Pathogenetic and Clinical Aspects of Endogenous Hypertriglyceridemia

Frits H.A.F. de Man

Stellingen behorende bij het proefschrift

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1. Er bestaan belangrijke verschillen in enzymkinetiek tussen lipoproteïne lipase in-oplossing en lipoproteïne lipase dat gebonden is aan heparan sulfaat proteoglycanen. Dit proefschrift.

2. Het succes van dieet in hypertriglyceridemie lijkt niet te worden bepaald door veranderingen in de verhoudingen van voedingscomponenten maar door vermindering van de absolute energie- en alcoholinname. Dit proefschrift.

3. Het hypolipidemisch effect van bezafibraat in hypertriglyceridemie wordt primair gemedieerd door opregulering van lipoproteïne lipase zonder effect op substraataffiniteit van VLDL voor lipoproteïne lipase en de LDL-receptor. Dit proefschrift.

4. Hypertriglyceridemie is geassocieerd met normale oxydatieve stress en een goede oxydatieresistentie van VLDL en LDL. Dit proefschrift.

5. Chronische hypertriglyceridemie is geassocieerd met een gestoorde endotheelfunctie. Dit proefschrift.

6. Het niet-overwegen van behandeling met HMG-CoA-reductase remmers bij patiënten met hart- en vaatziekten is een kunstfout. 2e Herziening Cholesterol Consensus 1998.

7. HMG-CoA-reductase remmers reduceren de kans op een cerebrovasculair accident bij patiënten met coronaire hartziekten. Daarnaast is het aannemelijk dat de afname van cognitieve functies en het ontwikkelen van dementie gunstig beïnvloed worden. Blauw GJ *et al.* Stroke 1997;28:946-50.

8. Microsomal transfer protein-inhibitors vormen een nieuwe groep krachtige cholesterolverlagende geneesmiddelen die een medicamenteuze oplossing zouden kunnen bieden voor patiënten met een ernstige hyperlipidemie die onvoldoende reageert op conventionele therapie. Wetterau JR *et al.* Science 1998;282:731-4.

9. Het suprasystolisch polsmanchet dat gebruikt wordt bij veneuze occlusie plethysmografie heeft het adagio "time is tissue" een geheel nieuwe dimensie gegeven.

10. Dikke mensen leven korter, maar ze zitten langer aan tafel. Stanislaw Jerzy Lec (1909).

11. Het beurs-AIO systeem, dat geïntroduceerd werd ter bestrijding van wachtgeld-problematiek, heeft op onverklaarbare wijze geleid tot halvering van het salaris van de promovendus.

12. Het boerenverstand moet je koesteren. Tobias Bruning (1999).

13. Na het schrijven van een proefschrift, mag de promovendus wel even achter de vetlap. De gezusters Kerkhof (1999).

14. Het is niet verdienstelijker 1000 boeken te hebben gelezen, dan 1000 akkers te hebben geploegd. W. Somerset Maugham (1874).

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Proefschrift

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Frederik Hendrik Andreas Franciscus de Man

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in 1967

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Cover: Transmission electron micrograph of VLDL particles negatively stained with 3% uranyl acetate. Magn. 285,000x produced at the Center for Electron Microscopy, Leiden, The Netherlands.

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1. General Introduction

1.1. Lipoprotein metabolism

Although lipids are considered to be a prerequisite for the development of atherosclerosis, cholesterol and triglycerides (TG) have important roles in the human body. Cholesterol is essential for the synthesis of cell membranes, steroid hormones and bile acids. TG are mainly used as energy source in peripheral tissues or stored in adipose tissue. As these lipids are hydrophobic compounds, transport in the hydrophilic surrounding of blood requires a special way of packaging: the lipoprotein. A lipoprotein is a spherical structure that contains a nonpolar core of cholesteryl esters and triglycerides, that is covered by a polar surface monolayer of phospholipids, free cholesterol and (apo)proteins. The lipoproteins can be divided by intrinsic density into 5 major classes: Chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These lipoproteins differ in density, size and composition as shown in table 1.

Table 1. *Physical properties and composition of plasma lipoproteins in humans.*

	CM	VLDL	IDL	LDL	HDL
Source	Intestine	Liver	VLDL	VLDL	Intestine, TRL
Density (g/mL)	< 0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Composition					
triglycerides (%)	86	55	23	6	4
cholesteryl ester	3	12	29	42	15
free cholesterol	2	7	9	8	5
phospholipids (%)	7	18	19	22	34
protein (%)	2	8	19	22	42
Apolipoproteins					
apoB	B-48	B-100	B-100	B-100	-
apoA	A-I, -II, -IV	-	-	-	A-I, -II, -IV
apoE	E	E	E	-	E
apoC	C-I,-II, -III	C-I,-II, -III	C-I,-II, -III	-	C-I,-II, -III

CM, chylomicron; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

The metabolism of lipoproteins can be divided in three pathways: 1. the exogenous lipid pathway: the metabolic route of dietary lipids; 2. the endogenous lipid pathway: the metabolic route of hepatically produced lipoproteins; and 3. the reverse cholesterol transport: the route of peripherally absorbed cholesterol to the liver. I will focus on the metabolism of triglycerides in the section below.

1.2. Normal metabolism of triglycerides

A schematic presentation of normal lipoprotein metabolism is presented in figure 1. Transport of triglycerides (TG) is carried out by chylomicrons from the intestine (exogenous route) and very low density lipoproteins (VLDL) from the liver (endogenous route), designated triglyceride-rich lipoproteins or TRL. TRL are large particles which consist of a lipid core of predominantly TG.

1.2.1. Endogenous route

The endogenous triglyceride transport is mediated by TRL from the liver. These hepatic TRL, known as VLDL, contain apoB-100 as structural protein, while chylomicrons contain the structural protein apoB-48. In contrast to rodents, humans demonstrate a distinct tissue-specificity of apoB-100 (liver) and apoB-48 (intestine) production. A newly discovered protein, microsomal triglyceride transfer protein (MTP) plays a critical role in the assembly and secretion of the apoB-containing lipoproteins: it mediates the early, co-translational transfer of lipids into nascent chylomicrons in intestinal cells, or VLDL in hepatocytes (1). It is hypothesized that the MTP-mediated lipid transfer stabilizes the apoB protein as it enters the endoplasmatic reticulum where, after folding into the proper structure, it can be lipidated as the first step in lipoprotein assembly (for review, see (2)). Upon secretion, nascent VLDL contain apoB-100 as structural protein as well as apoE and apoC. More apoE and apoC become associated to VLDL immediately after secretion in the blood.

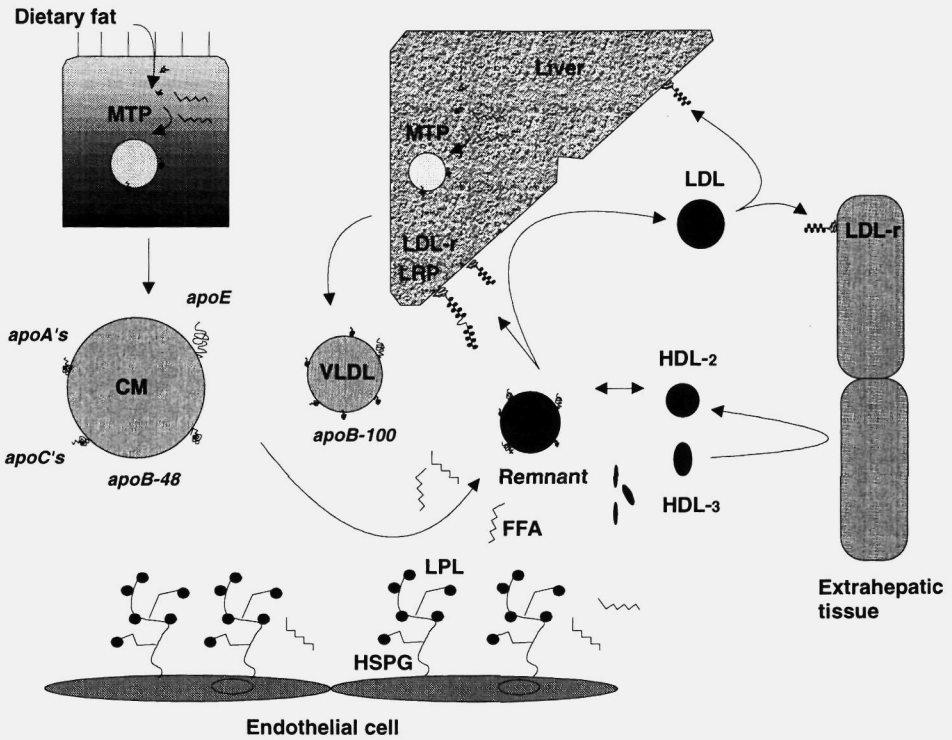

Figure 1. Schematic presentation of lipoprotein metabolism. *CM, chylomicron; FFA, free fatty acid; HSPG, heparan sulphate proteoglycan; LDL-r, LDL receptor; LRP, LDL receptor-related protein; LPL, lipoprotein lipase; MTP, microsomal transfer protein.*

The enzyme lipoprotein lipase (LPL) plays a key role in the catabolism of TRL (for review, see (3)). LPL is produced by various parenchymal tissues and binds to heparan sulphate chains on the luminal surface of capillary endothelium. A small amount (~ 10%) of LPL is associated with lipoprotein particles (4). Apolipoproteins have active roles in the lipolytic process. ApoC-II interacts directly with LPL and activates the enzyme. ApoB and apoE have heparan-binding sites and are thought to interact with the heparan sulphate chains (5). The heparan sulphate-bound LPL extends into the bloodstream to bind TRL. To allow interaction in vivo of TRL with endothelium-bound LPL and subsequent lipolysis, the particle has to reside transiently at the proteoglycan-LPL complex. Apolipoproteins are considered to play a role in this process. ApoE can bind to proteoglycans and may participate in the interaction between TRL and lipolytic sites (3,6). Also, apoC-II and apoB have been shown to contain LPL-binding sites and may be involved in this

interaction. After LPL has bound to apoC-II, attached to the surface of the particle, hydrolysis of triglycerides occurs and fatty acids are released in the circulation. Many LPL molecules are thought to act simultaneously on the chylomicron, holding the particle firmly at the endothelial binding sites. It is not clear how the particle dissociates from the endothelium, but it is thought that both the reduction in size and the accumulation of lipolysis products, particularly fatty acids, reduce its affinity to the surface (7,8). With regard to the role of apoE, Rensen *et al.* (9) elegantly showed that apoE-enrichment of artificial chylomicrons inhibited *in vivo* and *in vitro* lipolysis in a dose-dependent way, a finding that was confirmed by Jong *et al.* in transgenic mice overexpressing apoE (10). It was suggested that enrichment of TRL with apoE during lipolysis, results in dissociation of the TRL from HSPG-bound LPL, subsequently leading to avid uptake of the TRL remnants by apoE-specific lipoprotein receptors (9). The flow of fatty acids, released by LPL and derived from peripheral adipose tissue, is partly assimilated by extra-hepatic tissues. However, a substantial amount of fatty acids, either bound to albumin or still present in the remnant particles, is taken up by the liver and re-esterified to VLDL triglycerides in the hepatocyte. After hydrolysis, cholesterol- and apoE-enriched remnants remain. The high concentration of apoE on the surface of the remnant particle results in high affinity binding and rapid removal. Part of the IDL particles is processed to LDL particles by LPL and perhaps HL (11).

The receptor-mediated clearance accounts for a substantial TRL amount (10-60%) and the majority of TRL remnants that is removed from the plasma directly (12). So far, several TRL-binding receptors have been identified: the low density lipoprotein receptor (LDL-R), the low density lipoprotein-related protein (LRP), the VLDL receptor (VLDL-R), the lipolysis-stimulated receptor (LSR) and membrane-binding protein (MBP) 200 and 235 (for review, see (13)). Although these receptors have distinct functions and tissue-distribution, they all recognize TRL and, if necessary, can take over the function of other TRL-binding receptors. The LDL-R, or B/E-receptor, is mainly located in the liver and only a small number of LDL-receptors is found extra-hepatically. The LDL-R recognizes apoB-100 and apoE, but has a much greater affinity for apoE. Although the LDL-R plays an important role in VLDL clearance, there is evidence for effective alternative TRL removal pathways (14,15). This is illustrated by the fact that animals and humans who are homozygous for LDL-R mutations show normal VLDL and CR catabolism (16-18). The LRP, or remnant-receptor, is a large, apoE-binding receptor that

is located in various tissues including the liver. It is a multifunctional receptor that binds many ligands, such as tissue plasminogen activator and α_2-macroglobulin. Although several studies have demonstrated that LRP can bind TRL, its relevance in human lipoprotein metabolism remains to be elucidated. Animal studies, however, have provided interesting data. Blockade of the LRP by RAP (receptor-associated protein) does not affect lipid levels in normal mice (19,20). However, blockade of LRP in LDL-R deficiency causes marked hyperlipidemia, indicating that the LRP provides an efficient rescue mechanism for the clearance of TRL (19). Recently, Veniant *et al.* (21) demonstrated that RAP expression in LDL-R knock-out mice resulted in the accumulation of apoB-48 whereas apoB-100 levels did not change. These data suggest that, at least in mice, the LRP does not play a significant role in the clearance of VLDL. The VLDL-receptor (VLDL-R) is a novel non-hepatic receptor that recognizes apoE. Mice with a defective VLDL-R do not develop HTG, even after provocation with a carbohydrate-enriched diet (22). Therefore, the VLDL-R does not appear to play an important role in TRL clearance.

1.2.2. Exogenous route

Chylomicrons are synthesized in intestinal cells. After a meal, the ingested fat is mostly absorbed as monoglycerides. These monoglycerides are esterified to triglycerides inside the intestinal cell. Assembly of TG with phospholipids, cholesterol, apoB-48, A-I, A-II and A-IV results in chylomicron formation. The intestinal apoB isoform, apoB48, is the structural protein of chylomicrons. The chylomicrons are secreted into the lymph and enter the systemic circulation via the thoracic duct. They receive C and E apolipoproteins from HDL in exchange for apoA-I. After "digestion" of the chylomicron by LPL, a smaller cholesterol-rich remnant remains which will subsequently be eliminated by lipoprotein receptors. In addition, there is evidence that in normolipidemic man, direct removal of large chylomicron remnants from the plasma compartment occurs (23,24). Conversion to small remnants is therefore not a prerequisite for elimination from the circulation.

The uptake mechanism of chylomicron remnants (CR) has received a lot of attention recently. Although the majority of CR is removed by the liver, CM and CR are partly cleared by peripheral tissues (25). The low density lipoprotein (LDL) receptor participates in the clearance of CR. Illustrative is the fact that "apoB-48 only" mice present higher apoB-48 levels in case of LDL-R deficiency as compared to the presence of the LDL-R. However, *in vitro*

and *in vivo* studies do not provide evidence for an exclusive role of the LDL receptor (LDL-R) in this process, as discussed above (14,15). Several *in vitro* and *in vivo* studies have provided evidence that the LRP is involved in the remnant removal (19,26). An intriguing finding was the fact that chylomicrons are taken up by the liver only after lipolysis by LPL (20). This binding-enhancing effect was not dependent on lipolysis, but was due to the structural properties of LPL itself. Endothelial-bound proteoglycans play an important role in this process. Proteoglycans serve to concentrate LPL-lipoprotein complexes on the cell surface, thereby enhancing their interaction with the lipoprotein receptors (27,28).

1.2.3. Interrelations between triglyceride-rich lipoproteins and other lipoproteins and the reverse cholesterol transport

High density lipoproteins play an important role in the metabolism of TRL. They serve as a storage pool of apolipoproteins including apoE and apoC. When TRL enter the circulation, they receive apoC-II and apoE from HDL, facilitating lipolysis and remnant removal. During lipolysis of TRL, surface fragments are delivered to HDL, adding a substantial contribution to the HDL pool (29,30). Accordingly, the effectiveness of TRL catabolism has been shown to correlate positively with HDL-C concentrations (31,32). Small, protein-rich HDL particles (HDL-3) initiate reverse cholesterol transport, accepting free cholesterol from peripheral cells (33,34). Subsequently, the cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT). In plasma, cholesteryl ester transfer protein (CETP) mediates the transfer of triglycerides and surface fragments from lipoproteins to HDL-3 in exchange for CE, creating a lipid-enriched intermediate HDL particle (33). Subsequent esterification by lecithin:cholesterol acyltransferase (LCAT) completes conversion of HDL-3 in HDL-2. This exchange of lipids is most pronounced in the postprandial phase. HDL-2 is reconverted into HDL-3 by the action of hepatic lipase on the hepatic membranes. In this process, HDL cholesterol is adsorbed by the hepatocyte membrane, completing one route of reverse cholesterol transport. The second route, represented by the transferred CE inside apoB-containing lipoproteins, is completed when the remnant particles are degraded by the liver. Recently, an HDL receptor was identified by Kozarsky *et al.* (35). Overexpression in mice resulted in a rapid disappearance of HDL from the circulation and an increase in biliary cholesterol. This receptor appeared to belong to the scavenger receptor family (type BI) and

could be demonstrated in the liver and steroidogenic tissues such as the ovaries, testes and adrenal cortex.

LDL particles are produced as end products of VLDL metabolism. Because LDL particles are deprived of the majority of apoE, associated to the precursor IDL particles, its affinity to the LDL-R is relatively lower than that of other apoB-containing lipoproteins resulting in a long residence time of approximately 3 days. Eventually, the LDL particle binds to the LDL-R in the liver and extra-hepatic tissues and is degraded. There is evidence for lipid transfer between TRL and LDL particles. Schaefer *et al.* (30) demonstrated that during in vitro lipolysis of chylomicrons, particle constituents were transferred to LDL density. In man, TG enrichment of LDL depends on interactions with determinants of the removal pathways of TRL (36,37). LDL particle size is determined in part by genetic factors including CETP, the apoAI-CIII-AIV gene cluster, and the LDL-R locus (38).

1.3. Pathogenesis of endogenous hypertriglyceridemia

The oldest case-report of hypertriglyceridemia entitled "pure milk on the blood" dates from 1641, when it was originally described by the Dutch physician Nicolaes Tulp (1593-1674), who was famous as the demonstrator in Rembrandt's picture "The anatomical lesson of Dr Nicolaes Tulp" (39). In accordance with the current perspective that hypertriglyceridemia is a risk factor for cardiovascular disease, Tulp makes the connection between the milky serum of this obese patient and premature atherosclerosis. Although the diagnosis is a biochemical one, the HTG patient may have some characteristic features. The patient with hypertriglyceridemia is often moderately obese and insulin-resistant. The frequency of hypertension and gout is several fold higher than in a normal population. A more specific and visible clinical feature are the eruptive xanthomas. These papular, yellow-white cutaneous lesions are lipid deposits in the skin, which result from phagocytosis of TRL by phagocytes. Although these typical xanthomas are found only in the minority of HTG patients, these lesions are pathognomonic for a severe hypertriglyceridemia (type V hyperlipoproteinemia (HLP)). The most dangerous complication of HTG is acute pancreatitis. Although the underlying mechanism is not fully understood, animal studies have suggested that pancreatitis may result from toxic fatty acid levels generated within the

pancreatic capillaries (for review, see (40)). As a pancreatitis is a life-threatening disorder, institution of TG-lowering therapy is effective and advisable (41).

Endogenous hypertriglyceridemia is not a rare condition. In the Framingham study, 8-10% of the general population presented elevated plasma triglyceride levels (42). The prevalence is even higher in a population with established CHD. In the screening for the BIP study (a secondary prevention trail with bezafibrate in patients with low HDL-C in Israel)(43), 20% showed elevated plasma triglyceride levels of which a fourth part had TTG levels exceeding 3.4 mmol/L. The diagnosis endogenous hypertriglyceridemia is a laboratory diagnosis. The lipid profile characteristically shows a large VLDL pool, whereas HDL and LDL levels are low to normal (44). It encompasses type I, IV and V HLP of the original Fredrickson classification (45). Since type I HLP, caused by homozygous apoC-II or LPL-deficiency, is an extremely rare condition, the population of interest consists of both type IV and V HLP. Patients with type V differ from type IV with regard to a higher TG level (> 20 mmol/L) and/or the presence of fasting chylomicrons. Since the discrimination between both types is difficult (patients may present both phenotypes within a short period of time), rather artificial (what is the basis of the TG cutoff point of 20 mmol/L), and technically difficult (separation of CM from VLDL), type IV and V HLP are often combined.

It is generally accepted that the accumulation of TRL results from both an increased production as well as a delayed clearance (46-50). The overproduction of VLDL-TG is disproportionately higher than the increase in VLDL-apoB production, resulting in the generation of large VLDL particles (46,47). Although genetic factors predispose subjects to the development of HTG (51-53), environmental factors are considered to play an important role in the expression of hypertriglyceridemia (54). Obesity, insulin resistance, alcohol consumption and dietary habits are the principal exogenous factors involved (55-57). Other factors are considered to contribute predominantly to a delayed catabolism of TRL in HTG (48-50): First, HTG is associated with an extended TG pool, implicating saturation of TRL removal pathways. Second, LPL mutations and associated LPL deficiency are commonly found in HTG patients. As LPL is the key enzyme in TRL lipolysis, an impaired catabolism may result. And third, HTG is associated with high apoC-III concentrations that may lead to a decreased lipolysis and receptor-mediated clearance of TRL. In the following section I will discuss these factors in detail.

1.3.1. Genetic factors

With regard to the genetic predisposition, the lipoprotein lipase (LPL) gene has received a lot of attention as the key enzyme in triglyceride catabolism. The human LPL gene is located on chromosome 8 and consists of 10 exons. The mature LPL protein consists of 448 amino acids. Several functional domains have been identified, including a catalytic domain and binding sites for heparan sulphate proteoglycans and apoC-II. Amino acid substitutions have been found in most of the exons in the LPL gene. Homozygosity for a functional LPL mutation is rare and is associated with LPL deficiency and gross hypertriglyceridemia with fasting chylomicronemia (58). Few data are available on the prevalence of heterozygous LPL mutations. Mailly *et al.* (59) studied the prevalence of the LPL (Asp9-Asn) mutation in a randomly selected English-Scottish population of subjects without CAD. In this large population, 25 heterozygotes and 2 homozygotes were found yielding an average frequency of 3.5%. Plasma triglyceride levels were increased in the carrier group by 24% as compared to the noncarriers, while plasma cholesterol levels were not different. In patients with HTG and combined HLP, the prevalence of the LPL (Asp9-Asn) mutation was about 2-fold increased. In addition, Minnich *et al.* (53) studied the prevalence of four common LPL point mutations in exon 5 in a French-Canadian population of patients with endogenous HTG (type IV and V HLP). Interestingly, 15-20% of the patients proved to be carrier of one of these four LPL mutations while in the normolipidemic control group no carriers could be identified. Syvänne *et al.* (54) recently published an elegant study describing two Finnish kindreds with the LPL (Asn291-Ser) mutation. The carriers showed higher triglycerides (+154%) and lower post-heparin LPL activities (-23%) than noncarrying family members. More than half of the carriers (9/16) were hypertriglyceridemic. This study makes two important points: First, not all carriers express lipid disturbances. And second, LPL activities are only mildly decreased in the heterozygous state. Therefore it appears that LPL mutations predispose subjects to the development of HTG but other genetic and/or environmental factors are required for the expression of HTG.

ApoC-III is an important inhibitor of LPL. In transgenic mice, overexpression of apoC-III causes hypertriglyceridemia whereas the induction of apoC-III deficiency by means of knock-out mice is associated with reduced triglyceride levels and resistance to diet-induced hyperlipidemia (60,61). The apoC-III gene has therefore been regarded as an important candidate gene in hyperlipoproteinemias. The apoC-III encoding gene is located in the APOAI-

CIII-AIV gene cluster on chromosome 11. Numerous studies have demonstrated a strong relationship between the SstI restriction length polymorphism (RFLP) and the occurrence of hypertriglyceridemia (51,52,62,63). A recent study by Surguchow *et al.* (64) performed in a sample of the ARIC population demonstrated a frequency of the S2 allele of 14.2% in those with high TG levels and 5.2% in those with normal TG levels. In accordance, in a well-defined Dutch population of patients with endogenous hypertriglyceridemia, the Sst1 RFLP was 3-fold more common than in a normolipidemic control population (63). Although the Sst1 RFLP of all candidate genes has shown the best correlation with hypertriglyceridemia so far, its significance remains a matter of dispute. Since the Sst1 polymorphic site is located in the 3' untranslated region of the APOC-III gene, it is considered not to influence transcription or function of apoC-III, but merely indicative of a second, yet unknown, mutation related to hypertriglyceridemia.

The promoter region of the apoC-III is an important regulatory element of apoC-III transcription. Dammerman *et al.* (52) demonstrated that a combination of five polymorphic sites in the apoC-III promoter associates with an increased risk of hypertriglyceridemia. Hoffer *et al.* (63) studied the -455 and -482 RFLP in a group of patients with HTG from the outpatient lipid clinic in Leiden. Interestingly, an increased frequency was observed in the HTG group as compared to a normolipidemic control group. This result indicates that the promoter region of the apoC-III gene may be involved in the expression of HTG. How variations in the promoter region of apoC-III can be linked to HTG was reported by Li *et al.* (65). They performed a study to determine if the variant promoter DNA sequence would cause a change in transcriptional activity of the apoC-III gene. Interestingly, they demonstrated loss of the, normally suppressive, activity of insulin on apoC-III transcription. It was hypothesized that the regulatory dysfunction of insulin on the variant apoC-III promoter region may lead to an increased apoC-III production and subsequently hypertriglyceridemia. Whether this *in vitro* observation bears clinical significance remains to be determined by future research.

An other gene that may be involved in the pathogenesis of HTG is the APOE gene. The central role of apoE in receptor-mediated lipoprotein removal is generally accepted. Mutations in (or in the vicinity of) the receptor-binding domain (aa 130 - aa 150) result in dominantly heritable forms of familial dyslipidemia or type III HLP, which is characterized by the accumulation of remnant lipoproteins (66,67)(for review, see (68)). Theoretically, mutations in the C-terminal region of the APOE gene, which contains the heparin binding

(aa 214 - aa 236) and lipid-binding region (aa 246 - aa 266) may result in a defective lipolysis and subsequent HTG. Several studies have demonstrated an association between C-terminal mutations and HTG (68,69). Although several genetic factors for HTG have been identified, frequently no cause is found (70,71).

1.3.2. Insulin resistance

Patients with endogenous hypertriglyceridemia generally present insulin resistance, characterized by high fasting insulin and normal or mildly elevated glucose concentrations (72). In addition to the elevated cardiovascular risk of dyslipidemia, high fasting insulin concentrations appear to be an independent predictor of ischemic heart disease in men (73). It is known that insulin levels strongly correlate with hepatic VLDL-TG production (74,75). Although some authors have suggested that hyperinsulinemia may stimulate VLDL-TG synthesis, *in vitro* studies have demonstrated that insulin normally suppresses VLDL-TG and apoB production (76,77). Therefore, the most feasible explanation for enhanced VLDL-TG synthesis in hyperinsulinemia is the concept that the liver is less sensitive to the inhibitory action of insulin. In the normal postprandial situation, the hyperinsulinemia stimulates the uptake of glucose whereas the lipolysis of lipids in the tissue is suppressed. Thus, fuel utilization shifts postprandially from fatty acid oxidation to glucose oxidation (78). However, in the insulin resistant state, lipolysis is not suppressed sufficiently and fatty acids are released at a high rate. Indeed, in insulin resistance the ability of insulin to suppress FFA levels is impaired, and FFA concentrations are high (79). In addition, subjects with insulin resistance often have a large amount of visceral fat (abdominal obesity) (80). So, the impaired responsiveness to insulin as well as the increased number of adipocytes may contribute to a high turnover of FA. The FFA flux from visceral adipose tissue provides the liver with a surplus of FA that are re-esterified to triglycerides in the hepatocytes and VLDL particles (for review, see (81). In endogenous HTG, this mechanism is enhanced by a high activity of hormone-sensitive lipase (82).

The high levels of FFAs and glucose in combination with reduced sensitivity to the normally suppressive action of insulin, stimulate hepatic VLDL production, designated as the substrate driven VLDL overproduction (83,84). In addition, the elevated FFA levels inhibit VLDL lipolysis by a negative feedback mechanism (8). Post-heparin LPL-activity is decreased in patients with insulin resistance, as was recently shown by Knudsen *et al.* (85)

in first-degree relatives of NIDDM patients. Probably, there is a direct effect of insulin resistance on the expression of LPL. In conclusion, insulin resistance is an important factor involved in the development of hypertriglyceridemia.

Recently, Sniderman *et al.* (86) postulated an intriguing hypothesis regarding the role of adipocytes in the pathogenesis of hyperlipoproteinemias. In the normal situation, about half of the fatty acids released by lipolysis of triglyceride-rich lipoproteins are taken up (trapped) by adipocytes, myocytes and other tissues (figure 2A). The remaining half of fatty acids enter the systemic circulation and reach the liver where they are re-esterified to triglycerides, and to a smaller extent to phospholipids and cholesteryl esters. The two major determinants of fatty acid uptake by adipocytes are insulin and the acylation-stimulating protein (ASP). If fatty acid trapping by adipocytes is decreased, a cascade of negative sequels may follow (figure 2B). First, an increased amount of fatty acids reach the liver and stimulate VLDL production. Second, the high local amounts of fatty acids inhibit lipolysis and induce premature detachment of TRL from the endothelium. Thus, triglyceride production is increased whereas triglyceride catabolism becomes impaired. The most frequent clinical cause of reduced fatty acid trapping is omental obesity. These omental adipocytes differ from normal subcutaneous adipocytes in several respects. Lipolysis by hormone-sensitive lipase is more pronounced in the omental adipocyte, leading to a higher efflux of fatty acids in the fasting state. In addition, triglyceride synthesis appears to be reduced in the omental adipocyte, which implicates a reduced fatty acid trapping. All these adverse events predicted to occur from reduced fatty acid trapping have been demonstrated in patients with hypertriglyceridemia: elevated fatty acid levels, increased VLDL synthesis and decreased triglyceride catabolism.

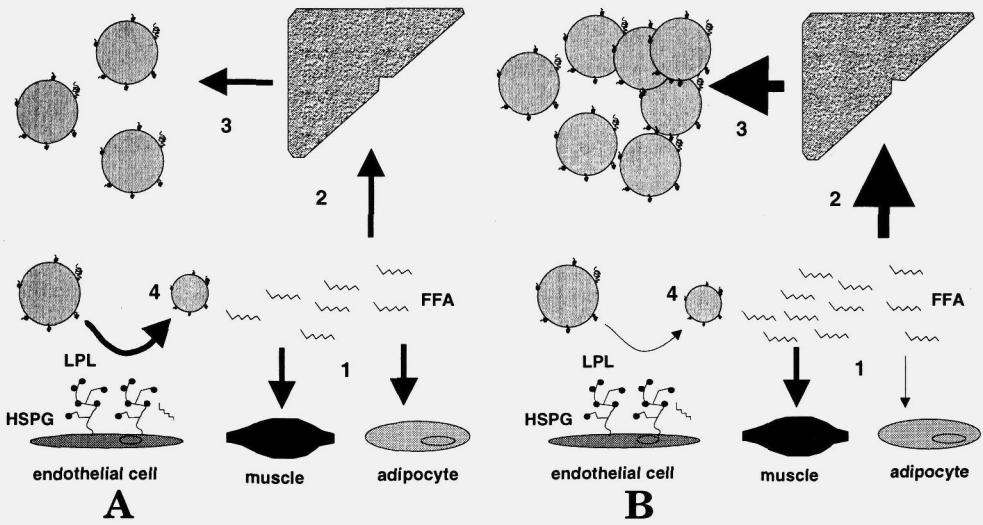

Figure 2. Schematic presentation of fatty acid trapping. *The normal situation is shown in panel A, the pathological situation of reduced fatty acid trapping is shown in panel B (see text above). FFA, free fatty acid; HSPG, heparan sulphate proteoglycan; LPL, lipoprotein lipase.*

1.4. Secondary causes of hypertriglyceridemia

A wide variety of secondary causes of hyperlipoproteinemia have been identified (87). It is crucial in the clinical evaluation of the hyperlipidemic patient to exclude the most important causes of hyperlipoproteinemia since it has major effect on the type of treatment. For example, hyperlipoproteinemia associated with hypothyroidism is very resistant to lipid-lowering drugs. The most effective therapy is adequate treatment of the underlying disease. The most important causes of secondary hypertriglyceridemia are summarized in the following section.

Alcohol. Immoderate alcohol consumption is associated with hypertriglyceridemia (for review, see (88)). Several mechanisms have been identified. First, alcohol consumption is known to stimulate the synthesis of endogenous lipoproteins. This is probably due to the fact that ethanol is metabolized in preference to fatty acids as source of energy. The accumulating fatty acids may be used for the production of triglycerides and incorporated in VLDL particles. Second, ethanol consumption may be associated with reduced lipolysis of TRL by LPL. Pownall *et al.* (89) demonstrated that addition of

alcohol to a fat load significantly increased the postprandial lipemia. Therefore, limitation of alcohol intake seems obligatory in every patient with HTG.

Diabetes mellitus. Poorly controlled diabetes mellitus (type I and type II) is associated with a high frequency of hyperlipidemia and premature atherosclerosis. If type I diabetes (insulin-dependent diabetes mellitus, or IDDM) is accurately treated, lipid and lipoprotein levels generally are within the normal range. The same applies to the insulin-resistant type, designated as type II or non-insulin dependent diabetes mellitus (NIDDM) (90). One of the most common lipid abnormalities in non-insulin-dependent diabetes mellitus is hypertriglyceridemia and low HDL cholesterol. The LDL cholesterol levels are normal or mildly elevated. The HTG is due to both overproduction of TRL and decreased catabolism. With regard to the overproduction, an increased supply of glucose and FFAs contribute to overproduction of very low density lipoproteins, increasing the burden of triglyceride-rich lipoproteins on the common lipolytic pathway at the level of lipoprotein lipase. Low lipoprotein lipase activity and increased amounts of lipolysis-inhibiting free fatty acids further impair lipolysis of postprandial lipoproteins. In addition, glycation of apolipoproteins (apoC-II, apoB and apoE) are considered to impair lipolysis and receptor-mediated lipoprotein catabolism (91). Correction of the lipid abnormalities in NIDDM is advisable since it may contribute to attenuation of the risk on premature atherosclerosis. When dietary measures and hypoglycemic agents have failed to achieve acceptable lipid levels, lipid-lowering drugs should be advised. Fibric acids and HMG CoA reductase inhibitors are the drugs of choice. The clinical efficacy of statin therapy was recently confirmed in a subgroup analysis of diabetic subjects from the 4S-study (92). Secondary prevention of CAD with simvastatin treatment resulted in a 60% reduction of coronary events in this high-risk group. Whether an aggressive lipid-lowering treatment is indicated in the primary prevention of CAD in diabetics remains to be established.

Hypothyroidism. Hypothyroidism is a frequent cause of hyperlipidemia. In a recent study at our department, the prevalence of subclinical hypothyroidism was studied in 1200 Dutch population-based subjects (manuscript in preparation). The overall prevalence of subclinical hypothyroidism was 1.9% in men and 7.6% in women. The prevalence of hypothyroidism appeared to be higher in the subgroups with the highest cholesterol levels. Previous studies have shown that 5-10% of all

hyperlipidemic subjects demonstrate apparent or subclinical hypothyroidism (93-95). Therefore, it is recommendable to screen all patients with hyperlipidemia for hypothyroidism (thyroid stimulating hormone and thyroxin). Hypothyroidism is not associated with a specific hyperlipidemia but can express different phenotypes. Although hypothyroidism has been associated with a decreased LPL activity and decreased LDL catabolism, the exact pathophysiological mechanism is not fully understood. Adequate treatment of the thyroid disease rapidly improves the lipid derangements.

Liver/renal disease. Liver disease may lead to lipid derangements in several ways. Cholestasis leads to hyperlipidemia when biliary lipids leak into the plasma. This may result in severe hypercholesterolemia and even the presence of cutaneous xanthomas. Interestingly, a rare lipoprotein can be identified in these subjects: lipoprotein X or Lp-X. This LDL-like particle does not contain apoB and contains high amounts of unesterified cholesterol. Inflammation of liver tissue can also lead to hyperlipidemia. However, in case of advanced hepatic disease, lipid levels tend to fall due to a reduced lipoprotein synthesis. Treatment of liver disease related hyperlipidemia is difficult since most drugs are metabolized by the liver and bear a potential risk for the remaining liver function. In addition, fibric acids should not be given in case of cholelithiasis. Renal failure is associated with hyperlipidemia and premature atherosclerosis. A decreased LPL activity is thought to be the basic pathophysiological defect in patients with chronic renal failure. This typically results in hypertriglyceridemia, low HDL cholesterol levels and small dense LDL. A renal transplantation is advisable, however, the extensive use of steroids and other drugs often sustains a mild hyperlipidemia. The nephrotic syndrome, characterized by an albuminuria > 3 g/day, is also associated with hyperlipidemia. It is thought that the hypoalbuminemia leads to increased hepatic production of albumin that is accompanied passively by an increased lipoprotein synthesis. Improvement of the proteinuria is associated with normalization of the hyperlipidemia.

Medication. Many drugs are known to affect plasma lipid levels. One of the most important drugs that interfere with the lipoprotein metabolism are the steroids. Corticosteroids can cause slight increases in serum lipid levels in normal individuals. However, in subjects prone to the development of hyperlipidemia, e.g. apoE2 homozygotes, corticosteroid therapy may result in a marked hyperlipidemia. Induction of insulin resistance is thought to be the underlying mechanism. Also, hormone preparations such as oral contraceptives may cause HTG (96). On the other hand, postmenopauzal

hormone replacement has been reported to improve plasma lipid levels. Many effects have been reported on different hormones in different age groups. Some of these effects are positive, some are negative, but the message is that hormones can effect lipid levels and it would be recommendable to discontinue hormone therapy in a hyperlipidemic patient to evaluate the drug effect. Beta blocking agents are known to elevate triglyceride levels and decrease HDL cholesterol levels mildly. There is no difference between the older aselective and newer class of selective beta blockers. Other drugs that affect lipid levels include retinoic acid, cyclosporin, and amiodarone.

1.5. Triglycerides and atherosclerosis

1.5.1. Epidemiological and clinical data

Although plasma triglycerides have not attracted the epidemiological attention as a risk factor for CHD as plasma cholesterol, LDL-cholesterol and HDL-cholesterol concentrations, several groups have emphasized the epidemiological evidence of an association between plasma triglyceride concentration and CHD (42,97-99). The controversial issue of this association is caused by the fact that an increased plasma triglyceride concentration is often seen in combination with a decreased HDL-cholesterol concentration and an increased concentration of small dense LDL particles. In multivariate statistical models the association between plasma triglyceride concentration and CHD becomes insignificant after adjustment of covariates, which has led to the conclusion that an elevated plasma triglyceride levels is not an independent risk factor for CHD. In a report from the Framingham Heart Study, Abbott *et al.* (100) pointed out that, when 2 items are associated both statistically and metabolically, the current mathematical models will grossly underestimate the contribution to risk of one of the items, thus representing a misapplication of the statistical models. By scoring the incidence of coronary artery disease during 14 years of the Framingham Study, Castelli (42) demonstrated that in the lowest HDL-cholesterol tertile (men: < 1.03 mmol/L; women < 1.29 mmol/L) the group of subjects in the highest triglyceride tertile (men > 1.57 mmol/L; women > 1.34 mmol/L) had a 4 times higher incidence of CHD for men and a 10 times higher incidence for women than those in the lowest triglyceride tertile (men > 1.02 mmol/L; women > 0.90 mmol/L).

