Statin therapy in diabetic dyslipidemia
Efficacy and mechanisms

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Statine therapie bij diabetische dyslipidemie
Efficiëntie en mechanismen

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Aan Luuk
Aan mijn ouders

List of abbreviations:

ApoA1	Apolipoprotein A1
ApoB-100	Apolipoprotein B
ApoC-III	Apolipoprotein C-III
A10	Atorvastatin 10mg
A80	Atorvastatin 80mg
BMI	Body Mass Index
CETP	Cholesterol Ester Transfer Protein
CHD	Coronary Heart Disease
DALI	Diabetes Atorvastatin Lipid Intervention study
FFA	Free Fatty Acid
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
HL	Hepatic Lipase
HMG-CoA	Hydroxymethylglutaryl coenzyme A
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
LIPC	Human hepatic lipase gene.
LpB:CIII	ApoC-III and apoB-containing lipoprotein particles
LPL	Lipoprotein Lipase
TC	Total cholesterol
TG	Triglyceride
TRL	Triglyceride-rich lipoproteins
VLDL	Very low-density lipoprotein
WHR	Waist-to-Hip ratio

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Chapter 1

Introduction

1.1 General Introduction

It has been estimated that in the year 2010 there will be more than 220 million patients with diabetes mellitus worldwide (1). About 90% of these patients will have diabetes mellitus type 2. In the Netherlands, it is expected that the number of patients will have doubled to 500.000 patients (2). Type 2 diabetes is associated with high morbidity and mortality from macro- and microvascular complications. Atherosclerosis is the cause of death in 75-80% of adults with type 2 diabetes (3). At diagnosis, 30% of the patients have already some evidence of established coronary heart disease (4). Diabetes itself is a strong, independent risk factor for death from cardiovascular disease (5), and besides hyperglycemia, specific diabetic risk factors like endothelial dysfunction, glycation of proteins, and coagulation abnormalities may contribute (6). Several other atherosclerotic risk factors are combined in type 2 diabetes, like dyslipidemia, hypertension and obesity (7).
Although diabetic dyslipidemia is an established risk factor for cardiovascular disease, there is still a lack of information about the pathogenetic mechanism and the effect of hypolipidemic treatment. HMG CoA reductase inhibitors (statins) effectively lower total cholesterol (TC), low-density lipoprotein (LDL) and triglycerides (TG) in type 2 diabetes. Besides a decrease in hepatic production of very low-density lipoprotein (VLDL) and LDL-C, statins reduce TC and LDL-C through a LDL-receptor-mediated clearance. The mechanism by which statins lower plasma TG is still not completely understood. Regulators of TG and HDL-C plasma levels and of HDL and LDL subclasses composition are lipolytic enzymes, apolipoproteins, lipid transfer proteins, receptors and cellular transporters. Is it possible that these enzymes and proteins are potential targets for lipid-lowering therapy and if so, by which mechanism? This thesis will mainly focus on the effect of atorvastatin on hepatic lipase (HL), lipoprotein lipase (LPL) and apolipoprotein (apo)C-III, that may be key factors in the pathogenesis of lipoprotein abnormalities in type 2 diabetes. Other important aspects of the influence of statin therapy in patients with diabetic dyslipidemia, will be evaluated as well. The effect of atorvastatin on LDL subfractions will be described, since qualitative differences in lipoproteins may lead, besides quantitative differences, to the increased risk of coronary heart disease (CHD) in type 2 diabetes. The intriguing question whether there are gender differences in etiology of diabetic dyslipidemia that may contribute to the equalization of cardiovascular disease risk in men and women, will also be evaluated. Furthermore, if diabetic dyslipidemia indeed contributes to cognitive impairment, hypolipidemic treatment may result in improvement of cognitive functioning in patients with type 2 diabetes. Since most of these patients will eventually receive hypolipidemic treatment, studying the possible beneficial side-effect of lipid-lowering is of clinical interest as well.

1.2 Diabetic dyslipidemia

Type 2 diabetic dyslipidemia is characterized by elevated TG, low HDL-C, excessive postprandial lipemia and accumulation of remnant lipoprotein particles. These lipid derangements are intrinsically related to the abnormal physiology produced by insulin resistance or inadequate insulin action and concomitant metabolic disturbances. A main cause of the elevated TG concentration is overproduction of VLDL in the liver, provoked by an increased flow of glucose and free fatty acids (FFA) to the liver. Additionally, there may be reduced catabolism of the triglyceride rich lipoproteins (TRLs), including VLDL and chylomicrons, due to the altered activity of LPL. LPL in adipose tissue is controlled by insulin and its activity may be reduced by prolonged insulin deficiency or decrease in insulin action (8). In a non-fasting state, there will be a competition between circulating VLDL particles and postprandially synthesized chylomicrons for the action of LPL. Together with the overproduction of VLDL in type 2 diabetes, this competition between TRLs may lead to enhanced postprandial lipemia (9,10).
In addition to qualitative effects, quantitative changes in lipoproteins have also been observed. The VLDL composition may be changed, the particles are larger and TG enriched. The larger VLDL particles are the precursors of the atherogenic small dense LDL particles that are more readily engulfed by macrophages and taken into the arterial wall. These small dense LDL particles are more atherogenic than the larger, more buoyant particles by increased oxidation, increased affinity for the arterial wall and decreased clearance via the LDL-receptor pathway (11). The modified LDL particles bind less well to the LDL-receptor on the liver and may leave the circulation via the LDL scavenger receptors present on endothelial cells and macrophages (12). Furthermore small dense LDL particles have a tendency to induce free radical release (13). Taskinen et al (14) showed an association between LDL particle size and endothelial dysfunction suggesting increased atherogenicity, even at normal levels of LDL-cholesterol in type 2 diabetes. Hypertriglyceridemia also has important consequences for HDL metabolism, since TG metabolism is strongly related to HDL levels. If TG levels increase, numbers of HDL particles decrease. This is partly explained by an increased catabolic rate of HDL. LPL, HL and cholesterol ester transfer protein (CETP) are the main modulators of HDL-C. These modulators may be affected in type 2 diabetes. The rate of cholesteryl ester transfer, mediated by CETP, between HDL and the apolipoprotein B-containing lipoproteins is enhanced during hypertriglyceridemia due to an elevated concentration of apolipoprotein B-containing lipoproteins (15,16). In diabetic dyslipidemia the HDL particles are often smaller and more dense. This is partly due

to the expanded VLDL pool and to CETP over-activity resulting in an increased neutral lipid exchange. This subsequently leads to TG enrichment of both HDL and LDL. The smaller and denser HDL particles have altered physiological functions. The reverse cholesterol transfer capacity may be diminished and the antioxidant potential may be reduced. This will eventually interfere with the anti-atherogenic potential of HDL. The effect of therapeutic intervention on some aspects of these pathophysiological alterations in diabetes that may lead to diabetic dyslipidemia will be evaluated in this thesis.

1.3 Treatment of lipid disorders in type 2 diabetes mellitus

Although there is controversy about how aggressively to treat cardiovascular risk factors in patients with diabetes, they are at high risk and it has been argued that diabetics should be treated as if they have established CHD (17). Besides hypertension, diabetic dyslipidemia has emerged as a prevalent and modifiable atherogenic risk factor. Optimal lipid lowering in type 2 diabetes should focus on lowering of LDL-C and plasma TG levels and increasing HDL-C levels, since increased plasma TG and LDL-C levels and decreased HDL-C levels are all associated with increased risk for cardiovascular morbidity and mortality (11,18). Statins, as well as fibrates are used for therapeutic treatment of diabetic dyslipidemia. Post-hoc analyses of large, clinical trials, have shown that statins reduce the incidence of cardiovascular events in diabetic patients with prior CHD (19-21). These secondary prevention trials differ in relative risk-reduction (19%-55%). Recently, results of the Heart Protection Study (22), showed a 12% reduction in total mortality, a 18% reduction in vascular mortality, a 24% reduction in CHD events, a 27% reduction in all strokes and a 16% reduction in non-coronary revascularisations in more than 20.5000 patients at high risk for CHD. There were 5963 patients with diabetes of whom 3985 had no history of CHD. Other large prevention trials in patients with diabetes are currently ongoing (23,24) and may probably raise evidence to lower target levels below those recommended in current treatment guidelines and whether or not aggressive treatment of dyslipidemia leads to primary prevention of cardiovascular events in patients with type 2 diabetes.
Although fibrates lower plasma TG and increase HDL-C levels, intervention studies using fibrate therapy in patients with diabetes, did not show conclusive evidence with respect to reduction of cardiovascular events (25,26).
Based on present evidence (19-21), the American Diabetes Association (ADA) and the National Cholesterol Education Program (NCEP) have extracted guidelines with target values for plasma lipid levels in patients with diabetes (27,28), LDL-C <

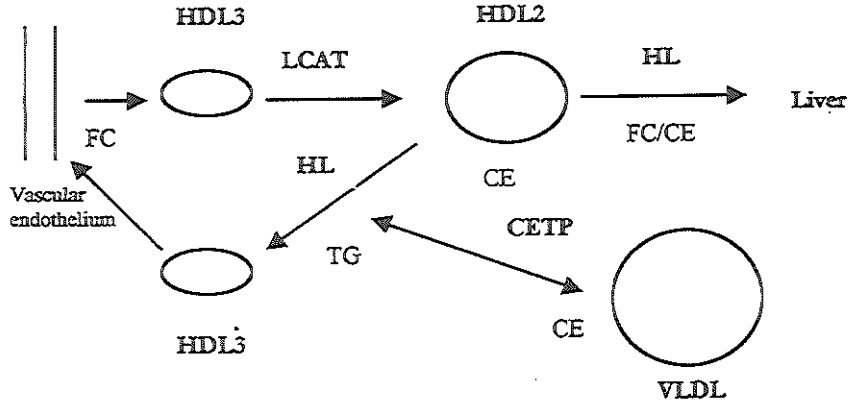
2.60 mmol/l, HDL-C >1.15 mmol/l and TG <2.3 mmol/l. To increase patient compliance, therapy should be simple and effective, so treatment with only one drug is preferred over a combination of drugs. Statins not only reduce LDL-C by 30-40%, but recent studies have shown that statins in adequate dosages lower TG levels and slightly increase HDL-C levels as well (29). These data are derived from small studies and most of these studies were done in subjects without diabetes. It is likely that newer and more potent statins and higher dosages of older statins with proven clinical efficacy, are beneficial for diabetic patients with dyslipidemia. Therefore, in this thesis the effect of low and high dose statin therapy on the diabetic lipid profile will be evaluated.
Moreover, the mechanisms of action of statins on lipid metabolism are not fully understood. Insight in these mechanisms may help to better understand the contribution of statins to cardiovascular risk reduction in patients with diabetes and may also add to a further improvement of therapeutic interventions. It is therefore that the physiological roles and the pathophysiology in diabetes of some key factors in lipid metabolism are under study in this thesis and will be discussed in the next paragraphs of this introduction.

