9). We have shown that ApoL1-3 genes are all TNF-responsive genes that suggest a role for these genes during inflammation (chapter 3). In fact, apoL1 deficiency was associated with susceptibility to Trypanosoma Evansi infection. The serum of the infected patient was found to have no trypanolytic activity, and the finding was linked to the lack of apoL1, which was due to frame-shift mutations in both apoL1 alleles. Trypanolytic activity was restored by the addition of recombinant apoL1 (10). Somehow, during evolution these genes are duplicated and have gained different functions.

Is there an evolutionary benefit of apoLs for primates? The only non-homologous region in ApoL gene cluster is the first intron of ApoL3, which also contains many additional Alu repeats. These repetitive DNA elements were used by Nishio et al. to analyze genomic
expansion of the albumin gene family on human chromosome 4. They found that the earliest gene during the course of the evolution of this gene family had the highest number of Alu repeats (11). Because ApoL3 is both the least homologous of the family members and has a much higher number of Alu repeats, we hypothesize that ApoL3 may be the ancestor gene in this gene cluster. Introduction and rapid duplication of a gene is indicative of pressure on gene dosage. Because the eukaryotic cell has only two alleles, the only way to upregulate the expression of a gene is gene duplication. This is best demonstrated by the ribosomal RNA (rRNA) duplication. The ribosome is a complex of rRNAs and proteins that is involved in translation of mRNA. Ribosomal proteins are the product of their mRNA where each mRNA molecule gives rise into a multiple protein molecules. Because the rRNA is the end product and thereby lacks the exponential increase of protein synthesis, the only way to keep up with the ribosomal protein supply is gene duplication of rRNA. Therefore, rRNA genes are organized in tandem repeats in five clusters on chromosomes 13, 14, 15, 21 and 22. It is conceivable that ApoL3 gene has been introduced into the animal genome and is duplicated later in the evolution. This duplication must have occurred due to inability of both apoL3 alleles to cope with the environmental demands. The normal evolution of duplicated genes is concerted evolution, silencing or gaining new functions. It has been suggested that selection and environmental pressure is likely to be one of the main factors determining the duration of concerted evolution of duplicated genes (12). Another evolutionary moment must have been the time when pressure on this gene family was dropped and allowed each specific gene to evolve separately and gain novel functions. ApoL4 has most probably lost its function because we were not able to find its expression in different human tissues. ApoL1 has changed dramatically and has become an extracellular protein associated with HDL particles. It is reasonable to assume that apoL1-3 genes are beneficial to primates, because they are still expressed and are not silenced.

Is there a role for apolipoprotein L2 and L3 in lipid metabolism? We have shown that apoL2 is an intracellular protein that co-localizes with PDI in the ER, where most of the enzymes in the TG metabolism reside. ApoL2 overexpression induced large TG droplets in cells capable of lipid loading, such as adipocytes, hepatocytes and macrophages but not in endothelial cells (chapter 4). Perilipin is a major lipid droplet protein that is also a target gene for PPARγ and has a PPARγ-responsive element in the promoter region. During foam-cell formation PPARγ is upregulated and induces the expression of many other genes, such as perilipin, involved in adipogenesis. ApoL2 has no responsive element for PPARγ, but its expression was coordinated in the same fashion as PPARγ, indicating its involvement in intracellular TG storage.

ApoL3 had the opposite effect on intracellular TG and was associated with the Golgi. While apoL2 and PPARγ were upregulated during foam-cell formation, the expression of apoL3 was completely repressed. Two highly homologous genes, both responsive to TNFα, with different intracellular localization thus have opposite effects on TG storage. The fact that apoL proteins are primate specific indicates that their role in TG storage is of importance.
for these species but not vital. Obviously, other mammals are capable of fat storage and breakdown without apoLs. Therefore, apoLs might be involved in the fine tuning of lipid metabolism during inflammation.