In the Helsinki Heart Study and the PROCAM study it was demonstrated that the combination of high triglyceride and low HDL-cholesterol represents the most unfavorable lipoprotein pattern with respect to risk of future CHD (101,102). In a recently published meta-analysis of 17 population-based prospective studies, Hokanson and Austin (99) calculated relative risks (RR) and 95% confidence intervals (CI) and standardized these figures with respect to a 1 mmol/L increase in triglyceride (figure 3). For men and women the univariate RRs for fasting plasma triglyceride levels were 1.32 (95% CI 1.26-1.39) and 1.76 (95% CI 1.50-2.07), respectively.

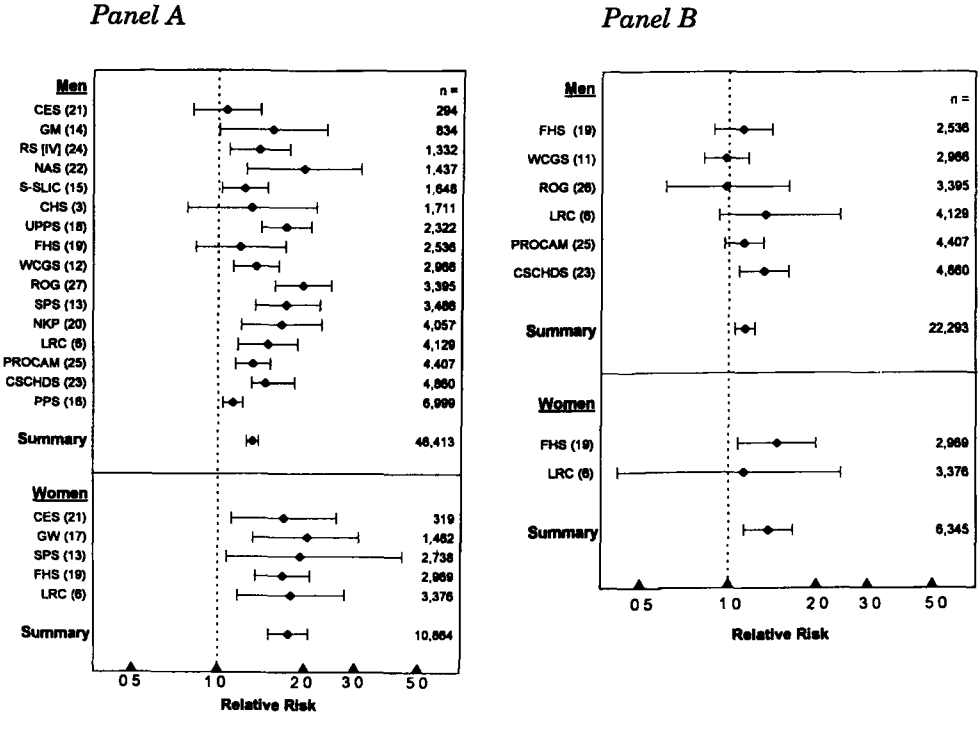

Figure 3. *Meta-analysis by Hokanson and Austin (99). Univariate relative risk estimates (panel A), multivariate relative risk estimates (panel B) and 95% confidence intervals for the association between the incident cardiovascular disease and a 1 mmol/L increase in triglyceride concentration, by gender. The relative risk values are given on the x-axis on a logarithmic scale. The y-axis lists the studies that were included (see table 1 in reference 99), ordered by sample size. Note that a relative risk of 1.0 (vertical dotted lines) represents no association, and confidence intervals that do not cover 1.0 indicate relative risks that are statistically significant at the p=0.05 level. (reprinted with permission).*

Adjustment of HDL-cholesterol and other risk factors (if provided in the published reports, these other risk factors include age, total cholesterol, LDL-cholesterol, smoking, body mass index, and blood pressure) attenuated these RRs to 1.14 (95% CI 1.05-1.28) and 1.37 (95% CI 1.13-1.66), respectively. The authors conclude from this meta-analysis that the fasting plasma triglyceride level is a risk factor for CHD for men and women in the general population, independent of HDL-cholesterol.

Also in case-control studies it was shown that an elevated concentration of total triglycerides or VLDL-triglycerides in plasma is a good discriminant between subjects with and without CHD (103,104). In a cross-sectional angiographic study, plasma triglyceride (in addition to LDL), HDL-2 and HDL-3 cholesterol proved independently predictive of the extent of coronary atherosclerosis (105). Others presented evidence that small VLDL remnants were associated with the presence or severity of CHD (106-108). VLDL remnants were found to predict progression of coronary atherosclerosis, as well as clinical events occurring up to 7 years later in a prospective study using quantitative coronary angiography at 2-year intervals (109). Accordingly, in MARS triglyceride-rich lipoproteins, particularly small VLDL, have been identified as being the most important lipoprotein to predict progression of coronary atherosclerosis in patients with mild-to-moderate coronary lesions (110). Although there is debate about the atherogenic potential of chylomicrons and normal buoyant VLDL particles, it is generally accepted that VLDL remnants and chylomicron remnants are atherogenic. Several groups have presented evidence that postprandial lipoproteins, and particularly chylomicron remnants, contribute to the risk of CHD (111-115) or to the risk of carotid atherosclerosis (116).

1.5.2. Mechanisms of atherogenicity of triglycerides

There is ongoing debate about the atherogenic potential of *VLDL*. Skeptics believed that VLDL particles were too large to penetrate the arterial wall. These rumours were silenced when Rapp *et al.* (117) demonstrated the presence of VLDL and VLDL remnants in atherosclerotic plaques. Over one third of the total cholesterol content of plaques were found to be derived from VLDL and VLDL remnants. In addition, macrophages are known to bind and internalize TRL-triglycerides. In this process, the macrophages can transform into "foam cells", a feature that is considered to play a central role in atherogenesis. Interestingly, VLDL from HTG patients proved to induce a several fold higher accumulation of lipids in macrophages as compared to

VLDL isolated from normolipidemic individuals (118). In conclusion, high concentrations of TRL may initiate and enhance atherogenesis 1) by penetration of the endothelium into the plaque and 2) by foam cell formation.

Hypertriglyceridemia is not only a derangement of the larger triglyceride-rich lipoproteins. It affects the entire lipoprotein metabolism and beyond. All classes of lipoproteins show abnormal size, lipid contents and apolipoprotein distribution. The primary affected class, namely the VLDL are relatively large, triglyceride-enriched and cholesterol-depleted. The apolipoprotein distribution shifts towards high apoC-III amounts, a condition which may be associated with delayed lipolysis and decreased receptor-mediated lipoprotein catabolism. The LDL particles in hypertriglyceridemia are typically small, dense and persistent. This atherogenic LDL pattern is a consequence of the overwhelming presence of TRL. Triglycerides are transported by CETP from the TRL to the other lipoproteins, e.g. LDL and HDL. These acceptor lipoproteins become enriched in triglycerides and relatively depleted of cholesteryl esters (CE). The CE are shuttled back to the TRL. As the LDL particles circulate for several days, they become progressively lipolyzed which results in small LDL particles. These *small dense LDL* have been suggested to be prone to oxidative modification (119). There is evidence that this LDL type is potentially atherogenic. Austin *et al* (120) demonstrated that small dense LDL particles were associated with a 3-fold increased risk of CHD in univariate regression analysis. Multivariate regression analysis demonstrated that both TG and HDL-C levels contributed to the risk associated with small dense LDL. The same mechanism is seen in the HDL fraction. In HTG states, triglycerides are transported to HDL in exchange for CE. This results in large, buoyant HDL-2 which simply have no more space to accept free cholesterol from peripheral tissues. The HDL cholesterol concentration decreases, which is known to be associated with an increased risk for CHD. Thus, the reverse cholesterol transport becomes saturated. The CE which originally were derived from peripheral tissues and were meant to be removed by the liver, end up in the potentially atherogenic, apoB-containing lipoproteins. In conclusion, the abundance of triglycerides saturates the normal lipoprotein removal pathways and the *reverse cholesterol transport* becomes impaired. These mechanisms may contribute to the accelerated atherosclerosis in hypertriglyceridemic patients.

Lipid values are determined in fasting subjects only. However, the general patient who consumes three meals a day spends most of the day and night in a postprandial phase. The postprandial lipid metabolism is receiving

a lot of attention recently. The postprandial TG rise has been found to be highly dependent on the fasting TG levels. The higher the fasting TG concentration, the longer and higher is the postprandial TG concentration. Therefore, all HTG states are associated with a *disturbed postprandial TG metabolism*. Although most people think that only chylomicrons are found in the first hours after a meal, Cohn *et al.* (121) demonstrated that both intestinally and hepatically derived TRL contribute to the triglyceride rise after ingestion of a meal. An exaggerated postprandial lipemia in HTG patients may contribute to the increased risk of CHD, since there is evidence that a disturbed postprandial TRL metabolism has atherogenic potential, as Zilversmit already postulated in 1979 (122). This has been overlooked for a long time, since most epidemiological studies of CHD examined fasting subjects only. Several studies support the concept that an exaggerated alimentary lipemia predisposes to coronary artery disease (CAD). Karpe *et al.* (32) compared the postprandial lipoprotein metabolism between healthy subjects and patients with CAD, and demonstrated an impaired postprandial lipoprotein metabolism in the patient group. Moreover, experimental and clinical studies have demonstrated that TRL remnants are atherogenic (112,123,124). Illustrative is the remnant removal disease, also called familial dysbetalipoproteinemia, which is due to insufficient ligand activity of apoE-2 to apoE-dependent lipoprotein receptors. This genetic lipid disorder, characterized by massive accumulation of remnant particles, leads to premature atherosclerotic disease. Remnants have been shown to exert their atherogenic effect in two ways. First, accumulation of remnants at the vascular endothelium leads to cholesterol deposition in the vessel wall and transforms macrophages into foam cells. Second, remnants have a direct cytotoxic effect on vessel wall cells, which in accordance with the response-to-injury-hypothesis, promotes atherogenesis. HDL protects against this cytotoxic effect. However, in the HTG patient, an abnormal HDL-subclass distribution and low HDL cholesterol levels are generally encountered. We infer from these observations that a prolonged postprandial exposure of the vessel wall to remnant particles, in combination with low plasma levels of HDL-C, may lead to accelerated atherogenesis.

Most hypertriglyceridemic patients demonstrate *insulin resistance*, a phenomenon that appears to precede the dyslipidemia. Although insulin resistance is associated with a wide variety of biochemical alterations, high fasting insulin concentrations appear to be an independent predictor of ischemic heart disease in men (73). The biochemical variations indicate a

clustering of cardiovascular risk factors. Characteristic features are: Low HDL cholesterol, high TG, small dense LDL, obesity, insulin resistance and hypertension. High triglyceride levels are frequently accompanied by insulin resistance (125), as Reaven (126) designated as "syndrome X", characterized by high triglyceride and low HDL-cholesterol levels, increased insulin resistance, and hypertension. The combination of these metabolic alterations has been given the name "insulin resistance syndrome" emphasizing the role of insulin resistance as the underlying mechanism (127). Some years earlier, Kaplan (128) suggested that this syndrome begins with central obesity, followed by dyslipidemia, insulin resistance, and hypertension, all combining to form the "deadly quartet". Austin *et al.* (120) have shown that patients with nonfatal myocardial infarction had LDL-cholesterol levels similar to those of control subjects, but were three times as likely to have a larger number of small, dense LDL particles, the so-called pattern B. Subjects with pattern B were also more obese and had higher triglyceride levels and lower HDL-cholesterol levels.

Although *fatty acids* (FA) are an important energy source for the human body, high FFA levels are considered to be potentially atherogenic. First, FFAs raise TG levels by stimulating VLDL-TG synthesis and inhibiting lipolysis. Second, FFAs have been demonstrated to cause increased oxidative stress (129,130). Third, FFAs may facilitate the transfer of LDL across the endothelial barrier into the intimal space (131). And finally, elevated FFA levels have been reported to cause endothelial dysfunction within a short period of time (132). These reports emphasize the atherogenic potential of fatty acids.

It is generally accepted that the *hemostatic and fibrinolytic system* play an important role in the occurrence of atherosclerosis and acute cardiovascular syndromes (133). It is also known that dyslipidemias can affect both counterbalancing systems. The most prominent changes in hypertriglyceridemia are increased factor VIIc and PAI-1 activity (134,135). Both have shown a positive correlation with plasma triglyceride levels. Silveira et al (136) demonstrated activation of factor VII after a fat-rich meal, emphasizing the relation between factor VII activation and TRL. PAI-1 is an important inhibitor of the fibrinolytic degradation of thrombus by t-PA. The finding that PAI-1 is increased in HTG indicates that the fibrinolytic capacity is decreased. Also other derangements of the hemostatic system have been reported in HTG patients. Plasma fibrinogen levels, known to be an important predictor of CAD, are increased (137). Tissue factor pathway inhibitor (TFPI) levels, an important inhibitor of the clotting system, are decreased in HTG

(135). These changes in the hemostatic system indicate a pro-thrombotic state in hypertriglyceridemic patients. Future research is needed to identify the metabolic links between hemostasis and lipoprotein metabolism.

1.6. Treatment

There has been, and still is, debate about the treatment of hypertriglyceridemia. The latest recommendations of the European Task Force in 1994 stated that HTG should be treated primarily by life style measures (138). In case of other risk factors, a total cholesterol to HDL-C ratio above 5 or a low HDL-C level, "the threshold for drug therapy will be lowered". However, if plasma TG exceed 5 mmol/L drug therapy is advised to reduce the risk of pancreatitis. Also in the latest (2^{nd}) cholesterol consensus, no specific guidelines are presented and physicians are advised to refer the patient to a lipid clinic if plasma TG exceed 4.0 mmol/L (139). There are no specific guidelines how and when to treat HTG. Nevertheless, there seems to be general agreement about the therapeutical path.

1. In parallel with blood pressure management, it is important to have several fasting blood levels in time, as plasma TG can show marked variations. In case of an established HTG, secondary causes of HTG should be excluded, e.g. diabetes, thyroid disease, liver/renal disease, alcohol abuse and medication. Then, a complete lipoprotein profile as well as apoE-phenotype and apoE-genotype should be determined.
2. As endogenous HTG is diagnosed, dietary and life style measures are first-line strategy. After approximately 2-3 months, the effectiveness is evaluated. Dependent on the outcome of the conservative measures, pharmacological therapy may be instituted.
3. Drug therapy is recommended in case of established cardiovascular disease or pancreatitis. With regard to primary prevention, drug therapy is advisable when other risk factors are present and conservative measures are not sufficient. To date, it seems questionable to treat mild HTG when no other risk factors are present.

1.6.1. Diet

Environmental factors are considered to play an important role in the expression of hypertriglyceridemia. Therefore, dietary and life-style measures

are first-line strategy in these patients diet (140). So far, 2 large studies have been performed to study the efficacy of dietary advise in patients with hypertriglyceridemia. In 1977, Gotto *et al.* (141) reported a mean TG-reduction of 30% (1.4 mmol/L) in over 100 patients with type IV HLP who had received dietary counseling for 2 years. With regard to short-term dietary counseling, Dallongeville *et al.* (142) demonstrated a TG-reduction of 21% in response to a American Heart Association (AHA) step I diet in 113 patients with hypertriglyceridemia in comparison with patients who did not receive counseling. However, only 20% of the patients achieved normal TG levels on dietary therapy alone. In the Netherlands, the following dietary guidelines have been developed: 1) Total fat 30-35 % of energy intake, 2) Saturated fat < 10% of energy intake, 3) Dietary cholesterol < 300 mg/d, and 4) Calorie reduction if body mass index > 26 kg/m^2. These dietary guidelines are similar to the AHA Step I diet (140), with the exception that not less than 30% but 30-35% of total energy intake should be derived from fat. In contrast to hypercholesterolemic patients (143), the efficacy of the dietary regimen has not been studied before in HTG patients in the Netherlands.

1.6.2. Fish oil

Diets enriched in polyunsaturated fatty acids and poor in saturated fatty acids reduce both triglyceride and total cholesterol levels. In the Zutphen Study, Kromhout *et al.* (144) demonstrated that habitual fish consumers (who eat, on average, 33 g of fish per day) had lower triglyceride levels than controls (who eat, on average 2 g of fish per day). The content of the n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), of the phospholipids of circulating lipoproteins was significantly higher among the habitual fish consumers than in controls. Patients with hypertriglyceridemia who receive large doses of n-3 PUFAs from fish oil have substantially reduced plasma triglyceride levels because of decreased triglyceride synthesis in the liver (145). Froyland *et al.* (146) recently demonstrated that n-3 fatty acids stimulate mitochondrial β-oxidation of fatty acids, thereby reducing the availability of substrate for VLDL-TG synthesis. When patients with hypertriglyceridemia were given fish oil (5 g per day) for 6 weeks, total triglyceride, VLDL-triglyceride, VLDL-cholesterol and total cholesterol concentrations in serum decreased by 54%, 56%, 40% and 15%, respectively. Serum LDL-cholesterol and HDL-cholesterol concentrations increased by 23% ($p < 0.05$) and by 14% (n.s.), respectively, whereas the LDL-cholesterol to HDL-cholesterol ratio did not change (147). An adverse effect of fish oil was the increased susceptibility to

oxidation of LDL and VLDL, which was correlated to the increased number of double bonds in PUFAs of LDL and VLDL. Although there is debate about the clinical relevance of copper-induced lipoprotein oxidations (148), fish oil should not be used in patients with HTG until its effectiveness has been established in clinical trials.

1.6.3. Nicotinic acid

Nicotinic acid treatment reduces plasma triglyceride and total cholesterol levels by decreasing the hepatic synthesis of VLDL-triglycerides. This effect of the drug on the liver is considered to be due to a reduced flow of circulating FFAs to the liver for triglyceride synthesis, which is caused by an inhibited mobilization of adipose tissue triglycerides. The nicotinic acid-induced decrease in plasma triglyceride levels is associated with a profound increase in HDL-cholesterol levels. This potent HDL-raising effect of nicotinic acid derivates makes it suitable for treatment of patients with severe hypoalphalipoproteinemia. Nicotinin acid has two important disadvantages: 1. Frequent side-effects (flushes and gastro-intestinal complaints), and 2. Impairment of glucose tolerance. Therefore, nicotinic acid therapy should not be prescribed to HTG, but restricted to patients with severe hypoalphalipoproteinemia (improvement of HDL-cholesterol) or patients with elevated lipoprotein(a) levels. (149). Acipimox is a derivative of nicotinic acid that does not have the disadvantages mentioned above and therefore may be of interest.

1.6.4. HMG-CoA reductase inhibitors

HMG-CoA reductase inhibitors have been proposed as the therapy of choice for patients with hypercholesterolemia or combined hyperlipidemia (150). However, in patients with hypertriglyceridemia, statin therapy is not regarded as therapy of choice since LDL-cholesterol levels, the primary target of statins, are normal or subnormal. High doses of statins, however, have been reported to reduce TG levels (151). This effect is considered to be mediated by 1. upregulation of LDL receptors, and 2. reducing the hepatic cholesterol pool that is necessary for VLDL assembly. A new and potent statin, atorvastatin, has been reported to suppress VLDL levels effectively and may therefore be effective in patients with endogenous HTG. Bakker Arkema *et al.* (152) reported a study with atorvastatin in patients with endogenous HTG. Atorvastatin was well tolerated and improved the lipid and lipoprotein levels in these patients significantly. At highest dosage (80 mg once daily), the following changes were observed: total triglycerides -46%, LDL-cholesterol -41%, VLDL-cholesterol -58% and HDL-

cholesterol +12%. In contrast to some other triglyceride-lowering drugs, fibrinogen and PAI-1 levels were not affected in this study. Although atorvastatin seems to be a good candidate drug for the treatment of HTG, a recent study reported that atorvastatin may increase fibrinogen levels (153). Currently, studies are being performed to solve this issue. Statin therapy in hypertriglyceridemia should be restricted to HTG patients with high LDL cholesterol levels (at baseline, or more likely after fibrate therapy) or if contra-indications for fibrates are present (e.g. gall bladder disease).

1.6.5. Fibrates

Efficacy. Fibrates are considered as drugs of choice in patients with endogenous hypertriglyceridemia. Although no prevention trials have been performed with fibrates in this particular patient group, there are several studies that have addressed the efficacy of fibrates in dyslipidemic patients. In the Helsinki Heart Study, 4081 men aged 40-55 yrs with non-HDL cholesterol > 5.2 mmol/L were included in this randomized, double-blind placebo-controlled trial with gemfibrozil 600 mg twice daily for 5 yrs (154). Although the gemfibrozil-treated group showed 34% less cardiovascular events, there were no differences in total mortality due to a higher incidence of accidents, violent deaths and intracranial hemorrhages in the gemfibrozil group. The highest efficacy was achieved in a subset of 10% of the study population who had a TG level > 2.3 mmol/L and a baseline LDL-HDL ratio > 5. In the BECAIT-study, 92 post-infarction patients under 45 yrs with high cholesterol, TG or both participated in this randomized, placebo-controlled secondary prevention trial with bezafibrate 200 mg 3 times daily for 5 yrs (155). Both the number of coronary events and angiographically determined minimum lumen diameter were significantly better in the bezafibrate group. Recently, the results of the Bezafibrate Infarction Prevention (BIP) study were presented at the XXth congress of the European Society of Cardiology in Vienna (hot line session). The BIP study is a secondary prevention trail with bezafibrate in patients with low HDL-C (lipid criteria: TC 4.7-6.5 mmol/L, TTG < 3.4 mmol/L and HDL-C < 1.16 mmol/L), conducted in 19 centers in Israel (43). There were 3122 male and female patients included in the BIP study. Interestingly, 20% of the total population that was screened showed elevated plasma triglyceride levels of which a fourth part had TTG levels exceeding 3.4 mmol/L. Unfortunately and incomprehensibly, the latter most interesting group of patients was not included. No significant effect on primary endpoints (fatal and non-fatal MI and sudden death) or mortality could be noted in the whole

group after a mean follow-up period of 6.26 years. However, in the subgroup with (mild) hypertriglyceridemia, a 40% reduction in the combined primary endpoints was seen. It is important to note that no increased frequency of adverse events such as malignancies could be observed between the placebo and bezafibrate group.

These studies indicate that fibrates improve cardiovascular outcome in dyslipidemic patients. However, the studies above included patients with varying types of HLP. In the Helsinki Heart Study, only 9% were patients with type IV HLP that we regard as the most suitable patient group for fibrate therapy. Therefore, fibrate therapy may even yield better results if subscribed to patients with high plasma TG, the lipid that is primarily affected by fibrates.

Pharmacological action. Fibrates effectively lower plasma triglyceride and cholesterol levels. The lipid-lowering effects depend upon the potency and dose of the fibrate used, as well as the patients pre-treatment lipid levels. The phenotype of the patient highly determines the observed lipid-lowering effects. In patients with hypercholesterolemia, both total cholesterol and triglyceride levels show modest reductions. On the other hand, in patients with a hypertriglyceridemia the most pronounced effect is the TG-lowering effect. Although VLDL-C levels are reduced, LDL- and HDL-cholesterol levels on the other hand increase (for review, see (156)). The increase in LDL and HDL cholesterol levels is a logical consequence of the reduction in TG, as TG are negatively associated with LDL-C and HDL-C levels. This association can be explained by the fact that TG are exchanged for CE from the HDL- and LDL-fraction, which results in a flux of cholesterol out of the HDL and LDL particles. The increase in HDL cholesterol levels can be attributed to the mechanism described above, and partly by increased production of apoA-I and A-II. The principal effects can be observed in the VLDL fraction. The VLDL mass is strongly reduced by 50-70%. As VLDL contain both TG and cholesterol, plasma TG and cholesterol levels decrease upon fibrate therapy. In addition, beneficial changes have also been reported on insulin resistance, fibrinogen and other components of the hemostatic system (157,158). A wide variety of fibrates is available, however, the most popular ones (with regard to safety, efficacy and tolerability) are bezafibrate (bezalip retard®, 1 dd 400 mg), ciprofibrate (modalim®, 1 dd 100 mg) and gemfibrozil (lopid®, 2 dd 600 mg). The differences between the fibrates are small (159).

Mechanisms of action. Several mechanisms have been identified that explain the hypolipidemic action of fibrate therapy: 1. Improval of lipolysis of lipoprotein triglycerides by upregulation of lipoprotein lipase and downregulation of apoC-III (160,161). Although it is known that apoC-III can inhibit lipolysis and receptor-mediated lipoprotein removal, the effects of fibrate therapy on these parameters have never been studied. The effects of fibrates are mediated by stimulation of the nuclear hormone receptor family PPAR (peroxisome proliferator-activated receptor) α (for review, see (162)). Activation of PPARs by fibrates, a synthetic ligand for PPAR, result in heterodimerisation with the retinoid X receptor (RXR) and consequent binding to responsive elements (peroxisome proliferator response elements) of DNA in the target cell nucleus. This eventually leads to activation or repression of target genes that are involved in lipoprotein metabolism, such as LPL, apoC-III, apoA-I and apoA-II. Fibrates increase the production of apoA-I and apoA-II in the human liver (not in rodents), that in combination with the reduction in exchangeable lipids, may contribute to the increase in HDL levels and improval of reverse cholesterol transport. 2. Fibrates increase β-oxidation of fatty acids in the mitochondrion, which results in a reduced amount of fatty acids available for triglyceride synthesis (146). Fibrates increase the hepatic fatty acid uptake and stimulate the conversion of fatty acids into acyl-coA, by inducing fatty acid transport protein (FATP) and acyl-coA synthase (ACS) (163,164). The resulting acyl-coA derivates in hepatocytes are then more efficiently oxidized by induction of fatty acid β-oxidation.

Recently, an interesting novel effect of fibrates was described. Staels *et al.* (165) demonstrated that fibrates can reduce the inflammation response via PPARα activation. They showed that fenofibrate therapy reduced the plasma levels of interleukin-6, fibrinogen and C-reactive protein in hyperlipidemic patients with and without coronary artery disease. In addition, Staels recently stated (personal communication) that fibrate therapy can induce apoptosis in human macrophages. Although the exact mechanism remains to be determined, part of the effect on macrophages appears to be mediated by inhibition of the apoptosis-inhibitor nuclear factor κB. This is very interesting as it suggests that fibrates may stabilize the atherosclerotic plaque by lowering the inflammation response in the fibrous cap via a lipid-independent mechanism.

1.7. Outline of this thesis

Since hypertriglyceridemia is associated with the development of atherosclerosis, it is important to expand our knowledge about the pathogenesis and treatment of this lipid disorder. Previous studies have demonstrated that triglyceride-rich lipoproteins in hypertriglyceridemic (HTG) patients are lipolyzed less efficiently *in vivo* as compared to controls. However, *in vitro* lipolysis experiments do not uniformly confirm the *in vivo* observations. We hypothesized that the currently available lipolysis assays with lipoprotein lipase (LPL) in solution do not provide the proper tool to study lipolysis since lipolysis *in vivo* is mediated by LPL that is bound to the endothelial surface of the vessel wall. In **chapter 2**, we describe the development of a novel lipolysis assay with heparan sulphate proteoglycan-bound LPL. In **chapter 3**, we apply the assay in a clinical setting, in order to study the effects of mutant apoE on lipolysis and binding of VLDL by proteoglycan-bound LPL. Exogenous factors are considered to play an important role in the expression of hypertriglyceridemia. Therefore, life style measures and dietary intervention are first line therapy. In **chapter 4**, we study the efficacy of short-term dietary counseling in hypertriglyceridemic patients. When life style measures can not achieve acceptable lipid levels in patients with hypertriglyceridemia, pharmacological therapy may be instituted. Fibrates are considered as treatment of choice. Although these drugs have been shown to be effective TG-lowering compounds, the mechanism of action is not fully understood. In **chapter 5**, we investigate the effects of bezafibrate therapy on factors involved in VLDL catabolism: LPL activity, VLDL lipolysis by proteoglycan-bound LPL and VLDL binding to the LDL receptor. Triglyceride-lowering therapy has been hypothesized to improve LDL particle size and density, and consequently may improve LDL susceptibility to oxidation. To elucidate this question, we study oxidation parameters in hypertriglyceridemia and the effects of fibrate therapy in **chapter 6**. Disturbances in TG metabolism have been demonstrated to affect the hemostatic system and thrombocytes. Previous reports have suggested that hypertriglyceridemia is associated with increased *ex vivo* platelet reactivity to various agonists. In **chapter 7**, we assess the *in vivo* platelet activation status directly by determination of activation-dependent platelet surface antigens by whole blood flow cytometry. The therapeutical arsenal for hypertriglyceridemia has recently been expanded with atorvastatin, a new and powerful HMG-CoA reductase inhibitor. In **chapter 8**, we study the

effects of atorvastatin therapy on lipid levels and endothelial function in patients with hypertriglyceridemia.

1.8. References

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Chapter 2

Lipolysis of Very Low Density Lipoproteins by Heparan Sulphate Proteoglycan-bound Lipoprotein Lipase

Frits H.A.F. de Man[1], Femke de Beer[2,3], Arnoud van der Laarse[1], Augustinus H.M. Smelt[2], and Louis M. Havekes[1,2,3]

Departments of [1]Cardiology and [2]Internal Medicine, Leiden University Medical Center, [3]TNO-Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands

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Summary

An *in vitro* assay to study lipolysis of very low density lipoproteins (VLDL) by heparan sulphate proteoglycan (HSPG)-bound lipoprotein lipase (LPL) was developed. Optimal conditions for VLDL lipolysis by HSPG-bound LPL were obtained by incubating plastic wells with 0.5 μg HSPG and 1.5 μg LPL, subsequently. Control experiments with heparinase indicate that at least 90% of the LPL activity is derived from LPL bound to heparan sulphate chains. For HSPG-LPL mediated lipolysis, the apparent Km and Vmax values were 0.36 ± 0.11 mmol/L VLDL-triglycerides and 46 ± 4 μmol free fatty acids/L.min, respectively. The mean intra-assay and inter-assay coefficients of variation were 5% and 8%, respectively.

Introduction

Lipoprotein lipase (LPL, EC 3.1.1.34) is the key enzyme involved in the hydrolysis of chylomicron and very low density lipoprotein (VLDL) triglycerides (1,2). After synthesis in parenchymal cells, LPL is secreted and transported across the endothelium where it binds to heparan sulphate proteoglycans (HSPG) at the luminal surface of endothelial cells (3,4). Although functional LPL acts *in vivo* as a proteoglycan-bound enzyme, its kinetics *in vitro* are commonly studied with LPL in solution. Only few studies with heparin-Sepharose immobilized LPL have been reported previously (5,6). In these studies, a higher Michaelis-Menten constant (Km) and lower maximum reaction velocity (Vmax) were noted for heparin-Sepharose immobilized LPL as compared to LPL in solution. This observation was in accordance with *in vivo* studies, showing that heparin-induced release of LPL from the vessel wall resulted in rapid clearance of plasma triglycerides (7,8), a phenomenon explained by increased accessibility of the enzyme for its substrate.

In addition to the observed difference in lipolysis rate, it is conceivable that the proximity of heparin or HSPG influences directly the substrate-enzyme interaction. As triglyceride-rich lipoproteins come into contact with the endothelium-bound LPL, the particle has to reside transiently at the lipolytic site. This interaction between the lipid particle and vessel wall components is considered to enhance the stability of the lipoprotein-LPL complex (9), a process probably mediated by apolipoproteins (apo). Specific binding sites for LPL have been reported on apoC-II and B-100 (10,11), whereas apoE is known to bind to heparan sulphate chains (9,12). Ji *et al.* (12) demonstrated that enrichment of ß-VLDL with apoE enhanced the binding of the ß-VLDL to liver cells 4-5 fold, an effect which was suggested to be mediated by the interaction of apoE with HSPG. In addition, they also found that mutant apoE showed reduced affinity to isolated HSPG, a finding which was confirmed by others (13). It was speculated that a defective interaction between apoE and HSPG may be associated with an impaired lipolysis of triglyceride-rich lipoproteins by HSPG-bound LPL (14,15). However, this hypothesis could not be tested so far as a lipolysis assay with HSPG-bound LPL was not available. The present paper presents a novel, reproducible and rapid *in vitro* assay for lipolysis of VLDL using HSPG-bound LPL.

Materials and Methods

Lipids and lipoproteins

Venous blood from healthy, normolipidemic apoE3 homozygotes was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. VLDL was isolated by ultracentrifugation as described by Redgrave *et al.* (16). Protein content of the VLDL samples was determined by the method of Lowry *et al.* (17). Triglyceride concentrations of the VLDL fraction was measured enzymatically using a test kit (Sigma Chemicals, St. Louis, MO). ApoE phenotyping was performed by isoelectric focusing according to Havekes *et al.* (18).

Lipoprotein lipase

LPL was purified from fresh bovine milk as described previously (19). The isolated fraction was resuspended in 20 mmol/L NaH_2PO_4, 50% glycerol and stored in aliquots at -80°C. Isolated LPL was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 4-20%) (figure 1) (20). Proteins were stained with Coomassie Brilliant blue or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were incubated with monoclonal antibody 5D2 (prepared by Dr. J.D. Brunzell *et al.* (21), University of Washington, Seattle), and rabbit anti-mouse IgG conjugated to peroxidase (DAKO, Glostrup, Denmark) was used as second antibody. A clear band with the approximate molecular weight of bovine LPL (56 kDa) was detected, which accounted for 51% of the total amount of protein in the sample (figure 1, panel A). This band reacted with the 5D2 antibody to bovine LPL (figure 1, panel B). The specific activity of the isolated LPL was 10.9 μmol FFA.min^{-1}.mg $protein^{-1}$.

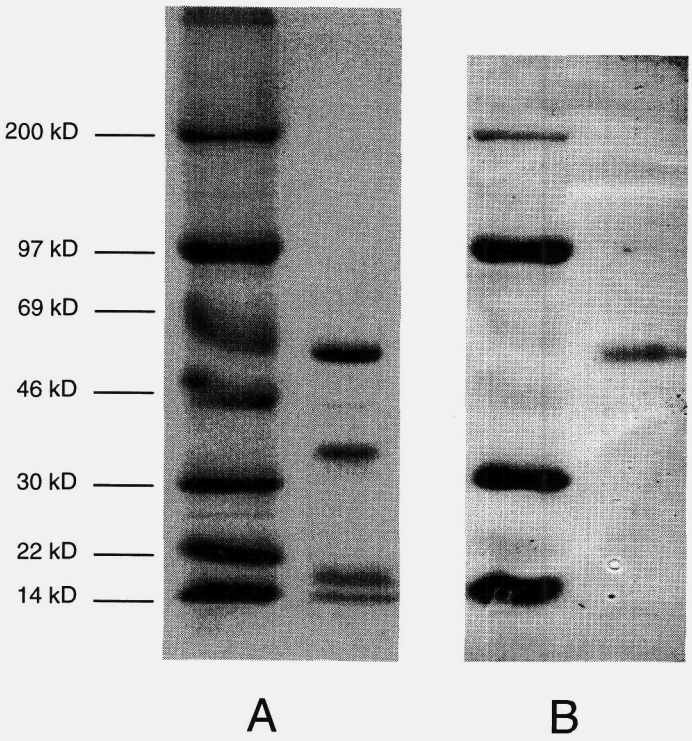

Figure 1. *Isolated bovine LPL was analyzed by SDS-PAGE gel electrophoresis (4-20%). Proteins were stained with Coomassie Brilliant blue (panel A) or transferred to nitrocellulose membranes. Blots were incubated with monoclonal antibody 5D2, and rabbit anti-mouse IgG conjugated to peroxidase was used as second antibody (panel B). Molecular weight standards are indicated in the left lanes of panel A and B.*

Assay of lipolysis with heparan sulphate proteoglycan-bound lipoprotein lipase

HSPG, isolated from basement membrane of mouse sarcoma cells, were purchased from Sigma. The assay was performed in 96-well microtiter plates (Greiner GmbH, Frichenhausen, Germany). Wells were incubated with different amounts of HSPG (as indicated) for 18 h at 4°C, washed three times with phosphate buffered saline (PBS) and subsequently blocked for 1 h at 37°C with PBS containing 1% (w/v) essentially free fatty acid (FFA)-free bovine serum albumin (BSA) (Sigma). Then, the wells were incubated with different amounts of LPL per well (as indicated), diluted in Tris-glycerol buffer (0.1 M Tris, 20% (v/v) glycerol, pH 8.5) for 1 h at 4°C. After washing the wells three times with Tris buffer (0.1 M Tris, pH 8.5), lipolysis was started by adding various amounts of VLDL (as indicated) to the preconditioned well in the presence of 1% (w/v)

essentially FFA-free BSA and placing the plate in a shaking incubator at 37°C. The rate of FFA release proved to be linear in time up to 10 minutes. The reaction was stopped after 10 min by the addition of Triton X-100 (1% (v/v), final concentration), followed by vortexing and cooling on ice. FFA concentrations were measured in triplicate using a NEFA C kit (Wako Chemicals GmbH, Germany).

As control, HSPG-LPL coated wells were incubated with a mixture of 2.4 U heparinase I/ml (EC 4.2.2.7.; Sigma) and 2.4 U heparinase III/ml (EC 4.2.2.8.; Sigma) in PBS for 20 min at 37°C. The wells were washed with Tris buffer and lipolysis was performed as described above.

To check whether LPL would detach from the HSPG-complexes in the presence of VLDL, the following control experiment was performed. Wells were preconditioned as described above, with the exception that ^{125}I-labeled LPL was used. Then, the wells were incubated with 0.6 mmol/L VLDL-TG or Tris buffer for 10 min at 37°C. After washing the wells three times with Tris buffer, the ^{125}I-labeled LPL bound to HSPG was dissolved in 0.2 N NaOH for quantitation.

Reproducibility was assessed by comparing the lipolysis of a VLDL sample freshly isolated from serum of a normolipidemic subject, and VLDL from the same subject but stored for 1 and 4 weeks, respectively. For storage, serum samples were brought to a final concentration of 10% (w/v) sucrose, 10 mmol/L EDTA, capped under nitrogen, snap-frozen in liquid nitrogen and stored at -80°C. Under these conditions, lipoprotein size and biological properties have been shown to remain intact for months (22).

Assay of lipolysis with lipoprotein lipase in solution

The VLDL samples were diluted in 0.1 M Tris, 1% (w/v) essentially FFA-free BSA, pH 8.5. The incubation was started by adding 7 ng LPL per well, followed by vortexing and incubation at 37°C. The reaction was stopped by the addition of 1% (v/v) Triton X-100, 0.1 M Tris, vortexing and cooling on ice. A blank sample was obtained by adding Triton prior to the addition of LPL and maintenance on ice. FFA concentrations were determined in triplicate. The rate of FFA release by LPL was linear for at least 6 min, as used in this assay.

Labeling of very low density lipoproteins and lipoprotein lipase

VLDL was iodinated using the ^{125}I-iodine monochloride method of Bilheimer *et al.* (23). ^{125}I-iodide (specific activity 15.5 mCi/μg) was purchased from Amersham (Buckinghamshire, UK). After iodination, VLDL was dialyzed extensively at 4°C against PBS for 24 h and stabilized with 1% (w/v) BSA (fraction V, Sigma).

Between the different ^{125}I-labeled VLDL samples the specific radioactivity ranged from 150-200 cpm/ng protein. The stabilized ^{125}I-labeled VLDL was stored at 4°C and used within two weeks.

LPL was iodinated using the IODO-BEADS® Iodination Reagent (Pierce, Rockford, IL). Free ^{125}I was removed by Sephadex G-25 gel filtration with 50 mmol/L Tris, 1 M NaCl, 0.01% Tween-80 as the eluent. The specific activity of ^{125}I-labeled LPL was 300 cpm/ng protein. ^{125}I-labeled LPL was stabilized with 0.1% (w/v) essentially FFA-free BSA and stored at -20°C.