1.4 The role of hepatic lipase (HL) in the lipoprotein metabolism

Hepatic lipase (HL) is a glycoprotein synthesized in hepatocytes. Breedveld et al (30) showed that a major portion of HL in the liver is located on luminal surfaces of sinusoidal endothelium and on the surfaces of hepatocytes, consistent with the functions of this enzyme in lipoprotein metabolism. HL is involved in the metabolism of several lipoproteins by catalysing the hydrolysis of lipoprotein TG and phospholipids (31).
HL, a key player in HDL metabolism, is usually inversely correlated with HDL-C levels (32,33) (Figure 1). Hydrolysis of phospholipids and TG by HL, leads to the conversion of large, buoyant HDL2 to small, dense HDL3 and may induce cholesterol (ester) flux to the liver (34,35). In this way HL may be involved in the reverse cholesterol transport (32). HL promotes the uptake of HDL-C, either directly and/or by facilitating uptake via lipoprotein receptors, and may therefore lower HDL-C levels (36,37). In hypertriglyceridemic conditions, such as type 2 diabetes, HDL is more enriched in TG, due to a transfer of cholesterol ester to apoB-containing lipoproteins in exchange for TG. HDL-TG is a substrate for HL and hydrolysis of HDL-TG by HL results in delipidation of HDL, which is more prone to degradation than lipid-rich HDL (31,37). Therefore, the effect of HL on the HDL metabolism during hypertriglyceridemia is unfavorable. HL plays an

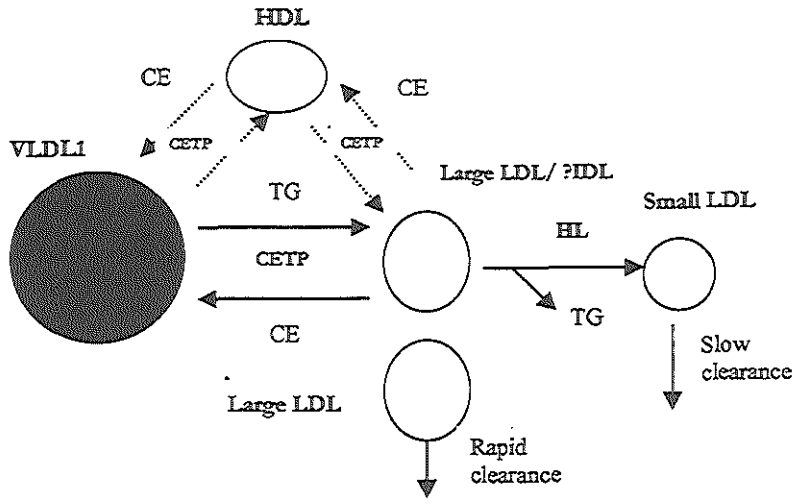
important role in the formation of LDL from small VLDL and atherogenic IDL (Figure 2). It modulates the compositional properties of LDL and contributes to the expression of the LDL subclass phenotype. In non-hypertriglyceridemic conditions, the amount of VLDL-TG exchange may be the limiting factor, determining LDL-size (38,39), but in hypertriglyceridemic conditions there is an excess of LDL-TG and small dense LDL may be formed as a result of lipolytic action of HL on TG-enriched LDL. Finally, HL is proposed to be involved in post-prandial lipid clearing (40-42). HL may influence chylomicron-remnant removal via hydrolysis of the remnant phospholipids, thereby enhancing the binding of the particles to apoE-recognizing receptors and by acting as a ligand protein for chylomicron-remnant binding to the liver.
In conclusion, the effect of HL activity as a determinant of HDL and LDL metabolism and phenotype, may differ in hypertriglyceridemic situations, like in type 2 diabetes, compared with normotriglyceridemic situations.

Figure 1 The role of hepatic lipase in HDL metabolism



Small HDL3 accepts cholesterol from tissues and LCAT converts free cholesterol (FC) to cholesterol ester (CE). HDL becomes larger and more spherical (HDL2). This large CE-rich HDL is a substrate for HL. HL stimulates HDL cholesterol uptake by hepatocytes. If TG-rich lipoproteins are present, denoted here as VLDL, CETP transfers cholesterol from HDL and TG from TG-rich lipoproteins. HL then hydrolyzes HDL2 TG and phospholipid and converts HDL2 back to smaller HDL3. Alternatively the HDL particle after HL and CETP action may be eliminated.

Figure 2 Formation and metabolism of small, dense LDL



If plasma TG exceeds levels > 1.5 mmol/L, there is sufficient CETP mediated TG movement from VLDL1 to LDL (possibly IDL) to promote hydrolysis of the LDL (or IDL) triglyceride core by HL. The resulting small, dense LDL has a relatively slow clearance from plasma and will accumulate. In the absence of high TG levels, LDL-TG is not a preferred substrate for HL and LDL remains larger and more buoyant. Large LDL will be rapidly cleared via the LDL-receptors. The dotted pathway indicates that TG transfer may occur through a HDL intermediary.

1.5 Factors influencing HL activity

HL activity shows large inter-individual variation. Several factors contribute to this variability like inheritance, sex steroid hormones, diabetes and adiposity (43-46).
In the human hepatic lipase gene (indicated as *LIPC*), variants are found that affect the lipase activity (47-49). Besides rather rare variants leading to complete HL deficiency (50-52), common base substitutions in the proximal *LIPC* promoter affect HL activity up to 2 fold (46, 53-55). There are two alleles, indicated as the *LIPC* C and T allele, that are associated with high and low HL activity, respectively.
The frequency of both alleles varies highly among different ethnic populations (53,56-59). The C allele is the most common allele in Caucasians, whereas the T allele is the major allele in black Americans. Asians contain an intermediate frequency.

Besides genetic variance, HL lipase activity is hormonally determined. In particular sex hormones influence *LIPC* expression. They are held responsible for lower HL activity in women than in men (43,60). Recently, Brunzell et al (61) showed that intra-abdominal fat is a major component of the gender difference in HL activity. HL activity is associated with omental fat mass, BMI, fasting insulin and fasting plasma triglycerides (62). These correlations are reflected in a high HL activity in type 2 diabetes. Baynes et al (63), also demonstrated that insulin resistance and hyperinsulinemia are both associated with higher levels of HL activity, which probably promotes aspects of the lipoprotein profile in patients with type 2 diabetes. Whether the increase in HL activity in type 2 diabetes mellitus is modulated by genetic factors is as yet unknown.

1.6 Lipid lowering therapy and the effect on HL activity

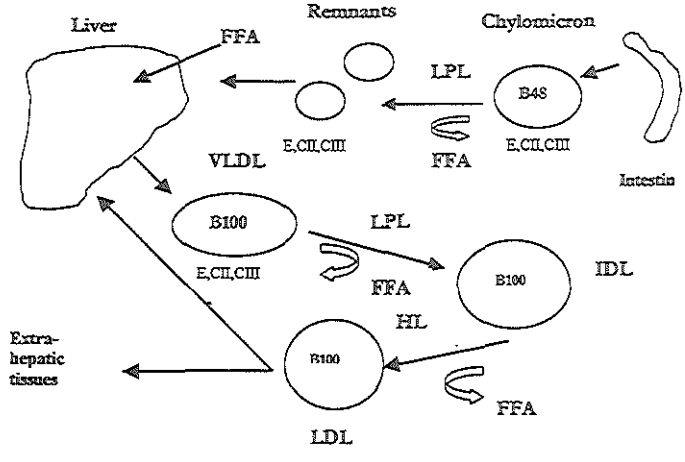
Intervention on HL activity is a potential therapeutic tool, since it may increase LDL buoyancy, increase HDL-C levels and modify HDL composition, resulting in amelioration of coronary risk in patients with diabetes mellitus. Some available hypolipidemic drugs have been shown to induce these changes in HL activity. Hoogerbrugge et al (64) found a decrease in HL activity during atorvastatin treatment of familial hypercholesterolemia. Zambon and coworkers (65) reported a drop in HL activity in participants of the FATS study, patients with documented coronary disease, upon treatment with lovastatin-colestipol and niacin-colestipol. Interestingly, the effect of the treatment was strongly dependent on the presence of the *LIPC* T allele. In carriers of this allele, the treatment effect on HL activity was less compared to non-carriers. Thus, the genotype of the *LIPC* promoter seems to determine the efficacy of the statin treatment. This is of interest as these authors also showed that lowering of the HL activity was accompanied by regression of the vascular disease. Zambon and coworkers, therefore, identified HL as a focal point for the development and treatment of CHD (66). Today, no data describing the effect of statins on HL activity in type 2 diabetes are available. In this thesis the effect of atorvastatin on HL activity in patients with type 2 diabetes will be described.

1.7 The role of lipoprotein lipase (LPL) in the lipoprotein metabolism

Lipoprotein lipase (LPL) is an enzyme bound to glycosaminoglycans on the surface of the endothelium in muscle and adipose tissue. The major sites of LPL synthesis

are the skeletal and cardiac muscle and adipose tissue, smaller amounts of LPL are produced in the kidney, brain, adrenals and in macrophages. LPL is the major enzyme responsible for conversion of lipoprotein TG into FFA and monoglycerides (67). LPL hydrolyzes TG in circulating chylomicrons after stimulation of apolipoprotein CII. The TG-depleted remnants are then degraded by the liver. LPL also hydrolyzes TG in VLDL particles, that convert initially to IDL and finally to LDL particles by HL (68,69) (Figure 3). The hydrolytic function of LPL is essential for the transfer of surface free cholesterol and phosholipids to HDL particles. LPL activity is positively correlated with HDL-C and therefore HDL levels may reflect LPL activity (70). LPL also has a nonenzymatic molecular "bridging" function, it acts as a ligand in lipoprotein-cell surface interactions, mediating cellular binding and uptake of lipoproteins (71-73). In summary, LPL influences both the plasma levels of lipoproteins and the interaction of atherogenic lipoproteins with the vascular wall. Factors influencing LPL activity may therefore interfere with these processes and thus are of clinical interest.

Figure 3 Role of LPL in the lipoprotein metabolism.



Chylomicrons are formed post-prandially in the small intestine. Chylomicron metabolism requires that these lipoproteins obtain apo CII after they enter the bloodstream from the thoracic duct. TG within the particles can then be hydrolysed by LPL. LPL is regulated by insulin, and its actions may be impaired in diabetes. TG-depleted remnants are primarily degraded in the liver.
VLDL-TG are secreted from the liver and after TG hydrolysis by LPL, converted initially to IDL, and finally to LDL. During these processes, surface lipid and apoproteins dissociate from these particles and transfer to HDL. ApoC-III interferes with the clearance of TRLs through inhibition of lipolysis by LPL. In diabetes two factors may increase VLDL production: poor insulinization results in increased FFA release from fat cells and increased apoB secretion in the liver.

1.8 Factors influencing LPL activity

LPL expression in adipose tissue is controlled by insulin, so that its activity is reduced by prolonged insulin deficiency or insulin resistance (74). In type 2 diabetes mellitus insulin action is often decreased in spite of increased plasma insulin levels. Therefore LPL activity may be decreased, resulting in an impaired lipolytic activity. Thus, in a condition of insulin insensitivity, the overproduction of VLDL TG in the liver in combination with the altered LPL activity may slow the clearance of plasma TG (8-10,75).
Although diminished LPL activity may result in part from poorly controlled diabetes and insulin deficiency, genetic defects in the LPL gene may also add to hypertriglyceridemia in patients with type 2 diabetes. About 80 naturally occurring mutations in the LPL gene have been described in humans. Most of them are missense mutations, but a number of structural mutations has been identified that diminish the lipolytic function of LPL. Genetic variants may affect the individual response to obesity and diabetes mellitus for the development of lipemia (76). Common LPL variants, resulting from amino acid substitutions, are the D9N, the N291S and the S447Stop mutation which have respectively a frequency in the Caucasian population of 2-3%, 5% and 5-20% (77-83). In vitro studies showed the D9N allele lowers LPL activity with 20% and the N291S allele with 50%. Advancing age might be another potential factor of influence to the LPL activity. This is only described in one study, that found a significant correlation between advancing age and decreasing LPL activity (84). In combination with factors like diabetes, obesity or genetic variation, changes in LPL activity influence the contribution to the atherogenic process.

1.9 Lipid lowering therapy and the effect on LPL activity

There is little information about the effect of statin therapy on post-heparin LPL activity. Hoogerbrugge et al (64), described a significant decrease in LPL activity with atorvastatin 80mg in males and females with Familial Hypercholesterolemia, –10% and-14% respectively. In males there was also a significant decrease (-13%) after atorvastatin 40mg therapy. On the other hand, Kobayashi et al (85) showed that atorvastatin 10mg effectively reduced plasma TG, but pre-heparin LPL mass was not changed. Also Heller et al (86) demonstrated in patients with mixed hyperlipidemia that atorvastatin did not affect LPL activity. They suggested that mechanisms other than enhancing lipolysis of TRLs by LPL contributed substantially to the TG reduction by atorvastatin. Simvastatin seems to increase

LPL activity in patients with combined hyperlipidemia in one study (87). Pravastatin did not affect LPL activity in patients with moderate hypercholesterolemia (88). In male New Zealand white rabbits neither atorvastatin nor simvastatin did change LPL activity (89). Studies investigating the effect of statins on LPL activity in patients with diabetes mellitus are lacking.