Is there a role for apoL proteins in lipid metabolism during inflammation? TNF-α has been shown to affect hepatic lipogenesis due to reducing the expression and activity of different nuclear hormone receptors such as PPARγ (13;14). TNF-α is an inflammatory cytokine that is mainly produced by macrophages. Adipocytes also express TNF-α and adipose tissue has been shown to be a major source of TNF-α locally and systemically. TNF-α was discovered not only as a soluble protein that induces the death of tumor cells but also as a molecule (cachectin) that causes hypertriglyceridemia and wasting of muscle and fat tissue (15). Inhibition of nuclear factor κB (NF-κB), the key transcription factor in TNFα-induced inflammatory response in monocytes, causes a decreased lipid loading after differentiation into macrophages. This is accompanied by increased expression of the transcription factor PPARγ (16). It seems that TNFα and PPARγ have opposing effects in TG metabolism. This effect is nicely demonstrated for an important protein in lipid metabolism, i.e. apolipoprotein E. ApoE is expressed in adipocytes and, besides its role in clearance of lipid particles in plasma, has an effect on TG storage inside the cells. Adipocytes from apoE−/− mice are smaller than those from wild-type mice and contain less TG (17). Adipocytes isolated from apoE−/− mice also accumulate significantly less TG after stimulation with PPARγ agonists, consistent with an important role for adipocyte apoE in mediating the effect of PPARγ agonists on adipocyte lipid metabolism. Furthermore, TNFα reduces the expression of apoE whereas, PPARγ induces its expression (18). The differential expression of apoL2 and apoL3 during foam-cell formation indicates their importance in TG storage. Although TNFα increases the expression of both apoLs in endothelial cells (chapter 3), in HepG2 cells, TNFα had no effect on apoL2 expression, whereas apoL3 mRNA expression was increased (unpublished data). Why would the apoL2 response to TNFα differ in different cell types? The liver has to cope with the tremendous energy demand that is required for the synthesis of acute phase proteins during inflammatory response. That might explain why “there is no place” for apoL2 in liver cells during inflammation and that other regulatory agents probably interfere with its expression to ensure sufficient energy supply. Future investigations are needed to understand the interplay between PPARγ and TNFα regarding the expression of apoLs. It would be interesting to investigate the effect of PPARγ activators on apoL2 and apoL3 expression in the presence of TNFα in different cell types. Obviously, such experiments should be performed in human cell lines, since these genes are primate specific. Although TG are essential for normal physiology, excess TG accumulation results in obesity and, particularly when it occurs in non-adipose tissues, is associated with insulin resistance. Obesity, type 2 diabetes and hyperlipidemia are coexisting conditions frequently associated with non-alcoholic fatty liver disease (NAFLD) (19). Because of the tremendous increase in the prevalence of obesity worldwide and the lack of efficacy of current medical therapies, efforts to develop novel therapies for obesity are needed. Medications currently available
for the treatment of obesity primarily act by decreasing energy input, by either suppressing appetite or interfering with lipid absorption in the gut. Other potential therapeutic strategies are inhibiting TG synthesis/storage and increasing energy usage by $\beta$-oxidation. In order to design novel therapeutic strategies, we need to understand the TG metabolism at the molecular level. In the past several years, pharmacological inhibition of Dacylglycerol:acyl-CoA acyltransferase 1 (DGAT1) has emerged as a potential strategy for inhibiting TG synthesis in obesity (20). We have shown that apoL2 overexpression causes steatosis only in cells capable of lipid loading. Maybe in steatotic human liver, the balance of gene expression is distorted and there is a higher apoL2 expression. Inhibition of apoL2, either by interfering with upstream regulatory mechanisms or direct gene silencing with anti-sense therapy, could reverse the steatotic process. Vice versa, apoL3 overexpression could reduce intracellular TG substantially. Therefore, apoL3 overexpression with adenoviral gene therapy could also reverse the steatotic process. Currently, we are investigating the endogenous expression of apoL2 and apoL3 in human liver samples and compare the expression profile in steatotic versus non-steatotic livers. If they are differentially regulated during inflammation, then either apoL3 overexpression or apoL2 inhibition could be used as a therapeutic strategy in the treatment of NAFLD or obesity.