Binding assays

Binding of VLDL

Plastic wells (96-well microtiter plates) were coated with 0.5 μg HSPG per well and subsequently incubated with 1.5 μg LPL per well, exactly as described above. After washing the plates two times with ice-cold PBS, the binding of VLDL to HSPG-bound LPL was determined by incubating the plates for 2 h at 4°C with the indicated amounts of ^{125}I-labeled VLDL, either in the presence or absence of a 20-fold excess of unlabeled VLDL. Thereafter, the plates were washed two times with ice-cold PBS containing 0.1% (w/v) BSA, and subsequently washed with PBS without BSA. The ^{125}I-labeled VLDL bound to the HSPG-LPL complex was dissolved in 0.2 N NaOH for quantitation of the binding. High affinity binding was calculated by subtracting the amount of labeled VLDL that was bound to the HSPG-LPL complexes after incubation in the presence of a 20-fold excess of unlabeled VLDL (aspecific binding) from the amount of labeled VLDL that was bound in the absence of unlabeled VLDL (total binding).

Binding of LPL

To measure the amount of LPL binding in the HSPG-LPL coated wells, LPL was iodinated as described above. Wells were incubated with 0.5 μg HSPG for 18 h at 4°C, washed three times with PBS and subsequently blocked for 1 h at 37°C with PBS containing 1% (w/v) essentially FFA-free BSA. Then, the wells were incubated with 1.5 μg ^{125}I-labeled LPL diluted in Tris-glycerol buffer for 1 h at 4°C. Thus, the pretreatment of the wells was performed exactly as described for the lipolysis assay with HSPG-bound LPL, with the exception that ^{125}I-labeled LPL was used. After washing the wells three times with ice-cold PBS, the ^{125}I-labeled LPL was dissolved in 0.2 N NaOH for quantitation. Of the initially added 1500 ng LPL, only 40 ng LPL proved to bind to the HSPG-coated

wells (2.7%). Similar results were obtained when heparin was used to release LPL from the wells instead of 0.2 N NaOH.

Results and Discussion

The present study was performed to develop an *in vitro* lipolysis assay using HSPG-bound LPL. The limited number of reports in literature, describing a lipolysis assay with immobilized LPL, were performed with heparin-Sepharose columns as adhesive surface for LPL (5,6). Since this design has been shown to be technically difficult and poorly reproducible, we used plastic microtiter plates as adhesive surface for coating with HSPG and LPL, subsequently.

The first objective was to determine the optimal incubation conditions for coating. Therefore, wells were incubated with increasing concentrations of HSPG (ranging from 0 - 3 µg/well) and LPL (ranging from 0 - 5 µg/well).

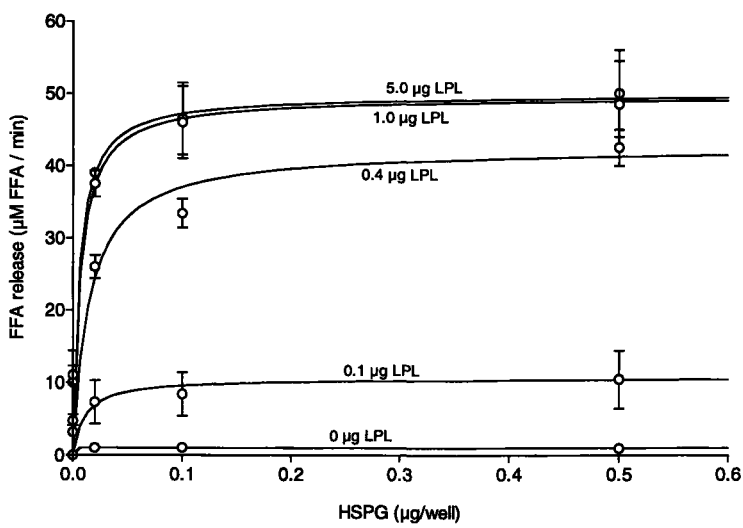

Figure 2. *Determination of optimal incubation conditions to study lipolysis of VLDL by HSPG-bound LPL. Plates were incubated with increasing concentrations HSPG and washed three times with PBS to remove unbound HSPG. Subsequently, the wells were incubated with 1% BSA in PBS to block aspecific binding sites. Then, the plates were incubated with different amounts of LPL, as indicated. Plates were washed three times to remove unbound LPL and the lipolysis assay was started by adding control VLDL (TG 1.0 mmol/L) to the preconditioned wells. After 10 min, the reaction was stopped by the addition of 1% Triton X-100. Free fatty acid release represent the mean ± S.D. for wells measured in triplicate.*

As shown in figure 2, the FFA release increases with increasing amounts of HSPG, but at HSPG-concentrations greater than 0.1 µg/well, lipolysis reaches a plateau indicating saturation. From figure 2 it is also obvious that lipolysis increases with increasing LPL concentrations in the second incubation step, showing saturation at a LPL concentration above 1.0 µg/well. All subsequent lipolysis experiments were carried out in the saturated parts of the curves, using a HSPG concentration of 0.5 µg/well in the first incubation step and a LPL concentration of 1.5 µg/well in the second step.

To determine the VLDL binding characteristics under these conditions, preconditioned wells were incubated with increasing concentrations of ^{125}I-labeled VLDL.

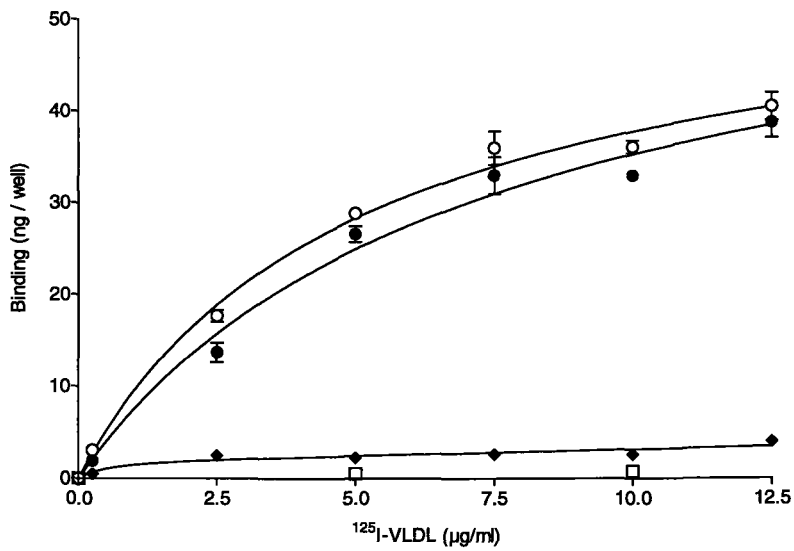

Figure 3. *Binding curve of ^{125}I-labeled VLDL to HSPG-bound LPL (●) and HSPG alone (□). After coating with 0.5 µg/well HSPG, aspecific binding sites were blocked with PBS,1% BSA and treated with 1.5 µg LPL or 0 µg LPL per well, respectively. Plates were then incubated for 2 h at 4°C with different amounts of ^{125}I-labeled VLDL as indicated, either in the presence or absence of a 20-fold excess of unlabeled VLDL. High affinity binding to HSPG-LPL (●) was calculated by subtracting the amount of labeled VLDL that was aspecifically bound to the HSPG-LPL complexes (◆) from the amount of labeled VLDL that was bound in the absence of unlabeled VLDL (total binding) (○). VLDL binding to HSPG-bound LPL is expressed as ng of lipoprotein per well. Each value represents the mean ± S.D. of triplicate measurements.*

Figure 3 clearly demonstrates that VLDL binds to the HSPG-LPL complexes of the preconditioned wells, whereas in the absence of LPL, VLDL binds very

poorly to the HSPG-coated wells. This indicates that the current lipolysis assay represents lipolysis of VLDL after binding to immobilized LPL.

In order to assess kinetic parameters of the novel lipolysis assay and compare these with the conventional assay, lipolysis experiments with HSPG-bound LPL and LPL in solution were carried out with increasing concentrations of VLDL-TG. Figure 4 shows the respective lipolysis curves.

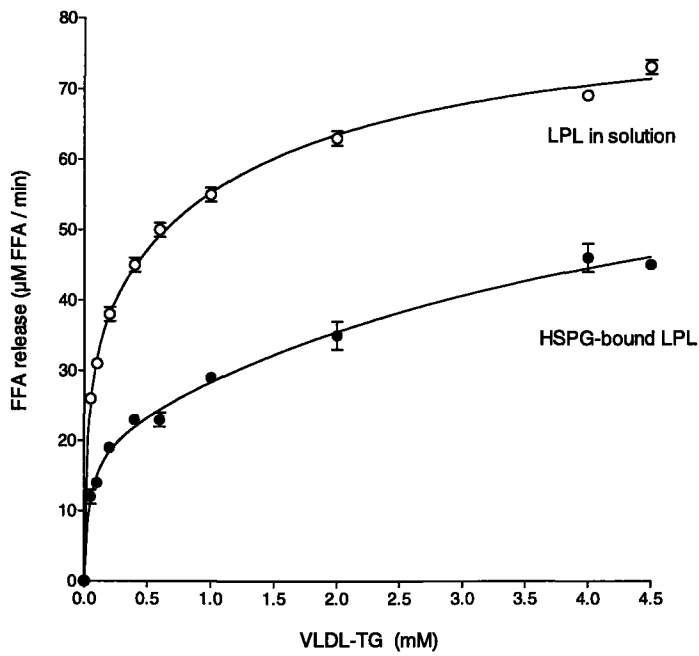

Figure 4. *Lipolysis experiments with HSPG-bound LPL (●) and LPL in solution (O) were carried out with increasing concentrations of VLDL-TG. With regard to the lipolysis assay with HSPG-bound LPL, wells were preconditioned with HSPG and LPL (materials and methods), washed and subsequently incubated with various amounts of VLDL-TG in the presence of 1% BSA for 10 min at 37°C. The lipolysis assay with LPL in solution was performed by incubating VLDL-TG samples with LPL in solution for 6 min at 37°C. The reactions were stopped by addition of Triton X-100, vortexing and cooling on ice. FFA concentrations were determined in triplicate.*

The apparent Km values, as calculated by Lineweaver-Burk analysis, of the lipolysis assay with LPL in solution and HSPG-bound LPL are 0.20 ± 0.03 mmol/L and 0.36 ± 0.11 mmol/L VLDL-TG, respectively. The apparent Vmax values of the lipolysis assay with LPL in solution and HSPG-bound LPL are 80 ± 3 µmol FFA/L.min and 46 ± 4 µmol FFA/L.min, respectively. Thus, the conventional assay with LPL in solution yields a lower Km and higher Vmax

value as compared to the novel assay with HSPG-bound LPL, which is in agreement with previous studies (5). Since lipolysis kinetic studies are performed preferably with substrate concentrations in the Km range, subsequent lipolysis experiments were performed in the VLDL-TG range of 0.2 - 0.6 mmol/L.

To determine whether under these assay conditions HSPG-LPL mediated lipolysis of VLDL occurs by LPL molecules that bound specifically to HSPG, preconditioned wells were incubated with heparinase in order to hydrolyze heparan sulphates (3,12). As shown in figure 5, treatment of HSPG-LPL coated wells with heparinase reduced lipolysis to approximately 10% of the normal lipolysis rate. These results indicate that at least 90% of the LPL activity is indeed derived from LPL bound to heparan sulphate chains.

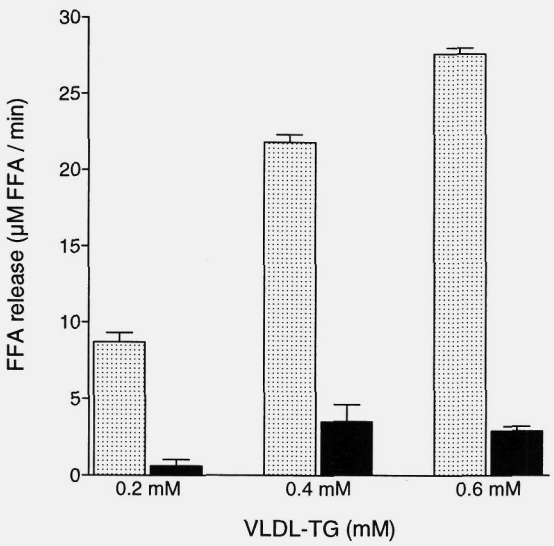

Figure 5. *Effect of heparinase treatment on HSPG-LPL mediated lipolysis. HSPG-LPL coated plates were incubated with PBS containing heparinase I/III (■), or PBS without heparinase (▒) at 37°C for 20 min. Then, the wells were washed three times. Lipolysis was carried out by incubating VLDL at three different VLDL-TG concentrations for 10 min at 37°C.*

It is known that LPL can detach from endothelium during lipolysis (24,25). To check whether LPL would detach from the HSPG-complexes in the presence of VLDL, the following control experiment was performed. Wells were preconditioned with HSPG and ^{125}I-labeled LPL, and subsequently incubated in the presence or absence of VLDL (see materials and methods). After washing the wells, the remaining amount of ^{125}I-labeled LPL was released from the HSPG-complexes for quantitation. No differences were noted between the wells incubated with VLDL-TG and the wells incubated with the lipoprotein-free buffer alone (data not shown). Thus, incubation of HSPG-LPL coated wells

with VLDL does not result in detachment of LPL from the HSPG-complexes under the conditions applied.

To test the reproducibility of the lipolysis assay, repetitive lipolysis experiments were performed with VLDL, isolated from a healthy normolipidemic subject. The first lipolysis experiment was carried out with freshly isolated VLDL; the second and third experiment with VLDL isolated from the same serum but stored for 1 and 4 weeks at -80°C (see materials and methods). Nearly identical rates of FFA release were observed in the three consecutive experiments (table 1).

Table 1. *Reproducibility and biological variation of VLDL-TG lipolysis using HSPG-bound LPL*

	Storage at -80 °C				Control subjects (n=4)				
VLDL-TG	0 wk	1 wk	4 wks	CV_{ia}[a]	A	B	C	D	CV_{ii}[b]
mmol/L	Rate of lipolysis (mmol/L FFA)				Rate of lipolysis (mmol/L FFA)				
0.20	0.19	0.16	0.16	11%	0.22	0.15	0.17	0.22	18%
0.40	0.33	0.27	0.29	10%	0.35	0.24	0.24	0.34	21%
0.60	0.42	0.40	0.41	3%	0.44	0.35	0.31	0.42	15%

Lipolysis rates are expressed as mmol FFA/l after 10 min incubation in HSPG-LPL coated wells. wk, week; [a]CV_{ia}, inter-assay coefficient of variation; [b]CV_{ii}, inter-individual coefficient of variation.

The inter-assay coefficients of variation ranged from 3 to 11%, depending on the substrate concentration used. Thus, the lipolysis assay with HSPG-bound LPL appears to be reproducible and storage at -80°C under well-defined conditions does not affect lipolysis rates. Inter-individual variation in lipolysis was assessed by performing lipolysis experiments with separate VLDL samples, isolated from four normolipidemic apoE3 homozygous subjects. The mean inter-individual coefficient of variation was calculated to be 18% (table 1). The intra-assay coefficient of variation was 5%. Although the variation between different lipolysis experiments (inter-assay coefficient of variation) is acceptable, it is recommendable to use an internal standard in each series of lipolysis experiments, e.g. a plasma pool stored at -80°C after cryopreservation from which VLDL can be isolated for each new series of lipolysis experiments.

The potential benefits of this novel lipolysis assay over the conventional assay with LPL in solution remain to be established. However, preliminary results indicated that VLDL isolated from patients with different apoE mutations show different lipolysis efficiencies by HSPG-bound LPL which are paralleled by differences in binding of VLDL to the HSPG-LPL complex (26). In

contrast, the conventional lipolysis assay with LPL in solution did not detect differences in lipolysis. Thus, the current assay with HSPG-bound LPL may provide the proper experimental tool to detect differences in lipolysis efficiency if an altered interaction between VLDL and HSPG-LPL complex is expected. This may be the case in endogenous hypertriglyceridemia. *In vitro* lipolysis experiments with hypertriglyceridemic VLDL using LPL in solution did not demonstrate an impaired lipolysis as compared to VLDL isolated from healthy subjects (27). However, since apoE and apoC may modulate the binding of triglyceride-rich lipoproteins to heparan sulphate (15), it is speculated that in patients with endogenous hypertriglyceridemia, whose VLDL contain an increased apoC content per particle, a reduced binding to HSPG-bound LPL and therefore a reduced lipolysis efficiency may be expected. The current assay with HSPG-bound LPL may provide the proper experimental tool to address this issue in the future.

The current lipolysis assay with HSPG-bound LPL presents a simple method to study lipolysis of VLDL. An important improvement is the preserved interaction between VLDL, LPL and HSPG. Although this system shows a better resemblance to the *in vivo* situation than the conventional assay with free LPL, there are still some important differences. FFAs and other lipolysis products can not be disposed into the underlying tissue but remain in the proximity of the enzyme-substrate complex. In addition, LPL is bound to HSPG alone whereas in the normal situation, LPL is bound to different proteoglycans and non-proteoglycan LPL-binding proteins (28,29). A lipolysis assay using LPL bound to endothelial cells, as has been described by Saxena *et al.* (30), therefore has theoretical advantages over the present cell-free system with HSPG-bound LPL. However, several practical problems have to be solved before this concept of endothelial-bound LPL can be used for measuring the kinetics of VLDL-TG lipolysis. In particular the exact number of cells in the system over a longer period of time (months to years) and the time- and cell phase-dependent expression of extracellular matrix proteins may affect the reproducibility of the assay (31).

We conclude that the current lipolysis assay presents a simple and reproducible method to study lipolysis of VLDL, whereby the interaction between VLDL, LPL and HSPG is preserved.

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Chapter 3

Effect of Apolipoprotein E Variants on Lipolysis of Very Low Density Lipoproteins by Heparan Sulphate Proteoglycan-bound Lipoprotein Lipase

Frits H.A.F. de Man[1], Femke de Beer[2,3], Arnoud van der Laarse[1], Augustinus H.M. Smelt[2], Jan A. Gevers Leuven[2,3], Louis M. Havekes[1,2,3]

Departments of [1]Cardiology and [2]Internal Medicine, Leiden University Medical Center, [3]TNO-Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands

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Summary

Background. Lipoprotein lipase (LPL) is bound to heparan sulphate proteoglycans (HSPG) at the luminal surface of endothelium. It is the key enzyme involved in the hydrolysis of very low density lipoproteins (VLDL). Prior to lipolysis by LPL, the lipoproteins are considered to interact with vessel wall HSPG. Apolipoprotein (apo) E is thought to mediate this interaction thereby enhancing the stability of the lipoprotein-LPL complex. We hypothesize that apoE mutations may cause a diminished interaction of VLDL with HSPG leading to impaired lipolysis of VLDL by HSPG-bound LPL.

Methods. VLDL was isolated from 8 normolipidemic apoE3 homozygous controls, 4 apoE2(Arg^{158}→Cys) homozygous patients, 4 patients with heterozygosity for the APOE*2 (Lys^{146}→Gln) allele and 4 heterozygous patients carrying the APOE*3-Leiden allele. The VLDL fractions were subjected to lipolysis experiments with HSPG-bound LPL and LPL in solution. Additional competition experiments were carried out to assess the binding affinity of VLDL to the HSPG-LPL complex.

Results. The mean lipolysis rates of VLDL, isolated from the apoE2(Lys^{146}→Gln) heterozygotes, apoE2(Arg^{158}→Cys) homozygotes and apoE3-Leiden heterozygotes were 92.3 ± 10.3% (n.s.), 77.3 ± 4.2% ($P < 0.05$) and 76.7 ± 10.0% ($P < 0.05$), respectively, of that of control VLDL (100.0 ± 9.7%). No differences in lipolysis were observed between VLDL from controls and VLDL from the same patients if LPL in solution was used. Thus, compositional differences itself can not explain the differences in lipolysis rates observed with HSPG-bound LPL. In competition experiments, the binding efficiency to HSPG-LPL of VLDL from the apoE2(Lys^{146}→Gln) heterozygotes, apoE2(Arg^{158}→Cys) homozygotes and apoE3-Leiden heterozygotes was 63% (n.s.), 41% ($P < 0.05$) and 35% ($P < 0.05$), respectively of that of control VLDL (100%).

Conclusion. VLDL isolated from apoE2 homozygotes and apoE3-Leiden heterozygotes display decreased lipolysis by HSPG-bound LPL due to a defective binding of these lipoproteins to the HSPG-LPL complex.

Introduction

Lipoprotein lipase (LPL, EC 3.1.1.34) is the key enzyme involved in the hydrolysis of chylomicron and very low density lipoprotein (VLDL) triglycerides. After synthesis in parenchymal cells, LPL is secreted and transported across the endothelium where it binds to heparan sulphate proteoglycans (HSPG) at the luminal surface of endothelial cells. To allow interaction *in vivo* of triglyceride-rich lipoproteins (TRL) with endothelium-bound LPL and subsequent lipolysis, the particle has to reside transiently at the HSPG-LPL complex. Apolipoprotein (apo) E, exposed at the surface of the lipoprotein particle, is considered to play an important role in the binding of VLDL to vessel wall proteoglycans (for reviews see (1,2)).

ApoE is a major apolipoprotein constituent of all lipoproteins except low density lipoproteins. There is substantial evidence that apoE plays an important role in lipoprotein metabolism by functioning as a ligand in receptor-mediated remnant removal (3,4). In humans the catabolism of chylomicron- and VLDL-remnants deranges if apoE is absent or defective in its binding, presenting lipid abnormalities referred to as type III HLP or familial dysbetalipoproteinemia (FD) (5,6). Several mutations in the APOE gene are associated with FD (7). Over 90% of the patients with FD are homozygous for the apoE2(Arg^{158}→Cys) allele. However, only 4% of all apoE2 homozygotes develop FD (8,9). Thus, expression of FD not only requires homozygosity for the apoE2(Arg^{158}→Cys) allele but also additional genetic and environmental factors. Mutations in or in the vicinity of the receptor-binding domain of apoE, which encompasses residues 130 to 150, result in dominantly heritable forms of FD with a high penetrance (10-14).

It is not clear why apoE2 homozygosity shows a penetrance much lower than that of the dominant variants, whereas the binding of the apoE2-variant to the LDL receptor is very low as compared to the dominant apoE-mutants (15-19). No correlation has been noted so far between the severity of the HLP and LDL-receptor binding defect of apoE-variants. Ji *et al.* (20) studied the binding affinity of apoE-variants to HSPG and found that normal apoE3 binds well to HSPG, whereas the apoE2(Arg^{158}→Cys)-variant also binds to HSPG, but to a lesser extent than apoE3. The apoE-variants associated with a dominant mode of inheritance showed poor binding to HSPG, whereby apoE3-Leiden showed the most severe binding impairment. Interestingly, a correlation was observed between the mode of expression of FD and the impairment of binding to HSPG (20), a finding which is

confirmed by others (18). However, it remains to be established whether this correlation also can be observed when assayed under physiological conditions. In the studies mentioned above (18,20), rabbit β-VLDL was enriched with the respective apoE-variant of interest, generating VLDL particles containing abnormally high amounts of the apoE-variant at the expense of endogenous apoE. This is highly unphysiological as compared to heterozygosity for an apoE-mutation, where the presence of normal apoE may compensate for the defective binding of its mutant counterpart.

Ji *et al.* (20) suggested that in the carriers with various apoE-mutations a defective binding to HSPG may have impact on the lipolytic processing of VLDL of these subjects. This hypothesis is sustained by our recent finding of an impaired *in vivo* lipolysis of VLDL in APOE*3-Leiden transgenic mice (21). However, this hypothesis has not been tested by an *in vitro* lipolysis assay using HSPG-bound LPL. In the present study, we found that the lipolysis by HSPG-bound LPL was decreased for VLDL isolated from FD subjects with different apoE-variants as compared to VLDL from apoE3 homozygote control subjects. These differences were not observed if the lipolysis experiments were carried out with LPL in solution. Parallel binding experiments suggest that differences in binding affinity of these VLDL samples to the HSPG-LPL complex may account for the observed differences in lipolysis rate.

Materials and Methods

Subjects

Eight normolipidemic apoE3 homozygous controls, four apoE2(Arg^{158}→Cys) homozygous patients, four patients with heterozygosity for the APOE*2 (Lys^{146}→Gln) allele and four heterozygous patients carrying the APOE*3-Leiden allele were included in this study. All patients were recruited from the outpatient lipid clinic of the Leiden University Medical Center and were classified as having a lipoprotein profile characteristic for FD (9). All heterozygous apoE2(Lys^{146}→Gln) and apoE3-Leiden patients carried the apoE3 allele as second allele, except one apoE3-Leiden patient who carried apoE2 as second allele. Three out of four apoE3-Leiden patients, and one out of four patients of both the apoE2(Arg^{158}→Cys) and apoE2(Lys^{146}→Gln) group were currently on HMG-CoA reductase inhibitor therapy. For ethical

reasons, medical therapy was not discontinued. Informed consent was obtained from all participants.

Lipids and lipoproteins

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. VLDL was isolated by ultracentrifugation as described by Redgrave *et al.* (22). Protein content of the VLDL samples was determined by the method of Lowry *et al.* (23). Triglyceride, total cholesterol, phospholipid and free cholesterol content of the VLDL fractions was measured enzymatically using commercially available kits (Boehringer, Mannheim, Germany). Cholesteryl ester (CE) content was calculated by subtracting the concentration free cholesterol from the concentration total cholesterol. VLDL diameter was determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, U.K.). Measurements were performed at 25 °C and a 90° angle between laser and detector. Particle number was calculated from the total lipoprotein mass and particle diameter, with the assumption that the particles were spherical in shape and their density was 1.006 g/ml. ApoE levels were determined using an enzyme-linked immunosorbent assay (ELISA) as described before (24). ApoE phenotyping was performed by isoelectric focusing according to Havekes *et al.* (25).

Lipoprotein lipase

LPL was purified from fresh bovine milk as described previously (26). The isolated fraction was resuspended in 20 mmol/L NaH_2PO_4, 50% glycerol and stored in aliquots at -80 °C. Isolated LPL was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 4-20%) (27). Proteins were stained with Coomassie Brilliant blue or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were incubated with monoclonal antibody 5D2 (prepared by Dr. J.D. Brunzell *et al.* (28), University of Washington, Seattle), and rabbit anti-mouse IgG conjugated to peroxidase (DAKO, Glostrup, Denmark) was used as second antibody. The specific activity of the isolated LPL was 10.9 μmol $FFA.min^{-1}.mg\ protein^{-1}$.

Assay of lipolysis with heparan sulphate proteoglycan-bound lipoprotein lipase

Heparan sulphate proteoglycans (HSPG), isolated from basement membrane of mouse sarcoma cells, were purchased from Sigma (H 4777, Sigma Chemicals, St. Louis, MO). The lipolysis experiments using HSPG-bound LPL were performed in 96-well microtiter plates (Greiner GmbH, Frichenhausen, Germany). The wells were incubated with 0.5 μg HSPG in 75 μl PBS for 18 h at 4°C. Aspecific binding sites were blocked by incubation with 1% (w/v) essentially free fatty acid (FFA) free bovine serum albumin (BSA) (Sigma) in 100 μl PBS for 1 h at 37°C. Subsequently, the wells were incubated with 1.5 μg LPL in 75 μl Tris-glycerol buffer (0.1 M Tris, 20% (v/v) glycerol, pH 8.5) for 1 h at 4°C. Unbound LPL was removed by washing the plates three times with Tris buffer (0.1 M Tris, pH 8.5). Subsequently, lipolysis was started by adding 50 μl VLDL-TG at a final concentration of 0.2, 0.4, and 0.6 mmol/L, to the preconditioned well in the presence of 1% (w/v) essentially FFA-free BSA and placing the plate in a shaking incubator at 37 °C. The reaction was stopped after 10 min by the addition of Triton X-100 (1% (v/v), final concentration) (Merck, Darmstadt, Germany), in Tris buffer (pH 8.5), vortexing and cooling on ice. FFA concentrations were determined in triplicate using the commercially available NEFA-C kit (WAKO Chemicals, Neuss, Germany).

Assay of lipolysis with lipoprotein lipase in solution

The VLDL samples were diluted in 0.1 M Tris (pH 8.5), 1% (w/v) essentially FFA-free BSA. The incubation was started by adding 0.28 μg LPL in 10 μl Tris-buffer (0.1 M Tris, pH 8.5) to 100 μl VLDL-TG, followed by vortexing and incubation in a shaking waterbath at 37 °C. The reaction was stopped after 6 min by the addition of Triton X-100 (1% (v/v), final concentration), vortexing and cooling on ice. A sample blank was obtained by adding Triton prior to the addition of LPL and maintenance on ice. FFA concentrations were determined in duplicate. The rate of FFA release by LPL was linear for 6 min in this assay. Lipolysis was performed with three VLDL-TG concentrations in the range 0.2 - 0.6 mmol/L, which is in the range of the apparent Km value.

Binding assay

In order to perform competition experiments, normal VLDL from homozygous apoE3 subjects was iodinated using the ^{125}I-iodine monochloride method of Bilheimer *et al.* (29). ^{125}I-iodide (specific activity 15.5 mCi/μg) was purchased from Amersham (Buckinghamshire, U.K.). After iodination, VLDL was dialyzed extensively at 4°C against PBS for 24 h and thereafter stabilized with 1% (w/v) BSA (fraction V, Sigma). The specific radioactivity ranged from 150-200 cpm/ng of protein. The stabilized ^{125}I-labeled VLDL was stored at 4°C and used within two weeks.

Plastic wells (96-well microtiter plates) were coated with 0.5 μg of HSPG per well and subsequently incubated with 1.5 μg LPL per well, exactly as described above. After washing the plates two times with ice-cold PBS, the binding of ^{125}I-labeled E3E3 VLDL to HSPG-bound LPL was determined by incubating the plates for 2 h on ice with 10 μg/ml of ^{125}I-labeled VLDL in the presence of increasing amounts of unlabeled VLDL of interest as competitor. Thereafter, the plates were washed two times with ice-cold PBS containing 0.1% (w/v) BSA, and, subsequently, washed once with PBS without BSA. The ^{125}I-labeled VLDL bound to the HSPG-LPL complex was dissolved in 0.2 N NaOH for quantitation of the binding. The concentration of competitor VLDL protein required to displace 50% of ^{125}I-labeled VLDL (IC_{50}-value) was calculated by logit-log plot analysis in order to estimate the binding affinity of competitor VLDL to the HSPG-LPL complex.

Statistical analyses

Results are presented as the mean ± S.D. Mean differences between the groups were calculated with the Mann-Whitney test. Correlation analysis was performed using the Spearman rank correlation analysis. Statistical analyses were performed with SPSSWIN 6.0 (SPSS, Chicago, IL, USA).

Results

Lipids and lipoproteins

At time of study entrance (table 1), the groups were comparable with regard to age and BMI. Serum cholesterol and triglyceride levels were significantly higher in the FD patients as compared to the control subjects. No significant difference was found in the serum HDL-cholesterol concentrations, although there was a tendency to lower concentrations in the three patient groups. Serum lipid levels were lower in the group of apoE3-Leiden heterozygotes as compared to the apoE2 homozygotes and apoE2(Lys^{146}→Gln) heterozygotes due to the fact that three out of four patients were receiving lipid-lowering medication. With regard to lipoprotein composition, VLDL particles from the FD subjects are cholesterol-enriched as expected (table 2). The phospholipid and protein content did not differ widely between the groups. In the patient groups, the number of apoE molecules per VLDL particle was significantly higher than in the control group. No significant differences in VLDL composition were observed between statin-treated and untreated FD patients, nor between male and female FD patients.

Table 1. *Patient characteristics at study entrance*

Subjects	n	Age yrs	Sex F / M	BMI kg/m²	Chol mmol/L	TG mmol/L	HDL-C mmol/L	Med [a]
Controls								
E3E3	8	47 ± 8	0 / 8	24.0 ± 2.9	4.33 ± 0.58	0.85 ± 0.14	1.32 ± 0.15	0 / 8
FD								
E2(Arg^{158}→Cys)	4	55 ± 6	1 / 3	26.7 ± 3.2	6.90 ± 1.38*	4.66 ± 2.19*	0.96 ± 0.34	1 / 4
E2 (lys^{146}→Gln)	4	39 ± 14	1 / 3	24.1 ± 3.3	9.34 ± 3.38*	5.64 ± 3.37*	1.02 ± 0.24	1 / 4
E3-Leiden	4	44 ± 13	2 / 2	24.5 ± 2.6	5.79 ± 0.66*	2.47 ± 0.96*	1.12 ± 0.13	3 / 4

BMI, body mass index; Chol, cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol.

Values are presented as the mean ± S.D. [a] Med, number of patients on HMG-CoA reductase inhibitor therapy.

**P < 0.05 compared to the corresponding value in control subjects (Mann-Whitney).*

Table 2. *Lipid composition and amount of apolipoprotein E per VLDL particle*

Subjects	n	TG	FC	CE	PL	Protein	Chol/TG	apoE
		%[a]	%	%	%	%	mol/mol	mol E/VLDL[b]
Controls	8	44 ± 4	5 ± 1	9 ± 2	25 ± 4	17 ± 4	0.76 ± 0.18	0.92 ± 0.25
E3E3								
FD								
E2(Arg^{158}→Cys)	4	40 ± 4	8 ± 1*	16 ± 3*	23 ± 1	13 ± 1*	1.35 ± 0.28*	3.80 ± 0.65*
E2 (Lys^{146}→Gln)	4	38 ± 5	7 ± 3	16 ± 5*	24 ± 1	14 ± 0	1.44 ± 0.40*	4.67 ± 1.78*
E3-Leiden	4	38 ± 3*	8 ± 1*	15 ± 1*	24 ± 2	16 ± 2	1.38 ± 0.22*	1.73 ± 0.36*

TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid.

[a] *Presented as percentage of total lipoprotein mass (mg/dl; sum of TG, FC, CE, PL and Protein).*

[b] *Presented as number of apoE molecules per VLDL particle.*

**P < 0.05 compared to the corresponding value in control subjects (Mann-Whitney).*

Lipolysis of VLDL samples

The amount of VLDL triglycerides, required to perform the lipolysis experiments, was not achieved in all VLDL isolations. Therefore lipolysis experiments were carried out with six out of eight controls, three out of four apoE2(Arg^{158}→Cys) homozygotes, all four apoE2(Lys^{146}→Gln) heterozygotes and three out of four apoE3-Leiden heterozygotes. Table 3 shows the results of the lipolysis experiments using both HSPG-bound LPL and LPL in solution. In case of HSPG-bound LPL, for all VLDL triglyceride concentrations applied, the lipolysis rates of VLDL, isolated from the apoE2(Arg^{158}→Cys) homozygotes and apoE3-Leiden heterozygotes were significantly lower than that of control VLDL. VLDL isolated from the apoE2(Lys^{146}→Gln) heterozygotes showed a tendency to a lower lipolysis efficiency, that did not reach statistical significance.

Table 3. *Lipolysis rates of E3E3 VLDL, E2E2 VLDL and VLDL from apoE2(Lys^{146}→Gln) and apoE3-Leiden heterozygous carriers*

	E3E3 (n=6) %[a]	E2E2 (n=3) %	E2(Lys^{146}→ Gln) (n=4) %	E3-Leiden (n=3) %
HSPG-bound LPL				
0.2 mmol/L VLDL-TG	100.0 ± 8.9	78.9 ± 2.3*	92.2 ± 11.0	77.1 ± 10.5*
0.4 mmol/L VLDL-TG	100.0 ± 11.7	79.2 ± 4.1*	93.1 ± 10.6	78.4 ± 11.4*
0.6 mmol/L VLDL-TG	100.0 ± 10.2	74.3 ± 4.7*	91.6 ± 12.4	74.5 ± 12.1*
mean[b]	100.0 ± 9.7	77.3 ± 4.2*	92.3 ± 10.3	76.7 ± 10.0*
LPL in solution				
0.2 mmol/L VLDL-TG	100.0 ± 6.9	98.2 ± 0.8	95.7 ± 5.1	98.3 ± 7.3
0.4 mmol/L VLDL-TG	100.0 ± 3.1	96.2 ± 0.9	90.6 ± 6.6	91.8 ± 8.5
0.6 mmol/L VLDL-TG	100.0 ± 3.8	101.4 ± 6.3	98.1 ± 4.0	96.6 ± 11.5
mean	100.0 ± 4.6	98.7 ± 4.2	94.8 ± 5.8	95.8 ± 5.7

*[a] Lipolysis rates are presented as the mean FFA-release (% of FFA release from normal E3E3 VLDL) after 10 min incubation in HSPG-LPL coated wells or 6 min incubation with LPL in solution, respectively. Each value represents the mean ± S.D. [b] Mean lipolysis rates were calculated from the respective individual lipolysis rates. *P < 0.05 as compared to the corresponding value in control subjects (Mann-Whitney).*

Studying the lipolysis rates using LPL in solution, no significant differences in lipolysis rate were observed between VLDL, isolated from control subjects,

and VLDL from the patients carrying the different apoE-variants (table 3). These results were not affected by differences in treatment and sex distribution between the study groups.

Binding of VLDL to HSPG-bound lipoprotein lipase

Competition experiments were performed to compare the binding of VLDL isolated from apoE2 homozygotes, apoE3-Leiden carriers and apoE2(Lys^{146}→Gln) carriers to that of VLDL from normolipidemic apoE3 homozygotes. Since the amount of isolated VLDL was not sufficient in all cases, binding experiments were carried out with VLDL isolated from six out of eight apoE3 homozygotes, all four apoE2(Arg^{158}→Cys) homozygotes, three out of four apoE2(Lys^{146}→Gln) and three out of four apoE3-Leiden carriers. As shown in figure 1, VLDL from the three apoE-variant groups is less efficient in competing with ^{125}I-labeled VLDL for the binding to HSPG-bound LPL than VLDL from apoE3 homozygous controls. The IC_{50} values of VLDL from apoE2(Arg^{158}→Cys) homozygotes and apoE3-Leiden carriers were significantly higher than that of control VLDL (Table 4).

Table 4. *Binding affinity of E3E3 VLDL, E2E2 VLDL and VLDL from apoE2(Lys^{146}→Gln) and apoE3-Leiden heterozygous carriers*

Subjects	n[a]	IC_{50}[b]	Binding affinity[c]
Controls			
E3E3	6	7.4 ± 2.1	100%
FD			
E2E2	4	18.0 ± 4.1*	41%*
E2 (Lys^{146}→Gln)	3	11.8 ± 4.2	63%
E3-Leiden	3	20.9 ± 9.7*	35%*

*[a] n = number of subjects studied. [b] Concentration of VLDL competitor required to displace 50% ^{125}I-labeled E3E3 VLDL, as calculated by logit-log plot analysis. [c] Binding affinity is presented as IC-50 control / IC-50 patient * 100%. Each value represents the mean ± S.D. *P < 0.05 as compared to the corresponding value in control subjects (Mann- Whitney).*

The IC_{50} value of VLDL from apoE2(Lys^{146}→Gln) carriers was slightly higher than that of control VLDL, but did not reach statistical significance. Comparable results were obtained after correction for differences in treatment and sex.