1.10 The role of apolipoprotein (apo)C-III in the lipid metabolism

Apolipoprotein (apo)C-III is a 8.8 kD glycoprotein, synthesized by the liver and intestine (90). It is a constituent of HDL, chylomicrons, VLDL and remnant particles. ApoC-III interferes with the clearance of TRLs through inhibition of lipolysis by LPL and diminished lipoprotein binding to proteoglycan and lipoprotein receptors (91,92). In normolipidemic subjects the majority of apoC-III is bound to HDL, while in hypertriglyceridemic subjects, the majority is bound to TRL (93). Direct evidence for causal involvement of apoC-III in hypertriglyceridemia has been obtained in studies with mice overexpressing apoC-III, in which TG rises in proportion to apoC-III mRNA (94). In patients with type 2 diabetes mellitus, hyperlipidemia or renal failure and in healthy individuals, plasma levels of apoC-III correlate with apoC-III- and apoB-containing lipoprotein particles (LpB:C-III), and TG levels (95-97). ApoC-III levels are significantly increased in obese individuals with borderline-high TG concentrations compared with controls (98). The increase in TRLs in visceral obese individuals with insulin resistance, probably arises from a decrease in catabolism of TRLs due to an increase in apoC-III. Lowering TG levels in these obese individuals seems preferable in order to reduce the amount of apoC-III (99).

Stable isotope studies demonstrate that in hypertriglyceridemic patients there is an increase in VLDL apoC-III levels. This increase is due to an increase in apoC-III production rather than a decrease in apoC-III catabolism (100). When the plasma concentration of apoC-III increases, apoC-III content in VLDL and HDL increases as well (101). Interestingly, the distribution of apoC-III among lipoproteins may affect the susceptibility to the atherogenic process (102).

Little is known about the mechanism of inhibition of remnant clearance, the interference with lipoprotein association with glycosaminoglycans and the inhibition of LPL by apoC-III. Shachter et al (103,104) proposed as predominant mechanism of hypertriglyceridemia in human apoC-II and apoC-III transgenic mice, a decrease in association between VLDL with cell-surface glycosaminoglycans, and a subsequently decreased lipolysis in-vivo. Another important proatherogenic action of apoC-III may be its interference with the clearance of apoB48 lipoproteins (105,106).

Several clinical studies have indicated apoC-III and LpB:C-III as risk factors for cardiovascular disease (107-110). The CLAS study (111) concluded that HDL apoC-III was the predominant risk factor predicting the probability of global coronary atherosclerosis progression in subjects treated with niacin-colestipol therapy. Data from the MARS study (112) suggested that in patients with mild and moderate atherosclerotic lesions, apoC-III was the predominant risk factor and that individuals with high levels of apoC-III in VLDL would be more likely to have progressive atherosclerotic disease.
Since apoC-III is a marker for the TRL-metabolism and a risk-indicator for cardiovascular disease, measurement of apoC-III in patients with type 2 diabetes seems important. It will identify the risk for coronary heart disease and it may be helpful for treatment strategies.

1.11 Lipid lowering therapy and the effect on apoC-III

In rats, it has been reported that HMG-CoA reductase inhibitors reduce plasma TG levels through modulation of apoC-III and LPL (113). Le et al (114) described an apoC-III reduction of 18-30% by atorvastatin 20mg and 80mg after 4 weeks in 27 patients with primary hypertriglyceridemia. Dallongeville (115) demonstrated an efficient reduction of LpB:CIII by atorvastatin 10mg in 305 patients with primary hypercholesterolemia. In contrast, lovastatin did affect Lipoprotein B (LDL), but not apoC-III nor LpB:C-III in patients with a coronary bypass graft (97).
In patients, atorvastatin may affect plasma TG concentration through decreased synthesis or secretion of VLDL from the liver by reducing insulin resistance (116) and/or through increased clearance of TRLs by lowering plasma apoC-III content. If and by which mechanism atorvastatin lowers the apoC-III content is still unknown.
From the clinical perspective, the high amount of apoC-III molecules on TRL's in diabetic dyslipidemia, resulting in inhibition of lipolysis of VLDL-TG by LPL (117) and interference with the hepatic uptake of TRLs by LDL receptors (118), may play a pivotal role in the delayed TG catabolism. Therefore, reduction of plasma apoC-III production might be crucial for maintaining a normal plasma lipid profile. Therapeutic intervention aimed at reducing apoC-III production seems to be a potentially powerful strategy for the treatment of hypertriglyceridemia.

1.12 HL, LPL and apoC-III in diabetic dyslipidemia as target for therapy

As described above HL, LPL and apoC-III are, among others, regulators of plasma TG and HDL-C levels and of LDL and HDL subclasses composition in diabetic dyslipidemia. Current knowledge of these key factors will now be placed within a model in order to elucidate targets for therapy.
In type 2 diabetes, there is due to poor insulinization, increased lipolysis in adipocytes. This results in increased FFA release from fat cells, which may cause an increase in VLDL secretion from the liver. Besides the enhanced secretion of VLDL, the clearance of VLDL is impaired as well. The VLDL particles are larger, due to a greater amount of TG. Most VLDL-TG particles return to the liver without complete conversion to LDL. Like the increase of apoB secretion in the liver, apoC-III secretion is elevated as well. This will also increase VLDL accumulation, by preventing the action of LPL and inhibiting lipoprotein uptake. Therefore reduction of the amount of apoC-III is a potential tool to restore LPL activity, thereby increasing clearance of TRLs and lipoprotein uptake. Atorvastatin may affect plasma TG concentration through decreased synthesis or secretion of VLDL TG from the liver, but it may also affect TG levels through apoC-III reduction. This last possibility will be studied and discussed in this thesis.
LPL is essential for the conversion of lipoprotein TG into FFA. LPL activity decreases plasma TG and TRLs, increases HDL-C and stimulates hepatic clearance of remnant particles. Its activity may be altered in diabetes. Therapy aiming to increase LPL activity in conditions where LPL activity is impaired, seems a logical strategy. Statins often seem to have no influence on LPL activity in dyslipidemic patients without diabetes. To date, there is no information about the effect of statins on LPL activity in patients with type 2 diabetes and this is a focus of study and discussion in this thesis as well.
Increased HL activity is often seen in type 2 diabetes. The increase in HL activity is possibly due to an upregulation of HL activity as a consequence of increased VLDL TG production. The long-term insulin resistance might eventually lead to failure of TG hydrolysis by HL, since the VLDL-TG supply exceeds the HL-hydrolysing capacity. In this situation high HL activity may be a marker of a pro-atherogenic lipid profile and might lead to the formation of an excess of atherogenic small, dense LDL and the formation of smaller and denser HDL-C. Reduction of HL activity may be necessary in patients with type 2 diabetes in order to restore a less atherogenic lipid profile. There are no data describing the effect of statins on HL activity currently available. Therefore the effect of atorvastatin on HL activity in patients with diabetic dyslipidemia will be described in this thesis.

1.13 Aim of the thesis

Diabetic dyslipidemia is an established risk factor for CHD and further elucidation of the lipoprotein metabolism and the effect of statin therapy seems necessary in order to better prevent atherosclerotic disease. Studying the role of lipolytic enzymes and apolipoproteins in diabetic dyslipidemia, may lead to more information of mechanisms involved and may reveal possible targets for therapeutic intervention. Results from the Diabetes Atorvastatin Lipid Intervention (DALI) study may deliver some of this information.

1.14 Description of chapters

The DALI study included patients with type 2 diabetes and diabetic dyslipidemia, expressed as hypertriglyceridemia in the range between 1.5 and 6.0 mmol/L. In Chapter 2, the effect of standard and aggressive lipid lowering therapy with atorvastatin 10mg and 80mg respectively, in these patients, will be described. The possibility of effective and safe treatment of diabetic dyslipidemia with single drug therapy might lead to an improvement of patients compliance and drug tolerability. In Chapter 3, 4 and 5, the role of HL, LPL and apoC-III in the lipid metabolism in type 2 diabetes as well as the influence of atorvastatin therapy on these regulators, is evaluated. HL activity, LPL activity and the amount of apoC-III were studied before and after atorvastatin therapy in the entire study group and in subgroups, like males, females and carriers of gene mutations. With this information, possible mechanisms of action of atorvastatin therapy in diabetic dyslipidemia were revealed and will be discussed.
In Chapter 6 the possible difference in etiology of diabetic dyslipidemia in men and women is discussed. Therefore the presence of sex differences in lipase activities before and after atorvastatin treatment is studied in order to explain possible gender differences in lipoprotein profiles. In Chapter 7 the effect of atorvastatin on LDL subfractions is presented. Besides the improvement of absolute levels of lipids and lipoproteins with statin therapy, the reduction of small dense LDL in patients with type 2 diabetes seems rewarding for reducing CHD risk, since the difference in risk for CHD between patients with and without type 2 diabetes is partly due to qualitative differences in lipoproteins. Diabetic dyslipidemia may contribute to cognitive functioning. According to the fact that treatment aimed at improvement of lipid profiles may change cognitive functioning, the effect of atorvastatin on cognitive functioning in patients with diabetic dyslipidemia, is evaluated in Chapter 8. Finally, in Chapter 9 the information from previous chapters is discussed.

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Chapter 2

The effect of aggressive versus standard lipid lowering by atorvastatin on diabetic dyslipidemia.

The DALI study: a double-blind randomized placebo-controlled trial in patients with type 2 diabetes mellitus and diabetic dyslipidemia.

The Diabetes Atorvastatin Lipid Intervention (DALI) study group

Diabetes Care 2001;24(8):1335-1341

Abstract

Objective

In patients with type 2 diabetes mellitus intensive glucose regulation, while effective for microangiopathy, has not shown unambiguous preventive effects on the occurrence of cardiovascular disease. Diabetic patients show a characteristic dyslipidemia (high triglycerides, low HDL-cholesterol). Aggressive lowering of triglycerides might be an effective method to reduce the cardiovascular risk in these patients.

Research design and methods

A double-blind placebo-controlled randomized study to assess the effect of 30 weeks atorvastatin 10mg and 80mg on plasma triglyceride levels in 217 patients with type 2 diabetes mellitus and fasting triglycerides between 1.5 and 6.0 mmol/L.

Results

Atorvastatin 10mg and 80mg resulted in significant reductions of plasma triglyceride of 25% and 35%, respectively (both $p<0.001$). The difference between 10mg and 80mg was not statistically significant ($p>0.5$). Atorvastatin 10mg provided significant reductions from baseline in total cholesterol (-30% ($p<0.001$), LDL cholesterol (-40%, $p<0.001$) and apoB (-31%, $p<0.001$), and significantly increased HDL cholesterol from baseline by 6% ($p<0.005$). Atorvastatin 80mg had a similar effect on HDL cholesterol (+5.2%, $p<0.005$), but decreased total cholesterol (-40%,$p<0.001$), LDL cholesterol (-52%, $p<0.001$) and apoB (-40%, $p<0.001$) significantly ($p<0.005$) more than atorvastatin 10mg. The side effects of atorvastatin 10mg and 80mg were similar and did not differ from the patients receiving placebo.

Conclusions

Atorvastatin 10mg and 80mg provide similar, significant reductions from baseline in triglycerides in patients with type 2 diabetes mellitus. A higher dose of atorvastatin improves cholesterol-related parameters. Both dosages were well tolerated in this patient population.