TG, PPAR$\gamma$ and atherosclerosis

Atherosclerosis, the major cause of death from cardiovascular disease (CVD) in industrialized countries, is characterized by the progressive accumulation of lipid and fibrous depositions in the vessel wall of large arteries. There is substantial evidence that high LDL-cholesterol and low HDL-cholesterol are both associated with CVD. Likewise, although debates continue regarding TG as an independent risk factor for CVD, the presence of hypertriglyceridemia confers a considerable increase in risk among subjects with otherwise similar ratios of LDL and HDL (21,22). Fibrates are drugs that specifically activate PPAR$\alpha$ and thereby reduce plasma TG levels. These drugs have been used for the treatment of dyslipidemia and have been shown to reduce the risk of CVD (23). Patients with metabolic syndrome due to obesity and diabetes have multiple risk factors for developing CVD. The term metabolic syndrome has been introduced to describe this cluster of metabolically related cardiovascular risk factors i.e obesity, insulin resistance, dyslipidemia and hypertension. During the past decades many novel drugs have been manufactured for each of these risk factors such as Rimonabant for treating obesity, Thiazolidinediones (TZDs) as anti-diabetic drugs and different fibrates as hypolipidemic drugs. TZDs are drugs that activate PPAR$\gamma$ and have been used for treatment of diabetes. Two widely used TZDs are Rosiglitazone and Pioglitazone. These compounds bind to the ligand binding domain of PPAR$\gamma$ and cause a conformational change that allows PPAR$\gamma$ to bind to DNA. Thereby, TZDs induce expression of array of genes that are involved in adipocytes differentiation and glycemic control. Both drugs have been used successfully in diabetic patients with improved metabolic parameters. Because of its beneficial effects on diabetes, it was expected to be also beneficial in preventing CVD. While information about the cardiovascular effects of these agents has gradually become available from a series...
of randomized controlled trials, it has required meta-analyses to better characterize their risks. Two separate meta-analysis on the effects of Rosiglitazone on cardiovascular endpoints showed that this drug was, however, associated with increased risk of myocardial infarction (24;25). How could we explain this paradox that Rosiglitazone improves glycemic control but increases the CVD risk? May be the best way to answer these questions is to look at a naturally occurring PPARγ insufficiency in patients with familial partial lipodystrophy. Lipodystrophies represent a heterogeneous group of diseases characterized by an abnormal, subcutaneous fat distribution that are associated with metabolic abnormalities comparable to the metabolic syndrome. Familial partial lipodystrophy type 3 (FPLD3) is a monogenic disorders that is caused by mutations in PPARγ gene (chapter 6 and 7 of this thesis). FPLD3 patients have a reduced expression of PPARγ due to haploinsufficiency of the PPARγ gene. From the currently described patients in the literature, none of them has been described to suffer from CVD. Conversely, FPLD2 patients with mutation in Lamin A (LMNA) gene have comparable clinical manifestations but do have CVD (26). Although, the number of patients is too small to draw any conclusion, it seems that patients with PPARγ deficiency, despite their metabolic abnormalities, have a lower risk of CVD compared to FPLD2. Comparing these data with the clinical data on Rosiglitazone, showing that activation of PPARγ increases the cardiovascular events one can hypothesize that PPARγ expression level is set to be in a specific range and that either low level of expression or too much activation is detrimental to the organism (Figure 1). PPARγ activation leads to metabolic improvement but increased CVD, whereas low expression of PPARγ in FPLD3 leads to metabolic abnormalities and protection from CVD. If the level of PPARγ should indeed be at a fixed biological range, then its activation with another TZD should have the same effect on CVD risk. Interestingly, in the same issue of JAMA, where a meta-analysis on Rosiglitazone was presented, Lincoff et al presented a meta-analysis on Pioglitazone (27). They showed that Pioglitazone was associated with a significantly lower risk of death, myocardial infarction, or stroke among a diverse population of patients with diabetes. How could one PPARγ activator (Rosiglitazone) be disadvantageous while another (Pioglitazone) is apparently beneficial in CVD risk lowering? There are two possibilities to explain this phenomenon. Either Rosiglitazone has additional detrimental side effects or Pioglitazone has additional positive effect. There is some evidence for the latter option. It has been shown that Rosiglitazone at concentrations used in the clinics is almost entirely restricted to activate PPARγ, whereas Pioglitazone has also PPARα activation capability (28). As already mentioned before, PPARα activation has been shown to reduce cardiovascular risk. Therefore, it is plausible that the CVD risk reduction with Pioglitazone is due to its PPARα-activating activity and the improved lipid profile and not for its PPARγ activity. To explore the above mentioned hypothesis, it would be interesting to define the atherogenic profile of FPLD2 and FPLD3 patients and compare it to obesity associated metabolic syndrome. We are planning to perform intima-media thickness (IMT) measurements, as a surrogate marker for atherosclerosis, in these patients. If the hypothesis is correct, we would find higher IMT measurements in obese subjects and FPLD2 patients.
when compared to PPARγ-deficient FPLD3 subjects. By comparing these two monogenic disorders, we can dissect the metabolic syndrome risk profile from PPARγ activation risk profile. Monogenic disorders are perfect human models to study the effect of one gene on the total body. Just a decade ago all lipodystrophies were referred as FPLD, until genetic mutations in these patients were determined by different groups. The terms FPLD2 and FPLD3 were introduced to define the genetic background of these patients. Later, it became clear that the phenotype of these two monogenic disorders were quite different that were not noticed before. For instance, the degree of lipoatrophy was shown to be significantly more in FPLD2 patients when compared to FPLD3 subject by means of MRI (29). Therefore, the term phenomics has been introduced to emphasize the importance of thorough phenotypical characterization of patients. Phenomics has been defined as systematic application of clinical, biochemical, and imaging methodologies/tools that are familiar to practicing clinicians and clinician investigators (30). Currently, we are examining patients with type 2 diabetes with extreme insulin-resistance to detect lipodystrophic patients that might have been overlooked because of the subtle clinical differences with the common type 2 diabetics. If, after a thorough examination, lipodystrophic features are present in these patients we will perform sequencing of the PPARγ- and LMNA genes to detect potentially novel mutations.