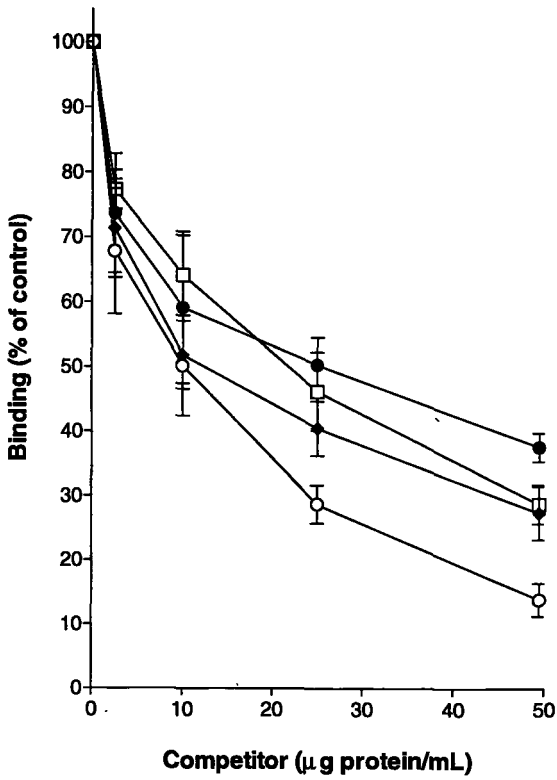

Figure 1. *Competition of VLDL isolated from E3E3 controls, E2E2 subjects, apoE3-Leiden and apoE2(Lys^{146}→Gln) carriers with ^{125}I-labeled E3E3 VLDL for the binding to HSPG-bound LPL. Plastic wells were incubated with 0.5 µg HSPG and 1.5 µg LPL per well, subsequently. The preconditioned wells were incubated for 2 h at 4°C with 10 µg/ml of ^{125}I-labeled E3E3 VLDL in the presence of the indicated amounts of unlabeled E3E3 VLDL* (O), *E2E2 VLDL* (□), *apoE3-Leiden VLDL* (●) *and apoE2(Lys^{146}→Gln) VLDL* (◆). *Values represent the binding expressed as percentage of the control binding, which is the binding in the absence of unlabeled lipoprotein (100%). Each value represents the mean ± S.D. Binding of each VLDL sample was carried out in triplicate at all concentrations applied.*

Discussion

Several studies have been performed to investigate which factors determine the expression of FD in various apoE mutations. Besides the defect in binding affinity to the LDL receptor, the impairment of apoE variants to bind to HSPG is suggested to be one of these factors (18,20). Since HSPG also play an important role in lipolysis of VLDL *in vivo* by anchoring LPL to the endothelium, we hypothesized that the presence of mutant apoE on VLDL particles may result in a defective binding to HSPG-bound LPL and, subsequently, impaired lipolysis.

In order to test this hypothesis, VLDL samples containing different apoE-variants were subjected to lipolysis using HSPG-bound LPL. Three different apoE-variants were selected. ApoE2(Arg^{158}→Cys) and apoE3-Leiden were chosen because of a reduced binding affinity of these apoE-variants to HSPG (20). In this respect, apoE2(Lys^{146}→Gln) has not been studied before, although a reduced heparin binding of an other mutation at the 146-position, the apoE1(Lys^{146}→Glu) mutation, has been reported previously (18). In addition, it has been found that the 142-147 amino acid region of apoE contains a heparin binding site (30).

Lipolysis experiments were performed at VLDL-TG concentrations around the apparent Km values of both assays (0.20 mmol/l and 0.36 mmol/l VLDL-TG for the LPL in solution and HSPG-bound LPL assay, respectively). While lipolysis by LPL in solution did not show differences in lipolysis rate between normal and apoE-variant VLDL, profound differences were observed using the lipolysis assay with HSPG-bound LPL.

Complementary to the lipolysis experiments, binding experiments were carried out. VLDL from the apoE2E2 subjects showed a binding affinity of 41% of normal VLDL (table 4), which is in close comparison with other studies (18,20). ApoE3-Leiden VLDL has been reported to show severely impaired binding to HSPG as compared to apoE2(Arg^{158}→Cys) (20). However, the present study (table 4) indicates that, in comparison to control VLDL, apoE3-Leiden VLDL is almost equally defective in binding to HSPG-LPL as apoE2(Arg^{158}→Cys) VLDL. In addition, our study shows that apoE2(Lys^{146}→Gln) VLDL shows almost normal binding capacity, whereas others observed an impaired binding for apoE-variants with mutations in this region of the APOE gene (18,20). These discrepancies can be explained by differences in experimental design: (i) In our study normal human VLDL was used, whereas others used isolated, lipid-free apoE-variants (18) or isolated rabbit ß-VLDL which was enriched with the apoE variant of interest at the expense of normal apoE (20). The VLDL particles we used in the present study contained physiological amounts of the apoE variant, and normal amounts of apoE3 in case of heterozygosity. (ii) In our experiments we studied binding of VLDL to HSPG-LPL complexes rather than to HSPG or heparin alone, which we consider as more relevant for extrapolation to the *in vivo* situation. We found that the presence of LPL in the binding assay stimulates the binding of VLDL to HSPG with several orders of magnitude, due to bridging comparable to the LPL-mediated stimulation of the binding of lipoproteins to cells (2,31,32).

The presence of excess apoE on the VLDL particles isolated from the FD patients may inhibit lipolysis. Rensen *et al.* (33) studied the role of apoE in lipolysis by enriching artificial lipid emulsions with recombinant apoE. Both *in vitro* and *in vivo* an inhibitory role of apoE in lipolysis could be demonstrated. However, their paper should be interpreted with caution for several reasons. Lipolysis kinetics were carried out with artificial chylomicron-like lipid emulsions which are different from normal VLDL. These lipid particles were relatively large and did not contain apolipoprotein B. In addition, the particles were enriched with large amounts of apoE, up to 62 apoE molecules per particle which is not a physiological concentration. From our results, inefficient lipolysis by excess of apoE molecules per VLDL particle seems less plausible since lipolysis experiments with LPL in solution did not show differences, as presented in table 2. We speculate therefore, that a defective binding to the HSPG-LPL is the primary cause of the less efficient lipolysis of apoE-variant VLDL, rather than differences in lipid or apolipoprotein composition of these VLDL samples.

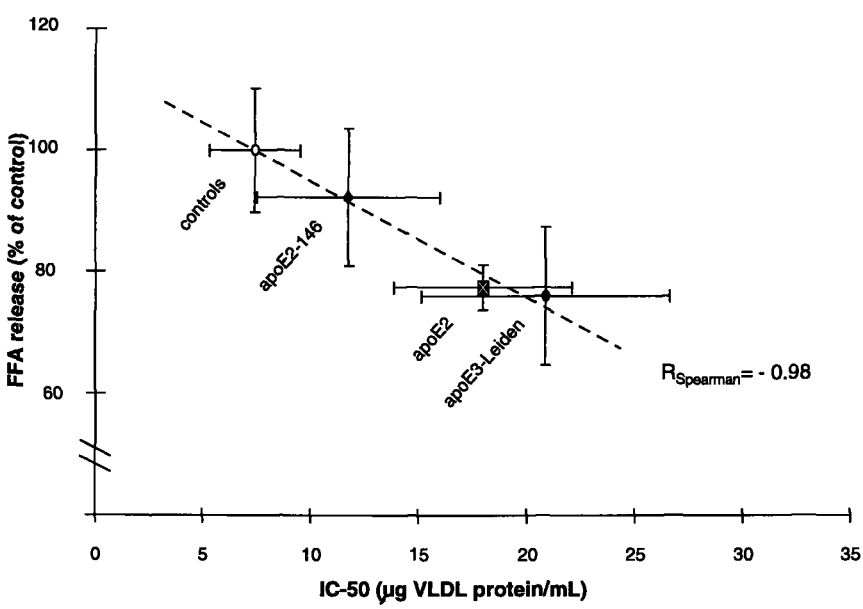

Figure 2. *Correlation between binding and lipolysis of VLDL using an assay with HSPG-bound LPL. IC_{50} values were calculated by logit-log plot analysis of the results from the binding experiments (table 4). IC_{50} values are presented as the mean of the corresponding apoE-variant group. Lipolysis rates are presented as the mean FFA-release ± S.D. after 10 min incubation in HSPG-LPL coated wells (% of control FFA release).*

Binding experiments showed the following hierarchy of binding: E3/E3 > E2(Lys^{146}→Gln)/E3 > E2/E2 > E3-Leiden/E3, which is parallel to the respective lipolysis rates by HSPG-bound LPL. Thus, a defective binding to the HSPG-LPL complex seems to be responsible for the lower lipolysis rates of VLDL containing an apoE-variant. This is clearly illustrated in figure 2 where the average IC_{50} values and respective lipolysis rates of the four groups were found to be highly correlated (Spearman rank correlation coefficient -0.98, $p<0.05$).

In conclusion, VLDL isolated from apoE2(Arg^{158}→Cys) homozygotes and apoE3-Leiden heterozygotes display impaired lipolysis by HSPG-bound LPL. These low lipolysis rates were paralleled by a defective binding of VLDL to the HSPG-LPL complex, thereby confirming the importance of the interaction between VLDL and the proteoglycan-LPL complex in VLDL metabolism.

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Chapter 4

Dietary Counseling Effectively Improves Lipid Levels in Patients with Endogenous Hypertriglyceridemia: Emphasis on Weight Reduction and Alcohol Limitation

Frits H.A.F. de Man[1], Arnoud van der Laarse[1], Erica G. Hopman[2], Jan A. Gevers Leuven[3,4], Willem Onkenhout[5], Geesje M. Dallinga-Thie[6], and Augustinus H. Smelt[3]

Departments of [1]Cardiology, [2]Dietetics and Nutrition, [3]Internal Medicine, and [5]Pediatrics, Leiden University Medical Center; [4]TNO-Prevention and Health, Gaubius Laboratory, Leiden; [6]Department of Internal Medicine, Utrecht University Hospital, The Netherlands

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Summary

Introduction. Environmental factors are considered to play an important role in the expression of hypertriglyceridemia. Accordingly, dietary counseling and weight loss are first-line therapy in these patients It is not clear whether improvements in lipid levels are related to energy restriction or the changes in macronutrient composition that accompany dieting. This study was designed to evaluate the short-term effect of dietary counseling in patients with endogenous hypertriglyceridemia and evaluate the effects of advised nutrient changes.

Methods. Forty-five patients with endogenous hypertriglyceridemia were included in a prospective dietary intervention study, according to the Dutch guidelines for a healthy diet. Before and after the dietary intervention period of 12 weeks, 24-hour food recalls were used to assess dietary intake and macronutrient composition. Effectiveness was evaluated by assessment of body weight, serum lipids, lipoproteins and insulin resistance parameters.

Results. A significant reduction in energy intake and body weight as well as changes in macronutrient composition were observed. Total serum triglyceride and cholesterol levels decreased by 31% and 15%, respectively. No effects were observed on serum glucose and insulin levels. Weight reduction was significantly correlated with reduction of total plasma triglyceride levels and inversely correlated with changes in HDL cholesterol levels. Of all nutrients assessed, only reduction of alcohol intake correlated with improvement of total serum triglycerides.

Conclusions. Short-term dietary counseling in patients with endogenous hypertriglyceridemia can effectively improve serum lipid and lipoprotein levels. With regard to the advised nutrient changes, weight loss and limitation of alcohol intake prove to be the best predictors of triglyceride reduction.

Introduction

Endogenous hypertriglyceridemia is a complex multifactorial lipid disorder characterised by increased plasma levels of very low density lipoprotein (VLDL) particles and by reduced concentrations of high-density lipoprotein cholesterol (HDL-C). There is increasing evidence that hypertriglyceridemia (HTG) is associated with an increased risk of coronary artery disease (1,2). In a recent meta-analysis, Hokanson *et al.* reported an odds ratio of 1.32 for men and 1.76 for women with HTG as compared to healthy population-based control subjects (3), suggesting that adequate triglyceride-lowering therapy would be mandatory in this patient group.

Although genetic factors predispose subjects to the development of HTG (4-6), environmental factors are considered to play an important role in the expression of hypertriglyceridemia (7). Obesity, insulin resistance, alcohol consumption and dietary habits are the principal exogenous factors involved (8-10). It is generally accepted that reduction of energy intake and qualitative improvement of dietary habits have an important effect on patients with endogenous hypertriglyceridemia. Accordingly, dietary counseling and weight loss are the first-line therapy in these patients (11). Several studies have demonstrated impressive improvements of the hypertriglyceridemia in response to dietary counseling (12,13). However, few data are available on the specific diet composition that should be recommended to these patients. It is not clear whether improvements in lipid levels are related to energy restriction or the changes in macronutrient composition that accompany dieting. This study was performed to evaluate the short-term effect of dietary counseling in patients with endogenous hypertriglyceridemia and to evaluate the effects of advised nutrient changes on serum lipid levels.

Materials and Methods

Subjects

Between January 1988 and December 1996, 45 unrelated patients with endogenous HTG were recruited from the outpatient Lipid Clinic of the Leiden University Medical Center. The patient population consisted of 43 males and 2 females, with a mean age of 46 ± 10 y. As expected, the patient group was mildly obese (mean BMI 28.2 ± 3.2 kg/m^2) and hypertension was common

(33%). Fifteen HTG patients (33%) had a medical history of symptomatic cardiovascular disease. At first presentation to the Lipid Clinic, 30 patients were non-smokers and 15 patients were smokers. The diagnosis of the lipoprotein disorder was based on the means of two fasting blood samples obtained at baseline with an interval of 3 weeks. The diagnostic criteria for endogenous hypertriglyceridemia were: total serum TG > 4.0 mmol/L, serum VLDL-C > 1.0 mmol/L, and LDL-C < 4.5 mmol/L. Additional exclusion criteria were the apolipoprotein E2E2 phenotype, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/L, and alcohol consumption of more than 40 g/d) and the use of lipid-lowering drugs. Informed consent was obtained from each patient and the protocol was approved by the institutional Medical Ethics Committee.

Study design

Subjects were selected from new, untreated patients with endogenous hypertriglyceridemia, referred to the outpatient Lipid Clinic of the Leiden University Medical Center in the period between January 1st 1988 and December 31st 1996. Baseline blood samples were taken at 2 separate occasions before dietary counseling. At the first visit they were interviewed by a registered dietician. The mean daily food intake was estimated by means of a dietary history. Energy and nutrient intake were calculated using the computerised version of the Netherlands food table. Subjects who already followed a healthy diet and did not need dietary advise were not included in this study. Thereafter an individual dietary advise according to "Guidelines for a Healthy Diet" from the Netherlands Nutrition Council was given (14), as described before (15). These dietary guidelines are similar to the American Heart Association Step I diet (16), with the exception that not less than 30% but 30-35% of total energy intake should be derived from fat. The diet was adapted to the dietary habits and lifestyle of the patient. The recommendations were: 1. 30-35 % of total energy derived from total fat. 2. A maximum of saturated fat of 10 % of total energy. 3. Cholesterol intake less than 300 mg per day. 4. As compensation for the reduced intake of saturated fat, increase of complex carbohydrate consumption upto 55% of total energy was advised when patients were not overweight. 5. In case of obesity (body mass index > 26 kg/m^2), an energy restriction of the typical daily energy intake by 500 kcal (2093 kJ) was advised and the patient was encouraged to increase physical activity. 6. Alcohol use was discouraged. No specific recommendations were given for the intake of monounsaturated and

polyunsaturated fat. Twelve weeks after start of the diet, a second dietary history was obtained from each patient by the same dietician and blood samples were collected from each patient at 2 separate time points. After this dietary intervention period, the effectiveness was evaluated and, if necessary, pharmacological therapy was instituted. Long-term efficacy of dietary counseling could not be evaluated as it was not considered to be ethical to withhold patients lipid-lowering drugs if indicated.

Analytical methods

Venous blood was collected from each patient after more than 12 hours of fasting. Serum was obtained by low speed centrifugation within 4 hours after collection. Three ml of fresh serum was ultracentrifuged for 15 hours at 232,000 g (75,000 rpm) at 15°C in a TL-100 tabletop Ultracentrifuge, using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA, USA). The ultracentrifugate was carefully divided in a density (d) < 1.006 (supranatant) and d 1.006 - 1.25 g/ml (infranatant) fraction, designated as the VLDL and LDL-HDL fraction, respectively. The triglyceride and cholesterol concentrations were measured enzymatically using test kits (Boehringer, Mannheim, Germany). High density lipoprotein-cholesterol was measured in the LDL-HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and $MgCl_2$. Serum total fatty acid composition was determined by gas chromatography on an Interscience 8160 gas chromatograph fitted with a cold on-column injector and a CP-Sil88 fused silica capillary column (Chrompack, Bergen op Zoom, the Netherlands) after methylation of the fatty acids (17). Glucose was measured with a Hitachi 747 analyzer, according to standard procedures (Boehringer, Mannheim, Germany). Insulin was measured with a conventional radio-immuno assay (Medgenix, Brussels, Belgium).

Statistical analysis

Results are presented as the mean ± SD. Differences between the patient group before and after dietary counseling were evaluated pairwise using the Wilcoxon paired signed-ranks test. Correlation analysis was performed using the Pearson correlation analysis (analysis of triglyceride levels was performed on logarithmic transformed data). Findings were regarded to be statistically significant when the probability of these data under the null hypothesis was less than 0.05. Statistical analyses were performed with SPSS/PC+™ software (SPSS Inc., Chicago, IL USA).

Results

Changes in energy and nutrient intake

Twenty-eight HTG patients (62%) were advised to reduce the intake of total fat, and 37 patients (82%) were encouraged to limit the intake of saturated fats. Reduction of dietary cholesterol intake was advised in 10 cases (22%). Although dietary counseling was associated with weight loss in almost all patients, an explicit advise to reduce energy intake was given in 27 patients (60%). In most cases, multiple changes in dietary habits were advised.

The short-term effects of dietary counseling on dietary habits and body weight are shown in table 1. A modest reduction in body weight was observed. The reported total energy intake was reduced by 21%. The intake of discouraged nutrients such as saturated fats, cholesterol and alcohol was reduced. On the other hand, the absolute intake of carbohydrates and proteins remained unchanged. So, according to the dietary histories, the patients adhered to the dietary advise.

To estimate the accuracy of the dietary histories and changes in dietary habits, the reported intake of different fatty acids was compared with the fatty acid composition in serum.

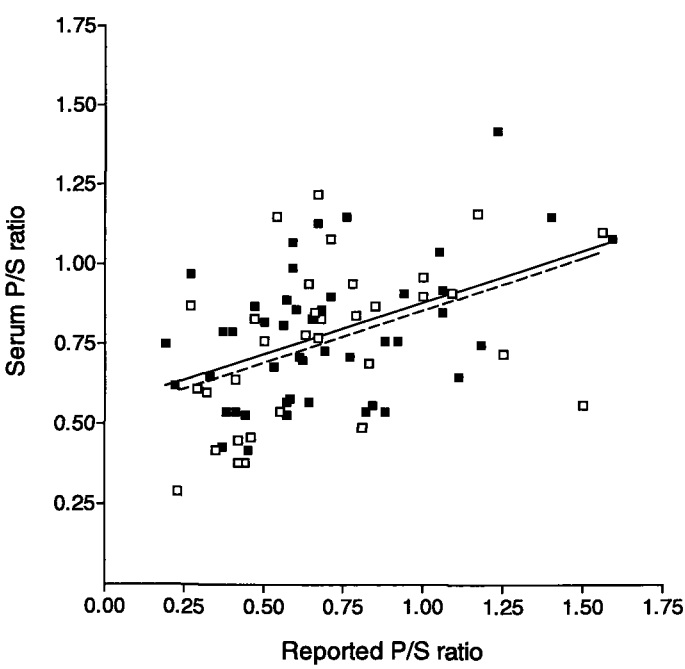

Figure 1. *The correlation between the reported ratio of polyunsaturated fatty acids to saturated fatty acids (P/S ratio) and the P/S ratio in serum ($r=0.45$, $p<0.001$), calculated from the pooled data of overweight (n=27, ■ solid line) and non-overweight (n=18, □ dashed line) subjects.*

As shown in figure 1, a significant correlation was found between the reported ratio of polyunsaturated fatty acids to saturated fatty acids (P/S ratio) and serum P/S ratio (r=0.45, p<0.001), both in the overweight (r=0.46, p=0.001) and non-overweight patients (r=0.44, p=0.009). A weak association was found between reduction of energy intake and weight reduction (r=0.32, p=0.042). Subdivision of the population in overweight and non-overweight patients resulted in disappearance of significant correlations in both groups.

Table 1. *Effect of dietary counseling on body weight and dietary habits*

	Dietary counseling		p-value[a]
	Before	During	
Number	45		
Weight (kg)	89.7 ± 13.7	88.2 ± 13.4	<0.001
BMI (kg/m²)	28.2 ± 3.2	27.4 ± 3.2	<0.001
Energy (kJ/d)	8762 ± 2531	6982 ± 1806	<0.001
Total fat (g/d)	78.3 ± 29.1	50.4 ± 16.8	<0.001
(E%)	33 ± 6 %	27 ± 6 %	<0.001
Saturated fat (g/d)	31.7 ± 11.5	18.6 ± 6.5	<0.001
(E%)	13 ± 3 %	10 ± 3 %	<0.001
Monounsaturated fats (g/d)	27.4 ± 10.9	15.9 ± 5.3	<0.001
(E%)	11 ± 3 %	9 ± 2 %	<0.001
Polyunsaturated fats (g/d)	17.6 ± 9.1	15.3 ± 6.5	0.043
(E%)	7 ± 3 %	8 ± 3 %	n.s.
P/S-ratio of diet	0.57 ± 0.25	0.87 ± 0.35	<0.001
Cholesterol (mg/d)	239 ± 88	157 ± 62	<0.001
Carbohydrates (g/d)	229 ± 77	207 ± 67	n.s.
(E%)	45 ± 8 %	50 ± 6 %	<0.001
Protein (g/d)	90.8 ± 24.1	84.3 ± 20.4	n.s.
(E%)	18 ± 3 %	21 ± 4 %	<0.001
Alcohol (g/d)	14.7 ± 18.7	5.3 ± 8.6	0.002
(E%)	4 ± 5 %	2 ± 3 %	0.007

Values are presented as mean ± SD. BMI, body mass index; E%, percentage of energy; P/S-ratio, polyunsaturated / saturated fatty acids ratio. [a]Statistical analyses were performed using the Wilcoxon paired signed-ranks test.

Changes in serum lipids, lipoproteins, insulin and glucose levels

Table 2 shows the corresponding effects of dietary intervention on serum lipids, lipoproteins and insulin resistance parameters. Impressive improvements were observed in serum lipids and lipoproteins. Total serum triglycerides decreased from 15.80 to 10.85 mmol/L (-31%, p=0.001). Also total serum cholesterol levels dropped from 8.99 to 7.68 mmol/L (-15%, p=0.002), an effect predominantly caused by a decrease of VLDL cholesterol (-38%, p=0.006). Both LDL cholesterol and HDL cholesterol increased modestly, while the ratio of LDL cholesterol to HDL cholesterol (LDL/HDL ratio) did not change. No effects were observed on serum glucose and insulin levels. In accordance with the reported change in dietary fatty acid composition, the serum P/S ratio increased from 0.72 to 0.83 (p=0.021).

Table 2. *Effect of dietary counseling on serum lipids, lipoproteins, glucose and insulin concentrations*

	Dietary counseling		p-value[a]
	Before	During	
Number	45		
Total cholesterol (mmol/L)	8.99 ± 3.37	7.68 ± 2.54	0.002
Total triglyceride (mmol/L)	15.80 ± 15.67	10.85 ± 10.30	0.001
VLDL-cholesterol (mmol/L)	5.65 ± 5.15	3.52 ± 3.24	0.006
VLDL-triglyceride (mmol/L)	14.62 ± 17.22	8.14 ± 9.82	0.001
LDL-cholesterol (mmol/L)	2.65 ± 1.00	2.95 ± 1.09	0.044
HDL-cholesterol (mmol/L)	0.62 ± 0.22	0.67 ± 0.19	n.s.
Glucose (mmol/L)	4.86 ± 1.01	4.86 ± 1.07	n.s.
Insulin (mU/L)	50.3 ± 54.9	48.2 ± 65.0	n.s.
Serum P/S-ratio	0.72 ± 0.21	0.83 ± 0.24	0.021

Values are presented as mean ± SD. P/S-ratio, polyunsaturated fats / saturated fatty acids ratio.

[a]Statistical analyses were performed using the Wilcoxon paired signed-ranks test.

In order to determine whether specific nutrient changes are accompanied by changes in serum lipids and lipoproteins, correlation analyses were performed. Weight reduction correlated significantly with reduction of total plasma triglycerides (r=0.35, p=0.03), VLDL cholesterol (r=0.41, p=0.021) and VLDL triglycerides (r=0.46, p=0.009). In accordance, an inverse correlation between changes in body weight and HDL cholesterol levels (r=-0.39, p=0.031) was observed. Of all nutrients assessed, only reduction of alcohol intake correlated with improvement of total serum triglyceride levels (r=0.33,

p=0.041). These correlations could not be noted in the overweight and non-overweight subgroups.

Differences between overweight and non-overweight patients

At baseline, there were some interesting differences between both groups. As shown in table 3, the overweight patients reported a higher energy intake than non-overweight patients (9408 vs. 7792 kJ, respectively; p=0.035). The higher energy intake in the overweight group was mainly derived from carbohydrates (p=0.011), although there was a tendency to a higher intake of all macronutrients. After the dietary counseling period, these differences had disappeared and the patient groups were consuming comparable diets. Accordingly, the energy reduction in the overweight group was more pronounced than in the non-overweight group (p=0.032). In the overweight group, the absolute intake of all macronutrients was reduced. In the non-overweight group, however, the absolute intake of polyunsaturated fats and carbohydrates remained unchanged.

No differences could be observed between the baseline metabolic variables of the overweight and non-overweight patients. The efficacy of the dietary intervention with regard to lipid levels appeared to be slightly better in the overweight group, however, none of the differences in efficacy between the overweight and non-overweight group reached statistical significance. Plasma triglyceride levels in the overweight group decreased from 14.94 to 8.86 mmol/L, whereas in the non-overweight group the corresponding levels decreased from 17.09 to 13.79 mmol/L. In accordance, both VLDL-cholesterol and VLDL-triglyceride levels tended to be reduced more effectively in the overweight group than in the non-overweight group. Total cholesterol, LDL-cholesterol and HDL-cholesterol levels showed comparable changes in the two groups.

Table 3. *Dietary counseling in the overweight and non-overweight group*

	Overweight group		Non-overweight group	
	Before	During	Before	During
Number	27		18	
Weight (kg)	95.4 ± 13.0	93.1 ± 13.0**	80.6 ± 9.3	80.1 ± 10.0#
Energy (kJ/d)	9408 ± 2643	7112 ± 2039***	7792 ± 2057	6796 ± 1449#
Total fat (g/d)	82.5 ± 31.4	50.2 ± 16.5***	71.9 ± 24.7	50.6 ± 17.8###
Saturated fat (g/d)	32.7 ± 12.2	18.5 ± 6.7***	30.1 ± 10.5	18.8 ± 6.5###
Monounsaturated fats (g/d)	29.4 ± 11.6	15.7 ± 5.4***	24.4 ± 9.1	16.1 ± 5.4##
Polyunsaturated fats (g/d)	19.1 ± 10.1	14.9 ± 6.1**	15.4 ± 7.0	15.8 ± 7.1
Dietary cholesterol (mg/d)	238 ± 83	147 ± 65***	240 ± 97	170 ± 56#
Carbohydrates (g/d)	254 ± 73	217 ± 77**	192 ± 68	192 ± 47
Protein (g/d)	93.4 ± 25.5	83.6 ± 20.1	87.0 ± 22.2	85.4 ± 21.5
Alcohol (g/d)	15.7 ± 20.4	5.2 ± 9.3*	13.2 ± 16.5	5.5 ± 7.8#
Plasma cholesterol (mmol/L)	8.77 ± 3.65	7.13 ± 2.00	9.32 ± 2.97	8.49 ± 3.06#
Plasma triglyceride (mmol/L)	14.94 ± 16.59	8.86 ± 4.26*	17.09 ± 16.00	13.79 ± 15.14#
VLDL-cholesterol (mmol/L)	5.51 ± 5.55	2.96 ± 2.05	5.82 ± 4.79	4.35 ± 4.42##
VLDL-triglyceride (mmol/L)	15.08 ± 20.03	6.09 ± 4.05**	14.06 ± 13.56	11.14 ± 14.38
LDL-cholesterol (mmol/L)	2.55 ± 0.95	2.91 ± 0.94	2.76 ± 1.01	3.00 ± 1.30
HDL-cholesterol (mmol/L)	0.63 ± 0.22	0.66 ± 0.16*	0.61 ± 0.23	0.68 ± 0.23

** $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to the corresponding value in the overweight group before dietary counseling.*

 # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to the corresponding value in the non-overweight group before dietary counseling.

Discussion

Dietary treatment is recommended as first-line therapy in patients with hypertriglyceridemia. Although others have demonstrated that dietary counseling in hypertriglyceridemia can be effective (12,13), no data are available about the response to the "Dutch Guidelines for a Healthy Diet" in this patient group, nor about the effectiveness of specific nutrient changes. In the present study, 45 new untreated patients with endogenous hypertriglyceridemia were included in a 12-week dietary intervention study. In contrast to previous studies, an attempt was made to evaluate the effects of changes in macronutrient composition on serum lipid and lipoprotein levels. A limitation of this study is the use of dietary histories. Other authors have reported a systematic underreporting of dietary intake (18), that appears to be dependent on the degree of obesity (19). In the present study, however, a significant correlation was observed between the reported dietary P/S ratio and the P/S ratio that was measured in serum. In addition, an association was found between reduction of energy intake and weight reduction. These results suggest that the dietary history may provide an adequate indication of food intake.

In response to the dietary counseling, we observed a significant reduction in body weight, energy intake and improvement of macronutrient composition. In parallel, total serum triglyceride and total serum cholesterol levels were reduced by 31% and 15%, respectively, which is in general agreement with previous studies (12,13). In contrast to the marked changes in lipid levels, no effects were observed on serum insulin and glucose concentrations. We speculate that the observed changes in body weight are too modest to detect an improvement of insulin resistance. The observed effectiveness of the current dietary advise in hypertriglyceridemic patients was more pronounced compared with patients with hypercholesterolemia or combined hyperlipidemia that have been described previously (15,20). This confirms the concept that hypertriglyceridemia is the lipoprotein disorder most susceptible to dietary intervention. Interestingly, 3 years and 8 months after completion of the dietary intervention study, body weight was still lower than at study entrance, suggesting that dietary habits were changed permanently. Despite impressive improvements of the reported dietary macronutrient composition, only alcohol reduction appeared to correlate with reduction of serum triglyceride levels. It is known that alcohol can stress triglyceride metabolism by increasing VLDL production (21). Our study

confirms the concept that alcohol intake should be limited in hypertriglyceridemic patients.

In the present study, no correlation could be noted between changes in lipid levels and the changes in dietary fatty acid composition. This is in general agreement with other studies (22). Several reports suggest that both saturated and monounsaturated fatty acids do not affect serum triglyceride levels, whereas only high amounts of polyunsaturated fatty acids have been reported to reduce serum triglycerides in hypertriglyceridemic subjects (23).The amount of polyunsaturated fatty acids that is required to reduce serum triglycerides is difficult to achieve with regular dietary measures and artificial supplementation seems obligatory. However, an increased *in vitro* VLDL and LDL oxidizability has been reported in response to fish oil therapy, a phenomenon explained by an increased number of double bonds in the fatty acid chains (24). Moreover, diets rich in polyunsaturated fats have been reported to increase glucose concentrations in diabetic patients (25). Long-term studies have to be performed to investigate whether the controversial effects of a polyunsaturated fat-rich diet may be outweighed by the overall protective effect through other mechanisms that favourably modify platelet function, inflammatory responses and the development of atherosclerosis in general (26-30).

Although the primary stimulus for hepatic VLDL production is the availability of free fatty acids, it is well established that dietary cholesterol can stimulate VLDL production as well (31). Limitation of cholesterol intake is a common dietary recommendation for hyperlipidemic patients, however, no data are available on the effect of dietary cholesterol in hypertriglyceridemic patients. In the present paper, no relation could be noted between reduction in cholesterol intake and lipid improvements.

In case of normal body weight, an isocaloric dietary advise seems suitable. There has been debate as to whether saturated fat should be replaced by carbohydrates or monounsaturated fat. So far, no comparative studies have been performed in patients with endogenous hypertriglyceridemia. Garg *et al.* (32) performed a meta-analysis of nine studies comparing a high-carbohydrate diet with a high-monounsaturated-fat diet in type 2 diabetes. The high-monounsaturated-fat diet proved to be superior with regard to lipid levels and glycemic control. In accordance with this view, the American Diabetes Association recently recommended a more moderate intake of carbohydrates in hypertriglyceridemic patients with non-insulin dependent diabetes mellitus (33). However, it has been suggested that

the beneficial effects of a high-monounsaturated-fat diet may not be related to the high monounsaturated fat quantity but to the reduction in carbohydrates, as the latter is the macronutrient of which the metabolism is primarily impaired in diabetes. In hypertriglyceridemia, not the metabolism of carbohydrates but the catabolism of fat is impaired. In the postprandial phase, the rise in triglyceride levels in hypertriglyceridemia is severe and prolonged as compared to control subjects (34). Therefore, restriction of all types of fat seems to be a reasonable recommendation in hypertriglyceridemia. On the other hand, diets rich in carbohydrates have been reported to increase serum triglyceride levels both in normolipidemic and in hypertriglyceridemic subjects (35,36). An enhanced hepatic synthesis of VLDL triglycerides is thought to be the underlying mechanism (36). So, there is good evidence that both macronutrients can stress triglyceride metabolism. Our study adds some interesting data to this discussion, particularly the observation that a hypocaloric low-fat diet in normal-weight hypertriglyceridemic individuals is effective. One might therefore consider a more vigorous weight reduction beyond the target BMI of 26 kg/m^2 instead of focusing on the discussion whether the calorie loss should be compensated with carbohydrates or monounsaturated fat.

Results of the present study confirm that dietary therapy is associated with significant improvements of serum lipid profiles in patients with hypertriglyceridemia. However, only a minority of the patients achieved normal lipid levels after 12 weeks of dietary therapy. In most cases, institution of lipid-lowering medication was needed. Although single dietary intervention may be sufficient to achieve normal lipid levels in patients with mildly elevated lipid levels who are highly motivated to adhere to strict dietary guidelines, dietary therapy should be maintained as adjuvant therapy to lipid-lowering medication in the majority of patients.

This study confirms the current concept that energy restriction and weight loss are key issues in the dietary management of hypertriglyceridemia. Novel findings are the observation that dietary intervention is also effective in non-overweight patients, and that alcohol limitation is effective in patients with endogenous hypertriglyceridemia with a normal, social alcohol use (less than 40 g/d). In conclusion, we have demonstrated that short-term dietary counseling in patients with endogenous hypertriglyceridemia can effectively improve serum lipid and lipoprotein levels. With regard to the advised nutrient changes, weight loss

and limitation of alcohol intake prove to be the best predictors of triglyceride reduction.

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Chapter 5

The Hypolipidemic Action of Bezafibrate Therapy in Hypertriglyceridemia is Mediated by Upregulation of Lipoprotein Lipase: No Effects on VLDL Substrate Affinity to Lipolysis or LDL Receptor Binding

Frits H.A.F. de Man[1], Femke de Beer[2,4], Arnoud van der Laarse[1], Hans Jansen[5], Jan A. Gevers Leuven[2,4], John H.M. Souverijn[3], Ton F.F.P. Vroom[2], Selene C.M. Schoormans[1], Jean-Charles Fruchart[6], Louis M. Havekes[1,2,4] and Augustinus H.M. Smelt[2]

Departments of [1]Cardiology, [2]Internal Medicine, [3]Clinical Chemistry, Leiden University Medical Center; [4]Gaubius laboratory, TNO-PG, Leiden; [5]Laboratory for Lipid Research, Erasmus University, Rotterdam, The Netherlands; and [6]Department d'Athérosclérose, Institut Pasteur, Lille Cédex, France

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Summary

Introduction. Fibrates are regarded as drugs of choice in hypertriglyceridemia. Downregulation of apoC-III transcription and upregulation of lipoprotein lipase (LPL) gene expression have been suggested to explain the mechanism of action. This study was designed to study the effects of bezafibrate therapy on very low density lipoprotein (VLDL) susceptibility to lipolysis, VLDL binding to the low density lipoprotein (LDL) receptor and postheparin LPL activities in patients with HTG.

Methods. Eighteen HTG patients were randomized to receive in a double-blind placebo-controlled cross-over fashion 400 mg bezafibrate once daily for 6 weeks. VLDL lipolysis was studied with heparan sulfate proteoglycan-bound LPL. Binding affinity of VLDL to the LDL receptor was determined in J774 cells with ^{125}I-labeled control LDL.

Results. In response to bezafibrate therapy, plasma triglyceride and apoC-III levels decreased by 69% and 42%, respectively. HTG VLDL was lipolyzed less efficiently compared to control VLDL and lipolysis did not improve by bezafibrate therapy. VLDL binding affinity to the LDL receptor was comparable between the control group and HTG group, and did not change upon bezafibrate therapy. However, the post-heparin LPL activity in the HTG patients increased from 153 to 192 U/L ($p=0.025$). A strong inverse relation was observed between the change in LPL activities and the change in triglyceride levels ($r=-0.62$, $p=0.006$).

Conclusions. The hypolipidemic action of bezafibrate therapy in HTG may be attributed to upregulation of LPL, whereas VLDL susceptibility to lipolysis and LDL receptor binding are not affected.

Introduction

Hypertriglyceridemia is an independent risk factor for cardiovascular disease in both men and women (1-3). This multifactorial lipid disorder is characterized by elevated levels of very low density lipoproteins (VLDL), low high density lipoprotein cholesterol (HDL-C) levels, small dense low density lipoproteins (LDL) and insulin resistance. Both overproduction and delayed clearance of VLDL have been demonstrated in hypertriglyceridemia (4). Obesity, insulin resistance and high-energy diets are considered to be involved in the increased triglyceride synthesis (5,6). Genetic factors are mainly associated with impaired triglyceride catabolism (7). Mutations in the lipoprotein lipase (LPL) gene are frequently found in HTG patients and are thought to predispose subjects to the development of hypertriglyceridemia (8). In addition, several genetic studies have demonstrated a relation between hypertriglyceridemia and polymorphisms in the apoC-III gene and apoC-III promoter region (9,10), which may be associated with the increased plasma apoC-III levels in these patients (11). Since apoC-III can inhibit lipolysis (12) and impair the receptor-mediated clearance of VLDL (13,14), high apoC-III levels are considered to play a key role in the delayed triglyceride catabolism in HTG patients.

Fibrates are regarded as drugs of choice in patients with hypertriglyceridemia (15). The hypolipidemic effect is mediated via two important mechanisms. [1]They increase the hepatic fatty acid uptake and stimulate the conversion of fatty acids into acyl-coA, by inducing fatty acid transport protein and acyl-coA synthase (16,17). Thus, fibrates increase β-oxidation of fatty acids in the mitochondrion, which results in a reduced availability of fatty acids for triglyceride synthesis and reduced hepatic triglyceride production (18). [2]Fibrates affect transcription rates of regulatory enzymes and apolipoproteins implicated in lipoprotein metabolism via activation of peroxisome proliferator-activated receptor (PPAR) α (19). Activation of these nuclear receptors results in the upregulation of the lipoprotein lipase (LPL) gene expression and reduction of apoC-III transcription (19,20).

It is speculated that a fibrate-induced reduction of the apoC-III content per VLDL particle may improve VLDL catabolism. The best direct evidence is derived from studies in transgenic animals. Transgenic mice overexpressing the apoC-III gene show a marked hypertriglyceridemia (21). In accordance, apoC-III knock-out mice have low triglyceride levels and

appear to be resistant to postprandial hypertriglyceridemia when their triglyceride metabolism is stressed by a high fat diet (22). Corresponding metabolic studies in these animals have demonstrated that apoC-III impairs receptor-mediated lipoprotein removal (14). A recent study in apoC-III transgenic mice demonstrated a diminished interaction between glycosaminoglycans and apoC-III-rich VLDL particles (23), suggesting that apoC-III may also impair lipolysis by proteoglycan-bound LPL. Thus, with regard to a fibrate-induced reduction of apoC-III levels, it is hypothesized that fibrates may improve VLDL as substrate for lipolysis and as ligand for receptor-mediated lipoprotein removal. However, this hypothesis has not been tested in humans. In the present study, we examined in patients with hypertriglyceridemia the effects of bezafibrate therapy on VLDL suitability as substrate for LPL, binding to the LDL receptor and post-heparin LPL activity.