Introduction

Patients with type 2 diabetes mellitus (DM2) have a two to four fold increased risk for cardiovascular morbidity and mortality. (1-5) Intensive glucose regulation in DM2, while effective for microangiopathy, has not shown unambiguous preventive effects on the occurrence of coronary heart disease, stroke and peripheral artery disease. (6) Besides hypertension, dyslipidemia has emerged as a prevalent and modifiable atherogenic risk factor in patients with DM2. LDL-cholesterol lowering strategies, with the use of HMG-CoA reductase inhibitors, have shown at least equal benefits for the diabetic subgroups in large secondary prevention trials. (7-9) In primary prevention trials the diabetic subgroups were too small to show significant results. (10;11) However, these were all post hoc analyses and diabetic patients included in these studies did not have the typical diabetic lipid profile, i.e. elevated triglycerides, decreased HDL-cholesterol, normal or slightly elevated LDL-cholesterol. (12) In diabetic patients increased plasma triglyceride levels are associated with an increased risk for cardiovascular morbidity and mortality. (13-17) As a consequence, optimal lipid lowering in DM2 should focus on lowering of LDL-cholesterol and plasma triglycerides. (14;15) HMG-CoA reductase inhibitors have been proven to effectively reduce total cholesterol and triglycerides in non-diabetic patients (18;19) Only one small randomized study on the effect of different doses of simvastatin on diabetic dyslipidemia has been published. (20) Because higher doses of statins are effective in more aggressive cholesterol lowering, (19) we hypothesized that higher doses of statins also result in additional improvement of the diabetic lipid profile.
We performed a double-blind, placebo-controlled, randomized study to assess the effect of atorvastatin 10mg (A10) and 80mg (A80) on the reduction of triglyceride levels in patients with DM2 and diabetic dyslipidemia. In addition, we studied the effects on other aspects of diabetic dyslipidemia.

Subjects and methods

Patients

The Diabetes Atorvastatin Lipid Intervention (DALI) study is a randomized double-blind, placebo-controlled, multi-center study, conducted in the Netherlands. Patients were recruited from outpatient clinics of the University Medical Centers of Leiden, Rotterdam and Utrecht and surrounding community hospitals. The medical ethical committees of the three participating institutions approved the study, and written informed consent was obtained from all patients. The participants, aged

45–75 years, were male or female type 2 diabetic patients with a duration of diabetes of at least 1 year and HbA1c 10% or lower. The main inclusion criteria were fasting total cholesterol level between 4.0 and 8.0 mmol/L and fasting triglycerides level between 1.5 and 6.0 mmol/L.
Patients were not included in the present study if they had a history of myocardial infarction, PTCA, CABG, proven manifest coronary artery disease, severe or unstable angina pectoris (> grade II of the Canadian Cardiovascular Society), clinically manifest heart failure (> grade II NYHA) and severe cardiac arrhytmias. Premenopausal women, patients with acute liver disease or hepatic dysfunction, impaired renal function (plasma creatinine > 150 mmol/l), a history of partial ileal bypass surgery, any surgical procedure or any systemic inflammatory disease within the last three months before randomization, malignancies, vasculitis, rheumatic arthritis, idiopathic lung fibrosis, ulcerative colitis or Crohn's disease were excluded. Patients who consumed more than 4 alcoholic drinks per day or who used systemic steroids, androgens, cyclosporin, other immunosuppressive drugs, erythromycin or mibefradil were also excluded. When applicable, lipid-lowering drugs were withdrawn at least 8 weeks before the start of the run-in phase.

Study design
Patients who met the in- and exclusion criteria started with a placebo run-in period. If the lipid levels were still within the inclusion range after two weeks, patients were randomized to treatment with atorvastatin 10mg, 80mg, or placebo, administered once daily in the morning. Patients randomized to A80 started with 40mg for four weeks after which the dose was increased to 80mg. The total treatment period was 30 weeks. Follow-up visits were scheduled at weeks 4, 10, 20 and 30, at which adverse events were recorded, study medication was counted, blood pressure was measured and fasting blood samples were drawn for safety parameters and lipid profile. There were no changes in concurrent treatment, including hypoglycemic medication during the study.

Clinical safety and laboratory analysis
At baseline a medical history was taken and physical examination was performed. During the follow-up visits, patients were interviewed regarding possible adverse events.
Routine hematology and blood chemistry were determined after an overnight fast (12 hours) at screening, at randomization and at the end of the study. All laboratory measurements, except for the safety parameters, were performed at the Lipid Reference Laboratory Rotterdam, the Netherlands. Standard plasma lipid variables

(total cholesterol, HDL-cholesterol (HDL-c), triglycerides (TG), free fatty acids (FFA), and ApoB) were measured at each visit. Total cholesterol and triglycerides were measured by enzymatic colorimetric methods (CHOD-PAP and GPO-PAP, Boehringer Mannheim, Mannheim, Germany) on a Hitachi 911 analyzer (Boehringer Mannheim, Mannheim, Germany). HDL-cholesterol was measured by a direct enzymatic HDL-cholesterol method, based on PEG-modified enzymes method (Boehringer Mannheim, Mannheim, Germany) on a Hitachi 911 analyzer. LDL-cholesterol was estimated by the Friedewald formula. (21) ApoB was determined on a Hitachi 917 analyzer, using immunoturbidimetric methods (Tina-quant apo B, Cat.Nr.1551779) from Boehringer (Boehringer Mannheim, Mannheim, Germany). Fasting free fatty acids were determined using an enzymatic colorimetric method (Wako, NEFA C, Cat. Nr. 994-75409 D). The size of the LDL particles was measured at baseline and at the end of the study by polycrylamide gradient gel electrophoresis. (22). Standardization was achieved by inclusion of LDL samples with known size donated by dr. R.M. Krauss. Based on their size LDL particles were divided into two classes: pattern A reflects the presence of predominantly large buoyant LDL (>25.5 nm) and pattern B of predominantly small dense LDL particles (< 25.5 nm). IF both patterns were equally present, patients were classified as pattern AB. (23). Lipoprotein lipase (LPL) activity was determined in plasma at baseline and at the end of the study after intravenous injection of heparin (50 IU/kg body-weight) using an immuno-chemical technique. In a sample of 35 healthy subjects (mean age 55.5 years) the LPL activity measured with this technique was 147.4 ± 36.1 U/L (range 89 – 231 U/L).
Safety laboratory tests, creatine kinase (CK), alanine transferase (ALT) and aspartate transferase (AST), were performed at all follow up visits at local laboratories. An increase of the ALT or AST levels to >3 times the upper limit of normal, or an increase of CK >10 times the upper limit of normal, verified by repeat testing after one week, were considered clinically important and reported as an adverse event.

Statistics

All data were analyzed by intention-to-treat. Because patients were randomized, baseline values were not statistically tested between treatment groups. In subjects who did not complete the final visit the "last observation carried forward" principle was applied. Mean differences between the study groups were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels and study location. Intervention effects were also further adjusted for additional potential confounders, using ANCOVA. If logarithmic transformation was applied to parameters with

skewed distributions, the same results were obtained. Sample size of the study was based to prove a minimal reduction of 0.4 mmol/L in triglyceride levels (compared to placebo), or a difference of 0.4 mmol/L between the two treatment groups (SD 0.8, a = 0.05, power 85%). Analyses were performed by SPSS for Windows (release 9.0).

Results

Baseline Characteristics

After a screening visit, 251 patients fulfilled the in- and exclusion criteria and entered the two-week placebo run-in period. At baseline 26 patients had normalized triglyceride levels (<1.5 mmol/L) and 8 patients refused to continue. Finally, 217 patients were randomized. The baseline characteristics of the study population are given in Table 1. In all three groups compliance with trial medication during the study was over 95%. Twenty patients (9.2%) did not complete the study because of adverse events (n=7), personal reasons (n=10), protocol violation (n=1) or loss to follow-up (n=2). Personal reasons were defined as not related to the study medication and were mostly due to inability to spend time for participating in the study.

Table 1.
Baseline characteristics of the patients in the Diabetes Atorvastatin Lipid Intervention study (DALI)

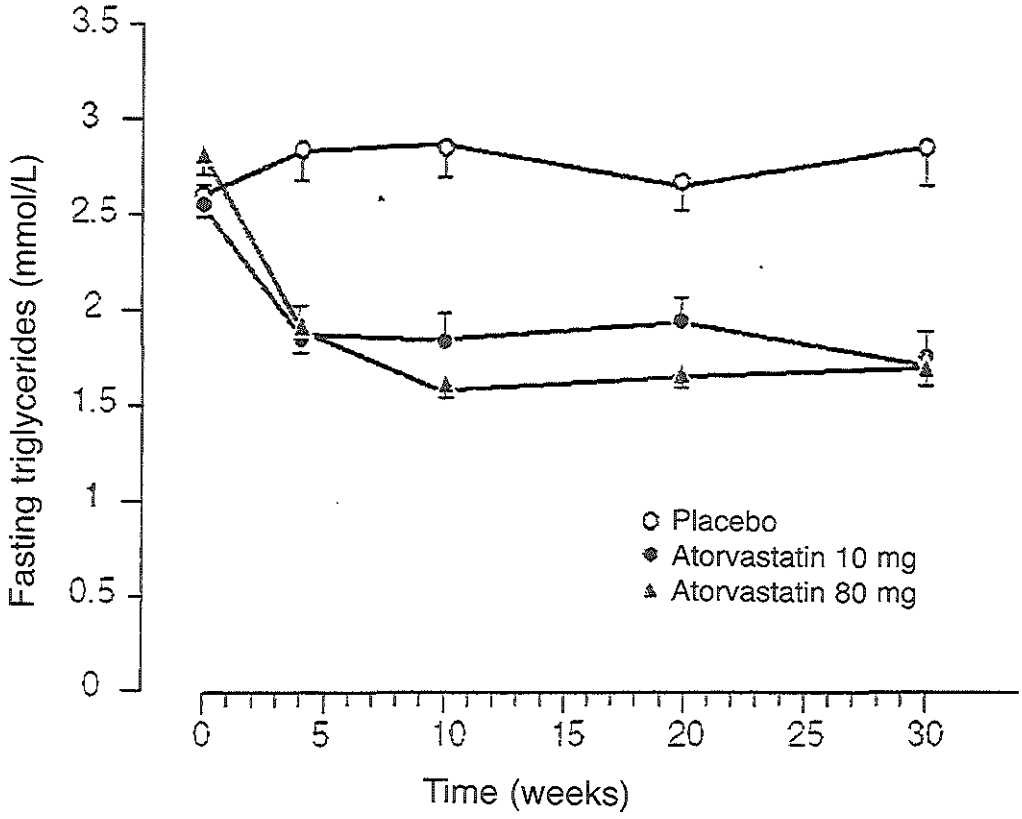
	Placebo	Atorvastatin 10mg	Atorvastatin 80mg
Number	72	73	72
Male gender (%)	46	60	53
Age (years)	58.5 ± 7.5	59.7 ± 7.6	60.1 ± 7.7
Caucasian ethnicity (%)	84	86	82
Diabetes duration (years)	8.2 ± 5.9	11.1 ± 7.6	12.2 ± 8.3
Diabetes treatment (number)			
Diet	2	3	0
Tablets	31	34	30
Insulin	21	19	21
Combination tablets/insulin	18	17	21
Neuropathy (%)	39	32	41
Retinopathy (%)	22	28	37
Body-Mass Index (kg/m2)	32.2 ± 6.0	30.0 ± 3.8	30.4 ± 4.5
Waist to Hip ratio	0.99 ± 0.1	1.00 ± 0.08	1.01 ± 0.1
Treated hypertension (%)	50	49	61
Blood pressure (mmHg)	144 ± 19/85 ± 9	146 ± 17/86 ± 10	145 ± 17/85 ± 9
Present smoking (%)	22	21	17
Fasting glucose (mmol/L)	10.5 ± 3.6	10.5 ± 3.0	10.6 ± 2.9
HbA1c (%)	8.3 ± 1.1	8.3 ± 1.2	8.4 ± 1.1

Continuous data are expressed as mean ± sd

Lipids and lipoproteins

Lipid and lipoprotein plasma values at baseline and after 30 weeks are shown in Table 2. In patients treated with A10 triglyceride levels were significantly lowered by 25% from 2.54 to 1.84 mmol/L (P<0.001). Treatment with A80 resulted in a significant reduction by 35% from 2.85 to 1.78 mmol/L (P<0.001). The difference in plasma triglycerides lowering between the two intervention groups was not statistically significant (Figure 1).