Figure 1: level of PPARγ activity and its effect on metabolic parameters and CVD risk

PPARγ is a nuclear transcription factor that has a specific physiological range. Either increased or decreased activity of PPARγ could be detrimental for the organism. In FPLD3 patients with low expression of PPARγ, many metabolic abnormalities such as those in metabolic syndrome are observed, but so far there is no evidence for increased CVD risk. On the other hand PPARγ activation with TZD leads to improved metabolic parameters but also increases the risk of CVD.
In summary, in this thesis, a subset of genes involved in TG metabolism has been characterized that enhances our understanding of this complex pathway. Obviously, future investigations are needed to characterize the remaining genes for further understanding of the (patho)physiology of TG metabolism in order to define novel therapeutic targets for common diseases such as obesity and steatohepatitis. Furthermore, applying phenomics to subjects with familial disorders that might have an inherited disorder is very important to pinpoint the genetic defect. Therefore, intensive collaboration between fundamental research scientists and clinicians is crucial.
Reference List


Summary
In Chapter 1, intra- as well as extracellular triglyceride metabolism is described at the molecular level. The role of lipases and apolipoproteins as well as transcriptional factors regulating these genes is illustrated.

In Chapter 2, we discuss the gene expression in atherosclerosis. This chronic process in the arterial wall is usually confined to specific sites where the blood flow is more turbulent and the shear stress is low. We focus on the role of each of three major cell types (i.e. endothelial cells, smooth muscle cells and monocyte/macrophages), in the arterial wall and their gene expression profile in healthy and atherosclerotic condition. Special attention is given to the vascular role of nuclear transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ) that is mainly known for its involvement in triglyceride storage in adipocytes.