Materials and Methods

Patients and control subjects

The study population consisted of 18 unrelated patients, 16 males and 2 females, with endogenous hypertriglyceridemia who were recruited from the outpatient lipid clinic of the Leiden University Medical Center. The diagnosis endogenous hypertriglyceridemia was based on the means of two fasting blood samples obtained after a dietary period of at least 8 weeks. The diagnostic criteria for endogenous hypertriglyceridemia were: total serum TG > 4.0 mmol/L, serum VLDL-C > 1.0 mmol/L, and serum LDL-C < 4.5 mmol/L. Additional exclusion criteria were the apoE2E2 genotype, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/L, and alcohol consumption of more than 40 g/day) and the use of lipid-lowering drugs. Patients with a medical history of cardiovascular disease were not included in the study. Twenty normolipidemic, age- and sex-matched control subjects, 18 males and 2 females, were recruited by a newspaper advertisement.

Study design and blood sampling

The patients were randomized to receive in a double-blind cross-over fashion a fixed dose of bezafibrate (slow-release), 400 mg once daily, or placebo for 6

weeks. The two periods in which medication was taken were separated by a wash-out period of 6 weeks. Before and after each treatment period of 6 weeks, fasting venous blood samples were obtained from the participants for lipid measurements. At the end of each treatment period, fasting venous blood samples were obtained which was followed by administration of an intravenous bolus of 50 IU of sodium heparin per kilogram body weight. After exactly 20 min, the post-heparin blood samples were drawn in ice-cooled heparin-coated tubes. From the control subjects, fasting blood samples were obtained at baseline under identical conditions. All tubes were kept on ice after sampling. Serum tubes were allowed to clot for 30 min at room temperature. Informed consent was obtained from each participant and the protocol was approved by the institutional Medical Ethics Committee.

Lipids and lipoproteins

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Three mL of fresh serum was ultracentrifuged for 15 hours at 232,000 g at 15°C in a TL-100 tabletop ultracentrifuge, using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA USA). The ultracentrifugate was carefully divided in a density (d) < 1.006 and d 1.006 - 1.25 g/mL fraction, designated as the VLDL and LDL-HDL fraction, respectively. HDL cholesterol was measured in the LDL-HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and $MgCl_2$.

Triglyceride, total cholesterol, phospholipid and free cholesterol concentrations were measured enzymatically using commercially available kits (Boehringer, Mannheim, Germany). Cholesteryl ester (CE) content was calculated by subtracting the concentration free cholesterol from the concentration total cholesterol. Protein was determined by the method of Lowry *et al.* (24). VLDL diameter was determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, Malvern, UK). Measurements were performed at 25°C and at a 90° angle between laser and detector. ApoB, apoE, and apoC-III levels were determined using an enzyme-linked immunosorbent assay (ELISA). ApoA-I was assessed by a turbidimetric assay using an automated Hitachi 911 analyzer (Boehringer-Mannheim/Hitachi, Mannheim, Germany). ApoE genotyping was performed as described by Reymer *et al.* (25).

Lipoprotein lipase (LPL) was determined by an immunochemical method as described by Jansen *et al.* (26), using a gum acacia-stabilized

[^{3}H]trioleolylglycerol substrate. LPL was assessed after inhibition of hepatic lipase (HL) with a goat antibody raised against HL purified from postheparin human plasma. The extraction efficiency of [^{3}H]-labeled nonesterified fatty acids liberated from the substrate during the assay was accounted for by priming the substrate with [^{14}C]oleate. In each series of determinations, pooled plasmas with high and low LPL activity were included as a reference. Activities are expressed as milliunits, 1 mU representing the release of 1 nmol fatty acid from the substrate in 1 minute.

The plasma samples for the *in vitro* experiments were stored in the following way: The samples were brought to a final concentration of 10% (w/v) sucrose, 10 mM EDTA, capped under nitrogen, snap-frozen in liquid nitrogen and stored at -80°C. Previous studies at our department have demonstrated that storage at -80°C under these conditions does not affect VLDL lipolysis kinetics nor VLDL oxidizability (27,28). Before the *in vitro* experiment, equal sample numbers of the study groups were thawed, VLDL was isolated and stored at 4°C until use. The contribution of apoB-48 to the total amount of apoB was less than 10% in the samples, which indicates that the contribution of chylomicron remnants to the isolated VLDL fraction was limited.

Assay of lipolysis with heparan sulfate proteoglycan-bound lipoprotein lipase

Lipolysis experiments were carried out with VLDL samples, isolated from ten representative HTG patients during placebo and bezafibrate therapy, and ten control subjects. The group of patients and control subjects were carefully selected in order to obtain baseline characteristics that were similar to the original groups. Non-responders were not included.

The lipolysis experiments with HSPG-bound LPL were performed in 96-well microtiter plates as described before (27). Briefly, the wells were incubated with 0.5 µg HSPG (Sigma Chemicals, St. Louis, MO) in 75 µl PBS for 18 h at 4°C. Aspecific binding sites were blocked by incubation with 1% (w/v) essentially free fatty acid (FFA) free bovine serum albumin (BSA) (Sigma) in 100 µl PBS for 1 h at 37°C. Subsequently, the wells were incubated with 1.5 µg LPL in 75 µl Tris-glycerol buffer (0.1 mol/L Tris, 20% (v/v) glycerol, pH 8.5) for 1 h at 4°C. Unbound LPL was removed by washing the plates three times with Tris buffer (0.1 mol/L Tris, pH 8.5). Subsequently, lipolysis was started by adding 50 µl VLDL-TG at a final concentration of 0.2, 0.4, and 0.6 mmol/L, to the preconditioned well in the presence of 1% (w/v) essentially FFA-free BSA and placing the plate in a

shaking incubator at 37°C. The reaction was stopped after 10 min by the addition of Triton X-100 (1% (v/v), final concentration) (Merck, Darmstadt, Germany), in Tris buffer (pH 8.5), vortexing and cooling on ice. FFA concentrations were determined in triplicate using the NEFA-C kit (WAKO Chemicals, Neuss, Germany).

LDL receptor binding assay

The LDL receptor binding experiments were carried out with VLDL samples, isolated from the same ten HTG patients during placebo and bezafibrate therapy, and ten control subjects that were studied in the lipolysis assay. J774 macrophages were cultured in 75-cm^2 flasks in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FCS (fetal calf serum), 0.85 g/L $NaHCO_3$, 4.76 g/L HEPES, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 24-well plates. The medium was refreshed every 3 days and used for experiments within 5 days of plating.

In order to perform competition experiments, low density lipoproteins (LDL) were isolated from a control subject using ultracentrifugation according to Redgrave *et al.* (29). LDL was immediately used for iodination by the ^{125}I-iodine monochloride method described by Bilheimer *et al.* (30). After iodination, LDL was dialyzed extensively at 4°C against PBS for 24 hours and thereafter stabilized with 1% (wt/v) BSA (fraction V, Sigma). The specific radioactivity ranged from 250-300 cpm/ng of protein. The stabilized ^{125}I-labeled LDL was stored at 4°C and used within two weeks.

J774 macrophages were cultured in 24-well plates as described above. Twenty-four hours before each experiment, cells were washed with DMEM containing 1% (wt/v) BSA and further incubated with DMEM containing 5% (v/v) of lipoprotein-deficient serum instead of FCS. The binding of ^{125}I-labeled LDL to J774 macrophages was determined after a 3-hour incubation at 4°C with 10 µg/mL ^{125}I-labeled LDL in the presence of increasing amounts of unlabeled VLDL of interest as competitor. Thereafter, the medium was removed and the cells were washed three times with ice-cold PBS containing 0.1% (wt/v) BSA and subsequently once with PBS without BSA. Cells were then dissolved in 1 mL of 0.2 mol/L NaOH. Protein content of the cell lysate was measured by the method of Lowry *et al.* (24). An aliquot of the cell lysate was counted for radioactivity. The binding of ^{125}I-labeled LDL in the absence of unlabeled VLDL was defined as 100%. The concentration of competitor

VLDL protein required to displace 50% of ^{125}I-labeled LDL (IC_{50}-value) was calculated by logit-log plot analysis in order to estimate the binding affinity of competitor VLDL to the LDL receptor present on J774 macrophages.

Statistical analyses

Results are presented as the mean ± S.D. Mean differences between the control group and patient group were calculated with the Mann-Whitney U test. Differences in categorical variables between the patient group and control group were assessed with the Fisher's exact test. Differences between the patient groups on placebo and bezafibrate therapy were evaluated pairwise using the Wilcoxon paired signed-ranks test. Correlation analysis was performed using the Spearman rank correlation analysis. Findings were regarded to be statistically significant when the probability of these data under the null hypothesis was less than 0.05. Statistical analyses were performed with SPSS/PC+™ software (SPSS Inc., Chicago, IL USA).

Results

Patient characteristics

As shown in table 1, the patient and control group were comparable with regard to age, sex and hypertension. The HTG patients had a higher BMI, as expected. Anti-hypertensive medication was more frequent in the HTG group: 3 patients were on beta-blocking agents and 3 patients received a combination of β-blockers, ACE-inhibitors, diuretics or calcium-antagonists. In the control group, 1 subject was using an ACE-inhibitor as anti-hypertensive therapy. The apoE genotype distribution and apoE allele frequencies were comparable in both groups. Serum triglyceride levels were 11-fold higher in the HTG patients as compared to the control subjects. Although both LDL-C and HDL-C levels were lower in the patient group, VLDL-C concentrations were markedly elevated and accounted for the elevation in total serum cholesterol concentrations. With regard to the apolipoproteins, serum apoC-III levels were 3-fold higher in the HTG group in comparison with the control group ($p<0.001$). Interestingly, the mean post-heparin LPL activities were similar although the HTG group showed a wider variation with extreme values both in the lower and upper range (range 19-449 U/L) compared with the control group (range 81-229 U/L).

Table 1. *Baseline characteristics*

	Controls	Hypertriglyceridemic patients	p-value[a]
Number	20	18	-
Age, y	48 ± 7	49 ± 9	n.s.
Sex, M/F	18 / 2	16 / 2	n.s.
BMI, kg/m^2	24.2 ± 3.3	28.0 ± 2.8	0.001
Hypertension, yes/no	5 / 15	7 / 11	n.s.
ApoE-2 allele frequence	0.125	0.222	
ApoE-3 allele frequence	0.700	0.639	n.s.
ApoE-4 allele frequence	0.175	0.139	
TTG, mmol/L	0.93 ± 0.43	10.21 ± 4.21	<0.001
TC, mmol/L	5.10 ± 0.94	7.82 ± 1.74	<0.001
VLDL-TG, mmol/L	0.55 ± 0.36	8.55 ± 3.75	<0.001
VLDL-C, mmol/L	0.26 ± 0.18	4.45 ± 2.10	<0.001
LDL-C, mmol/L	3.52 ± 0.87	2.62 ± 0.65	0.001
HDL-C, mmol/L	1.32 ± 0.29	0.76 ± 0.16	<0.001
apoA-I, g/L	1.32 ± 0.15	1.19 ± 0.11	0.002
apoC-III, g/L	0.08 ± 0.02	0.26 ± 0.08	<0.001
Lipoprotein Lipase, U/L	152 ± 40	153 ± 128	n.s.

BMI, body mass index; TC, total cholesterol; TTG, total triglycerides; VLDL-TG, very low density lipoprotein triglycerides; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol. Values are presented as the mean ± S.D. [a]Differences in categorical variables between the patient group and control group were assessed with the Chi-square test, while differences in numerical variables were assessed with the Mann-Whitney U-test.

Effect of therapy on serum lipid and lipoprotein levels

All subjects completed the study without any side-effects. No significant changes in body weight occurred. Treatment with placebo had no effect on serum lipid levels (data not shown). The effects of bezafibrate therapy are shown in table 2. Bezafibrate therapy resulted in a significant fall in serum TG (-69%, p<0.001) and serum cholesterol levels (-24%, p<0.001). The change of VLDL-TG (-71%, p<0.001) and VLDL-C levels (-67%, p<0.001) paralleled that of plasma TG. LDL-C increased by 34% (p=0.001) from 2.67 mmol/L to 3.58 mmol/L, which is comparable to the LDL-C level of 3.52 mmol/L in the controls.

Table 2. *Effects of bezafibrate therapy on lipoprotein and apolipoprotein levels and post-heparin lipoprotein lipase activities*

	Hypertriglyceridemic Patients		Difference	p-value
	Placebo	Bezafibrate		
Number	18			
TTG, mmol/L	12.28 ± 8.06	3.86 ± 1.44	-69%	<0.001
TC, mmol/L	7.75 ± 2.37	5.91 ± 1.24	-24%	0.001
VLDL-TG, mmol/L	10.03 ± 6.11	2.89 ± 1.07	-71%	<0.001
VLDL-C, mmol/L	4.37 ± 2.02	1.43 ± 0.62	-67%	<0.001
LDL-C, mmol/L	2.67 ± 0.64	3.58 ± 0.84	+34%	0.001
HDL-C, mmol/L	0.72 ± 0.13	0.91 ± 0.13	+26%	<0.001
apoA-I, g/L	1.08 ± 0.10	1.13 ± 0.11	+4%	0.017
apoC-III, g/L	0.24 ± 0.10	0.14 ± 0.06	-42%	<0.001
LPL, U/L	153 ± 128	192 ± 95	+25%	0.025

TC, total cholesterol; TTG, total triglycerides; VLDL-TG, very low density lipoprotein triglycerides; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Apo, apolipoprotein; LPL, lipoprotein lipase. All values are mean ± S.D. based on triplicate measurements.

HDL-C and apoA-I levels increased with 26% ($p<0.001$) and 4% ($p=0.017$), respectively. Interestingly, plasma apoC-III levels showed an impressive reduction (-42%, $p<0.001$) but remained elevated in comparison to the control group (0.14 vs 0.08 g/L). Thus, the lipoprotein profile changed markedly upon bezafibrate therapy towards that found in the normolipidemic reference group.

With regard to the VLDL size and composition, HTG VLDL was significantly larger than control VLDL (62 vs. 41 nm, $p<0.001$; table 3) and normalized upon therapy. Assuming that VLDL has a spherical shape, bezafibrate induced a 2.8-fold reduction in VLDL volume. In addition, HTG VLDL was cholesteryl ester-enriched and relatively poor in protein. During bezafibrate therapy, the VLDL protein content increased towards the reference range, but the cholesteryl ester enrichment remained. The number of apoC-III molecules per VLDL particle was 2 to 3-fold higher in the HTG group on placebo therapy as was the number of apoE molecules. Correction for VLDL size revealed that the effective number of these apolipoproteins (per 1000 nm^2 of VLDL surface) was comparable in the HTG and control group, and did not change upon bezafibrate therapy.

Table 3. *VLDL characteristics and apolipoprotein distribution*

	Controls	Hypertriglyceridemic Patients	
		Placebo	Bezafibrate
Diameter (nm)	41 ± 3***	62 ± 18	44 ± 4
VLDL-TG (%)	60 ± 4	62 ± 7	62 ± 5
VLDL-FC (%)	4 ± 1	5 ± 2	4 ± 1##
VLDL-CE (%)	6 ± 2**	9 ± 2	8 ± 3
VLDL-PL (%)	15 ± 2	14 ± 2	15 ± 1
VLDL-prot (%)	14 ± 3***	9 ± 2	11 ± 3##
VLDL-apoB, g/L	0.33 ± 0.18***	1.29 ± 0.50	1.38 ± 0.62
VLDL-apoC-III, g/l	0.02 ± 0.01***	0.14 ± 0.05	0.06 ± 0.04###
apoC-III / VLDL[a]	310 ± 126***	713 ± 430	293 ± 197##
apoC-III / 1000 nm^2 VLDL[b]	59 ± 25	64 ± 58	37 ± 20
apoE / VLDL[a]	18 ± 6**	41 ± 23	12 ± 9###
apoE / 1000 nm^2 VLDL[b]	3.5 ± 1.2	3.8 ± 2.8	1.9 ± 1.4
apoC-III / E ratio	19 ± 10	20 ± 9	26 ± 7

VLDL-TG, VLDL-FC, VLDL-CE, VLDL-PL, and VLDL-prot represent the relative weights of triglycerides, unesterified cholesterol, cholesteryl esters, phospholipids and protein, respectively. All values are presented as mean ± S.D. [a]number of apolipoprotein molecules per VLDL particle. [b]number of apolipoprotein molecules per 1000 nm^2 of VLDL surface.

p<0.01,p<0.001 control subjects vs. HTG patients on placebo. ##p<0.01, ###p<0.001, bezafibrate vs. placebo therapy.*

The activity of post-heparin LPL is shown in table 1 and 2. LPL activities at baseline were comparable in the HTG and control group. Upon bezafibrate therapy, LPL activity increased with 25% from 153 U/L to 192 U/L (p=0.025), which is significantly higher than the LPL activity of the reference group. Correlation analyses showed a strong relation between the increase in post-heparin LPL activity and the decrease in serum triglyceride levels (Spearman correlation coefficient -0.62, p=0.006), as shown in figure 1.

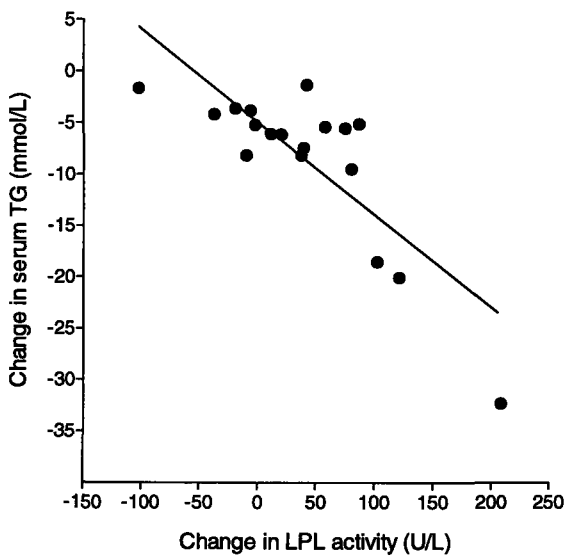

Figure 1. *Correlation between the change in post-heparin LPL activity and the change in serum TG levels in response to bezafibrate therapy (Spearman correlation coefficient: -0.62, p=0.006).*

Lipolysis of VLDL by HSPG-bound LPL

Figure 2 shows the results of the lipolysis experiments using HSPG-bound LPL. For all VLDL triglyceride concentrations applied, the lipolysis rates of VLDL, isolated from the control subjects, were higher than that of the HTG patients. The differences were most pronounced at the highest substrate concentration. Bezafibrate therapy did not affect the lipolysis rates of VLDL in the patient group. If the mean lipolysis rate of the reference samples is set at 100%, a lipolysis efficiency of 77 ± 17% is calculated for the HTG group on placebo and 75 ± 17% for the HTG group on bezafibrate therapy.

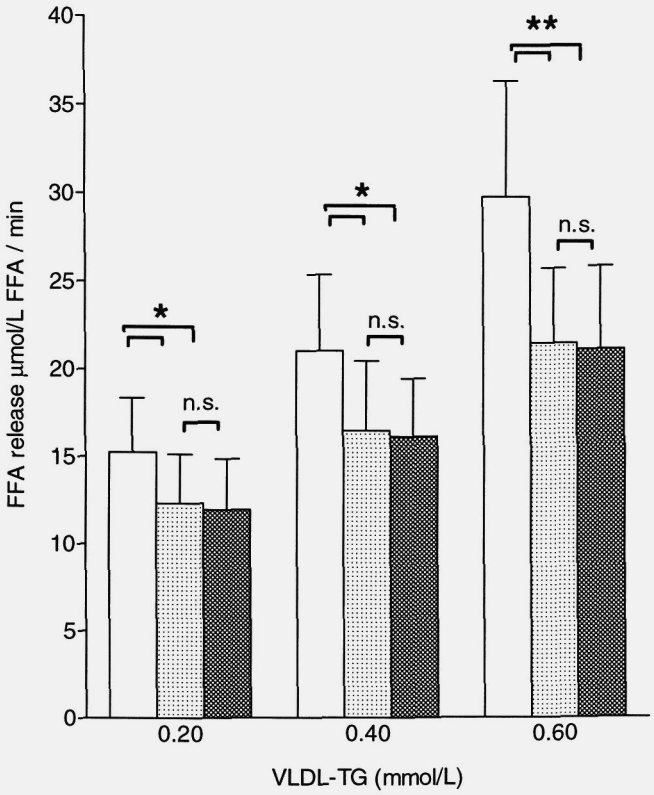

Figure 2. *Lipolysis experiments with HSPG-bound LPL were performed with VLDL samples isolated from healthy controls (□), HTG patients on placebo therapy (▒) and HTG patients on bezafibrate therapy (■). Plastic wells were preconditioned with HSPG and LPL, subsequently (Materials and Methods). After washing the preconditioned wells, lipolysis was carried out with three VLDL-TG concentrations in the presence of 1% BSA for 10 min at 37°C. The reactions were stopped by addition of Triton X-100 and cooling on ice. FFA concentrations were determined in triplicate. * $p<0.05$; ** $p<0.01$.*

LDL receptor binding assay

Competition experiments were performed to evaluate VLDL substrate affinity to the LDL receptor of J774 macrophages. As shown in figure 3, no differences were observed between controls and HTG patients on placebo therapy. In addition, bezafibrate therapy did not affect the binding affinity to the LDL receptor in the HTG group. The calculated IC_{50}-values of the control

subjects (34.6 ± 17.7 μg/mL), HTG patients on placebo (36.0 ± 25.2 μg/mL) and HTG patients on bezafibrate (53.0 ± 79.2 μg/mL) were comparable.

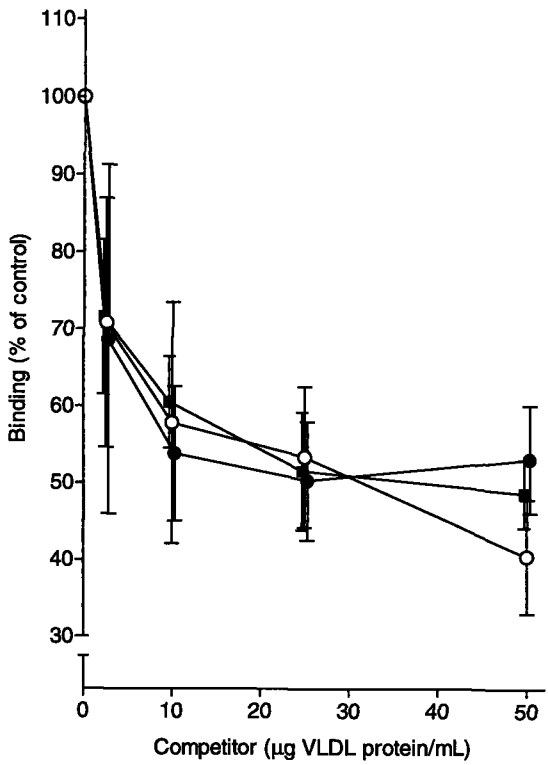

Figure 3. *Competition of VLDL, isolated from healthy control subjects (■) and HTG patients on placebo (O) and bezafibrate therapy (●), with ^{125}I-labeled control LDL for the binding to the LDL receptor in J774 macrophages. The J774 cells were incubated with 10 μg/mL ^{125}I-labeled LDL for 3 hours at 4°C in the presence of increasing quantities of unlabeled VLDL of interest as competitor. The binding of ^{125}I-labeled LDL in the absence of unlabeled VLDL was defined as 100%.*

Discussion

Although the ability of fibrates to lower plasma triglyceride levels in hyperlipidemic patients and animals is recognized for many years, the mechanism of action is not fully understood. It is known that fibrates can affect transcription rates of regulatory enzymes and apolipoproteins via activation of PPARs (19). Haubenwallner *et al.* (31) demonstrated that fibrates decrease the hepatic synthesis of apoC-III and corresponding plasma apoC-III levels. The degree of hepatic apoC-III mRNA reduction induced by different fibrates roughly correlated with the degree of triglyceride reduction. It has been speculated that fibrate therapy may reduce the

number of apoC-III molecules per VLDL particle and therefore improve VLDL suitability as substrate for lipolysis and receptor-mediated lipoprotein removal, however, this hypothesis has not been tested yet. In the present study we investigated the effect of bezafibrate on the three major components involved in VLDL metabolism, namely VLDL susceptibility to lipolysis by LPL, VLDL binding to the LDL receptor and the post-heparin LPL activity.

Since lipoprotein size and composition influence its metabolic fate, the VLDL particles were extensively characterized. We demonstrated that HTG VLDL are significantly larger than normal VLDL. In accordance with previous studies, HTG VLDL contained less protein whereas the amount of cholesteryl esters was significantly higher than control VLDL (32). Enrichment of VLDL by cholesteryl esters in HTG has been described before and is explained by the fact that high VLDL concentrations passively induce an increased transfer of TG to the LDL and HDL fraction in exchange for cholesteryl esters (33). With regard to the apolipoprotein distribution, we have focussed on apolipoproteins E and C-III as these proteins are important modulators of VLDL metabolism. HTG VLDL contained higher numbers of both apoE and apoC-III. However, the VLDL surface density of the respective molecules as well as the apoC-III to apoE ratio were comparable to those observed in control VLDL. Thus, HTG VLDL differed from control VLDL by size and composition, whereas apoC-III and apoE contents per unit of VLDL surface were comparable.

Bezafibrate therapy resulted in an impressive reduction of plasma triglyceride levels. Despite the drastic reduction in VLDL mass and size, the number of VLDL particles remained stable. In response to bezafibrate therapy, plasma apoC-III levels as well as the absolute number of apoC-III molecules per VLDL particle decreased. In order to test the hypothesis that the bezafibrate-induced reduction of apoC-III molecules per VLDL particle would result in improved lipolysis, VLDL samples obtained during placebo and bezafibrate therapy were subjected to lipolysis using HSPG-bound LPL (27). As expected, the VLDL samples isolated from the healthy volunteers showed higher lipolysis rates than the VLDL samples from the HTG subjects on placebo therapy. Although it is tempting to speculate that the high amount of apoC-III molecules on HTG VLDL is responsible for the impaired lipolysis in the HTG group, bezafibrate therapy did not result in improvement of VLDL susceptibility to lipolysis, despite a drastic reduction in the absolute number of apoC-III molecules per VLDL particle. However, if

we correct for differences in VLDL size, it appears that the effective number of apoC-III molecules per VLDL surface unit is in the same order of magnitude as in controls, and does not change upon bezafibrate therapy. Thus, fibrate therapy does not selectively decrease VLDL apoC-III but lowers apoC-III non-specifically like all other VLDL components. The low lipolysis efficiency of HTG VLDL can therefore not be explained by differences in apoC-III density. It seems more likely that the differences in VLDL composition (that persisted after bezafibrate) account for the differences in lipolysis rates between the HTG and control group. This is supported by Oschry *et al.* who demonstrated that CE-enriched VLDL particles, as in the HTG group, can not complete the lipolytic cascade into LDL efficiently (34).

VLDL binding affinity to the hepatic LDL receptor was assessed by competition experiments. No differences were observed between control VLDL and VLDL isolated from the HTG patients. In addition, bezafibrate therapy did not affect the binding of HTG VLDL to the LDL receptor. These results suggest that HTG VLDL display a normal binding affinity to the LDL receptor which is not influenced by fibrate therapy. Binding affinity of lipoproteins to the LDL receptor is, however, highly dependent upon the ratio of apoC-III to apoE on the lipoprotein (35). Transgenic mice overexpressing the apoC-III gene demonstrate high triglyceride levels, that can be normalized by simultaneous overexpressing apoE (35). Our finding that the VLDL apoC-III to apoE ratio was comparable in controls and HTG patients, both on placebo and bezafibrate therapy, sustains our observation that VLDL binding affinity to the LDL receptor is not affected by fibrate therapy. It should be emphasized, however, that other receptors may be involved in the catabolism of TG-rich lipoproteins, particularly LRP and the VLDL receptor. Therefore, additional competition experiments were performed in HepG2 cells with labeled VLDL, thereby focusing on the whole scala of hepatic receptors that recognize VLDL. In accordance with the competition experiments with LDL in macrophages, no differences were observed between the different groups (data not shown). These results are in line with the current concept that VLDL and VLDL-derived particles preferentially bind to the LDL receptor (36,37). Since LRP mainly binds chylomicron remnants, its contribution to the clearance of VLDL is considered to be limited (37,38). The VLDL receptor is located predominantly in extra-hepatic tissues such as adipose tissue and muscle and therefore will not contribute to the hepatic VLDL clearance.

Our hypothesis that bezafibrate therapy would improve VLDL suitability to lipolysis and LDL receptor binding by reducing apoC-III production was rejected in the present study. The primary working mechanism of bezafibrate in humans appears to be the increase in LPL activity. However, a reduction in VLDL-TG production may partly account for the hypotriglyceridemic effect of bezafibrate, as has been found previously in rats (31). A limitation of the present study is the lack of information about the quantity of LPL at the lipoprotein surface. It is generally accepted that addition of LPL to lipoproteins stimulates the binding affinity to lipoprotein receptors many-fold (39,40). Since fibrates can upregulate LPL transcription, one may speculate that this would lead to a higher amount of surface LPL. In the present study, however, no increased binding of VLDL to either macrophages or hepatocytes was observed in response to fibrates, suggesting that the amount of surface LPL was not changed substantially.

In conclusion, this study is the first to demonstrate that the primary effect of bezafibrate on VLDL catabolism may be attributed to upregulation of LPL activity, whereas VLDL susceptibility to lipolysis and VLDL binding to the LDL receptor are not affected.

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Chapter 6

Normal F2-Isoprostane Levels and Enhanced Lipoprotein Resistance to *In Vitro* Oxidition in Patients with Hypertriglyceridemia: Effects of Bezafibrate Treatment

Frits H.A.F. de Man[1], Arnoud van der Laarse[1], Augustinus H.M. Smelt[2], Wim van Duyvenvoorde[4], Rien Buytenhek[4], Jan A. Gevers Leuven[2,4], Willem Onkenhout[3] and Hans M.G. Princen[4]

Departments of [1]Cardiology, [2]Internal Medicine, and [3]Pediatrics, Leiden University Medical Center; [4]Gaubius Laboratory, TNO-PG, Leiden, The Netherlands

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Summary

Introduction. Although there is evidence that hyperlipidemia and predominance of small dense LDL are associated with increased oxidative stress, the oxidation status in patients with hypertriglyceridemia has not been studied in detail.

Methods. Plasma levels of F2-isoprostanes and susceptibility of VLDL and LDL to oxidation *in vitro* was studied in 18 patients with endogenous hypertriglyceridemia in comparison with 20 age- and sex-matched control subjects. In addition, the effects of 6 week treatment with bezafibrate were assessed in a double-blind, placebo-controlled, cross-over trial.

Results. Plasma levels of free F2-isoprostanes were comparable in the hypertriglyceridemic (37 ± 10 ng/mL) and normolipidemic group (41 ± 11 ng/mL, n.s.) and were not changed by fibrate therapy. Both VLDL and LDL of hypertriglyceridemic patients were more resistant to Cu^{2+}-induced oxidation and showed a lower oxidation rate compared to the control group. These differences appear to be related to differences in the ratio of polyunsaturated to saturated fatty acids. VLDL and LDL in hypertriglyceridemia presented a relative paucity of unsaturated fatty acids. Bezafibrate therapy reversed the oxidation resistance to the normal range, but did not influence the oxidation rate.

Conclusions. These results indicate that hypertriglyceridemia is associated with normal *in vivo* oxidative stress and enhanced *ex vivo* resistance of lipoproteins to oxidation.

Introduction

Hypertriglyceridemia has been recognized as an independent risk factor for cardiovascular disease in both men and women (1-3). This multifactorial lipid disorder is characterized by elevated levels of very low density lipoproteins (VLDL), low high density lipoprotein cholesterol (HDL-C) levels, small dense low density lipoproteins (LDL) and insulin resistance. Small dense LDL particles have been suggested to be prone to oxidative modification and may contribute to the increased cardiovascular risk in hypertriglyceridemia (4,5). There is evidence that several forms of hyperlipidemia are associated with increased oxidative stress (for review, see (6)). However, no studies have addressed the oxidation status in patients with hypertriglyceridemia in detail.

To date, oxidation can be assessed by determination of *in vivo* oxidation products or measurement of *ex vivo* oxidation behavior of lipoproteins. *Ex vivo*, the peroxidation process can be mimicked by incubating isolated lipoproteins with the pro-oxidant Cu^{2+} and by measuring the production of conjugated dienes from polyunsaturated fatty acids. In previous studies, we detected subtle changes in susceptibility of lipoproteins to oxidation after supplementation with vitamin E and fish oil (7,8). Although this method provides a precise assessment of the oxidation behavior of distinct lipoprotein fractions *ex vivo*, direct measurement of oxidation products is considered to be more indicative of *in vivo* oxidative stress. F2-isoprostanes have emerged as a promising marker of oxidative stress (for review, see (9)). In contrast to lipid hydroperoxides, F2-isoprostanes are chemically stable end-products of lipid peroxidation and present in significant concentrations in human plasma and urine (9). This class of prostaglandin $F_{2\alpha}$-isomers results from oxidative modification of arachidonic acid through a nonenzymatic, free radical-catalyzed mechanism. Both *in vitro* and *in vivo* studies have demonstrated that oxidative stress results in a dose-dependent elevation of F2-isoprostane levels (10,11). Previous studies have demonstrated increased F2-isoprostane levels in smokers (12), diabetics (13) and patients with hypercholesterolemia (14,15). However, to date there are no studies available that address F2-isoprostane levels in hypertriglyceridemia.

The present study was undertaken to compare plasma levels of F2-isoprostanes and susceptibility of VLDL and LDL to oxidation *in vitro* between patients with endogenous hypertriglyceridemia and control

subjects. In addition, the effects of triglyceride-lowering therapy by bezafibrate were studied.

Materials and Methods

Patients and control subjects

The study population consisted of 18 unrelated patients, 16 males and 2 females, with endogenous hypertriglyceridemia who were recruited from the outpatient lipid clinic of the Leiden University Medical Center. All patients received dietary advise according to "Guidelines for a Healthy Diet" from the Netherlands Nutrition Council as first-line therapy (16). The diagnosis endogenous hypertriglyceridemia was based on the means of two fasting blood samples obtained after the dietary period of at least 8 weeks. The diagnostic criteria for endogenous hypertriglyceridemia were: total serum TG > 4.0 mmol/L, VLDL-C > 1.0 mmol/L, and LDL-C < 4.5 mmol/L. Additional exclusion criteria were homozygosity for apoE2, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/L, and alcohol consumption of more than 40 g/day) and the use of lipid-lowering drugs. Patients with a medical history of cardiovascular disease were not included in the study. Eighteen normolipidemic, age- and sex-matched control subjects, 16 males and 2 females, were recruited in response to a newspaper advertisement. No subjects took vitamin supplementation during the study.

Study design

The patients were randomized to receive in a double-blind cross-over fashion a fixed dose of bezafibrate, 400 mg once daily, or placebo for 6 weeks. The two periods in which medication was taken were separated by a wash-out period of 6 weeks. Before and after each treatment period of 6 weeks, fasting venous blood samples were obtained from the participants for lipid measurements. From the control subjects, fasting blood samples were obtained at baseline under identical conditions. All tubes were kept on ice after sampling. Serum tubes were allowed to clot for 30 min at room temperature. Informed consent was obtained from each patient and the protocol was approved by the institutional Medical Ethics Committee.

Lipid and lipoprotein analyses

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Three mL of fresh serum was ultracentrifuged for 15 hours at 232,000 g at 15°C in a TL-100 tabletop Ultracentrifuge, using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA USA). The ultracentrifugate was carefully divided in a density (d) < 1.006 and d 1.006 - 1.25 g/mL fraction, designated as the VLDL and LDL-HDL fraction, respectively. HDL cholesterol was measured in the LDL-HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and $MgCl_2$.

Triglyceride, total cholesterol, phospholipid and free cholesterol concentrations were measured enzymatically using commercially available kits (Boehringer, Mannheim, Germany). Cholesteryl ester (CE) content was calculated by subtracting the amount of free cholesterol from the concentration of total cholesterol. Protein was determined by the method of Lowry *et al.* (17). VLDL diameter was determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, U.K.). Measurements were performed at 25°C and a 90° angle between laser and detector. LDL particle size was analyzed by gradient gel electrophoresis, as described by Mc Namara *et al.* (18) using 2-16% nondenaturing polyacrylamide gradient gels (Pharmacia LKB, Uppsala, Sweden) and a set of standard proteins with known hydrated diameters (high molecular weight marker electrophoresis calibration kit, Pharmacia, Piscataway, NJ, USA).

Fatty acid composition was determined by gas chromatography on an Interscience 8160 gas chromatograph fitted with a cold on-column injector and a CP-Sil88 fused silica capillary column (Chrompack, Bergen op Zoom, the Netherlands) after methylation of the fatty acids (19). The total number of double bonds in VLDL and LDL equaled the relative content of each FA with two or more double bonds times its number of double bonds. Monounsaturated FAs were not included in the calculation as they are less susceptible to oxidation than polyunsaturated fatty acids.

The vitamin E contents of plasma, VLDL and LDL was assessed by high-performance liquid chromatography with UV detection. ApoE phenotyping was performed by isoelectric focusing according to Havekes *et al.* (20).

Measurements of free F2-isoprostanes

Fasting venous blood was drawn in ice-cold Vacutainer tubes containing EDTA and centrifuged for 15 min at 1500 g at 4°C. The plasma was carefully isolated and a mixture of the following anti-oxidants was added to each plasma sample: reduced L-glutathione (final concentration 2 mg/mL; Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands), ascorbic acid (final concentration 1 mg/mL), butylated hydroxytoluene (final concentration 1.5 mg/mL) and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (OH-TEMPO, final concentration 1.5 mg/mL). The samples were gently mixed, capped under nitrogen, submerged in liquid nitrogen and stored at -80°C. Storage at -80°C does not affect F2-isoprostane levels (21).

The samples were processed within 6 months as one batch. The sample purification consisted of a protein precipitation and a solid phase extraction. After rapid thawing at 37°C, aliquots of 300 µl were put on ice and 25 µl tritium-labeled prostaglandin $F_{2\alpha}$ ([^{3}H]-PG $F_{2\alpha}$, 5000 cpm) was added as recovery standard. Subsequently, 1 mL ethanol was added, the samples were vortexed and allowed to stand on ice for 5 min. The samples were centrifuged at 4°C for 10 min at 15,000 rpm. The supernatant was carefully isolated and 8 mL formic acid-acidified water (pH=3.0) was added. Solid phase columns (Waters-Sep-Pak-C18 cartridges, Millipore Co./Waters, Milfort, MA, USA) were activated by rinsing with 5 mL methanol and 5 mL purified water. The sample was brought onto the column, followed by rinsing with 5 mL purified water and 5 mL hexane, subsequently. The F2-isoprostanes were eluted with 5 mL ethylacetate, containing 1% methanol. The solvent was evaporated by vacuum centrifugation and the samples were carefully dissolved in enzyme immunoassay buffer (Cayman Chemicals, Ann Arbor, MI, USA) at 4°C for at least 2 hours. Finally, a competitive enzyme immunoassay (EIA) was performed with a F2-isoprostane-acetylcholinesterase conjugate (tracer) and Ellman's reagent, as described by the manufacturer (Cayman Chemicals). The plates were read at 412 nm in a spectrophotometer. The recovery was calculated by dividing the radioactivity in the EIA sample by the radioactivity in the original sample. All samples were measured in triplicate. The variation coefficient was 13%.