Figure 1. Triglyceride levels in the DALI study. Values are mean ± SE



The effects on triglycerides were also analyzed in two strata of baseline plasma triglyceride levels to investigate whether the baseline levels of plasma triglycerides did influence the lipid lowering effect of atorvastatin. The first group included all patients with baseline plasma triglyceride levels >2.3 mmol/L (n=120), whereas the second group included 97 patients with baseline plasma triglyceride levels ≤2.3 mmol/L. In both groups the same results were obtained. In patients with high baseline triglyceride levels, A10 and A80 resulted in a triglyceride reduction of 29.6% and 23.4%, respectively (compared to placebo both $p<0.001$, A10 versus A80 $p>0.5$). The corresponding figures in patients with low baseline triglyceride levels were 23.4% and 27.9%, respectively (compared to placebo both $p<0.001$, A10 versus A80 $p>0.4$). Further adjustment for diabetes duration and BMI did not change the results.

LDL-cholesterol was dose-dependently improved to 2.2 mmol/L in A10 (-40.8%, $p<0.001$), and to 1.7 mmol/L in A80 (-52.3%, $p<0.001$). The difference between A10 and A80 was statistically significant ($p<0.001$). Like LDL-cholesterol, atorvastatin lowered apoB dose dependently (Table 2). A10 and A80 increased HDL-cholesterol significantly by 5-6%. Consequently, the total cholesterol/ HDL ratio improved, significantly more in patients treated with A80 ($p<0.005$) (Table 2).

At baseline relatively few patients had small, dense LDL particles. Pattern A, AB and B was present in 60.5%, 15.3% and 24.2%, respectively, which did not differ between the three intervention groups. There was no overall effect of atorvastatin on the LDL particle size. As a result, the number of patients with dense LDL particles (pattern B) did not differ at the end of the study between the intervention groups: 21.3%, 21.4% and 22.2% in the placebo, A10 and A80 groups, respectively. Atorvastatin had no effect on postheparin LPL activity. Further adjustment for diabetes duration and BMI did not change the results.

The effects of treatment were compared to target values defined by the American Diabetes Association (ADA). (24) In both atorvastatin treatment groups more than 75% reached the triglyceride treatment goals (79.5% in A10 and 76.4% in A80, NS). LDL-cholesterol treatment goals were reached in 71.2% of the patients treated with A10 and 84.7% of the patients treated with A80 compared to 11.1% in the placebo group. This difference was statistically significant between A10 and A80. HDL-cholesterol treatment goals were reached in 35.6% of the A10 group and 44.4% of the patients treated with A80 (NS).

Table 2. Lipids and lipoprotein at baseline and end of the DALI study.

		Placebo	Atorvastatin 10mg	Atorvastatin 80mg
Triglycerides (mmol/L)	Baseline	2.62 ± 0.11	2.54 ± 0.10	2.85 ± 0.13
	30 weeks	2.88 ± 0.22	1.84 ± 0.10*	1.78 ± 0.16*
	Change (%)	10.0 (-1.7 to 21.7)	-25.4 (-31.9 to -18.9)*	-34.6 (-42.7 to -26.5)*
Total cholesterol (mmol/L)	Baseline	6.0 ± 0.1	5.9 ± 0.1	6.0 ± 0.1
	30 weeks	6.0 ± 0.1	4.1 ± 0.1*	3.6 ± 0.1* $
	Change (%)	0.5 (-2.0 to 2.0)	-29.8 (-32.4 to -27.2)*	-39.2 (-43.3 to -35.1)* $$
LDL-cholesterol (mmol/L)	Baseline	3.8 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
	30 weeks	3.6 ± 0.1	2.2 ± 0.1*	1.7 ± 0.1* $
	Change (%)	-2.7 (-7.0 to 1.7)	-40.8 (-43.6 to -37.9)*	-52.3 (-58.9 to -45.7)* $
HDL-cholesterol (mmol/L)	Baseline	1.05 ± 0.02	1.05 ± 0.03	1.03 ± 0.03
	30 weeks	1.04 ± 0.03	1.10 ± 0.04	1.09 ± 0.04
	Change (%)	-0.9 (-3.7 to 1.9)	6.0 (3.6 to 8.6)**	5.2 (1.8 to 8.6)**
TC/HDL ratio	Baseline	5.9 ± 0.2	5.9 ± 0.1	6.1 ± 0.2
	30 weeks	6.0 ± 0.1	3.9 ± 0.2*	3.5 ± 0.2*
	Change (%)	+2.6 (-1.5 to 6.8)	-33.7 (-36.3 to -31.1)*	-42.0 (-46.3 to -37.8)* $$
Free Fatty Acids (mmol/L)	Baseline	0.67 ± 0.03	0.64 ± 0.03	0.69 ± 0.03
	30 weeks	0.72 ± 0.04	0.57 ± 0.03**	0.61 ± 0.03***
	Change (%)	18.6 (2.7 to 34.4)	-5.4 (-14.4 to 3.6)***	-3.2 (-13.7 to 7.2)***
Apo B (mg/100ml)	Baseline	1.27 ± 0.02	1.22 ± 0.02	1.24 ± 0.03
	30 weeks	1.25 ± 0.02	0.84 ± 0.02*	0.74 ± 0.03* $$
	Change (%)	-1.5 (-4.3 to 1.2)	-30.7 (-33.0 to -28.4)*	-40.2 (-44.2 to -36.1)* $
LDL particle size (nm)	Baseline	26.0 ± 0.1	26.1 ± 0.1	25.9 ± 0.1
	30 weeks	26.0 ± 0.1	26.1 ± 0.1	26.0 ± 0.1
	Change (%)	0.3 (-0.3 to 0.9)	-0.03 (-0.5 to 0.5)	0.5 (-0.04 to 1.0)
Lipoprotein lipase (mU/ml)	Baseline	140.4 ± 5.8	142.6 ± 5.3	138.7 ± 5.3
	30 weeks	137.7 ± 6.2	136.1 ± 5.4	133.6 ± 6.2
	Change (%)	2.3 (-7.6 to 12.1)	-1.5 (-8.4 to 5.5)	-2.0 (-9.6 to 5.6)

Values are mean ± SE, or percentage change with the 95% confidence interval. Test for difference versus placebo, adjusted for baseline value and study location: * $p < 0.001$; ** $p < 0.005$; *** $p < 0.05$ Test for difference versus atorvastatin 10mg, adjusted for baseline value and study location: $ $p < 0.001$; $$ $p < 0.005$; $$$ $p < 0.05$

Safety and metabolic control parameters

Adverse events are summarized in Table 3. There was no difference in the number of patients reporting adverse events between the three treatment groups. Twelve serious adverse events were reported. One patient receiving placebo (non-fatal myocardial infarction), 1 patient treated with A10 (benign neoplasm of skin) and 1 patient treated with A80 (depressive episode) reported a serious adverse event that was considered 'possibly related' to the study drug. The other serious adverse events, not considered related to the study drug, included self-limiting gastroenteritis (n=1) and trauma (n=2) in patients receiving placebo; self-limiting gastroenteritis (n=1), diabetes dysregulation (n=1) and malignant bronchuscarcinoma (n=1) in patients using A10; admission for hip replacement surgery (n=1), depressive episode (n=1) and benign neoplasm of the skin (n=1) in patients using A80.

Atorvastatin 80mg was associated with a slight increase in HbA1c concentration from 8.4% to 8.6% after 30 weeks (p=0.06). In both placebo and A10 groups a small decrease in HbA1c was observed after 30 weeks. After 30 weeks, HbA1c in the A80 group differed significantly from either placebo and A10 (both $p<0.05$). Fasting glucose and blood pressure remained stable during the study in all treatment groups.

Table 3. Adverse Events and Safety parameters in the DALI study

		Placebo	Atorvastatin 10mg	Atorvastatin 80mg
Adverse events	Gastrointestinal disorder	8 (1)‡	11	9 (1) ‡
	Mood disturbances	3 (1) ‡	1	3
	Headache	3	3	3
	Heart diseases	3 (1) ‡	0	2
	Respiratory tract disorder	6 (1) ‡	4	4
	Joint disorder/myopathy	9	10	7
	Urinary tract disorder	10	13	9
	Malaise	6 (1) ‡	11 (1) ‡	1
	Other	6	19	15
AST (U/L)	Baseline	24 ± 9	28 ± 12	26 ± 9
	30 weeks	25 ± 7	26 ± 8	28 ± 10
ALT (U/L)	Baseline	28 ± 11	36 ± 21	33 ± 19
	30 weeks	28 ± 11	33 ± 14	37 ± 15
CK (U/L)	Baseline	117 ± 89	121 ± 73	118 ± 64
	30 weeks	112 ± 66	126 ± 96	133 ± 96*
HbA1c (%)	Baseline	8.3 ± 1.1	8.3 ± 1.2	8.4 ± 1.1
	30 weeks	8.1 ± 1.1*	8.0 ± 1.2	8.6 ± 1.3 $ ##
Fasting glucose	Baseline	10.5 ± 3.6	10.5 ± 3.0	10.6 ± 2.9
(mmol/L)	30 weeks	10.2 ± 2.5	10.3 ± 2.5	11.0 ± 3.2
Blood pressure	Baseline	144 ± 19/85 ± 9	146 ± 17/86 ± 10	145 ± 17/85 ± 9
(mmHg)	30 weeks	144 ± 21/86 ± 11	140 ± 20/84 ± 11	143 ± 16/84 ± 10

Continuous data are expressed as mean ± standard deviation.
Test for difference versus baseline: * $p < 0.05$
Test for difference versus placebo: $ $p < 0.05$
Test for difference versus atorvastatin 10mg: ## $p<0.005$
‡ withdrawn from the study due to adverse event

Discussion

Atorvastatin 10mg and 80mg treatment resulted in significant reductions of plasma triglyceride and LDL cholesterol, and an increase of HDL cholesterol levels. in patients with type 2 diabetes mellitus. A80 had a significant larger effect on cholesterol related variables than A10. The side effects of A10 as well as A80 did not differ from placebo.

The lower limit of plasma triglyceride levels for inclusion was 1.5 mmol/L. Levels above 1.5 mmol/L are associated with an increased risk for cardiovascular disease in diabetic subjects in the Paris Prospective Study and the Bezafibrate Infarction Prevention Registry. (14,25) Moreover, 98% of the participants had dyslipidemia according to the ADA guidelines. (24) The results of the analyses stratified by baseline plasma triglyceride levels indicate that the effects of treatment are independent of baseline plasma triglyceride levels, as is also seen for plasma LDL-cholesterol reduction by statins. Our results show that in DM2 patients high dose atorvastatin (80mg) does not have a significant additional effect on triglyceride lowering compared to a standard dose of 10mg. The triglyceride-lowering efficacy of atorvastatin in our study is in agreement with previous small studies with 80mg atorvastatin in non-diabetic patients with primary dyslipidaemia (18,19,26) Among the limited number of studies with high doses of other HMG-CoA reductase inhibitors (mainly simvastatin) in diabetic patients, only one double-blind study was published, showing a 15% reduction in triglycerides after using simvastatin 40mg for 24 weeks in 42 patients with DM2. (20)

The mechanism by which atorvastatin lowers plasma triglycerides is not known. Diabetic hypertriglyceridemia is often ascribed to overproduction of VLDL triglycerides as well as impairment of triglyceride clearing due to decreased LPL activity. (12,27) In the present study LPL was in the normal range, and not affected by atorvastatin treatment. Therefore, it is likely that the hepatic triglyceride secretion is affected. Plasma FFA is the main precursors for hepatic triglyceride synthesis and secretion. Since atorvastatin lowered plasma FFA, it may be that hepatic triglyceride synthesis and secretion are attenuated.

The decrease in plasma triglycerides and rise in HDL was not accompanied by an increase in LDL size. It should be noted that LDL size was relatively large. Moreover, based on epidemiological studies it was found that presence of small dense LDL coincides with plasma TG levels above 1.6 mmol/L. In order to obtain a reversal of LDL size towards more buoyant particles an even lower plasma TG level might be necessary. Micronised fenofibrate has shown to improve LDL-size in patients with DM2 who suffered higher fasting triglycerides and total cholesterol

levels and a predominance (52%) of small, dense LDL at baseline compared to our study. (28).