In Chapter 3, a novel gene family that is expressed in the endothelial cells of human aorta is described. The apolipoprotein L gene cluster is located at chromosome 22q31 and consists of 4 homologous genes. We show that this gene cluster has emerged recently in the evolution as the result of tandem gene duplication. ApoL3 is shown to most probably represent the ancestral gene, with ApoL2 as closest relative at the DNA level. Interestingly, amino-acid sequences diverge more than DNA sequences, hinting at a fast functional divergent evolution. Most significantly, ApoL1 contains a signal peptide and is therefore secreted into plasma and is associated with HDL-particles. Presumably, apoL4 has lost its function during the evolution and has become a pseudogene because of the lack of gene expression in examined tissues.

Chapter 4 describes the function of apoL2 and apoL3. The divergent intracellular localization of these two proteins, ER for ApoL2 versus Golgi for ApoL3, is illustrated by specific antibodies as well as by Green fluorescent protein (GFP) fusion proteins. Although, these two genes are highly homologous, we found that they have opposite effects on intracellular triglyceride levels in the different cell types studied. ApoL2 over-expression increases intracellular triglyceride levels and apoL3 decreases it. Both genes are also expressed in cells specialized in triglyceride storage such as macrophages and adipocytes. In vivo, over-expression of apoL2 induced steatosis in mice livers, whereas apoL3 reduced the level of steatosis.

Chapter 5 describes a novel gene, Lipase H (LIPH) that is homologous to triglyceride lipase family. LIPH is localized on human chromosome 3q27–q28 and it was mainly expressed in intestine, lung, and pancreas. Lipase H is a secreted protein with an apparent molecular weight of 63 kDa. Although its structure resembles that of other triglyceride lipases, its substrate was not identified. Like other members of this gene family, LIPH may be involved in lipid and energy metabolism, but its precise function is yet to be determined.

Chapter 6 describes a patient with a mutation in the PPARγ gene. This nuclear transcription factor is involved in adipocyte differentiation and triglyceride storage. Activating this gene
by specific drugs has been widely used in the clinic for treating diabetic patients. Although this particular subject was not obese, she had all the characteristic features of metabolic syndrome such as diabetes with extreme insulin resistance, dyslipidemia and hypertension due to mutation in the DNA binding domain of PPARγ. We show that this specific mutation indeed disrupts PPARγ function, leading to PPARγ haplo-insufficiency.

Chapter 7 describes all types of lipodystrophy and the associated metabolic abnormalities in affected patients. The generalized lipodystrophies are often diagnosed by pediatricians because of their striking appearance, whereas partial lipodystrophies become apparent during adult life. Therefore, these patients are often not recognized and are treated as metabolic syndrome patients. We emphasize the importance of recognizing the patients with lipodystrophy due to genetic abnormalities, because of the therapeutic consequences.