Oxidation of VLDL and LDL

Fasting venous blood was drawn in ice-cold Vacutainer tubes containing EDTA and centrifuged within 1 hour for 15 min at 1500 g at 4°C. The plasma samples were brought to a final concentration of 10% (w/v) sucrose, capped

under nitrogen, submerged in liquid nitrogen and stored at -80°C. Under these conditions, lipoprotein characteristics and oxidation behavior have been shown to remain stable (22,23). The samples were analyzed within 6 months. Lipoproteins were separated by ultracentrifugation at 4°C, using standard methods adapted from Redgrave *et al.* (24) as described previously (8). Cu^{2+}-induced lipoprotein oxidation was measured by serial measurement of the conjugated dienes formed. A detailed description of the procedure and lipid peroxidation of LDL has been given before (7,23). The same procedure was applied to VLDL, with the exception that a lower protein concentration (0.03 mg/mL) was used in the oxidation assay to avoid turbidity (8). The formation of conjugated dienes was measured by continuously monitoring the change in absorbance at 234 nm in a spectrophotometer at 37°C. The time-dependent absorption curve can be divided in 3 distinct phases: A lag phase, during which absorption hardly increases, indicative for the lipoprotein resistance to oxidation. This is followed by a propagation phase, during which absorbance increases rapidly to a maximum value. And finally, after reaching the maximum value the conjugated dienes slowly decrease by decomposition to aldehydes. To quantify these phases, a tangent was drawn to the steep part of the curve and extrapolated to the horizontal (time) axis. The interval between the addition of Cu^{2+} and the intersection point is defined as the lag time and expressed in min. The propagation phase equals the slope of the tangent of the propagation phase and is expressed as nanomoles of dienes formed per minute per milligram of VLDL or LDL protein. The oxidation maximum was defined as the total quantity of conjugated dienes in nanomoles formed per milligram of VLDL or LDL protein. The VLDL and LDL samples of a control subject and a patient, during placebo and bezafibrate therapy, were oxidized on the same day in 3 consecutive oxidation runs.

Statistical analyses

Results are presented as the mean ± S.D. Mean differences between the control group and the patient group were calculated with the Mann-Whitney U test. Differences in categorical variables between the patient group and control group were assessed with the Fisher's exact test. Differences between the patient group on placebo and bezafibrate therapy were evaluated pairwise using the Wilcoxon paired signed-ranks test. Findings were regarded to be statistically significant when the probability of these data under the null hypothesis was less than 0.05. Statistical analyses were performed with SPSS/PC+™ software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

As shown in table 1, the patient and control groups were comparable with regard to age, sex, hypertension and smoking habits. The HTG patients had a higher BMI, as expected. Serum triglyceride levels were 13-fold higher in the HTG patients as compared to the control subjects ($p<0.001$, table 2). Although both LDL-C and HDL-C levels were lower in the patient group, VLDL-C concentrations were markedly elevated and accounted for the elevation in total serum cholesterol concentrations.

Table 1. *Baseline characteristics*

	Controls	Hypertriglyceridemic patients	p-value[a]
Number	20	18	n.s.
Age, y	47.9 ± 7.4	48.5 ± 8.8	n.s.
Sex,M/F	18 / 2	16 / 2	n.s.
BMI, kg/m^2	24.2 ± 3.3	28.0 ± 2.8	0.001
Hypertension, yes/no	5 / 15	7 / 11	n.s.
Smoking, yes/no	2 / 18	7 / 11	n.s.

Values are presented as the mean ± S.D. [a]Differences in categorical variables between the patient group and control group were assessed with the Fisher's exact test, while differences in numerical variables were assessed with the Mann-Whitney U-test.

Effect of bezafibrate therapy on serum lipids and lipoproteins

All subjects concluded the study without any side-effects. No significant changes in body weight occurred. Treatment with placebo had no effect on serum lipid levels (data not shown). Therefore, only the values obtained at the end of both treatment periods were compared (table 2).

Bezafibrate therapy resulted in a significant fall in serum TG (-69%, $p<0.001$) and serum cholesterol levels (-24%, $p<0.001$). The change of VLDL-C levels (-67%, $p<0.001$) paralleled that of plasma TG. LDL-C increased with 34% ($p=0.001$) from 2.67 mmol/L to 3.58 mmol/L, which is comparable to the corresponding value of 3.52 mmol/L in the controls. HDL-C increased with 26% ($p<0.001$). Thus, the lipoprotein profile changed markedly upon bezafibrate therapy towards that found in the normolipidemic reference group.

Table 2. *Serum lipids and lipoproteins in control subjects and hypertriglyceridemic patients*

	Controls	Hypertriglyceridemic Patients	
		Placebo	Bezafibrate
TTG, mmol/L	0.93 ± 0.43***	12.28 ± 8.06	3.86 ± 1.44###
TC, mmol/L	5.10 ± 0.94***	7.75 ± 2.37	5.91 ± 1.24##
VLDL-C, mmol/L	0.26 ± 0.18***	4.37 ± 2.02	1.43 ± 0.62###
LDL-C, mmol/L	3.52 ± 0.87**	2.67 ± 0.64	3.58 ± 0.84##
HDL-C, mmol/L	1.32 ± 0.29***	0.72 ± 0.13	0.91 ± 0.13###

*TC, total cholesterol; TTG, total triglycerides; VLDL-C, very low density lipoprotein cholesterol, LDL-C, low density lipoprotein cholesterol, HDL-C, high density lipoprotein cholesterol. All values are mean ± S.D. based on triplicate measurements. *p < 0.05; ** p < 0.01; *** p < 0.001 comparing the control group with the HTG group on placebo therapy. #p < 0.05; ## p < 0.01; ### p < 0.001 comparing the HTG group on placebo therapy with the HTG group on bezafibrate therapy.*

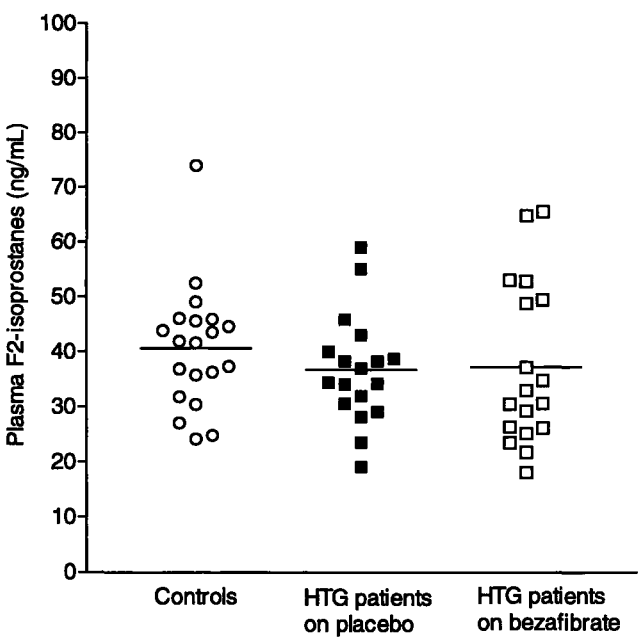

Figure 1. Plasma F2-isoprostane levels. *Levels of free circulating F2-isoprostane levels in control subjects (○; n=20), and hypertriglyceridemic patients on placebo (■, n=18) and bezafibrate therapy (□, n=18). Each dot represents a different subject. The small line represents the mean of the respective group. No significant differences were observed between the groups.*

F2-isoprostane levels and lipoprotein oxidation parameters

Free F2-isoprostanes. As shown in table 3 and figure 1, the levels of free F2-isoprostanes in plasma from patients with hypertriglyceridemia (37 ± 10 ng/mL) were comparable to those measured in the well-matched control subjects (41 ± 11 ng/mL, n.s.). A 6-week treatment with bezafibrate 400 mg once daily did not change the free F2-isoprostane levels in the patient group.

Table 3. *F2-Isoprostane Levels, in Vitro Lipoprotein Oxidizability and vitamin E levels*

	Controls	Hypertriglyceridemic patients	
		Placebo	Bezafibrate
	n=20	n=18	
Free F2-isoprostanes, ng/mL	41 ± 11	37 ± 10	37 ± 15
VLDL			
lag phase[a]	143 ± 30***	226 ± 43	195 ± 43#
propagation rate[b]	12.0 ± 3.1*	10.0 ± 3.0	9.7 ± 3.5
maximal dienes[c]	1098 ± 227**	1459 ± 397	1192 ± 238##
LDL			
lag phase	89 ± 7***	108 ± 17	95 ± 9#
propagation rate	12.1 ± 1.1***	9.5 ± 1.8	9.9 ± 1.9
maximal dienes	539 ± 39***	406 ± 50	468 ± 44##
Plasma vitamin E, μmol/L	26.9 ± 5.7***	93.3 ± 33.5	50.1 ± 13.0###

*[a]in min; [b]in nmol dienes.min^{-1}.mg protein^{-1}, [c]in nmol dienes.mg protein^{-1}. All values are mean ± S.D. based on triplicate measurements. *p < 0.05; **p < 0.01, *** p < 0.001 comparing the control group with the HTG group on placebo therapy. #p < 0.05, ##p < 0.01, ###p < 0.001 comparing the HTG group on placebo therapy with the HTG group on bezafibrate therapy.*

VLDL oxidation. Oxidation characteristics of VLDL and LDL are presented in table 3 and figure 2. The lag time of VLDL oxidation in the patient group (226 ± 43 min) was higher than in the control group (143 ± 30 min, p<0.001). The propagation rate of VLDL oxidation in the patient group (10.0 ± 3.0 nmol dienes.min^{-1}.mg protein^{-1}) was lower than that in the control group (12.0 ± 3.1 nmol dienes.min^{-1}.mg protein^{-1}, p=0.04). On the other hand, the maximum diene formation was elevated in the HTG group (1459 ± 397 nmol dienes.mg protein^{-1}) as compared to the control group (1098 ± 227 nmol dienes.mg protein^{-1}, p=0.002). Bezafibrate therapy induced a significant

reduction in the lag time and maximum diene production towards the normal range. The propagation rate of VLDL was not affected by fibrate therapy.

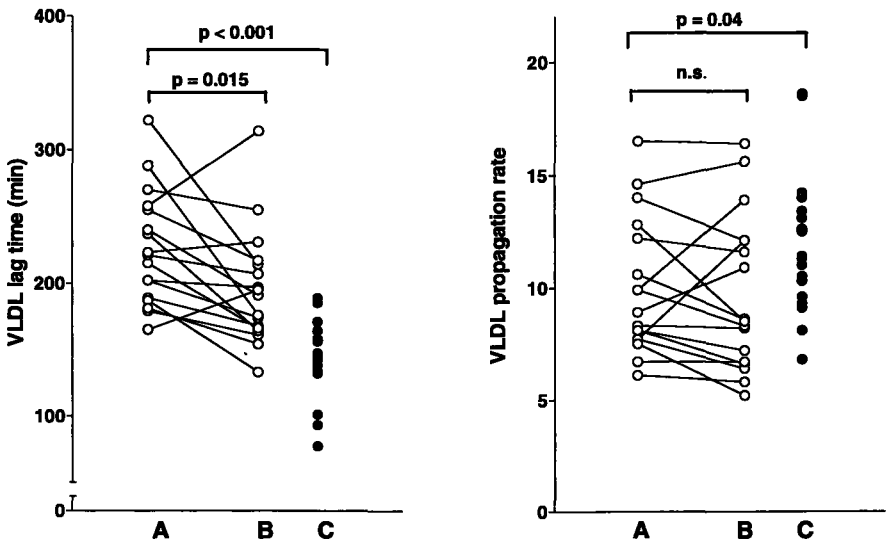

Figure 2A. Ex vivo lipoprotein oxidizability. *The VLDL lag times and propagation rates are shown. Group A, B and C represent the hypertriglyceridemic patients on placebo, the hypertriglyceridemic patients on bezafibrate and the control subjects, respectively.*

LDL oxidation. Oxidation characteristics of LDL paralleled that of VLDL. The lag time of LDL oxidation in the patient group (108 ± 17 min) was higher compared to the control group (89 ± 7 min, $p<0.001$). The propagation rate of LDL oxidation in the patient group (9.5 ± 1.8 nmol dienes.min^{-1}.mg protein^{-1}) was lower than that in the control group (12.1 ± 1.1 nmol dienes.min^{-1}.mg protein^{-1}, $p<0.001$). The maximum diene formation was lower in the patient group (406 ± 50 nmol dienes.mg protein^{-1}) as compared to the control group (539 ± 39 nmol dienes.mg protein^{-1}, $p<0.001$). Upon bezafibrate therapy, the lag time of LDL oxidation was reduced from 108 ± 17 to 95 ± 9 min ($p=0.02$), whereas the propagation rate did not change. The maximum diene formation increased from 406 ± 50 to 468 ± 44 nmol dienes.mg protein^{-1} ($p=0.001$).

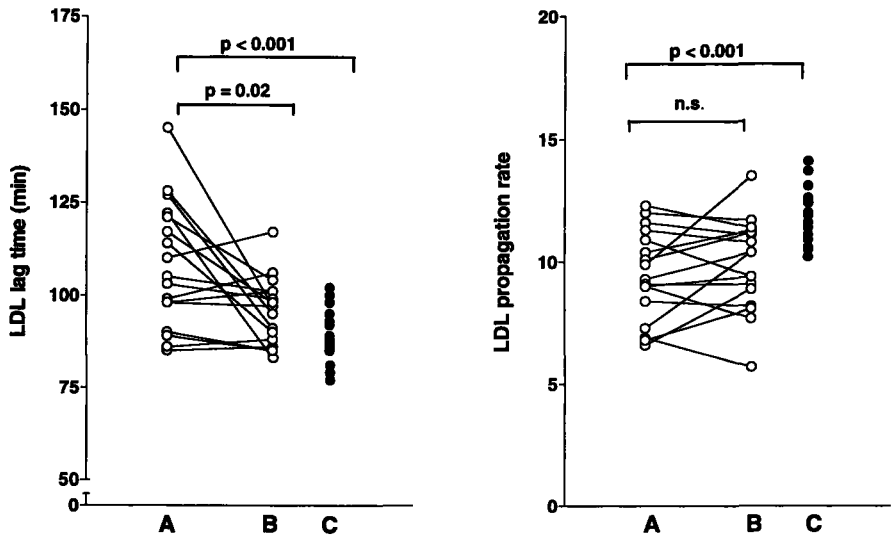

Figure 2B. Ex vivo lipoprotein oxidizability. *The LDL lag times and propagation rates are shown. Group A, B and C represent the hypertriglyceridemic patients on placebo, the hypertriglyceridemic patients on bezafibrate and the control subjects, respectively.*

Determinants of *ex vivo* oxidation parameters

To investigate which variables were determinants of the differences in *ex vivo* oxidation parameters between the patient and control groups, lipoprotein and fatty acid composition and α-tocopherol content were analyzed.

VLDL oxidation. As shown in table 4, there were marked differences in VLDL size and composition between the patient and control groups. VLDL particle size correlated with the lag time ($r=0.65$, $p<0.001$) and maximum diene formation ($r=0.52$, $p=0.001$). As expected, the large VLDL particle size in hypertriglyceridemia was associated with an increased α-tocopherol quantity, which decreased upon bezafibrate therapy. The α-tocopherol content of VLDL tended to correlate with the lag time ($r=0.36$, $p=0.068$).

VLDL of the hypertriglyceridemic group contained more saturated fatty acids and less polyunsaturated fatty acids than the control group. In accordance, the ratio of polyunsaturated to saturated fatty acids was lower in the patient group (0.71 ± 0.19) compared to the control group (0.96 ± 0.30, $p=0.04$). The contribution of the individual fatty acids is presented in figure 3. HTG VLDL contained more palmitic acid (C16:0) and stearic acid (C18:0) than control VLDL, whereas the amount of γ-linolenic acid (C18:3 ω6), docosapentaenoic acid (C22:5 ω3) and docosahexaenoic acid (C22:6 ω3) was

lower compared to control VLDL. Bezafibrate therapy did not alter the relative contribution of saturated and polyunsaturated fatty acids, nor the number of double bonds.

In accordance with a previous report (8), the lag time of VLDL oxidation was inversely correlated to the total number of double bonds (pooled data: $r=-0.72$, $p<0.001$; patient group on placebo: $r=-0.57$, $p=0.021$; control group: $r=-0.65$, $p=0.022$). In addition, a positive correlation was found between the propagation rate of VLDL oxidation and the total number of double bonds (pooled data: $r=0.78$, $p<0.001$; patient group on placebo: $r=0.74$, $p=0.001$; control group: $r=0.59$, $p=0.045$). These results indicate that the differences in VLDL oxidation parameters between the patient group and control group are related to differences in fatty acid composition.

LDL oxidation. The LDL particles of the hypertriglyceridemic patients were significantly smaller (23.5 ± 0.6 nm) than control LDL (25.2 ± 0.7, $p<0.001$; table 4). Bezafibrate therapy resulted in an increase of LDL particle size from 23.5 ± 0.6 nm to 24.4 ± 1.1 nm ($p=0.003$). The α-tocopherol content was comparable in the different study groups and did not change upon bezafibrate therapy. No significant correlations were noted between LDL size, composition and any of the oxidation parameters in both groups.

The fatty acid composition of LDL demonstrated differences between the patient and control groups that were in line with the VLDL fatty acid composition. LDL of the patient group contained more saturated fatty acids ($p=0.018$) and less polyunsaturated fatty acids ($p=0.001$) than the control group. In accordance, the ratio of polyunsaturated to saturated fatty acids was lower in the patient group (1.45 ± 0.45) compared to the control group (1.66 ± 0.21, $p=0.01$). HTG LDL showed a tendency to more myristic acid (14:0), palmitic acid (C16:0) and stearic acid (C18:0) than control LDL (figure 3). However, these differences did not reach statistical significance. The low amount of polyunsaturated fatty acids in HTG LDL was mainly attributable to linoleic acid (C18:2 ω6). In accordance with VLDL, bezafibrate therapy did not affect the ratio of polyunsaturated to saturated fatty acids nor the number of double bonds. The lag time of LDL oxidation was inversely correlated with the total number of double bonds only in the group of pooled data ($r=-0.65$, $p<0.001$). No correlations were found in the individual groups (patient group on placebo: $r=-0.30$, $p=0.338$; control group: $r=-0.39$, $p=0.165$).

Table 4. *Lipoprotein size and composition*

	VLDL			LDL		
	Controls	Hypertriglyceridemic Patients		Controls	Hypertriglyceridemic Patients	
		Placebo	Bezafibrate		Placebo	Bezafibrate
Particle size, nm	40.9 ± 3.0***	62.2 ± 17.6	44.4 ± 4.3###	25.2 ± 0.7***	23.5 ± 0.6	24.4 ± 1.1##
FC, mass (%)	4.1 ± 1.4	5.4 ± 2.2	4.3 ± 1.1##	6.5 ± 3.2	7.2 ± 1.9	11.0 ± 4.1##
CE, mass (%)	6.3 ± 2.1**	9.1 ± 2.5	8.1 ± 2.8	32.3 ± 5.7	28.2 ± 9.8	24.8 ± 11.0
TG, mass (%)	60.4 ± 4.3	61.8 ± 6.5	61.8 ± 4.9	6.1 ± 3.6	10.2 ± 8.5	7.9 ± 7.4##
PL, mass (%)	15.2 ± 2.2	14.4 ± 2.0	15.1 ± 1.4	24.3 ± 4.1**	16.5 ± 8.9	20.9 ± 7.6##
Prot, mass (%)	14.0 ± 2.6***	9.3 ± 2.3	10.7 ± 2.5##	30.9 ± 6.2*	37.9 ± 11.9	35.4 ± 11.4
Vit E/protein, μmol/g	19.1 ± 12.9*	30.2 ± 13.6	22.4 ± 12.3#	12.2 ± 3.7	10.9 ± 3.8	10.1 ± 1.6
SFAs, %	34.6 ± 4.4**	39.4 ± 3.0	39.5 ± 3.3	30.4 ± 2.2*	32.9 ± 2.3	31.8 ± 3.1
MUFAs, %	33.3 ± 4.5	33.0 ± 3.3	34.9 ± 2.8#	19.6 ± 2.6*	23.6 ± 3.6	24.0 ± 3.9
PUFAs, %	32.2 ± 6.7	27.6 ± 5.4	25.6 ± 4.5	50.0 ± 3.4**	43.5 ± 4.9	44.2 ± 5.9
Double bonds, %[a]	85.0 ± 16.9*	69.9 ± 12.1	67.6 ± 11.6	126.9 ± 9.1**	113.5 ± 15.0	115.2 ± 15.6

*All values are presented as mean ± S.D. PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid. FC, CE, TG, PL, and prot represent the relative weights of unesterified cholesterol, cholesteryl esters, triglycerides, phospholipids and protein, respectively. [a]The total number of double bonds in VLDL and LDL equaled the relative content of each FA with 2 or more double bonds times its number of double bonds. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing the control group with the HTG group on placebo therapy. #$p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ comparing the HTG group on placebo therapy with the HTG group on bezafibrate therapy.*

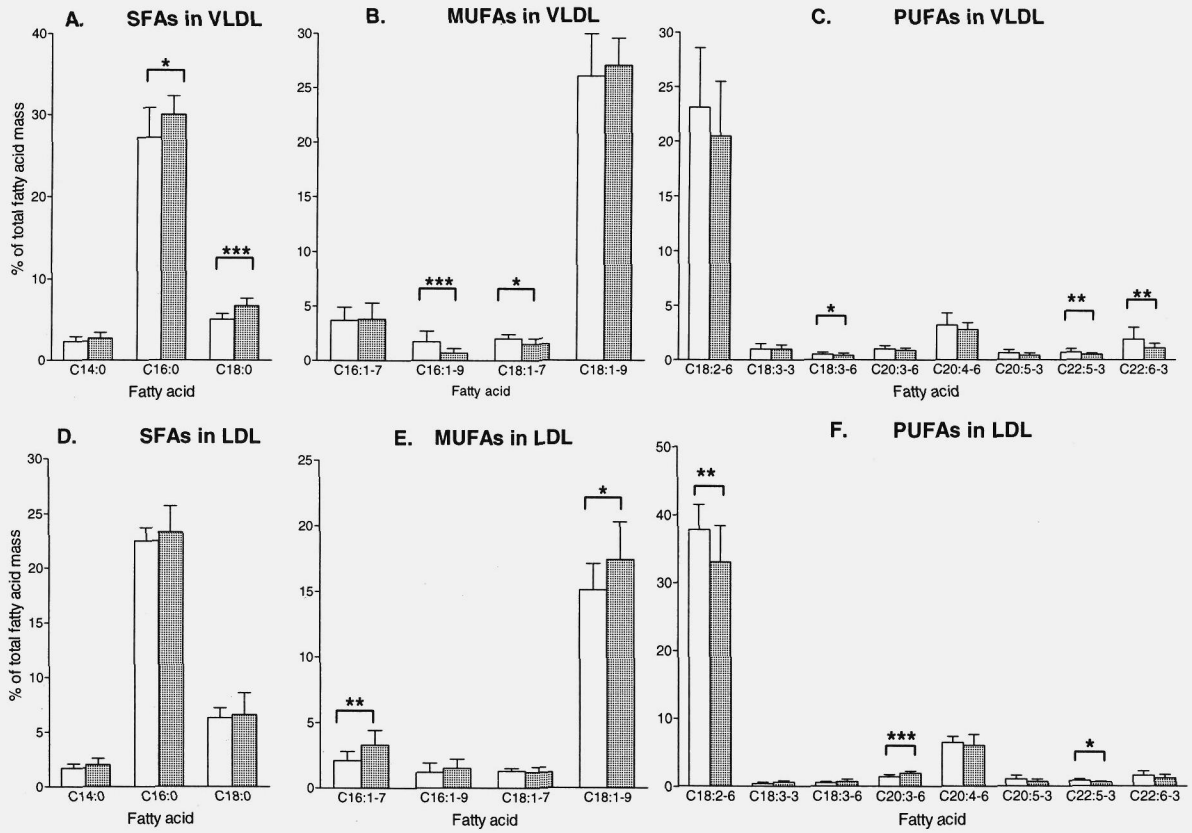

Figure 3. Fatty acid distribution. *Comparison of fatty acid distribution of VLDL and LDL between control subjects (□) and hypertriglyceridemic patients (▩). Results ar expressed as molar percentages (mean ± S.D.). The upper pannels (A, B and C) represent the VLDL fatty acid composition, whereas the lower panels (D, E and F) show the LDL fatty acid composition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing the control group with the hypertriglyceridemic group on placebo therapy by the Mann-Whitney U test.*

In accordance with VLDL, a correlation was noted between the propagation rate of LDL oxidation and the total number of double bonds (pooled data: r=0.61, p=0.001; patient group on placebo: r=0.64, p=0.025; control group: r=-0.11, p=0.970).

Discussion

The present study was designed to gain more insight in the oxidation status of patients with hypertriglyceridemia. Therefore, *in vivo* oxidation products as well as *ex vivo* oxidation behavior of lipoproteins were assessed. F2-isoprostanes have emerged as a sensitive, novel marker of oxidative stress in humans (9). Our finding that plasma levels of free F2-isoprostanes were comparable between hypertriglyceridemic patients and age- and sex-matched normolipidemic control subjects provides compelling evidence that oxidative stress in patients with hypertriglyceridemia is not increased. This conclusion is strengthened by the observation that vigorous triglyceride-lowering therapy by bezafibrate did not affect the F2-isoprostane levels. This finding is in sharp contrast with hypercholesterolemia which has been associated with increased levels of F2-isoprostanes (14,15).

In the present study, we observed 3.5-fold higher plasma α-tocopherol levels in the hypertriglyceridemic patients compared to controls. Vitamin E, or α-tocopherol, is a lipophylic anti-oxidant compound that is carried by plasma lipoproteins (25,26) and hyperlipoproteinemias have been associated with increased plasma levels of α-tocopherol (26). It may be hypothesized that the high α-tocopherol levels and the relative enrichment in saturated fatty acids in hypertriglyceridemia protect against the increased oxidative stress that is associated at least with elevated plasma cholesterol levels (14,15), which are also observed in hypertriglyceridemic patients. On the other hand, the plasma cholesterol levels in hypertriglyceridemic patients are only mildly elevated as compared to the hypercholesterolemic patients described by Davi *et al.* (14) and Reilly *et al.* (15) and distributed differently among lipoproteins, leaving open the possibility that there is no increased oxidative stress in hypertriglyceridemia. Although we have demonstrated normal oxidative stress in hypertriglyceridemia *in vivo*, we can not rule out the possibility that the oxidation status in the subendothelial space may be different from plasma. Macrophages, isolated from hypertriglyceridemic

individuals, have shown an increased superoxide production upon stimulation with oxidative compounds compared to control macrophages (27,28).

In order to estimate the oxidizability of separate lipoprotein fractions in hypertriglyceridemia, we determined *ex vivo* oxidation characteristics in response to Cu^{2+}. Both VLDL and LDL in the hypertriglyceridemic group demonstrated prolonged oxidation resistance times (lag times) and lower oxidation rates (propagation times) in comparison with the control group. These results are considered to be indicative of an increased resistance to oxidative stress in hypertriglyceridemia. The maximum diene production appeared to be a reflection of the quantity of oxidizable lipid per lipoprotein, resulting in a higher maximum for VLDL and a lower maximum for LDL in the hypertriglyceridemic group as compared to the control group.

The α-tocopherol contents of the isolated lipoproteins paralleled lipoprotein size. Although α-tocopherol is regarded as a strong lipoprotein-carried anti-oxidant, a relatively weak correlation was noted between the α-tocopherol content and the lag time in VLDL, whereas no correlation was noted in the LDL fraction. The latter is in accordance with numerous reports showing the same results in unsupplemented healthy control groups (7,25,29-31). It was demonstrated that the degree of unsaturation of fatty acids is a more important determinant of the susceptibility of lipoproteins to oxidation (31-35). In accordance with previous work of our group (8), we observed strong correlations between the number of double bonds in the lipoprotein-fatty acids and oxidation parameters. Unexpectedly, we found a different fatty acid distribution between the hypertriglyceridemic and control group. Both VLDL and LDL, isolated from the hypertriglyceridemic patients, showed a higher relative amount of saturated fatty acids and a lower relative amount of polyunsaturated fatty acids than the control subjects. Consequently, there was a relative paucity of double bonds in the hypertriglyceridemic patients that may explain the higher resistance of VLDL and LDL to oxidative stress compared to the control subjects.

The relative enrichment in saturated fatty acids in lipoproteins of the hypertriglyceridemic group was a rather unexpected finding that has not been reported before as far as we know. So far, only one study has addressed this issue. Agheli et al. (36) reported a normal fatty acid composition in hypertriglyceridemia, however, the study population consisted of only 5 patients. Differences in the dietary fatty acid composition do not appear to be a plausible explanation. The hypertriglyceridemic group has received dietary

counseling with the advice to increase the intake of polyunsaturated fatty acids at the expense of saturated fatty acids as first-line therapy. Therefore, not a lower but a higher intake of polyunsaturated fatty acids would be expected in the HTG group compared to the population-based control subjects (37,38). There are some indications that may explain these differences. Hepatocytes have been shown to synthesize preferably simple, saturated fatty acids over more complex unsaturated fatty acids (39). Accordingly, it may be hypothesized that an increased supply of substrates to the liver leads to a higher incorporation of saturated fatty acids as compared to polyunsaturated fatty acids in triglycerides, especially under conditions of VLDL overproduction as encountered in hypertriglyceridemia. In addition, polyunsaturated fatty acids decrease VLDL production which may cause the liver to incorporate polyunsaturated fatty acids at a slower rate than saturated fatty acids (40,41). Indeed, a decreased VLDL production has been reported in humans fed a diet rich in polyunsaturated fatty acids (42).

A second explanation may be preferential lipolysis of triglycerides that are rich in polyunsaturated fatty acids. Botham *et al.* (43) studied the effect of fatty acid composition on lipolysis by feeding rats fish oil (PUFAs), olive oil (MUFAs) or palm oil (SFAs). The triglyceride-rich lipoproteins were isolated and subjected to *in vitro* lipolysis. It was shown that saturated fatty acids in triglycerides were hydrolyzed at a slower rate than polyunsaturated fatty acids. So, in case of stressed triglyceride-removal pathways as is seen in hypertriglyceridemia, polyunsaturated fatty acids may be preferred over saturated fatty acids, resulting in a lower ratio of polyunsaturated to saturated fatty acids in plasma and lipoproteins.

The LDL particles in the hypertriglyceridemic patients were smaller than control LDL. To our surprise, we found that this small dense LDL was associated with an increased resistance to oxidative stress, as reflected by a longer lag time and lower propagation rate. Results from the present study indicate that differences in fatty acid composition of VLDL and LDL may explain the observed differences in oxidation behavior between hypertriglyceridemic and normolipidemic subjects.

There is controversy regarding the effects of fibrate therapy on lipoprotein oxidizability. Some groups have reported an enhanced resistance to oxidative stress (4,44,45), whereas others found no effect (46,47). In the present study, plasma levels of free F2-isoprostanes were not affected by fibrate therapy. We infer from these results that bezafibrate does not affect the oxidation status in hypertriglyceridemic patients.

In conclusion, we have found normal plasma levels of free F2-isoprostanes in the hypertriglyceridemic group, which were not influenced by vigorous triglyceride-lowering therapy by bezafibrate. In addition, an enhanced resistance of VLDL and LDL to Cu^{2+}-induced oxidation was noted in the hypertriglyceridemic group, a phenomenon that may be explained by a low ratio of polyunsaturated to saturated fatty acids. These combined *in vivo* and *in vitro* oxidation data provide strong evidence that hypertriglyceridemia is associated with normal oxidative stress.

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Chapter 7

Activated Platelets in Patients with Hypertriglyceridemia: Effects of Triglyceride-Lowering Therapy

Frits H.A.F. de Man[1], Rienk Nieuwland[2], Arnoud van der Laarse[1], Fred Romijn[2], Augustinus H.M. Smelt[3], Jan A. Gevers Leuven[3,4], Augueste Sturk[2]

Departments of [1]Cardiology, [2]Clinical Chemistry and [3]Internal Medicine, Leiden University Medical Center, Leiden; [4]TNO-Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands

Submitted

Summary

Background. Platelet activation has been associated with classical cardiovascular risk factors. The effects of triglycerides on platelet function are controversial. This study was designed to compare the *in vivo* platelet activation status between patients with hypertriglyceridemia and age- and sex-matched control subjects, and to evaluate the effects of triglyceride-lowering therapy.

Methods. Sixteen patients with endogenous hypertriglyceridemia were included in a double-blind, placebo-controlled cross-over trial with 400 mg bezafibrate once daily. Analysis of the platelet activation was performed by double label flow cytometry, using monoclonal antibodies against GP53, P-selectin, and platelet-bound fibrinogen as platelet surface activation markers.

Results. Comparison of baseline platelet analysis showed that the surface expression of the lysosomal membrane protein GP53 was significantly higher in the hypertriglyceridemic patients (16.3 ± 4.8%) as compared to the group of age- and sex-matched controls (8.9 ± 3.4%, $p<0.001$). No differences in the expression of P-selectin and fibrinogen binding were observed. In response to bezafibrate therapy, the expression of GP53 in the patient group decreased from 16.3 ± 4.8% to 13.1 ± 4.1% ($p=0.018$). The expression of P-selectin and fibrinogen binding was not affected by bezafibrate therapy.

Conclusion. Patients with hypertriglyceridemia have an increased *in vivo* platelet activation status which can be improved by bezafibrate therapy.

Introduction

Platelets play an important role in the pathogenesis of atherosclerosis and acute coronary syndromes (1,2). The beneficial effects of anti-platelet agents in secondary prevention trials support this hypothesis (3). Activation of platelets has been demonstrated in patients with unstable angina pectoris and acute myocardial infarction (4,5). Also the classical cardiovascular risk factors such as diabetes, smoking and hyperlipoproteinemia have been associated with an increased platelet reactivity or platelet activation (6-8).

Hypertriglyceridemia (HTG) is a risk factor for cardiovascular disease in both men and women (9). In addition to the characteristic lipoprotein profile, which includes low levels of high density lipoprotein (HDL) cholesterol and the presence of atherogenic small, dense low density lipoprotein (LDL) particles, hypertriglyceridemia has been associated with various derangements of the hemostatic system. Plasma fibrinogen levels are often elevated in hypertriglyceridemic patients. Also, activated factor VII and plasminogen activator inhibitor-1 levels are elevated in these patients and correlate with total plasma triglyceride levels (10,11). These results indicate that hypertriglyceridemia is associated with a hypercoagulable and hypofibrinolytic state. The effects of triglycerides on platelet function are less uniformly described (7,12). Previous studies used platelet aggregometry to indirectly assess platelet reactivity *ex vivo*. This technique is difficult to perform in turbid plasma samples. The development of whole blood flow cytometry (13) provided a tool to address the platelet activation status directly and specifically, by measuring the presence of activation antigens on the platelet surface.

As platelets become activated, changes occur at the platelet surface (14). Activation is initiated by the binding of agonists to specific platelet receptors. As a result, the glycoprotein (GP) IIb-IIIa complex changes its conformation, thereby revealing the binding site for fibrinogen. In addition, the membranes of the α-granules, dense granules and lysosomes fuse with the platelet cell-membrane to release the granular contents. As a consequence, novel activation-dependent membrane glycoproteins become exposed, which in the resting platelet were present only in the granule membranes. In the present study, three of these activation-dependent antigens were determined: P-selectin (CD62P), an α-granule-derived transmembrane protein; GP53 (CD63) a lysosomal membrane protein; and platelet-bound fibrinogen.

The present study was designed to directly assess the platelet activation status in patients with hypertriglyceridemia as compared to healthy age- and sex-matched control subjects. In addition, the effects of triglyceride-lowering therapy were studied in a double-blind, placebo-controlled trial with 400 mg bezafibrate once daily.

Materials and Methods

Patients and control subjects

The study population consisted of 16 unrelated patients (14 males and 2 females) with endogenous hypertriglyceridemia who were recruited from the outpatient lipid clinic of the Leiden University Medical Center. The diagnosis endogenous hypertriglyceridemia was based on the means of two fasting blood samples obtained after a dietary period of at least 8 weeks. The diagnostic criteria for endogenous hypertriglyceridemia were: total serum TG > 4.0 mmol/L, very low density lipoprotein (VLDL) cholesterol > 1.0 mmol/L, and LDL-C < 4.5 mmol/L. Exclusion criteria were the apoE2E2 phenotype, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/L, and alcohol consumption of more than 40 g/day) and the use of lipid-lowering drugs. Patients with a medical history of cardiovascular disease were not included in the study. Seventeen normolipidemic, age- and sex-matched control subjects (16 males and 1 female) were recruited in response to a newspaper advertisement.

Study design and blood sampling

The patients were randomized to receive in a double-blind cross-over fashion a fixed dose of bezafibrate, 400 mg once daily, and placebo for 6 weeks. The two periods in which medication was taken were separated by a wash-out period of 6 weeks. Before and after each treatment period of 6 weeks, fasting venous blood samples were obtained from the participants for lipid measurements. The platelet activation status was assessed at the end of the placebo and treatment periods. From the control subjects, fasting blood samples were obtained at baseline under identical conditions. Informed consent was obtained from each patient and the protocol was approved by the institutional Medical Ethics Committee (protocol number P183/96).

Lipids and lipoproteins

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Three mL of fresh serum was ultracentrifuged for 15 hours at 232,000 g at 15°C in a TL-100 tabletop Ultracentrifuge, using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA USA). The ultracentrifugate was carefully divided in a density (d) < 1.006 and d 1.006 - 1.25 g/mL fraction, designated as the VLDL and LDL-HDL fraction, respectively. The triglyceride and cholesterol concentrations were measured enzymatically using test kits (Boehringer, Mannheim, Germany). HDL cholesterol was measured in the LDL-HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and $MgCl_2$. ApoE phenotyping was performed by isoelectric focusing according to Havekes *et al.* (15).

Preparation of whole blood samples for flow cytometry

Blood was obtained from an antecubital vein through a butterfly needle (1.0 mm) with a light tourniquet. After discarding the first 2 mL of blood, a 1.8 mL sample of blood was collected in 0.2 mL of 3.2% tri-sodium citrate. Within five minutes, 5 µL aliquots of the citrated blood were diluted in 30 µL of HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 20 mmol/L HEPES, 1 mg/mL bovine serum albumin, 3.3 mmol/L NaH_2PO_4, pH 7.4; buffer A), 5 µL of biotinylated anti-GPIb and 5 µL of a second fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (MoAb). Final concentrations were 0.75 µg/mL biotinylated anti-GPIb, 5 µg/mL FITC-anti-GP53, 5 µg/mL FITC-anti-P-selectin, 16 µg/mL FITC-anti-fibrinogen (platelet-bound fibrinogen) and 5 µg/mL FITC-labeled control antibody (IgG_1 isotype). After carefully mixing and 15 minutes incubation at room temperature in the dark, 5 µL of 10-fold diluted phycoerythrin-conjugated streptavidin was added. Incubation continued for another 15 minutes at room temperature. Finally, 2.5 mL of buffer A was added, containing 0.2% formaldehyde (final concentration).

Antibodies

The following murine Abs were used. Anti-GPIb (CLB-MB45), directed against the α-chain of GPIb, was obtained from the Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service (Amsterdam, The Netherlands). Anti-GPIb was biotinylated as described by Hnatowich *et al.* (16). FITC-labeled MoAbs against P-selectin (CD62P) and GP53 were obtained

from Immunotech (Marseille, France). FITC-labeled chicken anti-human fibrinogen polyclonal antibodies were obtained from Biopool AB (Umeå, Sweden) and FITC-labeled IgG_1 control antibodies were purchased from Becton & Dickinson (San Jose, CA, USA). Phycoerythrin-conjugated streptavidin was obtained from Dakopatts (Glostrup, Denmark).