In the current study, total cholesterol, LDL-cholesterol and apoB levels were significantly reduced in a dose dependent manner. This reduction is of the same magnitude as found in studies with atorvastatin in non-diabetic hypercholesterolemic and hypertriglyceridemic patients. (18,19) Compared to studies with simvastatin (40mg) in patients with DM2, we found a larger reduction in total cholesterol (39% versus 30%) and LDL-cholesterol (52% versus 24-42%). (20,29)

The significant increase from baseline in HDL-cholesterol by atorvastatin was consistent with that reported in previous studies. Even small increases in HDL-cholesterol may be clinically relevant since they contribute to the reduction of the total cholesterol/HDL ratio. This ratio was one of the best predictors of future cardiovascular events in the Framingham study. (30) Since treatment with A80 resulted in a significant lower cholesterol/HDL cholesterol ratio than A10, the high dose may have an additional protective effect on future cardiovascular events.

The number of serious adverse events and side effects were the same in the three intervention groups. The present study is the first study showing the safety of A80 for a longer treatment period in patients with DM2 who concomitantly were using a wide variety of other medications. Possible limitations of the study are differences at baseline between the placebo and atorvastatin groups. These include duration of diabetes, which is not likely to influence the results since there is no relation between the duration of diabetes and the metabolism of triglycerides, and body mass index. Because visceral adipocytes are the main source for FFA supplied to the liver as substrates for triglycerides, the waist to hip ratio and FFA levels are more important than BMI (.31) Moreover, adjustment for diabetes duration or BMI did not change the results.

Atorvastatin 80mg induced a small increase in HbA1c values, while HbA1c in patients using A10 and placebo slightly decreased. Other studies have shown inconsistent results of lipid lowering therapy on glycemic control in diabetic patients, and several clinical studies reported an increase of HbA1c with either fluvastatin or simvastatin. (20,32-34)

Conclusions

In conclusion, atorvastatin 10mg and 80mg are both effective in the treatment of diabetic dyslipidemia (elevated triglyceride, normal to elevated LDL cholesterol, low HDL cholesterol) in patients with type 2 diabetes mellitus. Both doses were well tolerated in this patient population.

Appendix

DALI-study group (In alphabetical order): Erasmus Medical Center Rotterdam, Department of Internal Medicine (I.I.L. Berk-Planken, N. Hoogerbrugge, H. Jansen); Erasmus University Rotterdam, Departments of Biochemistry and Clinical Chemistry (H. Jansen); Gaubius Laboratory TNO-PG, Leiden (H.M.G. Princen); Leiden University Medical Center (M.V. Huisman, M.A. van de Ree); University Medical Center Utrecht, Julius Center for General Practice and Patient Oriented Research (R.P. Stolk, F.V. van Venrooij); University Medical Center Utrecht, Division of Internal Medicine (J.D. Banga, G.M. Dallinga-Thie, F.V. van Venrooij).

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Chapter 3

Atorvastatin decreases hepatic lipase activity in type 2 diabetes mellitus dose-dependently. Effect of gender and the LIPC promoter variant.

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Abstract

Objective

Hepatic lipase (HL) is involved in the metabolism of several lipoproteins and may contribute to the atherogenic lipid profile in type 2 diabetes. Little is known about the effect of cholesterol synthesis inhibitors on HL activity in relation to gender and the hepatic lipase gene, the *LIPC* promoter variant in type 2 diabetes. Therefore, we studied the effect of atorvastatin 10mg (A10) and 80mg (A80) on HL activity in 198 patients with type 2 diabetes.

Research design and methods

Patients, aged 45-75 years, without manifest coronary artery disease and a total cholesterol of 4.0-8.0 mmol/L and fasting triglycerides (TG) of 1.5-6.0 mmol/L were included in a double-blind, randomized, placebo-controlled trial for 30 weeks (DALI study).

Results

HL activity at baseline was significantly higher in our population compared with an age-matched control group without type 2 diabetes (406 ± 150 vs 357 ± 118 U/L). HL activity in males vs females (443 ± 158 vs 358 ± 127 U/L), in carriers of the *LIPC* C/C allele vs carriers of the T/T allele (444 ± 142 vs 227 ± 96 U/L) and in Caucasians vs blacks (415 ± 150 vs 260 ± 127 U/L), all differed significantly ($p<0.001$). Atorvastatin decreased HL dose-dependently (A10 -11%, A80 -22%, both $p<0.001$). Neither gender or the *LIPC* C>T variation influenced the effect of atorvastatin on HL activity.

Conclusions

Gender, *LIPC* promoter variant and ethnicity significantly contribute to the baseline variance in HL activity. Atorvastatin treatment in diabetic dyslipidemia results in a significant dose-dependent decrease in HL activity, regardless of gender or the *LIPC* promoter variant.

Introduction

Hepatic lipase (HL) is involved in the metabolism of several lipoproteins (1) and is a key player in the HDL metabolism (2,3). Hydrolysis of phospholipids and triglycerides by HL, leads to the conversion of large, buoyant HDL2 to small, dense HDL3 and may induce cholesterol (ester) flux to the liver (4,5). In this way HL is involved in the reverse cholesterol transport and is a major determinant of plasma HDL concentration (6,7). HL also plays a role in the formation of small dense LDL and contributes to the expression of the LDL subclass phenotype (8,9). Finally, HL is proposed to be involved in post prandial lipid clearing (10). Thus, HL activity seems to be central in the metabolism of lipoproteins strongly associated with CAD risk in type 2 diabetes mellitus. Zambon and coworkers identified HL as a focal point for the development and treatment of coronary artery disease (11).

Genetic variation, gender and abdominal fat mass affect HL activity in humans (12-15). In the human hepatic lipase gene (denoted as *LIPC*), variants are found that affect the lipase activity (16-18). Besides rather rare variants leading to complete HL deficiency (19,20), common base substitutions in the proximal *LIPC* promoter affect HL activity up to 2 fold (15, 21, 22). Four base substitutions, -250 G-to-A, -514 C-to-T, -710 T-to-C, and -763 A-to-G, were found to be completely linked (23). Together, they constitute two alleles, indicated as the *LIPC* C and T allele, after the C-to-T variant at -514 bp, which is also indicated as C-480T. The C and T alleles are associated with high and low HL activity, respectively. The frequency of both alleles varies highly among different ethnic populations (21,24,25). The C allele is the most common allele in caucasians, whereas the T allele is the major allele in black Americans. Asians contain an intermediate frequency. Beside genetic variance, HL lipase activity is also hormonally affected. In particular sex hormones influence *LIPC* expression and are believed to be responsible for gender differences in HL activity being lower in women than in men (13, 25, 26). An increase in HL activity is associated with an increase in abdominal fat mass, BMI and fasting insulin and fasting plasma triglycerides (27). These correlations are reflected in a high HL activity in type 2 diabetes.

A change in HL activity may also be induced by hypolipidemic drugs. In males, but not in females, we found a decrease in HL activity during atorvastatin treatment of familial hypercholesterolemia (28). Zambon reported a drop in HL activity in participants of the FATS study during treatment with lovastatin-colestipol and niacin-colestipol (29). Interestingly, in both groups the effect of treatment on HL activity was strongly dependent on the presence of the *LIPC* T allele. HL activity was lowered less in carriers of the T allele compared to non-carriers (30). So, gender

and the genotype of the *LIPC* promoter may determine the efficacy of the statin treatment. Earlier studies (31-34), showed a beneficial effect of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors on the incidence of cardiovascular events in type 2 diabetes. Also although atorvastatin, a powerful statin, effectively reduces total cholesterol and triglyceride concentrations, no data describing its effect on HL activity in type 2 diabetes are currently available. Therefore, we conducted a double-blind, placebo-controlled randomized 30-weeks study to evaluate the effect of atorvastatin 10mg versus 80mg on HL activity in subjects with type 2 diabetes mellitus. Gender and the *LIPC* C>T variation as possible modificators of the atorvastatin treatment effect, were considered in this study.

Patients and methods

Study population

This study is part of the Diabetes Atorvastatin Lipid Intervention (DALI) study. DALI is a randomized double-blind, placebo-controlled, multi-center study conducted in the Netherlands. The subjects and methods are described in detail earlier (35).

In short, 217 patients, aged 45 to 75 years, with type 2 diabetes mellitus participating in the DALI study were randomized to placebo, atorvastatin 10mg (A10) or atorvastatin 80mg (A80) during 30 weeks, to evaluate the effect on lipid metabolism, endothelial function, coagulation and inflammatory parameters. The main inclusion criteria were plasma triglycerides between 1.5 and 6.0 mmol/l, total cholesterol between 4.0 and 8.0 mmol/l and no history of coronary heart disease. Post heparin lipase activity blood samples of 198 DALI-patients were available and evaluated in the present study.

Analytical methods

Blood samples were drawn after 12 h of fasting at baseline and after 30 weeks, the end of the study. Standard lipid variables (Total cholesterol (TC), HDL-cholesterol (HDL-c) and triglycerides (TG)), free fatty acids (FFA), plasma glucose, HbA1c and HL activity were measured. Cholesterol and TG were determined by enzymatic colorimetric methods on a Hitachi 911 automatic analyzer (Boehringer Mannheim, Mannheim, Germany). Plasma HDL cholesterol was measured by a direct enzymatic HDL-cholesterol method, after precipitation of very-low density lipoprotein and LDL by addition of manganese chloride. LDL-cholesterol was estimated by the Friedewald formula (36). Fasting plasma glucose was determined

on a Hitachi 917 analyzer using an UV-hexokinase method (Cat. Nr.18766899) from Boehringer Mannheim, Mannheim, Germany. HbA1c was determined by HPLC, using the BIO-RAD Variant TM method (Cat.Nr.270-0003.Bio-rad).

Postheparin plasma HL activity

HL activity was measured using an immunochemical method as described previously (37) in plasma collected 20 min after contralateral intravenous administration of heparin (50 IU/kg body-weight). Leo Pharmaceutical Products, Weesp, The Netherlands. HL controls consisted of 93 male and female volunteers, aged 45 to 75 years, without type 2 diabetes mellitus and hypertriglyceridemia.

DNA Analysis

Genotyping of the *LIPC* gene for the C>T variance was carried out by determination of the G>A base substitution at –250 bp (38). Primers for the polymerase chain reaction amplification were 5-GAT ACT TTG TTA GGG AAG ACT Gcc-3' and 5-GGA TCA CCT CTC AAT GGG TC –3'. Amplification was carried out in a 25 µL reaction mixture with an initial denaturation at 95°C for 5 minutes, followed by 31 cycles of amplification at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C using Goldstar Taq polymerase (Eurogentec). Fifteen µL of the polymerase chain reaction mix was digested with the restriction enzyme DraI during 2 hrs at 37°C, followed by electrophoresis on a 2% agarose gel. The non digested PCR product had a length of 560 bp. After digestion the –250G variant yielded two products of 449 and 111 bp. Digestion of the -250A variant yielded a major product of 335 bp and minor products of 114 and 111 bp. It was shown previously that the –250G variant is 100% linked to the C variant at –514 and the –710T and –763A variants upstream in the *LIPC* promoter (18). These variants collectively form the *LIPC* C allele. The variant sequence containing -250 A, -514 T, -710 C, and -763 G, will be indicated as *LIPC* T allele.

Statistical analysis

Analyses were performed by SPSS for Windows (release 9.0). Mean differences between the study groups were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels of each treatment group and study location. Intervention effects were also further adjusted for additional potential confounders, using ANCOVA. The student T-test was used to test significance between baseline and 30 weeks follow-up. Continuous variables are presented as mean values with the standard deviation (SD). P values < 0.05 were considered statistically significant.