Finally Chapter 8 summarizes the findings in this thesis and the role of genes involved in triglyceride metabolism. Fat storage in adipose tissue as well as in non-adipose tissue is discussed. Special attention has been given to the significance of communication between fundamental researchers and clinicians to improve our understanding of the pathophysiology of diseases to be able to define new therapeutic targets and design new drugs. Therefore, bidirectional transfer of information, i.e from bench to bedside as well as from bedside to bench is of utmost importance.
Samenvatting
In dit proefschrift worden een aantal recent ontdekte genen beschreven die een rol blijken te spelen in het triglyceride (TG) metabolisme. Patiënten met aangeboren dyslipidemie hebben een afwijkend lipidenprofiel als gevolg van een genetisch defect. De best onderzochte aangeboren dyslipidemie is familiare hypercholesterolemie (FH) waarbij de patiënten een sterk verhoogde risico op hart en vaat ziekten (HVZ) hebben. Het ontrafelen van het genetisch defect bij FH heeft er toe geleid dat onze kennis over dit ziektebeeld enorm is toegenomen en de patiënten kunnen met specifieke geneesmiddelen behandeld worden waardoor hun overlevingskans sterk is toegenomen. Dit voorbeeld laat zien dat het zeer van belang is om het genetische defect bij verschillende patiënten op te sporen om in de toekomst nieuwe therapieën te kunnen ontwikkelen. Het totale aantal humane genen wordt geschat op ongeveer 35000 waarbij de funktie van veel van deze genen vooralsnog onbekend is. De laatste jaren zijn door de technische vooruitgang in het genetisch onderzoek steeds meer genen ontdekt die een rol spelen in zowel het intracellulaire als het extracellulaire lipidenmetabolisme. Er zijn steeds meer aanwijzingen dat hoge plasma TG gehaltes ook een verhoogde kans op HVZ geven. TGs zijn niet alleen de belangrijkste energie bron voor het organisme, maar tevens de voorlopers van vele andere moleculen die een structurele functie in de cel hebben. Door de ongezonde levensstijl in westerse landen wordt overgewicht steeds een groter probleem. Patiënten met obesitas hebben naast onderhuidse vetstapeling ook last van vetstapeling in andere organen, o.a. in de lever. Hierdoor ontstaat er op de lange termijn leverinsufficiënte. Overigens hebben deze patiënten ook een verhoogde kans op HVZ. Naast levensstijl en omgevingsfactoren zijn er ook genetische defecten die aan dit ziektebeeld kunnen bijdragen. Het begrijpen van het normale TG metabolisme op moleculair niveau kan ons verder helpen in begeleiden en behandelen van deze patiënten.