Flow cytometric analysis

Double label flow cytometry was performed as described previously (17,18). After collection, blood samples were analyzed in a FACScan flow cytometer with CellQuist software (Becton Dickinson, San Jose, CA, USA). Forward light scatter and sideward light scatter were set at logarithmic gain. Platelets were identified by analyzing the phycoerythrin-GPIb content of particles at 585 nm. Regions were identified, corresponding to platelet-derived microparticles (R1), platelets (R2) and platelet-platelet, platelet-leukocyte, platelet-derived-microparticle(s)-leukocyte or platelet-erythrocyte complexes (R3). The surface expression of activation markers was determined on a population of 5,000 platelets (R2) by analyzing the fluorescence intensity of the FITC-labeled antibodies at 515 nm. The threshold for platelet activation was set at 2% fluorescence-positive platelet activation with a FITC-labeled IgG_1 control antibody. Platelet count and volume were measured on a Sysmex™ SE-9000 analyzer (TOA Medical Electronics, Kobe, Japan).

To verify our method of platelet activation analysis by expression of the activation markers, whole blood obtained from 8 healthy volunteers, and coagulated with 0.10 mmol/L sodium citrate (final concentration) was stimulated with 10 μmol/L adenosine diphosphate *in vitro*. The mean fluorescence intensity (MFI) for FITC-labeled anti-P-selectin (CD62P) increased from 3.6 ± 0.5 to 10.8 ± 4.3 and MFI for FITC-labeled anti-GP53 (CD63) increased from 5.6 ± 0.8 to 9.7 ± 1.5 (mean ± S.D.). These data indicate that the procedure used to label and fix the platelets can detect platelet activation by increased antigen expression of activation markers.

Statistical analyses

Results are presented as the mean ± S.D. Mean differences between the control group and patient group were calculated with the Mann-Whitney U test. Only differences in sex distribution between the patient group and control group were calculated with the Fisher's exact test. Differences between the patient group on placebo and bezafibrate therapy were evaluated pairwise using the Wilcoxon paired signed-ranks test. Findings were regarded to be

statistically significant when the probability of these data under the null hypothesis was less than 0.05. Statistical analyses were performed with SPSS/PC+™ software (SPSS Inc., Chicago, IL USA).

Results

Patient characteristics

As shown in table 1, the patient and control group were comparable with regard to age and sex. Serum triglyceride levels were significantly higher in the HTG patients as compared to the control subjects. Although both LDL-C and HDL-C levels were lower in the patient group, VLDL-C concentrations were markedly elevated and accounted for the elevation in total serum cholesterol concentrations.

Table 1. *Baseline characteristics*

	Controls	Hypertriglyceridemic patients	p-value[a]
Number	17	16	-
Age, y	47 ± 8	49 ± 9	n.s.
Sex, M/F	16 / 1	14 / 2	n.s.
TTG, mmol/L	0.99 ± 0.43	10.21 ± 4.44	< 0.001
TC, mmol/L	5.03 ± 0.99	7.79 ± 1.84	< 0.001
VLDL-C, mmol/L	0.29 ± 0.18	4.47 ± 2.23	< 0.001
LDL-C, mmol/L	3.48 ± 0.93	2.57± 0.64	0.002
HDL-C, mmol/L	1.25 ± 0.26	0.76 ± 0.16	< 0.001

Values are presented as the mean ± S.D. TC, total cholesterol; TTG, total triglycerides; VLDL-C, very low density lipoprotein cholesterol, LDL-C, low density lipoprotein cholesterol, HDL-C, high density lipoprotein cholesterol. [a] *Numeric variables were analyzed with the Mann-Whitney U test. Sex distribution was analyzed with the Fisher's exact test.*

Effect of therapy on serum lipid and lipoprotein levels

All subjects concluded the study without any side-effects. The effects of bezafibrate and placebo are shown in table 2. Placebo therapy did not influence serum lipid and lipoprotein levels. Bezafibrate therapy resulted in a significant reduction in serum TG (-66%, $p<0.001$), serum cholesterol levels

(-27%, p<0.001) and VLDL-C levels (-68%, p<0.001). LDL-C and HDL-C levels increased with 29% (p=0.003) and 12% (p=0.011), respectively.

Table 2. *Effects of Treatment with Placebo and Bezafibrate on Serum Lipids and Lipoproteins*

	Before placebo	During placebo	Before bezafibrate	During bezafibrate
Number	16	16	16	16
TTG, mmol/L	10.48 ± 5.31	12.20 ± 8.58	11.41 ± 7.77	3.83 ± 1.51***
TC, mmol/L	8.01 ± 2.01	7.65 ± 2.46	8.08 ± 2.75	5.90 ± 1.32**
VLDL-C, mmol/L	4.35 ± 2.25	4.29 ± 2.13	4.52 ± 3.06	1.45 ± 0.66***
LDL-C, mmol/L	2.86 ± 1.04	2.65 ± 0.61	2.76 ± 0.52	3.55 ± 0.89**
HDL-C, mmol/L	0.80 ± 0.20	0.72 ± 0.13	0.81 ± 0.13	0.91 ± 0.13*

*Values are presented as the mean ± S.D. TC, total cholesterol; TTG, total triglycerides; VLDL-C, very low density lipoprotein cholesterol, LDL-C, low density lipoprotein cholesterol, HDL-C, high density lipoprotein cholesterol. All values are mean ± S.D. based on triplicate measurements. * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to the corresponding value before bezafibrate therapy using the Wilcoxon paired signed-ranks test.*

Platelet analyses

The first objective was to compare the expression of GP53, P-selectin and platelet fibrinogen binding between control subjects and HTG patients. Since placebo therapy did not influence serum lipid levels in the patient group, the platelet activation data obtained during placebo therapy were used as "baseline" values and compared with the platelet activation data of the control group. As shown in table 3 and figure 1, no differences in the expression of P-selectin and fibrinogen binding were found between the control subjects and HTG patients (during placebo therapy). However, the expression of GP53 was significantly higher in the HTG patients (16.3 ± 4.8%) as compared to the group of control subjects (8.9 ± 3.4%, p<0.001). Platelet count and mean platelet volume did not differ between the HTG patients and control subjects (table 3).

Table 3. *Analysis of Platelet Activation Markers*

	Controls	Hypertriglyceridemic patients	
		Placebo	Bezafibrate
Number	17	16	
Platelet count, 10^9/L	231 ± 51	204 ± 45	221 ± 45 #
Mean platelet volume, fL	9.91 ± 0.50	10.03 ± 1.20	9.98 ± 1.17
GP53, %[a]	8.9 ± 3.4***	16.3 ± 4.8	13.1 ± 4.1#
P-selectin, %[a]	2.4 ± 0.7	1.4 ± 1.1	1.6 ± 1.1
Fibrinogen, %[a]	12.3 ± 3.9	11.2 ± 4.3	12.0 ± 4.4

*Values are presented as the mean ± S.D. [a] presented as percentage activated platelets. *** $p < 0.001$ as compared to the corresponding value in the hypertriglyceridemic group on placebo therapy using the Mann-Whitney U test. ; # $p < 0.05$ as compared to the corresponding value on placebo therapy using the Wilcoxon paired signed-ranks test.*

Bezafibrate therapy resulted in an improvement of the lipoprotein profile in the patient group (table 2). The mean effects of this therapy on the expression of platelet activation markers are shown in table 3. The expression of P-selectin and binding of fibrinogen did not change in response to bezafibrate therapy. During bezafibrate therapy, the high expression of GP53 in HTG patients slightly normalized on average from 16.3 ± 4.8% to 13.1 ± 4.1% (p=0.018). Although mean platelet volume was not affected by both therapy modalities, the platelet number increased in response to bezafibrate therapy (+8%, p=0.011; table 3). The individual changes in GP53, P-selectin and platelet-bound fibrinogen expression upon bezafibrate therapy are presented in figure 1. The expression of GP53 increased in 4 patients, remained stable in 1 and decreased in 11 patients with originally the highest GP53 expression. The P-selectin expression did not change substantially within the patients and was similar to the values obtained in the healthy controls. The changes in platelet-bound fibrinogen were variable.

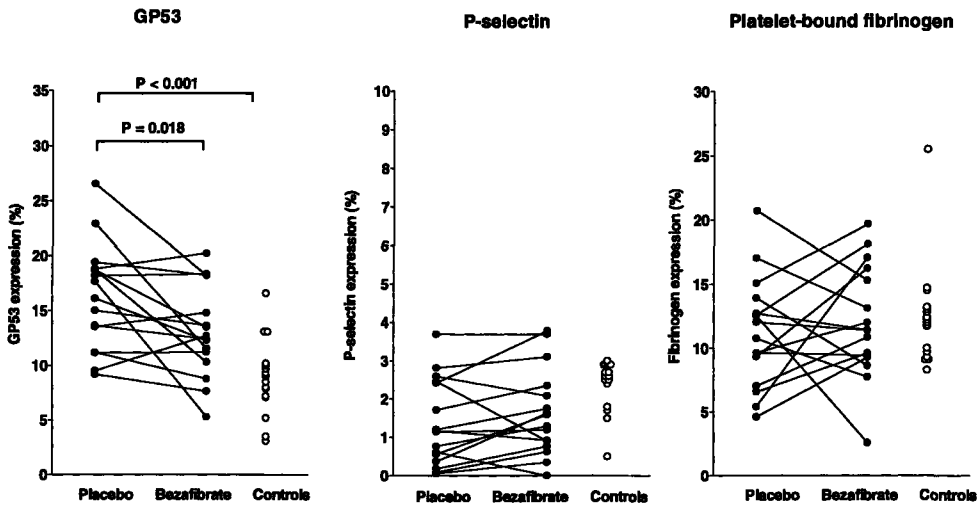

Figure 1. Expression of platelet activation markers. *Expression of GP53 (left panel), P-selectin (middle panel) and platelet-bound fibrinogen (right panel) on platelets obtained from 16 hypertriglyceridemic patients (solid circles) during placebo and bezafibrate therapy compared with 17 age- and sex-matched control subjects (open circles). Analysis of the platelet activation markers was performed by double label flow cytometry, using antibodies against GP53, P-selectin and platelet-bound fibrinogen. The surface expression of the lysosomal membrane protein GP53 was significantly higher in the hypertriglyceridemic patients as compared to the group of controls. No differences in the expression of P-selectin and fibrinogen binding were observed. In response to bezafibrate therapy, the expression of GP53 in the patient group decreased, whereas the expression of P-selectin and fibrinogen binding was not affected.*

Discussion

In this study we investigated the *in vivo* platelet activation status in hypertriglyceridemic patients directly by flow cytometric analysis of activation-dependent platelet surface antigens. In previous studies, platelet reactivity was assessed *in vitro* by platelet aggregometry (7,12). A major problem of this technique is that light transmission measurements are influenced by turbidity of plasma, a common phenomenon in this patient group. Other investigators have tried to avoid this problem by defining an upper limit of the serum triglyceride level (19) or using an impedance

aggregometer technique (12). A second problem of platelet aggregometry is the fact that the *in vivo* platelet activation status is not assessed directly but is merely implied from platelet reactivity *in vitro*. In contrast, the flow cytometric analysis of activation-dependent platelet surface antigens is not influenced by turbidity of blood samples and directly reflects the platelet activation status *in vivo*. This is therefore the first report describing the *in vivo* platelet activation status of hypertriglyceridemic patients.

Our first objective was to compare the platelet activation status of HTG patients with age- and sex-matched control subjects. The surface expression of GP53 was higher in the patient group as compared to the group of healthy volunteers. The platelet-activation markers P-selectin and platelet-bound fibrinogen were comparable in both groups. Thus, only one out of three platelet activation markers showed a higher expression in the HTG group. GP53 is a member of the lysosome-associated membrane proteins (LAMP) which become exposed on the platelet surface upon activation as the membrane of the lysosomal granules fuse with the plasma membrane (20,21). Several studies have shown that LAMPs become exposed at the platelet surface when platelets are activated *in vitro* (22). In addition, an increased platelet expression of LAMPs has been demonstrated in clinical syndromes like metastatic cancer and preeclampsia which are associated with thrombo-embolic complications (18,22). These studies indicate that GP53 and other LAMPs are good markers for platelet activation. Accordingly, the increased expression of GP53 in the HTG group indicates that hypertriglyceridemia is associated with an increased platelet activation status. The results of the triglyceride-lowering therapy with bezafibrate on both the improvement of lipid parameters and the reduction of GP53 expression further support this hypothesis. It should be emphasized, however, that despite a considerable reduction in plasma TG levels, normotriglyceridemia was not achieved. In accordance, the extent of increased GP53 expression was reduced but it did not reach the normal range. In contrast to GP53, no differences were found in expression of P-selectin between platelets obtained from HTG patients and healthy volunteers. P-selectin is a transmembrane protein of α-granules and only becomes expressed on the platelet surface upon activation. Michelson *et al.* (23) showed that retransfusion of activated platelets that expressed P-selectin resulted in a rapid loss of P-selectin *in vivo*. This decrease was paralleled by an increase in soluble P-selectin. Thus, P-selectin is exposed transiently at the platelet surface after activation and dissociates from the platelet, implying that the expression of P-selectin may not be a good marker

of platelet activation *in vivo*. Also in contrast to GP53, no differences were found in the amount of fibrinogen bound to the surface of patient versus control platelets. Upon platelet activation, fibrinogen binds to the activated GPIIb-IIIa complex. Resting platelets do not bind fibrinogen, but when platelets are activated the GPIIb-IIIa complex changes its conformation and thus becomes eligible to bind fibrinogen. This change in the fibrinogen receptor conformation is highly reversible (24). Therefore, the extent of surface binding of fibrinogen may be a transient phenomenon, which makes it less suitable as a marker for the platelet activation status. The fibrinogen receptor conformation of the GPIIb-IIIa complex can also be measured with the MoAb PAC-1. We did not find differences in PAC-1 binding between patients and controls (data not shown), which confirms the fibrinogen data. Finally, the finding that different platelet activation markers provide seemingly discrepant results is not uncommon in clinical studies. A number of studies have reported increased expression of GP53 only, or of one of the other markers, in a variety of diseases (18,25-27). We conclude that an increased platelet activation status, as measured by surface GP53 exposure, is present in hypertriglyceridemic patients.

Several mechanisms may explain the increased platelet activation status in patients with hypertriglyceridemia. First, platelet activity in hyperlipidemias may be related to changes in the lipid composition of platelet membranes. Increased plasma cholesterol levels have been shown to decrease the platelet membrane fluidity (28). These cholesterol-enriched, rigid platelet membranes show an enhanced platelet responsiveness by increasing the number and affinity of platelet thrombin receptors (29). Malle *et al.* (30) studied platelet membrane fluidity in other types of dyslipidemia. Interestingly, platelets from HTG patients demonstrated an increased membrane fluidity as compared to healthy control subjects. So, platelet membrane rigidity does not seem to be a plausible explanation for the enhanced platelet activation status in the HTG group.

A second mechanism that may be involved is oxidative stress. Several studies have demonstrated that oxidative stimuli may activate platelets (31,32). A characteristic lipoprotein pattern that can be observed in hypertriglyceridemia includes relatively low concentrations of LDL and HDL cholesterol, which are caused by exchange of lipids between the VLDL pool on one hand and the LDL-HDL pool on the other. In this process, the LDL particles become triglyceride-enriched and relatively cholesterol ester-depleted. As the LDL particles are progressively lipolyzed in the circulation,

the triglycerides are degraded and a small, dense particle remains. These small, dense LDL particles are regarded as potentially atherogenic since they show a low resistance to oxidative modification (33). Although no data are available about the total *in vivo* oxidative stress in HTG patients, the presence of small, dense LDL particles in these patients is associated with an increased tendency to oxidative modification (33,34) and, consequently, may be associated with an increased platelet activation (35).

A third explanation may be the platelet activating potential of VLDL, the lipoprotein fraction that primarily accumulates in hypertriglyceridemia. Several studies have demonstrated that VLDL stimulates platelet aggregation (36,37). Van Willigen *et al.* (38) studied the effect of LDL on fibrinogen binding to the GPIIb-IIIa complex. Interestingly, incubation of platelets with LDL induced a rapid and dose-dependent increase in fibrinogen binding to platelets. No increase was observed after modification of the lysine residues of LDL, and therefore the effect was suggested to be receptor-mediated. It was speculated that LDL changes the exposure of the GPIIb-IIIa complex into a more active configuration. The principal candidate ligand of LDL is apolipoprotein (apo) B-100, which mediates the clearance of LDL from the circulation as ligand for the LDL-receptor. Interestingly, apoB-100 contains a substantial number of lysine residues that play an important role in the receptor-mediated clearance (39). In hypertriglyceridemia, however, LDL concentrations are low and the principal lipoprotein fraction that accumulates is VLDL. Since VLDL, like LDL, contain apoB-100, VLDL may influence the platelet activation status as well by changing the conformation of the GPIIb-IIIa complex via apoB-100. Another study, however, found that LDL augments platelet reactivity via a receptor-independent mechanism, by inhibiting the Na^+/H^+ antiport in the platelet membrane (40). Whether this ion exchanger is influenced by VLDL as well remains to be determined. Further studies are needed to elucidate the mechanisms that could be involved in the interaction between VLDL and platelets.

In conclusion, our data indicate that hypertriglyceridemia is associated with an increased expression of the lysosomal membrane protein GP53, which is indicative of an increased *in vivo* platelet activation status. Triglyceride-lowering therapy by bezafibrate results in an impressive improvement of the serum lipid levels and a modest reduction in GP53 expression.

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Chapter 8

Not Acute but Chronic Hypertriglyceridemia is Associated with Impaired Endothelium-Dependent Vasodilation: Reversal upon Lipid-Lowering Therapy by Atorvastatin

Frits H.A.F. de Man[1], Annelies W.E. Weverling-Rijnsburger[2], Arnoud van der Laarse[1], Augustinus H.M. Smelt[2], J. Wouter Jukema[1], and Gerard J. Blauw[2]

Departments of [1]Cardiology and [2]Internal Medicine,
Leiden University Medical Center, Leiden, The Netherlands

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Summary

Background. Endothelial dysfunction has been associated with most cardiovascular risk factors. There is controversy regarding the relation between hypertriglyceridemia (HTG) and endothelial function. This study was designed to investigate endothelial function in a patient group with chronic hypertriglyceridemia, before and during lipid-lowering therapy by atorvastatin. In addition, the effects of acute hypertriglyceridemia on endothelial function were studied in normolipidemic individuals.

Methods. We studied forearm blood flow (FBF) responses to brachial artery infusions of serotonin as endothelium-dependent vasodilator, and sodium-nitroprusside as endothelium-independent vasodilator by venous occlusion plethysmography. Eight male patients with chronic HTG were studied before and after 6 weeks of lipid-lowering treatment with 80 mg atorvastatin once daily. Ten age-matched control subjects were studied at baseline and immediately after a high-dose infusion of artificial triglycerides.

Results. The HTG patients had 15-fold higher serum triglyceride levels compared to the control group. In the HTG group, the FBF response to serotonin was attenuated compared to the controls (63% vs. 103% at the highest dose, respectively, $p<0.001$), whereas the FBF response to nitroprusside was comparable to that observed in the control group. In response to atorvastatin therapy, serum triglyceride and cholesterol levels decreased significantly by 43% ($p=0.017$) and 38% ($p=0.012$), respectively. After 6 weeks of treatment, the FBF response to serotonin improved from 63% to 106% ($p<0.001$). Induction of acute hypertriglyceridemia in the control subjects did not affect the FBF responses to serotonin and nitroprusside.

Conclusions. Patients with chronic hypertriglyceridemia have an impaired endothelium-dependent vasodilation, that is normalized after 6 weeks of lipid-lowering therapy by atorvastatin. Unlike chronic hypertriglyceridemia, acute hypertriglyceridemia does affect neither endothelium-dependent nor endothelium-independent vasodilation.

Introduction

Endothelial dysfunction is regarded as an early feature of atherosclerosis. Most cardiovascular risk factors like hypercholesterolemia, smoking, hypertension, and age have been shown to be associated with an impaired endothelium-dependent vasodilation (1-5). However, the effects of hypertriglyceridemia on endothelial function are still subject to discussion. Incubation of aortic rings with triglyceride-rich lipoproteins inhibited the endothelium-dependent vasodilation to the same extent as low density lipoproteins (LDL) did (6). In accordance, oral as well as intravenous triglyceride loads in healthy subjects induced an impaired endothelial response within hours (7,8), suggesting that hypertriglyceridemia is a risk factor for endothelial dysfunction. In sharp contrast with this concept, Chowienczyk *et al.* (9) showed normal endothelium-dependent responsiveness in hypertriglyceridemic patients. It should be noted, however, that these patients were characterized by lipoprotein lipase (LPL) deficiency, which represents only a small subset of the hypertriglyceridemic population (10). The present study was designed to investigate endothelial function in a group of non-LPL-deficient patients with chronic hypertriglyceridemia, and to evaluate the effects of lipid-lowering therapy by atorvastatin. In addition, the acute effects of hypertriglyceridemia on endothelial function were studied in healthy normolipidemic subjects.

Materials and Methods

Subjects

The study population included 8 patients with chronic hypertriglyceridemia (Fredrickson type IV-V) and 10 healthy normolipidemic control subjects. The patients with hypertriglyceridemia were recruited from the outpatient lipid clinic of the Leiden University Medical Center. Patients with hypertriglyceridemia were eligible if they had a total serum triglyceride > 4.0 mmol/L, VLDL-cholesterol > 1.0 mmol/L, and LDL-cholesterol < 4.5 mmol/L on two separate occasions under fasting conditions. Exclusion criteria were the apoE2E2 phenotype, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/L, and alcohol consumption of more than 40 g/day) and the use of lipid-lowering drugs. All individuals were non-smoking,

normotensive and without history of cardiovascular disease. The HTG group had a mean post-heparin LPL activity that was not significantly different from the control group (223 ± 66 vs. 158 ± 10 U/L, p=0.77). The study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center and all subjects gave informed consent.

Biochemistry

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Three mL of fresh serum was ultracentrifuged for 15 hours at 232,000 g at 15°C. The ultracentrifugate was carefully divided in a density < 1.006 and density 1.006-1.25 g/mL fraction, designated as the VLDL and LDL-HDL fraction, respectively. HDL cholesterol was measured in the LDL-HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and $MgCl_2$. Triglyceride, cholesterol and FFA concentrations were measured enzymatically using commercially available kits. ApoB and ApoA-I were assessed by a turbidimetric assay using an automated Hitachi 911 analyzer (Boehringer-Mannheim/Hitachi, Mannheim, Germany). Lipoprotein lipase (LPL) was determined in venous blood samples, obtained exactly 20 minutes after administration of a bolus of 50 IU of sodium heparin per kilogram body weight. The post-heparin blood samples were drawn in ice-cooled heparin-coated tubes. LPL was determined by an immunochemical method as described by Jansen *et al.* (11), using a gum acacia-stabilized [^{3}H]trioleolylglycerol substrate.

Procedures

All subjects were studied under fasting conditions in a quiet room with a constant temperature of 21 - 23 °C. Alcohol and caffeine containing beverages were withheld at least 24 hours before the study. During the experiments, the subjects were in supine position with the nondominant arm stabilized slightly above the level of the heart. After local anesthesia of the skin, a 20-gauge polyethylene catheter (Ohmeda, Swindon, UK) was inserted in the brachial artery of the nondominant arm for determination of blood pressure and infusion of drugs with a Graseby 3200 constant rate infusion pump (Graseby, Watford, UK). Heart rate was recorded from a triple lead electrocardiogram. The subjects rested for 20 minutes after the insertion of the intra-arterial catheter to achieve a stable baseline.

Forearm blood flow was measured by computerized, R-wave triggered, venous occlusion plethysmography, using mercury in silastic strain gauges and a rapid cuff inflator (Hokanson Inc., Bellevue, USA) as has been described previously (12). During the measurements of forearm blood flow, the hand was excluded from the circulation, using a small wrist cuff inflated to 40 mmHg above the systolic blood pressure. Endothelium-dependent vasodilation was determined during cumulative dose-infusions of serotonin (0.3 and 0.9 ng. $kg^{-1}.min^{-1}$). Sodium nitroprusside (30 and 90 $ng.kg^{-1}.min^{-1}$) was infused as endothelium-independent vasodilator. The drugs were given in a randomized order and each dose was infused for 5 minutes. A wash-out period of 20 minutes was applied between the different cumulative-dose infusions in order to allow the blood flow to return to baseline levels. Forearm blood flow, blood pressure, and heart rate were measured during two minutes immediately prior to the start of each intra-arterial infusion, and during the last two minutes of each infusion step. Forearm blood flow was expressed as ml.100 mL^{-1} forearm tissue.min^{-1}.

Drugs and solutions

The following compounds were used for intra-arterial infusions: 5-hydroxytryptamine·HCl (ICN Pharmaceuticals, Costa Mesa, Ca, USA), sodium-nitroprusside (Merck, Darmstadt, Germany) and L-arginine (Bufa BV, Uitgeest, NL). All drugs were dissolved in 0.9% saline, except for nitroprusside which was dissolved in 5% glucose. Intralipid (20% Fat Emulsion; Pharmacia and Upjohn, Woerden, NL) was used as artificial triglyceride emulsion for intravenous administration. The solutions were prepared from sterile stock solutions and ampoules on the day of the study, and stored at 4°C until use. Atorvastatin (Lipitor®) tablets of 40 mg were provided by Parke Davis (Hoofddorp, NL).

Study Protocol

Hypertriglyceridemic patients. The patients with chronic hypertriglyceridemia were studied on 2 separate occasions: at baseline and after 6 weeks of treatment with atorvastatin 80 mg once daily. At each occasion, forearm blood flow responses to serotonin and nitroprusside were assessed during simultaneous infusions of saline and L-arginine in a dose of 0.2 $mg.kg^{-1}.min^{-1}$. The order of drug administration was randomized. Saline and L-arginine infusions were started 5 min before the start of the serotonin and nitroprusside infusions. Drug compliance was assessed by tablet counting.

Control subjects. The control subjects were studied before and during the induction of an acute, systemic hypertriglyceridemia. After assessment of the baseline forearm blood flow responses to the intra-arterial infusions of serotonin and nitroprusside, a cannula was inserted in an anticubital vein of the contralateral arm. Subsequently, a bolus of 0.25 $g.kg^{-1}$ Intralipid was administered in 2 min, followed by a graded infusion at a dose of 0.40 $g.kg^{-1}.min^{-1}$. Immediately after reaching a stable level of hypertriglyceridemia, the forearm blood flow measurements were repeated and completed within 70 min after the onset of acute hypertriglyceridemia. To assess the effects of the Intralipid infusions on the lipoprotein profile, blood samples of 3 representative subjects were drawn before and at the end of the Intralipid administration. Then, the plasma lipoproteins in these samples were isolated by ultracentrifugation according to Redgrave *et al.* (13). L-arginine infusions were not performed in the control subjects, because previous studies have demonstrated that L-arginine does not increase endothelium-dependent vasodilation in healthy subjects (14).

Statistical Methods

Values are expressed as mean ± S.E.M. Comparisons between groups were made with the Mann-Whitney U test and analysis of variance (ANOVA). Effects of atorvastatin therapy were analyzed pairwise with the Wilcoxon paired signed-ranks test and the repeated-measures ANOVA. Statistical significance was accepted at the 95% confidence level. The statistical analyses were performed with SPSS/PC+™ software (SPSS Inc, Chicago, IL USA).

Results

Baseline characteristics

Table 1 provides characteristics of the study groups. All subjects were normotensive, non-diabetic, non-smoking males. There were no significant differences in age. The HTG group demonstrated 15-fold higher serum TG levels than the control group. Also the serum cholesterol levels were elevated due to the high VLDL levels in the patient group. The HDL-C and LDL-C concentrations were lower than in the control group. Interestingly, FFA levels

were elevated in the HTG group, whereas post-heparin LPL activities were in the same order of magnitude as in the control group.

Table 1. *Baseline characteristics of control subjects and the untreated patients with chronic hypertriglyceridemia*

	Control subjects	Hypertriglyceridemic patients	p-value
Number	10	8	-
Age, y	46 ± 7	51 ± 9	n.s.
Heart rate, min^{-1}	55.9 ± 2.9	68.3 ± 5.4	n.s.
SBP, mmHg	128.0 ± 4.7	121.5 ± 4.4	n.s.
DBP, mmHg	61.6 ± 4.2	52.3 ± 2.1	n.s.
Baseline FBF, $ml.100\ mL^{-1}.min^{-1}$	2.25 ± 0.26	2.69 ± 0.27	n.s.
TTG, mmol/L	0.80 ± 0.11	12.05 ± 4.02	<0.001
TC, mmol/L	4.85 ± 0.35	7.83 ± 1.55	0.021
VLDL-C, mmol/L	0.18 ± 0.03	4.72 ± 1.53	<0.001
LDL-C, mmol/L	3.29 ± 0.30	2.42 ± 0.16	0.034
HDL-C, mmol/L	1.38 ± 0.09	0.70 ± 0.06	<0.001
apoA-I, g/L	1.30 ± 0.05	1.09 ± 0.04	0.004
apoB, g/L	1.01 ± 0.07	1.10 ± 0.12	n.s.
FFA, mmol/L	0.53 ± 0.06	1.02 ± 0.32	0.043

Data are presented as mean ± S.E.M. SBP, systolic blood pressure; DBP, diastolic blood pressure; FBF, forearm blood flow; TTG, total triglycerides; TC, total cholesterol; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FFA, free fatty acids. To convert mmol/L cholesterol to mg/dL, multiply by 38.7; to convert mmol/L triglycerides to mg/dL, multiply by 88.5.

Lipids and lipoproteins

Atorvastatin therapy. In accordance with previous studies (15), atorvastatin therapy at a dose of 80 mg once daily induced a major improvement of the lipoprotein profile in patients with chronic hypertriglyceridemia. As shown in table 2, both serum triglyceride and cholesterol levels decreased considerably within 6 weeks of treatment. The main lipid-lowering effects were observed in the VLDL and LDL-fractions. Although HDL-C levels tended to increase, this increase did not reach statistical significance. Serum FFA levels, being twice as high as in controls, decreased upon therapy.

Table 2. *Effects of atorvastatin therapy in the patients with chronic hypertriglyceridemia*

	Hypertriglyceridemic patients		p-value
	Baseline	Atorvastatin	
Number	8		
Heart rate, min^{-1}	68.3 ± 5.4	69.5 ± 5.3	n.s.
SBP, mmHg	121.5 ± 4.4	127.8 ± 4.1	n.s.
DBP, mmHg	52.3 ± 2.1	58.5 ± 3.0	n.s.
Baseline FBF, $ml.100\ mL^{-1}.min^{-1}$	2.69 ± 0.27	2.59 ± 0.39	n.s.
TTG, mmol/L	12.05 ± 4.02	6.92 ± 2.89	0.017
TC, mmol/L	7.83 ± 1.55	4.87 ± 1.02	0.012
VLDL-C, mmol/L	4.72 ± 1.53	2.37 ± 1.14	0.012
LDL-C, mmol/L	2.42 ± 0.16	1.72 ± 0.16	0.025
HDL-C, mmol/L	0.70 ± 0.06	0.78 ± 0.10	n.s.
apoA-I, g/L	1.09 ± 0.04	1.09 ± 0.04	n.s.
apoB, g/L	1.10 ± 0.12	0.74 ± 0.05	0.012
FFA, mmol/L	1.02 ± 0.32	0.72 ± 0.27	0.036

Data are presented as mean ± S.E.M. SBP, systolic blood pressure; DBP, diastolic blood pressure; FBF, forearm blood flow; TTG, total triglycerides; TC, total cholesterol; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FFA, free fatty acids. To convert mmol/L cholesterol to mg/dL, multiply by 38.7; to convert mmol/L triglycerides to mg/dL, multiply by 88.5.

Artificial hypertriglyceridemia. The systemic intra-venous infusion of Intralipid resulted in a rapid, 15-fold increase in serum triglycerides to levels that remained stable during the graded infusion scheme (figure 1A). Total serum cholesterol levels did not change. Analysis of the lipoprotein cholesterol profiles, before and during this acute hypertriglyceridemia, demonstrated that the LDL- and HDL-fraction were not affected (figure 1B). The corresponding lipoprotein triglyceride profiles showed a sharp peak in the $d < 1.006$ g/mL region indicative of the artificial chylomicronemia, whereas the LDL- and HDL-fraction were not affected (data not shown). These results indicate that an acute and stable hypertriglyceridemia has been established without affecting other lipoprotein fractions up to 70 min after the start of the Intralipid infusion.

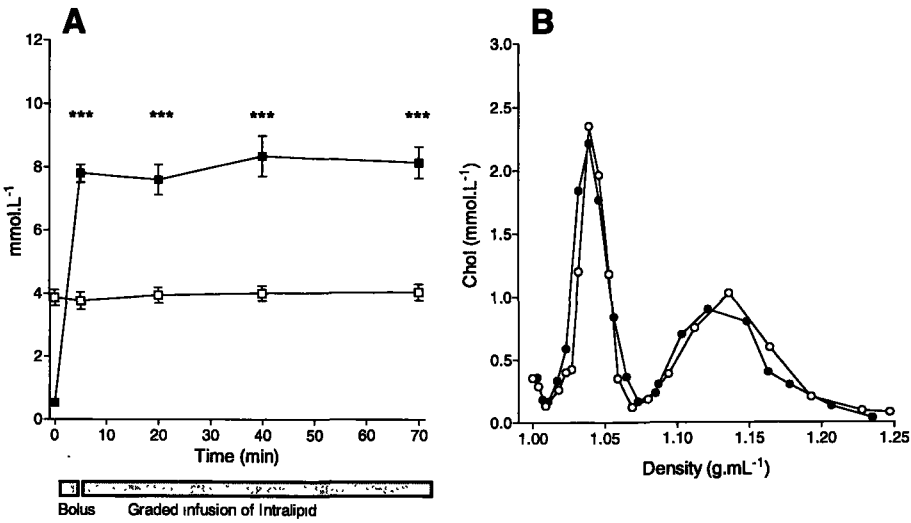

***Figure 1. Panel A** shows the changes in total plasma triglyceride (■) and cholesterol (□) levels after a systemic intravenous administration of a bolus of 0.25 g.kg^{-1} Intralipid in 2 min, followed by a graded infusion at a dose of 0.40 g.kg^{-1}.h^{-1}. Data are presented as mean ± S.E.M. *** $p < 0.001$, as compared to the baseline value at t = 0 min. **Panel B** shows lipoprotein profiles from 3 representative control subjects before (O) and 70 min after (●) the Intralipid infusion. The first peak (1.019 < density < 1.063 g.mL^{-1}) represents the LDL-fraction. The second peak (1.063 < density < 1.21 g.mL^{-1}) represents the HDL-fraction. No significant changes in lipoprotein cholesterol were observed during Intralipid infusions.*

Hemodynamic measurements

The local intra-arterial infusions of serotonin, sodium nitroprusside, and L-arginine did not induce any significant changes in intra-arterial blood pressure and heart rate (data not shown). Therefore the forearm blood flow changes can be interpreted as local vascular effects of the vasoactive substances used (12).

Vascular responses at baseline. The cumulative dose infusions of serotonin and nitroprusside induced a significant dose-dependent vasodilation in both study groups (figure 2). The forearm blood flow responses to serotonin were significantly lower in the HTG group as compared to the control group, 63 ± 10% vs. 103 ± 10% at the highest dose, respectively ($p<0.001$; figure 2A). The concomitant infusion of L-arginine in the HTG group significantly improved the vasodilator responses to serotonin from 63 ± 10% to 113 ± 9% at the highest dose ($p<0.001$). The forearm blood flow responses to the

endothelium-independent vasodilator nitroprusside were comparable in the HTG patients and control subjects as is shown in figure 2B. At the highest dose, the forearm blood flow increased to 292 ± 42% in the HTG group versus 348 ± 42% in the reference group (p=0.14). The infusion of L-arginine did not alter the forearm blood flow responses to nitroprusside (p=0.92).

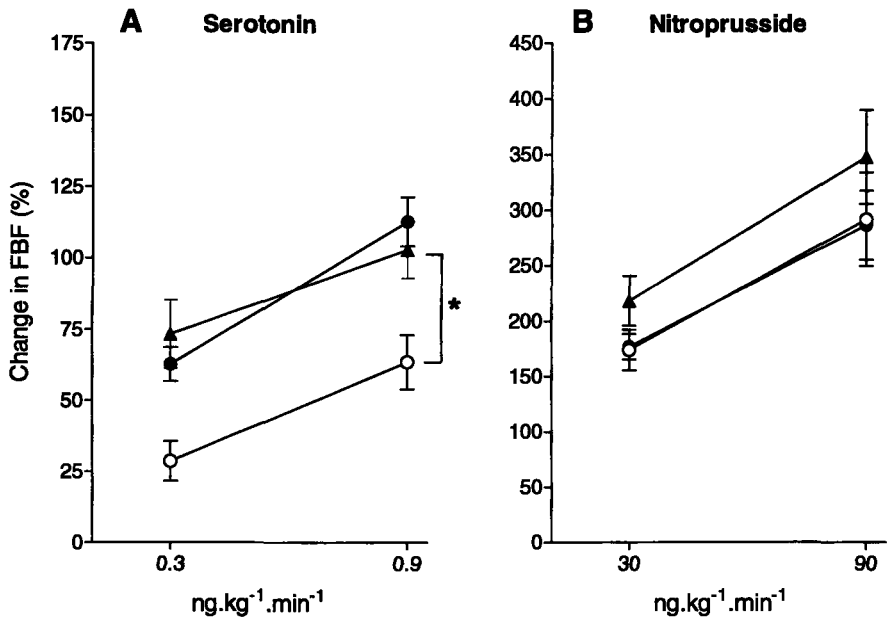

Figure 2. Differences in forearm blood flow responses between controls and hypertriglyceridemic patients. *Forearm blood flow responses to the intra-arterial infusions of serotonin (panel A) and nitroprusside (panel B) in healthy subjects* (▲) *and HTG patients during concomitant saline* (O) *and L-arginin* (●) *infusion. Data are presented as mean ± S.E.M.* * *p<0.001 controls vs HTG patients at baseline.*

Vascular responses after atorvastatin therapy. After 6 weeks of atorvastatin therapy at a dose of 80 mg once daily, the vasodilator responses to serotonin were significantly improved compared to baseline (figure 3A). The maximum increase in forearm blood flow was 106 ± 7% at the highest dose (p<0.001). The infusion of L-arginine did not influence vasodilator effects of serotonin compared to saline (p=0.43; figure 3A). The forearm blood flow responses to nitroprusside did not change significantly after atorvastatin therapy, although there was a tendency towards an increased forearm blood flow response compared to the forearm blood flow response to nitroprusside at baseline (p=0.052; figure 3B).