Results

Patient characteristics

The baseline characteristics of the 198 patients are shown in Table 1.
There were no significant differences in the patient characteristics between the three groups, except for the duration of diabetes, which was shorter in the placebo compared to the A80 group ($p<0.05$). Fasting glucose and HbA1c at baseline were relatively high, but equal in all groups. Fasting glucose did not change during the study. The A80 group had a slight increase in HbA1c levels (8.4 ± 1.1 % to 8.6 ± 1.3 %, $p<0.05$), whereas in patients using A10 and placebo, the HbA1c levels decreased slightly (both 8.3 ± 1.2 % to 8.1 ± 1.2 %, $p<0.05$).

Atorvastatin effect on lipids and lipoproteins.

At baseline plasma lipids and lipoproteins were similar in all three groups (Table 1). As described before (35), administration of A10 and A80 resulted in significant reductions (25 and 35%, respectively) of plasma triglyceride levels (both $p<0.001$), in significant, dose-dependent, reductions (30% and 40% respectively, both $p<0.001$) of total cholesterol and LDL (41% and 52% respectively, both $p<0.001$). HDL cholesterol levels significantly increased, dose-independently, during atorvastatin treatment by 5 to 6% ($p<0.01$).

Table 1. Baseline characteristics

	Placebo (n=65)	Atorvastatin 10mg (n=67)	Atorvastatin80mg (n=66)
Male Gender (%)	48	62	54
Age (years)	58.5 ± 7.3	59.4 ± 7.6	60.5 ± 7.8
Diabetes duration (years)	9.5 ± 6.1	12.2 ± 7.7	13.2 ± 8.5*
Diabetes treatment (%)			
Diet	3.1	1.5	0
Tablets	40.6	50.0	43.9
Insulin	31.3	27.3	27.3
Combination	25.0	21.2	28.8
Body-Mass Index (kg/m2)	31.9 ± 6.1	30.1 ± 3.8	30.6 ± 4.5
Waist to Hip ratio	0.99 ± 0.1	1.00 ± 0.1	1.00 ± 0.1
Hypertension (%)	48	49	62
Current smoking (%)	58	66	70
Fasting glucose (mmol/l)	10.5 ± 3.6	10.4 ± 3.0	10.6 ± 3.0
HbA1c (%)	8.3 ± 1.2	8.3 ± 1.2	8.4 ± 1.1
Total cholesterol (mmol/l)	6.1 ± 0.9	5.9 ± 0.8	6.0 ± 1.0
LDL cholesterol (mmol/l)	3.7 ± 0.9	3.6 ± 0.8	3.7 ± 0.9
HDL cholesterol (mmol/l)	1.05 ± 0.2	1.06 ± 0.3	1.04 ± 0.2
Triglycerides (mmol/l)	2.64 ± 0.9	2.50 ± 0.9	2.82 ± 1.1

Continuous data are expressed as mean values ± standard deviation
*p <0.05 between atorvastatin 80mg and placebo group

HL activity in type 2 diabetes mellitus.

Baseline HL in the DALI population was significantly higher than in an age-matched, non-diabetic controls without hypertriglyceridemia, 406 ± 150 U/L (n=198) versus 357 ± 118 U/L (n=93), p<0.001. This was due to a significant increase in HL activity in the male patients compared with the male controls, 443 ± 158 vs 397±.125 U/L (p<0.001). Female DALI patients had a comparable HL activity compared with female control patients (358 ± 127 vs 328 ± 105 U/L). Gender, *LIPC* genotype, ethnicity and the BMI were all significant and independent predictors of HL activity, demonstrated by stepwise linear regression. The *LIPC* genotype contributed 13%, gender 8%, ethnicity 4% and BMI 3% to the variance in HL activity (adjusted R2 of the model = 0.28, p<0.0001).

Atorvastatin effect on postheparin lipase activity.

Mean HL activity at baseline was comparable between the three study groups. In the placebo group there was no significant reduction in HL activity at the end of the study. HL activity decreased in the A10 group by 11%, p<0.001 and in the A80 group by 22%, p<0.001 (Table 2). The additional reduction in HL activity by A80 compared to A10 was highly significant (p<0.005).

Table 2. Hepatic lipase activity (U/L) after 30 weeks treatment.

Patients		Placebo	Atorvastatin 10mg	Atorvastatin 80mg
All	Baseline	406 ± 156	402 ± 152	410 ± 138
n=198	30 weeks	382 ± 146	361 ± 149*$	313 ± 112*#^
Males	Baseline	449 ±138	429 ± 154	451 ± 143
n=107	30 weeks	425 ± 128	390 ± 150*$	343 ± 98*#^
Females	Baseline	358 ± 131	360 ± 155	356 ± 109
n=91	30 weeks	343 ± 125	318 ± 138*$	278 ± 120*#^

Continuous data are expressed as mean values ± standard deviation
*p<0.001, significantly different from baseline in each treatment group.
#p<0.002, significantly different, adjusted for baseline, from placebo after 30 weeks treatment.
$p<0.05, significantly different, adjusted for baseline, from placebo after 30 weeks treatment.
^p<0.005, significantly different, adjusted for baseline, from A10 after 30 weeks treatment.

Atorvastatin effect on postheparin lipase activity in males and females.

Since HL activity in the DALI participants was significantly higher in males compared to females, 443 ± 158 U/L (n=107) versus 358 ± 127 U/L (n=91), p<0.001, we analyzed males and females separately. The effect on HL activity by atorvastatin was not influenced by gender differences. Treatment with atorvastatin 10 or 80 mg reduced HL activity similarly 11% and 22% in both males and females (Table 2).

Atorvastatin effect on hepatic lipase activity in the LIPC promoter variant.

The C>T variance in the *LIPC* promoter is a strong, significant predictor of the HL activity, therefore we analyzed carriers and non-carriers of the T allele separately. In the DALI study population 55.0% of the patients were homozygote for the *LIPC* C allele, 37.4% were heterozygote for the T allele and 7.6% homozygote for the T allele, leading to a frequency of the T allele of 0.262.

At baseline the presence of the T variant significantly affected HL activity (Table 3). In heterozygote carriers of the T allele, HL activity was 19% lower and in

homozygote carriers of the T allele 49% lower than C allele homozygotes, p<0.0001. The T-allele lowered the baseline HL activity in males and females. Male heterozygote and homozygote T allele carriers had respectively a 20% and 57% lower HL lipase activity than C homozygotes. In females this was respectively 16% and 37% (Table 3). In males and females the frequency of the T allele was comparable, 0.252 versus 0.278.

Table 3. The effect of the *LIPC* C>T variance on HL activity (U/L) at baseline.

	Total	Males	Females
CC genotype	444 ± 142	489 ± 131	385 ± 137#
	n=109	n=61	n=48
CT genotype	358 ± 135*	390 ± 158*	324 ± 95*##
	n=74	n=38	n=36
TT genotype	227 ± 96*	212 ± 56*	244 ± 131*
	n=15	n=8	n=7

* p<0.001, compared to non-carriers.
# p<0.001, compared to males.
p=0.03, compared to males

Homozygote carriers of the C allele and heterozygote carriers of T allele showed similar percentage reductions in HL activity after atorvastatin treatment (Table 4). In both groups, A10 resulted in a 10-12% decrease and A80 in a 21-22% reduction in HL activity (p<0.001 compared to placebo effect and dose-dependent, p<0.005). Four of the fifteen T/T homozygotes were assigned to the A10 treatment group, three to the A80 group and eight patients received placebo. Post atorvastatin treatment HL activities were only available in five patients. A10 treatment lowered HL activity in two cases (249 U/L to 216 U/L (-15%) and 349 to 259 U/L (-25%), respectively). In one case, A10 did not reduce HL activity (319 U/L to 349 U/L (+9%). In both subjects assigned to A80, HL activity was lowered (248 U/L to 218 U/l (-12%) and 266 U/l to 200 U/L (-25%). A10 lowered HL activity to a similar degree in male and female C/C and C/T carriers (10-13%). A80 resulted in a further reduction of 16% in the female C/C homozygotes and to 31% in the female C/T heterozygotes. In males the additional decrease was respectively 27% and 19%, not significantly different from the female values.

Table 4. *LIPC* C>T variance and HL activity (U/L) after 30 weeks treatment.

Genotype		Placebo	Atorvastatin 10mg	Atorvastatin 80mg
CC genotype	Baseline	437 ± 124	448 ± 140	447 ± 129
n=109	30 weeks	441 ± 139	402 ± 156*$	348 ± 98*#
CT genotype	Baseline	352 ± 144	357 ± 121	366 ± 157
n=74	30 weeks	349 ±137	319 ± 128*$	280 ± 128*#
TT genotype	Baseline	224 ± 7	306 ± 104	257 ± 76
n=15	30 weeks	236 ±137	273 ± 34	209 ± 45

* p<0.001, significantly different from baseline in each treatment group.
#p<0.001, significantly different, adjusted for baseline, from placebo after 30 weeks treatment.
&p<0.05, significantly different, adjusted for baseline, from placebo after 30 weeks treatment

Ethnic differences, LIPC polymorphism and HL activity.

It has been reported that the prevalence of *LIPC* C and T alleles, thereby affecting HL activity, strongly varies among subjects with different ethnic offspring. So, we studied the influence of ethnic heterogeneity in our study population. Mean HL activity at baseline was significantly lower among Asians (n=10), Mediterranean (n=9) and black (n=11) patients compared to Caucasians (n=168), respectively 350 ± 95 U/L, 269 ± 124 U/L, 260 ± 127 U/L vs 415 ± 150 U/L (p<0.01). The T allele frequency was very high in the black (0.590) and the Asian patients (0.400). Caucasians and Mediterranean patients had a T allele frequency of 0.235 and 0.222, respectively. At baseline HL activity was strongly influenced by the *LIPC* gene variance in all ethnic groups. HL activity was significantly higher in CC homozygotes than in CT heterozygotes or TT homozygotes. To study a possible effect of the ethnic background, Caucasians were separately analyzed for the effect of atorvastatin in relation with the *LIPC* genotype. Atorvastatin had similar effect in caucasians as in the whole study population (data not shown). The other groups were not analyzed separately, because of the small numbers.

Discussion

This prospective, randomized study demonstrated a decrease in HL activity, dose-dependently by atorvastatin in type 2 diabetes mellitus patients. HL activity decreased 11% and 22% after treatment with atorvastatin 10 and 80mg, respectively (p<0.001). In type 2 diabetes mellitus HL is considered an important factor in the

development of the atherogenic lipid profile. Our study results are the first published effects in type 2 diabetes mellitus of low versus high dose statin influencing the HL activity. Gender and the *LIPC* C>T polymorphism both influence HL activity. Males have a higher HL activity than females. The higher HL activity in males is due to a number of endogenous factors, like central adiposity and sex steroid hormones (14,15,26,39, 40). Recently, Brunzell et al (41) also demonstrated that intra-abdominal fat is a major component of the gender difference in HL activity. Also in our diabetic study population males exhibited a higher HL actvity than females. While the male patients tended to have a higher HL activity compared to controls without diabetes, in the female patients HL activity was similar to control values. Baseline HL activity correlated significantly with WHR (R= 0.20, p=0.004). Males had a higher WHR compared to females ($p<0.001$) and the increased HL activity in the males may be partially explained by this increased WHR. Atorvastatin treatment abolished the correlation between HL activity and WHR. In preliminary results we found that fatty acids may stimulate *LIPC* expression in HepG2 cells and that this effect is abolished by atorvastatin (27). Our present results are in line with these observations and suggest that increased HL activity in our male patients may be due to stimulation of HL expression by increased supply of fatty acids derived from a higher abdominal fat mass as reflected in the high WHR. By interfering with the stimulation of HL by FFA, atorvastatin may then lower HL activity with the consequent loss of the association between HL activity and WHR. Besides gender, genetic variation of the *LIPC* promoter strongly affected HL activity at baseline and contributed 13% to the variance in HL activity. In the whole study population, *LIPC* T/T homozygotes had a 50% lower HL activity than the C/C homozygotes. Atorvastatin lowered HL activity in all subjects independent of gender and genotype. Zambon and coworkers (30), found an attenuating effect of the T-allele on HL lowering by hypolipidemic drug treatment in hyperlipidemic patients. In the present study already the low dose of statin decreased HL activity in all genotypes similarly. It is possible that atorvastatin has a higher intrinsic capacity to lower HL than other hypolipidemic drugs. Alternatively, HL activity in type 2 diabetes may be affected in a different way than in patients without diabetes. HL activity also varies among different ethnic groups. Our results showed that the allele frequency for the *LIPC* T allele in black patients was much higher than in Caucasian patients consistent with earlier studies (21). This higher prevalence of the T allele in black patients completely accounted for the lowered HL activity at baseline in these patients compared to the Caucasian patients (260 U/L vs 415 U/L). However, Nie et al (24) described that 97% of African Americans have at least one HL allele that is associated with low HL activity. The effect of ethnic background on the frequency

of the T allele underscores the importance of this factor, when *LIPC* allele frequency between groups, with or without diabetes, is compared. The T allele frequency in our Caucasian subjects was 0.235. Jansen et al (16) reported a frequency of 0.189 in a healthy, non-diabetic, Caucasian population. Without correction for ethnicity, T allele frequency in the patients with type 2 diabetes, appeared to be higher than in Caucasians without diabetes, 0.262 versus 0.189.
The clinical significance of our findings needs further investigation. HL seems to be involved in the metabolism of almost all lipoprotein classes. It may, among others, modulate LDL and HDL metabolism and postprandial lipid clearing. How and to what extend HL lowering by atorvastatin in type 2 diabetes affects these processes, and thereby CAD risk, is presently unknown.