Hart en vaatziekten zijn de nummer één doodsoorzaak in de westerse landen hetgeen vooral wordt veroorzaakt door de wijdverbreide onderliggende pathologische verandering van de vaatwand, genaamd atherosclerose (aderverkalking). Er is een duidelijk verband tussen atherosclerose en ontsteking. Russel Ross was een van de pioniers op dit gebied en maakte duidelijk dat atherosclerose een inflammatoire ziekte van de vaatwand is. Inmiddels is dit een algemeen geaccepteerd concept geworden, waarbij meerdere studies het belang van inflammatie in dit chronische proces hebben laten zien. Daarnaast is het ook reeds lang bekend dat dyslipidemieen de kans op HVZ en atherosclerose vergroten. In hoofdstuk 1 wordt uitgelegd hoe lipidenmetabolisme en inflammatie een rol spelen in atherosclerose. In hoofdstuk 2 wordt de genexpressie bij atherosclerose beschreven. De vaatwand is uit meerdere cellen opgebouwd die ook verschillende functies hebben. De endotheel cellen bedekken de binnenkant van de vaatwand. Niet alleen inflammatoire processen, maar ook de zogenaamde lokale “shear stress” kan de genexpressie beïnvloeden. Bijvoorbeeld bij bifurcaties van de vaten bestaat een turbulent flow die voor een afwijkend genexpressie patroon in die cellen zorgt. Door deze veranderde (atherogene) genexpressie zijn deze plaatsen veel gevoeliger voor het ontwikkelen van atherosclerotische plaques die uiteindelijk leiden tot vaatvernavuingen en HVZ.
Hoofdstuk 3 en 4 worden een nieuwe genfamilie beschreven; genaamd apolipoprotein L genfamilie. Dit gencluster bestaat uit 4 homologe genen (apoL1-4) op chromosoom 22q31. We hebben aangetoond dat deze genfamilie zeer recent in de evolutie is ontstaan. Daarom wordt het apolipoprotein L gencluster ook alleen bij primaten gevonden. ApoL3 is hoogst waarschijnlijk het moedergen, dat later gedupliceerd is. Overigens vertonen deze genen op DNA niveau meer homologie dan op eiwit niveau. Dit betekent dat ze in relatief korte tijd verder geëvolueerd zijn om nieuwe functies te verwerven. Dit wordt vooral duidelijk bij apoL1 dat een extra exon heeft waardoor het N-terminale gedeelte een nieuwe extensie heeft gekregen. Dit gedeelte heeft een signaal peptide wat er voor zorgt dat apoL1 wordt uitgescheiden uit de cel. In het plasma is apoL1 dan ook geassocieerd met HDL partikels. Een andere groep heeft aangetoond dat apoL1 de lang gezocht trypanosoma lytische factor is. Trypanosoma’s kunnen door HDL gelyseerd worden, maar het was lange tijd niet duidelijk welke eiwit hiervoor verantwoordelijk was. Wij hebben aangetoond dat apoL1 door Tumor Necrosis Factor α (TNF-α) wordt opgereguleerd waarbij een rol van apoL1 in inflammatie wordt gesuggereerd. We konden in alle weefsel die we onderzocht hebben geen expressie van apoL4 aantonen, wat heel aannemelijk maakt dat apoL4 een pseudogen is dat in de loop van de evolutie zijn functie heeft verloren. We laten ook zien dat apoL2 en apoL3 betrokken zijn bij het intracellulaire TG metabolisme. We tonen aan dat deze eiwitten, hoewel ze op elkaar lijken, een verschillende intracellulaire lokalisatie hebben. ApoL2 is namelijk gelokaliseerd in het endoplasmatisch reticulum (ER), terwijl apoL3 alleen in het Golgi complex werd aangetoond. De meeste genen die betrokken zijn bij de TG synthese bevinden zich in het ER, waardoor de rol van apoL2 in het TG metabolisme aannemelijk wordt. Overexpressie van apoL2 in adipocyten veroorzaakt een significante stijging van de intracellulaire TG inhoud. Tevens lieten onze in vivo studies in muizen zien dat overexpressie van apoL2 in de lever tot leververvetting leidt. ApoL3 overexpressie in de lever liet juist het tegenovergestelde zien, namelijk een significante afname van de intracellulaire TG inhoud. De endogene expressie van apoL3 was bovendien geremd tijdens vetstapeling in humane macrofagen, terwijl die van apoL2 juist sterk verhoogd was parallel met de inductie van PPARγ. Dat betekent dat apoL2 het intracellulaire TG verhoogt, terwijl apoL3 juist verlaagt. Blijkbaar is er in de revolutie een tijdelijke behoefte (omgevingsdruk) geweest aan hogere expressie van apoL3, het moedergen. Deze omgevingsdruk is waarschijnlijk later weggevallen zodat apoL2 en apoL1 zich verder hebben aangepast. Hierdoor heeft apoL2 het tegenovergestelde effect op TG ontwikkeld om de intracellulaire TG balans in stand te houden. ApoL1 heeft een compleet nieuwe functie gekregen buiten de cel. Het zou interessant zijn om erachter te komen wat die specifieke omgevingsdruk geweest is die ongeveer 35 miljoen jaar geleden ertoe heeft geleid dat apoL3 zich heeft gedupliceerd. Gezien de rol van apoL2 en apoL3 in het TG metabolisme is het heel goed denkbaar dat het modifiseren van de expressie van deze genen gebeukt kan worden in de behandeling van leververvetting en obesitas. Verder onderzoek naar de precieze functie van deze genen in de toekomst is daarom noodzakelijk.
In hoofdstuk 5 beschrijven we een nieuw gen genaamd lipase H (LIPH), dat zeer homoloog is aan andere leden van de triglyceride lipasefamilie. Dit gen ligt op humaan chromosoom 3q27-q28 en komt vooral tot expressie in darm, long en alvleesklier. Gezien de homologie met andere lipases en het expressiepatroon in verschillende weefsels die betrokken zijn bij het lipidemetabolisme, hebben we lang gezocht naar het lipidsubstraat van dit enzym. We konden echter geen duidelijke lipase activiteit aantonen voor LIPH. Een andere groep heeft later aangetoond dat mutaties in dit gen geassocieerd zijn met familiaire kaalheid. Hoewel de associatie met kaalheid zeer aannemelijk is, weten we nog steeds de precieze functie van dit gen niet. Immers, in de darm hoeven we geen haargroei te verwachten waardoor nog veel ruimte blijft voor onderzoek naar de potentiële functie van LIPH in het TG metabolisme.