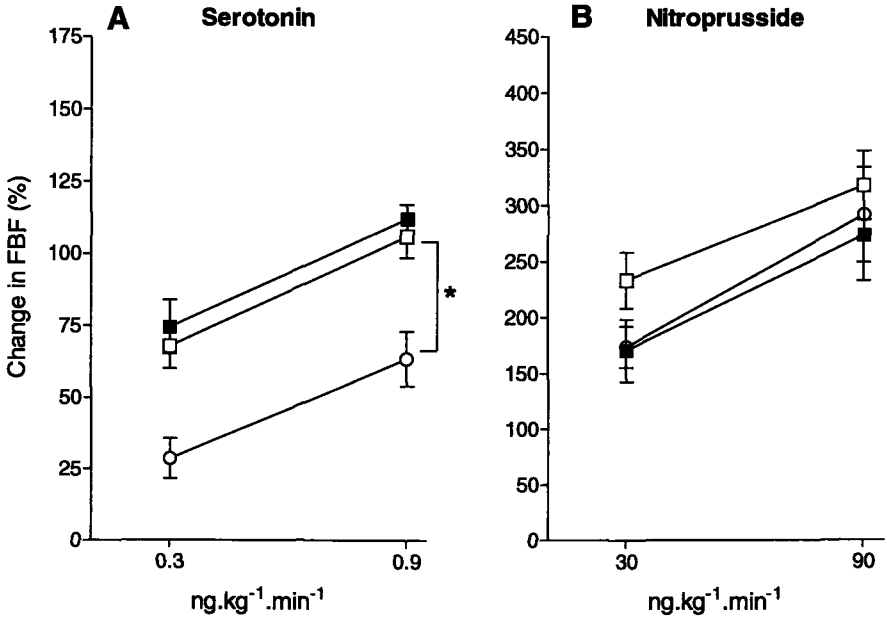

Figure 3. Effect of atorvastatin therapy on forearm blood flow responses in hypertriglyceridemia. *Forearm blood flow responses to the intra-arterial infusions of serotonin (panel A) and nitroprusside (panel B) in the HTG patients at baseline during concomitant saline* (O) *infusion, and after atorvastatin therapy during concomitant saline* (□) *and L-arginin* (■) *infusion. Data are presented as mean ± S.E.M. * p<0.001 HTG patients at baseline vs HTG patients during atorvastatin therapy.*

Vascular responses during acute hypertriglyceridemia. Immediately after the establishment of the hypertriglyceridemia in control subjects, the forearm blood flow measurements were carried out with a randomly chosen order of the serotonin and nitroprusside infusions. As shown in figure 4, the systemic Intralipid infusion did not affect the forearm blood flow responses to serotonin and nitroprusside. During this artificial hypertriglyceridemia, the forearm blood flow response to the highest doses of serotonin and nitroprusside were 117 ± 21% and 409 ± 67%, respectively, compared to 103 ± 10% and 348 ± 42%, respectively, during the saline infusion.

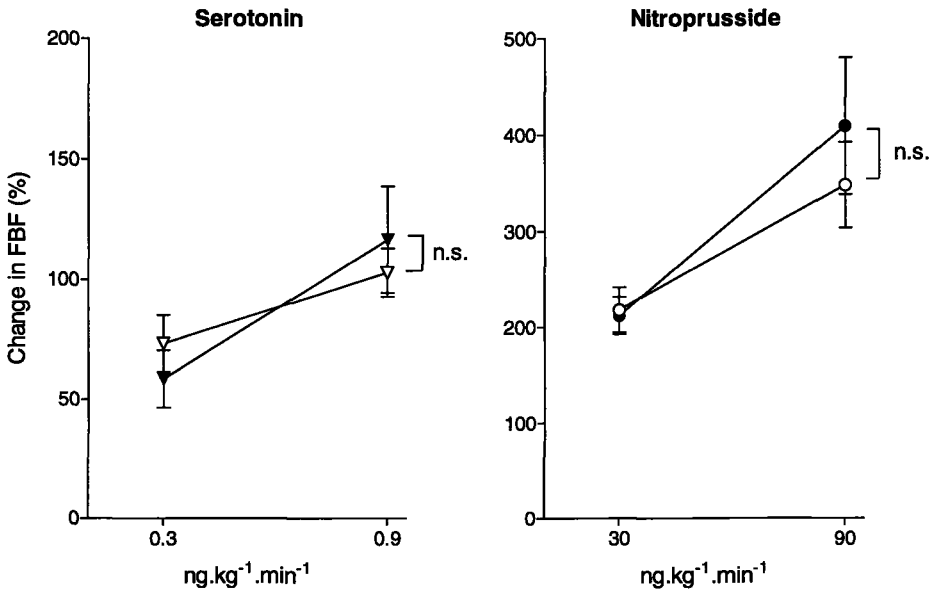

Figure 4. Effects of an acute hypertriglyceridemia on forearm blood flow responses. *Forearm blood flow responses of the control subjects before (open symbols) and during (closed symbols) the intralipid infusion. No significant differences were observed. Data are presented as mean ± S.E.M.*

Discussion

We have investigated the effects of acute and chronic hypertriglyceridemia on endothelial function. The study reveals the following new findings: (a) Patients with chronic HTG have an impaired endothelium-dependent vasodilation, whereas the endothelium-independent response is normal. (b) The endothelium-dependent vasodilation in chronic HTG can be normalized by infusion of L-arginine. (c) A 6-week treatment period with 80 mg atorvastatin once daily reverses the endothelium-dependent response to normal. (d) An acute artificial hypertriglyceridemia affects neither endothelium-dependent nor endothelium-independent vasodilation in normolipidemic controls.

The main finding of the present study is, that hypertriglyceridemia is associated with an impaired endothelial function, which corroborates the current concept that hypertriglyceridemia is an independent risk factor for cardiovascular disease (16,17). Endothelial dysfunction is regarded as an early feature of atherosclerosic disease, and has been found to be associated with

several other classical risk factors (1-5,18). In contrast with our findings, Chowienczyk *et al.* (9) have reported normal endothelial responsiveness in patients with chronic hypertriglyceridemia. Our study differs from the study of Chowienczyk with regard to the patient group as well as the choice of vasoactive compounds. The patients studied by the group of Chowienczyk were characterized by LPL deficiency, which represents only a small subset of the HTG population (10). This consideration is important since patients with LPL deficiency may have lower plasma FFA levels than non-LPL deficient HTG patients, caused by lower lipolysis rates. High plasma FFA levels have been reported to impair endothelial-dependent vasodilation (19), indicating that LPL deficiency and the corresponding low FFA levels may have masked the deleterious effects of HTG in the patient group that was studied by Chowienczyk *et al.* (9). Another explanation of the discrepancy might be the fact that different vasoactive compounds were used: In the present study serotonin was used as endothelium-dependent and nitric oxide-mediated vasodilator, whereas Chowienczyk used acetylcholine for this purpose. It has been shown that acetylcholine-induced vasodilation is, at least partly, independent of the nitric oxide pathway, which might explain the different results (20). A similar discrepancy between the endothelium-dependent vasodilators acetylcholine and bradykinin has been reported in hypercholesterolemic patients (21).

The present finding, that exogenous administration of the nitric oxide precursor L-arginine improves the endothelium-dependent vasodilation to serotonin in chronic HTG, suggests that low nitric oxide availability may play a pivotal role in this impaired responsiveness. This low nitric oxide availability can be caused by several mechanisms. One possibility is that nitric oxide production is normal, but that it is metabolized at a higher rate due to circulating lipoproteins, lipolysis products or oxidation products. Our observation that during acute HTG the endothelium-dependent vasodilation remained unchanged, strongly suggests that the lipids do not interfere with the nitric oxide availability directly. The fact that others have reported that a triglyceride load for a longer period of time results in impaired endothelial-dependent vasodilation, a phenomenon that could be prevented by antioxidant therapy with vitamin E and C, indicates that oxidative mechanisms may be involved (7,8,22). An alternative explanation could be that nitric oxide is formed at a lower rate, due to a decreased availability of L-arginine, by paucity of co-factors, or structural defects in the endothelial cell itself. It is not likely that the endothelial cells are depleted of L-arginine, since the

intracellular concentration of L-arginine has shown to be many times higher than necessary for optimal activity of nitric oxide synthase (23). Whether deficiency of the co-factor tetrahydrobiopterin plays a role in HTG, as has been demonstrated in diabetes, smoking and hypercholesterolemia, remains to be elucidated (24-26). Finally, it is conceivable that high amounts of triglyceride-rich lipoproteins accumulate in endothelial cells, as was recently demonstrated in endothelial cells from atherosclerotic plaques in human coronary arteries (27). Lipid accumulation is associated with structural changes in the cell that may impede its function. Since vasodilatory effects prevail over vasoconstrictive effects in normal endothelial cells, we hypothesize that accumulation of triglycerides in endothelial cells may lead to impairment of vasorelaxation.

The observation that atorvastatin restores endothelial function in HTG patients, indicates that treatment with this HMG-CoA reductase inhibitor might reduce the risk for cardiovascular disease. The mechanism underlying this beneficial effect of statins on endothelial function remains to be elucidated. However, it seems likely that the reduction of the circulating lipid burden by lowering plasma triglyceride and cholesterol levels, and the improvement of the LDL subclass pattern, play an important role. Furthermore, there is evidence that lipid-independent mechanisms, namely upregulation of nitric oxide synthase expression, may be involved (28).

In conclusion, we have demonstrated that patients with chronic hypertriglyceridemia have an impaired endothelium-dependent vasodilation, mediated by the nitric oxide pathway, which is reversed upon lipid-lowering therapy by atorvastatin. The observation that induction of an acute artificial HTG does not influence endothelial vasodilation, suggests that lipids do not interfere directly with nitric oxide availability.

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Chapter 9

Summary

Triglycerides play an important role in energy metabolism, synthesis of steroid hormones and the uptake of lipophilic vitamins. Triglycerides are derived from dietary fat in the gut (exogenous route), but can also be produced by the liver (endogenous route). Transport is carried out by lipoproteins, which consist of a lipophilic core of triglycerides and cholesteryl esters and a surface layer of apoproteins, phospholipids and free cholesterol. Triglycerides are hydrolyzed by lipoprotein lipase (LPL), a dimeric enzyme that is attached to heparan sulphate proteoglycans (HSPG) at the luminal surface of endothelial cells. Part of the triglyceride-rich lipoproteins is removed from the circulation via a direct receptor-mediated mechanism. In case of an increased triglyceride synthesis and/or decreased catabolism, hypertriglyceridemia (HTG) may develop.

An elevated plasma triglyceride level is not a rare condition. The Framingham study has shown that 8-10% of the normal population is hypertriglyceridemic, whereas in a population with a history of cardiovascular disease up to 20% has hypertriglyceridemia. Since hypertriglyceridemia is an established cardiovascular risk factor, it is important to treat the lipid disorder appropriately. First-line therapy is based on life style measures such as weight loss and healthy diet. Additional pharmacological therapy with fibrates or statins can be indicated after careful assessment of the cardiovascular risk profile. The purpose of this dissertation is to study the pathogenesis as well as therapeutical regimens in hypertriglyceridemia.

Chapter 1. General introduction
The first chapter of the thesis gives an extensive overview of the current knowledge regarding lipoprotein metabolism in general. In addition, the various pathogenetic as well as therapeutical aspects of endogenous hypertriglyceridemia are discussed.

Chapter 2. Lipolysis of very low density lipoproteins by heparan sulphate proteoglycan-bound lipoprotein lipase
In this chapter, we describe the development of a novel lipolysis assay to study substrate suitability to proteoglycan-bound LPL. Although functional LPL acts

in vivo as a proteoglycan-bound enzyme that is attached to the luminal surface of the endothelium, its kinetics *in vitro* are commonly studied with LPL in solution. The novel assay was performed as follows. Plastic wells were incubated with heparan sulphate proteoglycans, subsequently blocked with albumin, and finally incubated with LPL. Control experiments with heparinase indicate that at least 90% of the LPL activity is derived from LPL bound to heparan sulphate chains. The intra-assay and inter-assay variations were limited.

Chapter 3. Effect of apolipoprotein E variants on lipolysis of very low density lipoproteins by heparan sulfate proteoglycan-bound lipoprotein lipase

This study was performed to apply the novel lipolysis assay in a clinical setting with VLDL of patients with different apoE-variants. Lipoproteins are considered to interact with vessel wall HSPG prior to lipolysis by endothelium-bound LPL. ApoE is thought to mediate this interaction thereby enhancing the stability of the lipoprotein-LPL complex. It was hypothesized that apoE mutations may cause a diminished interaction of VLDL with HSPG, leading to impaired lipolysis of VLDL by HSPG-bound LPL. In order to test this hypothesis, lipolysis and binding experiments were performed with VLDL, isolated from patients with various apoE mutations, using HSPG-bound LPL. VLDL, isolated from apoE2 homozygotes and apoE3-Leiden heterozygotes, displayed reduced lipolysis by HSPG-bound LPL due to a defective binding of these lipoproteins to the HSPG-LPL complex. These differences could not be detected with the conventional assay with LPL in solution.

Chapter 4. Dietary counseling effectively improves lipid levels in patients with endogenous hypertriglyceridemia: emphasis on weight reduction and alcohol limitation

Exogenous factors are considered to play an important role in the expression of hypertriglyceridemia. Dietary counseling and weight loss are the first-line therapy in these patients. A prospective dietary intervention study was performed according to the Dutch guidelines for a healthy diet to evaluate the short-term effect of dietary counseling in patients with endogenous hypertriglyceridemia and to evaluate the effects of advised nutrient changes on serum lipid levels. Before and after the dietary intervention period of 12 weeks, 24-hour food recalls were used to assess dietary intake and macronutrient composition. A significant reduction in energy intake and body

weight as well as changes in macronutrient composition were observed. Total serum triglyceride and cholesterol levels showed impressive reductions. Weight reduction and alcohol limitation correlated with reduction of total plasma triglyceride levels. This study confirms the current concept that energy restriction and weight loss are key issues in the management of hypertriglyceridemia. Novel findings are the observation that a hypocaloric, low-fat diet is also effective in non-overweight patients, and that alcohol limitation is effective in patients with endogenous hypertriglyceridemia with a moderate alcohol use.

Chapter 5. The hypolipidemic action of bezafibrate therapy in hypertriglyceridemia is mediated by upregulation of lipoprotein lipase: no effects on VLDL substrate affinity to lipolysis or LDL receptor binding

Fibrates are regarded as drugs of choice in hypertriglyceridemia. Downregulation of apoC-III transcription and upregulation of LPL gene expression have been suggested to explain the mechanism of action. This study was designed to investigate the effects of bezafibrate therapy on VLDL susceptibility to lipolysis, VLDL binding to the LDL receptor and postheparin LPL activities in patients with endogenous HTG. Eighteen HTG patients were randomized to receive in a double-blind placebo-controlled cross-over fashion 400 mg bezafibrate once daily for 6 weeks. In response to bezafibrate therapy, plasma triglyceride and apoC-III levels decreased by 69% and 42%, respectively. HTG VLDL was lipolyzed less efficiently compared to control VLDL and lipolysis did not improve by bezafibrate therapy. VLDL binding affinity to the LDL receptor was comparable between the control group and HTG group, and did not change upon bezafibrate therapy. However, the post-heparin LPL activity in the HTG patients increased and a strong inverse relation was observed between the increase in LPL activities and the decrease in triglyceride levels. In conclusion, the hypolipidemic action of bezafibrate therapy in HTG may be attributed to upregulation of LPL, whereas the susceptibility of VLDL to lipolysis and LDL receptor binding are not affected.

Chapter 6. Normal F2-isoprostane levels and enhanced lipoprotein resistance to in vitro oxidition in patients with hypertriglyceridemia: effects of bezafibrate treatment

There is evidence that several forms of hyperlipidemia are associated with increased oxidative stress. However, no studies have addressed the oxidation

status in patients with hypertriglyceridemia in detail. The present study was undertaken to compare plasma levels of F2-isoprostanes and *ex vivo* VLDL and LDL oxidation behavior between patients with endogenous hypertriglyceridemia and control subjects, and to evaluate the effects of fibrate therapy. F2-isoprostane levels were comparable in the patients with hypertriglyceridemia and control subjects and did not change upon fibrate therapy. However, both VLDL and LDL in the hypertriglyceridemic group demonstrated prolonged *ex vivo* oxidation resistance times and lower oxidation rates in comparison with the control group. Bezafibrate therapy reversed these oxidation parameters in hypertriglyceridemia towards the normal range. In conclusion, hypertriglyceridemia appears to be associated with normal F2-isoprostane levels and an increased resistance of VLDL and LDL to oxidation.

Chapter 7. Activated platelets in patients with hypertriglyceridemia: effect of triglyceride-lowering therapy

The effects of triglycerides on platelet function are controversial. This study was designed to compare the *in vivo* platelet activation status between patients with hypertriglyceridemia and control subjects, and to evaluate the effects of triglyceride-lowering therapy. Analysis of platelet activation was performed by double label flow cytometry, using monoclonal antibodies against GP53, P-selectin, and platelet-bound fibrinogen as platelet surface activation markers. Baseline platelet analyses demonstrated that the surface expression of the lysosomal membrane protein GP53 was significantly higher in the hypertriglyceridemic patients as compared to the control group. No differences in the expression of P-selectin and fibrinogen binding were observed. In response to bezafibrate therapy, the expression of GP53 in the patient group decreased, whereas the expression of P-selectin and fibrinogen binding was not affected. In conclusion, patients with hypertriglyceridemia have an increased *in vivo* platelet activation status which can be improved by bezafibrate therapy.

Chapter 8. Not acute but chronic hypertriglyceridemia is associated with impaired endothelium-dependent vasodilation: reversal upon lipid-lowering therapy by atorvastatin

Although endothelial dysfunction has been associated with most cardiovascular risk factors, there is controversy regarding the relation between hypertriglyceridemia and endothelial function. Eight male patients with chronic HTG were studied before and after 6 weeks of lipid-lowering

treatment with 80 mg atorvastatin once daily. Ten age-matched control subjects were studied at baseline and immediately after a high-dose infusion of artificial triglycerides. Endothelial function was assessed by venous occlusion plethysmography. The forearm blood flow (FBF) response to serotonin was attenuated in the HTG group compared to the controls, whereas the FBF response to nitroprusside was comparable to that observed in the control group. Atorvastatin therapy was able to reverse the endothelium-dependent vasodilation to normal. Induction of acute hypertriglyceridemia in the control subjects did not affect the FBF responses to serotonin and nitroprusside. This chapter demonstrates that unlike acute hypertriglyceridemia, chronic hypertriglyceridemia is associated with an impaired endothelium-dependent vasodilation.

Future perspectives

Although a substantial number of factors that contribute to the expression of hypertriglyceridemia have been identified, the pathogenesis of hypertriglyceridemia remains obscure. An important issue that remains to be solved is the well-recognized association between the apoC-III gene polymorphisms and hypertriglyceridemia (1). This finding, in combination with elevated plasma apoC-III levels in hypertriglyceridemia, has resulted in the concept that hypertriglyceridemia may be caused by excess apoC-III. In the present thesis, we have shown that hypertriglyceridemic VLDL contain a normal density of apoC-III on the lipoprotein particle surface. High apoC-III levels in hypertriglyceridemia and the association between plasma apoC-III and plasma triglyceride levels appear to be a logical consequence of the fact that triglyceride-rich lipoproteins contain a substantial quantity of apoC-III. However, it has been demonstrated that apoC-III gene polymorphisms render the apoC-III-encoding gene less sensitive to the inhibitory effect of insulin, which may lead to an increased production of apoC-III (2). Assuming that the production of the various VLDL components is closely coupled to one another, triglyceride synthesis may be increased concomitantly. Future studies are needed to confirm our data and study the mechanism postulated above.

Free fatty acids are considered to play an important role in the pathogenesis of insulin resistance. In hypertriglyceridemia, the plasma levels of free fatty acids are characteristically elevated and there is a close relation between free fatty acid and triglyceride levels. Although the generation of free fatty acids by lipolysis of triglycerides is decreased in hypertriglyceridemia, plasma free fatty acid levels are increased due to reduced fatty acid

assimilation and increased fatty acid release by adipocytes (3). It would be of interest to study this mechanism in detail in hypertriglyceridemia in order to identify novel treatment strategies. With regard to the latter, the recently developed PPARγ agonists have an important effect on adipocyte differentiation and insulin sensitivity that may be effective in hypertriglyceridemia (4).

It should be emphasized that, to date, no primary or secondary prevention trial has been performed to study the effect of lipid-lowering therapy in patients with endogenous hypertriglyceridemia. The main intervention trial that are referred to are the Helsinki Heart Study (5), BECAIT (6) and BIP study (7), which were conducted in dyslipidemic and not hypertriglyceridemic patients. The relevance of treatment was only shown in subgroup-analyses. Considering the high prevalence of hypertriglyceridemia, especially in a population with coronary heart disease, I would like to stress the need for a prevention trial with fibrates or statins in patients with hypertriglyceridemia. These trials are essential for the development of guidelines that are evidence-based.

Recently, Koenig and Staels (8) reported that fibrates appear to have anti-inflammatory effects. Moreover, fibrates have been suggested to induce apoptosis of macrophages. This may imply that fibrates have a lipid-independent effect on prevention of cardiovascular disease by enhancing plaque stability. Additional studies are needed to elucidate a possible anti-inflammatory effect of fibrates and to assess the differences between the various fibrate derivates.

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Chapter 10

Samenvatting

Triglyceriden spelen een belangrijke rol in de energiehuishouding, de synthese van steroïdhormonen en de opname van vetoplosbare vitamines. Triglyceriden zijn afkomstig uit diëtair vet in de darm (exogene route), maar worden ook geproduceerd door de lever (endogene route). Transport vindt plaats door lipoproteïnen, welke bestaan uit een lipofiele kern van triglyceriden en cholesterol esters, en daaromheen een schil van apoproteïnen, fosfolipiden en vrij cholesterol. Triglyceriden worden gehydrolyseerd door lipoproteïne lipase, een enzym dat gebonden is aan heparan sulfaat proteoglycanen aan de luminale zijde van endotheelcellen. Een deel van de triglyceriderijke lipoproteïnen wordt verwijderd uit de circulatie door een direct, receptor-gemediëerd mechanisme. In geval van een verhoogde triglyceride synthese en/of een verminderd katabolisme kan hypertriglyceridemie ontstaan.

Een verhoogde plasma triglyceride-concentratie is geen zeldzaam fenomeen. Het Framingham onderzoek heeft aangetoond dat 8-10% van de normale populatie hypertriglyceridemisch is, terwijl in een populatie met een cardiovasculaire voorgeschiedenis zelfs 20% hypertriglyceridemie heeft. Aangezien hypertriglyceridemie een bewezen cardiovasculaire risicofactor vormt, is het belangrijk om deze lipidenafwijking adequaat te behandelen. De initiële behandeling is gebaseerd op leefstijlveranderingen zoals gewichtsverlies en goede voedingskeuzen. Aanvullende farmacologische behandeling met fibraten of statines kan geïndiceerd zijn na zorgvuldige afweging van het cardiovasculair risicoprofiel. Het doel van deze dissertatie is meer helderheid te verschaffen in de ontstaanswijze en behandeling van hypertriglyceridemie.

Hoofdstuk 1. Introductie

Het eerste hoofdstuk van dit proefschrift geeft een uitgebreid overzicht van het lipoproteïnenmetabolisme in het algemeen. Daarnaast wordt een aantal pathogenetische en therapeutische aspecten van endogene hypertriglyceridemie besproken.

Hoofdstuk 2. Lipolyse van very low density lipoproteïnen door heparan sulfaat proteoglycaan-gebonden lipoproteïne lipase

In dit hoofdstuk wordt de ontwikkeling van een nieuwe lipolyse-assay ter bestudering van de subtraataffiniteit voor proteoglycaan-gebonden lipoproteïne lipase (LPL) beschreven. Hoewel functioneel LPL *in vivo* werkzaam is als een proteoglycaan-gebonden enzym dat geassocieerd is met de luminale zijde van endotheel, wordt de kinetiek *in vitro* doorgaans bestudeerd met LPL in-oplossing. De nieuwe assay werd als volgt uitgevoerd. Plastic wellen werden geïncubeerd met heparan sulfaat proteoglycanen (HSPG), vervolgens geblokt met albumine, en tenslotte geïncubeerd met LPL. Na toevoeging van verschillende concentraties VLDL kon door bepaling van de hoeveelheid vrijgekomen vrije vetzuren de lipolyse worden gekwantificeerd. Controle experimenten met heparinase wezen uit dat tenminste 90% van de LPL-activiteit afkomstig bleek van heparan sulfaat-gebonden LPL. De intra- en interassay variatie was gering.

Hoofdstuk 3. Effecten van apolipoproteïne E varianten op lipolyse van very low density lipoproteïnen door heparan sulfaat proteoglycaan-gebonden lipoproteïne lipase

Dit onderzoek is uitgevoerd om de nieuwe lipolyse-assay toe te passen in een klinische setting en de effecten te bestuderen van verschillende apoE-varianten op VLDL-lipolyse. Men neemt aan dat lipoproteïnen interactie vertonen met endotheliale proteoglycanen alvorens lipolyse door endotheel-gebonden LPL kan plaatsvinden. ApoE zou deze interactie mediëren door de stabiliteit van het lipoproteïne-LPL complex te bevorderen. Wij veronderstelden dat apoE-mutaties aanleiding zouden kunnen geven tot een verminderde interactie tussen VLDL en HSPG en, dientengevolge, tot een verminderde lipolyse van VLDL door HSPG-gebonden LPL. Om deze hypothese te toetsen werden lipolyse- en bindingsexperimenten uitgevoerd met VLDL, geïsoleerd uit patiënten met diverse apoE mutaties, door gebruik te maken van HSPG-gebonden LPL. VLDL, afkomstig van apoE2 homozygoten en apoE3-Leiden heterozygoten, bleek minder goed afbreekbaar door HSPG-gebonden LPL ten gevolge van een verminderde binding van deze lipoproteïnen aan het HSPG-LPL complex.

Hoofdstuk 4. Dieetadvies verbetert plasma-lipiden in patiënten met endogene hypertriglyceridemie: nadruk op gewichtsverlies en alcoholreductie

Exogene factoren spelen een belangrijke rol in de expressie van hypertriglyceridemie. Dieetadvies en gewichtsverlies vormen de eerste-lijns behandeling bij deze patiënten. Een prospectieve interventiestudie werd uitgevoerd volgens "de Nederlandse richtlijnen gezonde voeding" met als doel de korte-termijneffectiviteit te evalueren in patiënten met endogene hypertriglyceridemie. Ook werd getracht om de effecten van de verschillende voedingsbestanddelen op plasma-lipiden te onderzoeken. Voor en na de dieet-interventieperiode van 12 weken werden 24-uurs voedingsanamneses afgenomen om de voedingsinname en -samenstelling vast te stellen. Na het dieetadvies werd een aanzienlijke reductie in energie-inname en lichaamsgewicht, evenals een verbetering in samenstelling van voedingsbestanddelen, waargenomen. De totale plasma triglyceride- en cholesterol-concentraties toonden significante dalingen. Gewichtsverlies en alcoholreductie correleerden met afname van de totale plasma triglyceride-concentratie. Dit onderzoek bevestigt het huidige concept dat energie-restrictie en gewichtsverlies een belangrijke plaats innemen in de behandeling van hypertriglyceridemie. Nieuwe bevindingen zijn de observaties dat een hypocalorisch, vetarm dieet ook effectief is in niet-obese patiënten, en dat alcoholbeperking ook effectief is in patiënten met endogene hypertriglyceridemie met een sociaal alcoholgebruik.

Hoofdstuk 5. De hypolipidemische werking van bezafibraattherapie in hypertriglyceridemie wordt gemediëerd door opregulering van lipoproteïne lipase: geen effecten op substraataffiniteit van VLDL voor lipolyse of LDL-receptor binding

Fibraten worden beschouwd als geneesmiddel van eerste keus bij hypertriglyceridemie. Men veronderstelt dat het werkingsmechanisme berust op downregulering van apoC-III-transcriptie en opregulering van LPL-gen expressie. Dit onderzoek is opgezet om de effecten te bestuderen van bezafibraattherapie op de gevoeligheid van VLDL voor lipolyse, binding van VLDL aan de LDL-receptor en postheparine LPL-activiteiten in patiënten met endogene HTG. Achttien HTG patiënten werden gerandomiseerd om, in een dubbelblind, placebo-gecontroleerde cross-over studie, 400 mg bezafibraat per dag te gebruiken gedurende 6 weken. Bezafibraattherapie resulteerde in een afname van plasma triglyceride- en apoC-III-concentraties met resp. 69% en

42%. HTG-VLDL werd minder efficiënt gelipolyseerd dan controle-VLDL, hetgeen niet verbeterde tijdens therapie. De VLDL-bindingsaffiniteit voor de LDL-receptor was vergelijkbaar tussen de controle- en HTG-groep en veranderde niet tijdens bezafibraattherapie. Echter, de post-heparine LPL-activiteit van de HTG patiënten nam toe onder invloed van bezafibraat, en een sterke omgekeerde relatie werd gezien tussen de toename in LPL-activiteit en de afname van de triglyceride-concentratie. Samenvattend, de hypolipidemische werking van bezafibraattherapie in HTG blijkt te berusten op opregulering van LPL, terwijl de gevoeligheid van VLDL voor lipolyse en LDL-receptor binding niet wordt beïnvloed.

Hoofdstuk 6. Normale F2-isoprostaan-concentraties en verhoogde lipoproteïne-resistentie tegen *in vitro* oxydatie in patiënten met hypertriglyceridemie: effecten van bezafibraattherapie

Er zijn aanwijzingen dat verschillende hyperlipidemieën geassocieerd zijn met verhoogde oxydatieve stress. Er zijn echter geen studies voorhanden met gegevens over de oxydatieve status van patiënten met hypertriglyceridemie. De huidige studie werd verricht om plasmaspiegels van F2-isoprostanen en *ex vivo* VLDL- en LDL-oxydatiegedrag van patiënten met endogene hypertriglyceridemie te vergelijken met controlepersonen. Tevens werden de effecten van fibraattherapie bestudeerd. F2-isoprostaan-concentraties in plasma waren vergelijkbaar tussen de patiënten met hypertriglyceridemie en de controlepersonen en veranderde niet tijdens fibraattherapie. Echter, zowel hypertriglyceridemisch VLDL als LDL vertoonden een grotere *ex vivo* oxydatieresistentie en lagere oxydatiesnelheden in vergelijking met de controlegroep. Bezafibraattherapie induceerde veranderingen in deze oxydatieparameters richting waarden van de referentiegroep. Concluderend, hypertriglyceridemie is geassocieerd met normale F2-isoprostaan-concentraties en een verhoogde resistentie van VLDL en LDL tegen oxydatieve stress.

Hoofdstuk 7. Geactiveerde bloedplaatjes in patiënten met hypertriglyceridemie: effecten van triglyceride-verlagende therapie

De effecten van triglyceriden op plaatjesfunctie zijn controversieel. Dit onderzoek werd opgezet om de *in vivo* plaatjesactivatiestatus te vergelijken tussen patiënten met hypertriglyceridemie en controlepersonen, en de effecten van triglycerideverlagende therapie te bestuderen. Analyse van plaatjesactivatie werd verricht door middel van dubbel-label flowcytometrie

met antilichamen tegen GP53, P-selectine en plaatjesgebonden fibrinogeen als plaatjesactivatie markers. De uitgangsanalyses toonden aan dat de oppervlakte-expressie van het lysosomale membraaneiwit GP53 significant hoger was in de hypertriglyceridemische patiënten dan in de controlegroep. Er werden geen verschillen gezien in de expressie van P-selectine en fibrinogeen-binding. De expressie van GP53 in de patiëntengroep nam af tijdens bezafibraattherapie, terwijl de expressie van P-selectine en fibrinogeen-binding niet werden beïnvloed. Samenvattend, patiënten met hypertriglyceridemie hebben een verhoogde *in vivo* plaatjesactivatiestatus die verbeterd kan worden door bezafibraattherapie.

Hoofdstuk 8. Niet acute maar chronische hypertriglyceridemie is geassocieerd met verminderde endotheelafhankelijke vaatverwijding: verbetering door lipidenverlagende therapie met atorvastatine

Hoewel endotheeldysfunctie geassocieerd is met cardiovasculaire risisofactoren, bestaat er geen eenduidigheid over de endotheelfunctie in patiënten met hypertriglyceridemie. Acht mannelijke patiënten met chronische HTG werden bestudeerd voor en na een 6 weken durende behandeling met 80 mg atorvastatine per dag. Daarnaast werden 10 leeftijd-gematchte controlepersonen bestudeerd vóór en onmiddellijk na infusie van een hoge dosering artificiële triglyceriden. De endotheelfunctie werd gemeten door middel van veneuze occlusieplethysmografie. De verandering in onderarmsdoorbloeding op serotonine was verminderd in de HTG-groep in vergelijking met de controlegroep, terwijl de reactie op nitroprusside vergelijkbaar was in beide groepen. Atorvastatinetherapie bleek in staat de verminderde endotheelafhankelijke vaatverwijding te normaliseren. Introductie van een acute hypertriglyceridemie in de controlepersonen had geen effect op de reacties in onderarmsdoorbloeding op serotonine en nitroprusside. Dit hoofdstuk toont aan dat, in tegenstelling tot een acute hypertriglyceridemie, chronische hypertriglyceridemie geassocieerd is met een verminderde endotheelfunctie.

Toekomstperspectieven

Hoewel een aantal factoren die kunnen bijdragen aan de expressie van endogene hypertriglyceridemie geïdentificeerd is, blijft de ontstaanswijze onduidelijk. Een belangrijke vraag die beantwoord dient te worden is waarom er een associatie bestaat tussen polymorfismen in het apoC-III-gen en het vóórkomen van hypertriglyceridemie (1). Deze bevinding in combinatie met

verhoogde apoC-III-concentraties in hypertriglyceridemie heeft geleid tot de hypothese dat hypertriglyceridemie veroorzaakt zou kunnen worden door een overmaat aan apoC-III. In dit proefschrift is aangetoond dat hypertriglyceridemisch VLDL een normale dichtheid aan apoC-III bevat op het lipoproteïne oppervlak. Hoge apoC-III-concentraties in hypertriglyceridemie en de associatie tussen plasma apoC-III- en plasma triglyceride-concentraties lijken een logisch gevolg te zijn van het feit dat triglyceriderijke lipoproteïnen een aanzienlijke hoeveelheid apoC-III bevatten. Echter, men heeft aangetoond dat polymorfismen in het apoC-III-gen samengaan met een verminderde gevoeligheid voor de remmende werking van insuline, hetgeen zou kunnen leiden tot een verhoogde productie van apoC-III (2). Ervan uitgaande dat de productie van de afzonderlijke VLDL-bestanddelen nauw gekoppeld is, zou de triglyceride-synthese, parallel aan de apoC-III synthese, verhoogd kunnen zijn. Aanvullend onderzoek is nodig om onze resultaten te bevestigen en de hierboven gepostuleerde hypothese te toetsen.

Vrije vetzuren lijken een belangrijke rol te spelen in de pathogenese van insulineresistentie. De concentraties van vrije vetzuren in plasma zijn karakteristiek verhoogd in hypertriglyceridemie en er bestaat een nauwe relatie tusen plasma vrije vetzuur- en triglyceridespiegels. Hoewel het vrijkomen van vrije vetzuren tijdens lipolyse van triglyceriden verlaagd is in hypertriglyceridemie, blijken de vrije vetzuur-concentraties in plasma verhoogd te zijn ten gevolge van een verlaagde opname door de weefsels en een verhoogde productie door adipocyten (3). Met het oog op de ontwikkeling van nieuwe behandelingsstrategieën zou dit mechanisme uitvoerig onderzoek verdienen in de toekomst. De recent ontwikkelde PPARγ agonisten, die een belangrijke invloed hebben op de differentiatie van adipocyten en insuline resistentie, vormen een interessante groep geneesmiddelen die effectief zou kunnen zijn in de behandeling van hypertriglyceridemie (4).

Er dient benadrukt te worden dat tot op heden geen primaire of secundaire preventiestudies verricht zijn naar de effecten van lipidenverlagende therapie in patiënten met endogene hypertriglyceridemie. De belangrijkste interventiestudies waaraan gerefereerd wordt zijn de Helsinki Heart Study (5), BECAIT (6) en BIP (7), die alle uitgevoerd werden in dyslipidemische en niet primair hypertriglyceridemische patiënten. De relevantie van behandeling van hypertriglyceridemie werd dan ook alleen aangetoond in subgroep-analyses. Gezien de hoge prevalentie van hypertriglyceridemie in de populatie patiënten met hart- en vaatziekten, dient het belang van preventiestudies met fibraten of statines in patiënten met

hypertriglyceridemie benadrukt te worden. Deze onderzoeken zijn absoluut noodzakelijk voor het ontwikkelen van richtlijnen die "evidence-based" zijn.

Recent hebben Koenig en Staels (8) aangetoond dat fibraten anti-inflammatoire effecten hebben. Ook werd gesuggereerd dat fibraten apoptose van macrofagen kunnen induceren. Deze bevindingen impliceren dat fibraten een lipiden-onafhankelijk effect zouden kunnen hebben op de behandeling van cardiovasculaire ziekte door stabilisering van de atherosclerotische plaque. Aanvullende onderzoek is nodig om de mogelijke anti-inflammatoire effecten van fibraten in kaart te brengen en te bekijken of er verschillen zijn tussen de diverse derivaten.

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Abbreviations

Apo	Apolipoprotein
BSA	Bovine serum albumin
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
CR	Chylomicron remnant
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBF	Forearm blood flow
FC	Free cholesterol
FD	Familial dysbetalipoproteinemia
FFA	Free fatty acid
HDL	High density lipoprotein
HL	Hepatic lipase
HLP	Hyperlipoproteinemia
HSPG	Heparan sulfate proteoglycan
HTG	Hypertriglyceridemia
IDL	Intermediate density lipoprotein
Km	Michaelis-Menten constant
LCAT	Lecithin:cholesteryl acyltransferase
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LRP	Low density lipoprotein-related protein
MTP	Microsomal transfer protein
NO	Nitric oxide
PBS	Phosphate-buffered saline
RFLP	Restriction fragment length polymorphism
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Sodium-nitroprusside
TG	Triglyceride
Tris	Tris(hydroxymethyl)-aminomethane
TRL	Triglyceride-rich lipoprotein
VLDL	Very low density lipoprotein
5-HT	5-Hydroxytryptamine, serotonin

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Curriculum Vitae

Frederik Hendrik Andreas Franciscus de Man werd geboren op 18 november 1967 te 's-Hertogenbosch. Na het behalen van het Gymnasium-β-diploma aan het Bisschoppelijk College te Weert, begon hij in 1986 aan de studie Geneeskunde aan de Rijksuniversiteit te Utrecht. Daarnaast volgde hij in het kader van het Utrechtse "excellent tracée" de verkorte opleiding Medische Biologie van 1990 tot 1992. Als bijvak werd onderzoek gedaan op de afdeling Experimentele Cardiologie van het Academisch Ziekenhuis Utrecht naar vaatwandbeschadiging in minivarkens. De hoofdvakstage werd doorlopen op het Laboratorium van Vetstofwisseling in het Academisch Ziekenhuis Utrecht met als doel meer inzicht te krijgen in de afbraakgevoeligheid van lipoproteïnen door lipoproteïne lipase. Na het behalen van het doctoraalexamen Medische Biologie zette hij de studie geneeskunde voort, hetgeen in 1995 resulteerde in het behalen van het artsexamen. Aansluitend was hij 10 maanden werkzaam als assistent-geneeskunde-niet-in-opleiding Cardiologie in het Groot Ziekengasthuis te 's-Hertogenbosch onder leiding van dr. H.J.M. Dohmen. Van oktober 1995 tot maart 1999 was hij, aanvankelijk als beurs-promovendus en vervolgens als assistent-in-opleiding, werkzaam op de afdeling Cardiologie (opleider prof. dr. A.V.G. Bruschke) van het Leids Universitair Medisch Centrum. Daar werd onder leiding van prof. dr. A. van der Laarse in nauwe samenwerking met de afdeling Algemene Interne Geneeskunde (dr. A.H.M. Smelt en dr. J.A. Gevers Leuven) en het Gaubius Laboratorium van TNO-PG te Leiden (prof. dr. L.M. Havekes en dr. H.M.G. Princen) het in dit proefschrift beschreven onderzoek uitgevoerd. Sinds maart 1999 is hij, in het kader van de opleiding tot cardioloog, werkzaam als arts-assistent op de afdeling Interne Geneeskunde van het Academisch Ziekenhuis Utrecht onder leiding van prof. dr. D.W. Erkelens.