In hoofdstuk 6 wordt een patiënt beschreven met duidelijke kenmerken van lipodystrofie. patiënten met een aangeboren lipodystrofie hebben een afwijkende verdeling van het subcutane vet met daarbij ook alle kenmerken van metabool syndroom. Ze hebben diabetes met extreme insuline resistentie, dyslipidemie en hypertensie. De patiënt die we in hoofdstuk 6 beschrijven is een 31 jarige vrouw met alle bovengenoemde kenmerken en bleek een mutatie te hebben in het PPARγ gen. PPARγ is een nucleaire transcriptie factor die betrokken is bij vetceldifferentiatie en TG stapeling. Nucleaire transcriptie factoren werken als intracellulaire antennes die de signalen uit de omgeving opnemen en na activatie meerdere genen tot expressie brengen als respons op de stimulus. De mutatie bij deze patiënt was in het DNA bindend domein van PPARγ en zorgt voor het onvermogen van dit eiwit om aan DNA te binden en de genexpressie in gang te zetten. Hierdoor heeft ze een haploinsufficiëntie, wat inhoudt dat ze alleen één werkzaam allele heeft en dat het gemuteerde allele wel het eiwit maakt maar geen effect heeft op genexpressie. Blijkbaar is de zogenaamde “gene dosage” in dit geval van belang waardoor 50% minder expressie al voldoende is om tot een ziekebeeld te leiden.

In hoofdstuk 7 worden alle aangeboren vormen van lipodystrofie beschreven, waarbij onderscheid wordt gemaakt tussen de partiële en de gegeeneraliseerde vorm. Deze laatste zorgt voor een extreem fenotype waarbij de diagnose veelal op de kinderleeftijd wordt gesteld, terwijl de partiële vorm tot volwassenen leeftijd ongemerkt kan blijven. Bij meeste patiënten met partiële lipodystrofie wordt het klinisch beeld niet herkend en de patiënten worden beschouwd als type 2 diabetes.

In hoofdstuk 8 worden de consequenties van de bevindingen in de eerdere hoofdstukken nader toegelicht. Speciale aandacht is besteed aan de evolutie van genen betrokken bij het lipidemetabolisme. Naast apolipoproteine L zijn er ook andere genen die specifiek in bepaalde species voorkomen. Verder wordt ingegaan op de rol van PPARγ in atherosclerose. Hoewel PPARγ agonisten zijn gebruikt als middel tegen diabetes wordt hun gunstige rol in atherosclerose in twijfel getrokken. Recente analyses hebben laten zien dat Rosiglitazone, een PPARγ agonist, geassocieerd is met een verhoogde sterfte aan HVZ, terwijl juist het
tegenovergestelde werd verwacht. Een mogelijke verklaring hiervoor wordt aangedragen. Verder wordt het belang van directe communicatie tussen basale wetenschappers en clinici benadrukt. Nieuw ontdekte genen en pathways moeten ook in een klinische setting onderzocht worden (from bench to bedside), maar ook andersom is het van belang om patiënten met specifieke fenotypes, verder genetisch te karakteriseren (from bedside to bench). We zijn momenteel bezig om de genexpressie van apoL2 en apoL3 in patiënten met leververvetting te analyseren (from bench to bedside), terwijl we ook patiënten met extreme insuline resistantie opzoeken om bij hun PPARγ mutaties op te sporen.
Dankwoord
Dankwoord

Er wordt gezegd dat het dankwoord het meest gelezen hoofdstuk is van proefschriften maar ik ga ervan uit dat je alle hoofdstukken hebt gelezen en dat je nu de laatste pagina’s van dit boek niet ongelezen wilt laten!! Als dat niet het geval is, kan je vooralsnog beginnen bij hoofdstuk 1.

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