Conclusions

Gender, *LIPC* promoter variant and ethnicity greatly contribute to the baseline variance in HL activity in type 2 diabetes mellitus. This should be taken into account in studies evaluating the effect of lipid lowering therapy on HL expression. Atorvastatin treatment results in a dose-dependent decrease in HL activity, regardless of gender or the *LIPC* promoter variant.

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Chapter 4

Genetic variation in the lipoprotein lipase gene in type 2 diabetes and the effect of atorvastatin on lipid profiles.

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Submitted

Abstract

Objective

Lipoprotein lipase (LPL) gene variants may influence LPL activity and alter HDL-cholesterol (HDLc) and triglyceride (TG) concentrations, thereby affecting atherosclerotic risk. Genetic variants may modulate the efficacy of drugs.

Research design and methods

We investigated the effect of 3 most common variants in the LPL gene (D9N, N291S and S447Stop) on plasma lipids and atorvastatin treatment effects in 217 patients with type 2 diabetes.

Results

At baseline, patients (n=217, aged 45-75 years) had a total cholesterol of 4.0-8.0 mmol/L and fasting triglycerides (TG) of 1.5-6.0 mmol/L. They were treated for 6 month with atorvastatin (10 or 80 mg/daily) for 30 weeks in a double-blind, randomized, placebo-controlled trial (DALI study). Allele frequencies of the LPL gene variants D9N, N291S and S447Stop were 0.026, 0.028 and 0.097, respectively. Carriers of the D9N variant had a lowered LPL activity (-9.3%), HDLc (-16%) and ApoA-1 (-9.4%), and increased plasma TG (+21%). The N291S variant did not affect LPL activity nor lipid levels. The S447Stop variant showed an allele-dose dependent increase in LPL, apoA-I, HDL cholesterol and decrease in plasma TG. Atorvastatin did not substantially affect LPL activity in patients without the LPL D9N, N291S or S447Stop mutation (non-carriers). In patients carrying the LPL S447Stop mutation, LPL activity after atorvastatin treatment was significantly higher than in the non-carriers (145 ± 55 Units/L to 120 ± 49 Units/L, $p<0.05$). The favorable lipid profile in carriers of the S447Stop mutation persisted under atorvastatin treatment.

Conclusions

LPL gene mutations influence lipids, lipoproteins and LPL activity in type 2 diabetes. The S447Stop mutation modulates the response of LPL activity to atorvastatin.

Introduction

Lipoprotein lipase (LPL) is the major enzyme responsible for the clearance of plasma triglycerides (TG) (1). On the luminal endothelial surface, LPL catalysis the hydrolysis of TG in circulating chylomicrons and very low density lipoprotein (VLDL) particles after stimulation by apolipoprotein CII. In this process the surplus of surface material, phospholipid and unesterified cholesterol, transfers to high density lipoproteins (HDL). Consequently, HDL levels reflect LPL activity (2). Besides this enzymatic function, LPL has a non-enzymatic "bridging" function mediating cellular binding and uptake of lipoproteins (3-6). Due to its effects on plasma lipoproteins and lipoprotein metabolism, LPL may affect atherosclerotic disease risk. LPL activity may lower atherogenic risk by decreasing plasma TG and TG-rich lipoprotein (TRLs) levels, increasing HDLc levels and stimulating hepatic clearance of remnant particles (7). Low LPL may therefore constitute a pro-atherogenic condition. Indeed, in a large cohort of patients with angiographically proven coronary artery disease (REGRESS population), LPL activity was lower than in healthy controls (8). The lowered post-heparin LPL activity was associated with increased plasma TG levels and lowered HDLc levels. Moreover, LPL activity was inversely correlated with the severity of angina pectoris (9).

Insulin is a powerful stimulatory regulator of LPL in adipose tissue (10) and decreased LPL has been implicated in the pathogenesis of dyslipidemia in conditions associated with insulin resistance (11-13). During insulin resistance overproduction of very low-density lipoproteins (VLDL) in the liver may be compensated for by insulin-mediated stimulation of LPL activity with subsequent maintenance of normal plasma TG levels (14,15). If this system fails, like for instance in type 2 diabetes mellitus, hypertriglyceridemia might ensue. Couillard et al (16) showed that the duration and the extent of hypertriglyceridemia in 43 men with visceral obesity were dependent on LPL activity.

Variants in the LPL gene may affect the catalytic activity of LPL. Three LPL variants with frequencies in Caucasians of 2-3% (D9N), 5% (N291S) and 5-20% (S447Stop mutation), may have an effect at a population level (8, 17-19).

In non-diabetic patients, the risk of coronary disease is increased in carriers of D9N and N291S mutations (20,21). The D9N variant is associated with higher TG, lower HDL and a trend towards a lowered LPL activity. The changes in lipids could account for the increased risk in cardiovascular disease among carriers of the LPL D9N mutation (17,22,23). The LPL N291S allele appears to increase the susceptibility to lipid abnormalities and cardiovascular disease in combination with other genetic and environmental factors, like low density lipoprotein (LDL)

receptor mutations, familial combined hyperlipidemia, apolipoprotein E2, diabetes and obesity (24). In contrast, in carriers of the LPL S447Stop mutation lower plasma TG is associated with higher LPL activity (25-27).
Little information is available on the effects of LPL gene variants on diabetic lipid metabolism. Zhang et al (28) studied 18 patients with type 2 diabetes and severe diabetic dyslipidemia for mutations in the LPL gene. Eight of these patients contained 7 different genetic variants at the lipoprotein lipase gene locus and four of these patients probably had a functional mutant. Zhang and coworkers concluded that genetic variants at the lipoprotein lipase locus are common in subjects with type 2 diabetes mellitus, obesity and hyperlipidemia and that mutations in the LPL gene may affect the individual susceptibility for the development of hyperlipidemia. LPL gene variants not only determine baseline levels of LPL activity, but also affect the response to various drugs in non-diabetic patients (23,25,29). In the DALI study (30) patients with type 2 diabetes were treated with atorvastatin, a HMG CoA reductase inhibitor. Atorvastatin did not affect mean LPL activity (30), but this does not exclude an effect of the LPL gene variants on the response to atorvastatin. We now report on the effect of atorvastatin on post-heparin LPL activity, lipids and lipoproteins in non-carriers and carriers of LPL gene mutations (D9N, N291S and S447Stop).

Patients and methods

Study population

This study is part of the Diabetes Atorvastatin Lipid Intervention (DALI) study. DALI is a randomized double-blind, placebo-controlled, multi-center trial conducted in the Netherlands. The subjects and methods are described in detail earlier (30).
In summary, 217 patients, aged 45 to 75 years, with type 2 diabetes mellitus were randomized to placebo, atorvastatin 10mg (A10) or atorvastatin 80mg (A80) for 30 weeks, to evaluate the effect on lipid metabolism, endothelial function, coagulation and inflammatory parameters. The main inclusion criteria were plasma TG between 1.5 and 6.0 mmol/l, total cholesterol between 4.0 and 8.0 mmol/l and no history of coronary heart disease. Patients were recruited at centers in Leiden, Rotterdam and Utrecht in the Netherlands. The study protocol was approved by the Ethical Committees of the participating centers and all procedures followed were in accordance with institutional guidelines. Written informed consent was obtained from all subjects.

Analytical methods

Blood samples were drawn after 12 h of fasting at baseline and after 30 weeks, at the end of the treatment period. Standard lipid variables (Total cholesterol (TC), LDLc, HDLc, TG, apolipoprotein A-I (ApoA-I), apolipoprotein B-100 (Apo B-100)), plasma glucose and HbA1c were measured. Cholesterol and TG were determined by enzymatic colorimetric methods on a Hitachi 911 automatic analyzer (Boehringer Mannheim, Mannheim, Germany). Plasma HDLc was measured by a direct enzymatic HDLc method, after precipitation of VLDL and LDLc by addition of manganese chloride. LDLc was estimated by the Friedewald formula (31). ApoA-1 and ApoB-100 were determined on a Hitachi 917 analyzer, using immunoturbidimetric methods (Tina-quant apoA, Cat.Nr.1551680, apo B, Cat.Nr.1551779; Boehringer Mannheim). Fasting plasma glucose was determined on a Hitachi 917 analyzer using an UV-hexokinase method (Cat. Nr.18766899) from Boehringer Mannheim, Mannheim, Germany. HbA1c was determined by HPLC, using the BIO-RAD Variant TM method (Cat.Nr.270-0003.Bio-rad).

Postheparin plasma lipase activity

LPL activity was measured using an immunochemical method as described previously (32) in plasma collected 20 min after contralateral intravenous administration of 50 IU/kg body-weight heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). LPL controls consisted of 185 male and female volunteers, aged 45 to 75 years, without type 2 diabetes mellitus and hypertriglyceridemia. Of 217 DALI patients, 198 blood samples for evaluation of post heparin LPL activity were available. Eleven samples were excluded from the analysis because very low activities of LPL and HL in post heparin plasma indicated insufficient heparin delivery. Of eight patients no blood sample for post heparin lipase activity was available.

DNA Analysis

For DNA analysis of the LPL gene, 212 bloodsamples were available. DNA from all patients was subjected to mutation analysis by polymerase chain reaction (PCR) and density gradient gel electrophoresis (DGGE). To identify carriers for the D9N mutation, PCR analysis was performed with primers: 5'-CTC ATA TCC AAT TTT TCC TT -3'; 5'-ATA AAT ATA AAA TAT AAA TAG GGG TCA GGG CAA ATT TAC TTT CAA TG –3' (33). Amplification yielded a non-digested PCR product with a length of 114 bp. After digestion with Taq1, the D9N mutant allele yielded two products of 74 and 40 bp. To identify carriers for the N291S mutation, PCR analysis was performed with primers:5'- GCC GAG ATA CAA TCT TGG TG –3':

5' –CTG CTT CTT TTG GCT CTG ACT GTA –3' (34). Amplification yielded a non-digested PCR product with a length of 238 bp. After digestion with RSA1, the N291S mutant allele yielded two products of 215 and 23 bp. To identify carriers for the S447 Stop mutation, PCR analysis was performed with primers: 5'-CAT CCA TTT TCT TCC ACA GGG -3'; 5'-GCC CAG AAT GCT CAC CAG ACT –3' (33). Amplification yielded a non-digested PCR product with a length of 120 bp. After digestion with HinfI, the S447 Stop mutant allele yielded two products of 98 and 22 bp.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (release 9.0). Mean differences between the study groups were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels. Continuous variables are presented as mean values with the standard deviation (SD). P values < 0.05 were considered statistically significant.

Results

Patient characteristics

The baseline characteristics of the DALI patients have been described before (30). According to the inclusion criteria, 217 patients were randomized. There were no significant differences in the patient characteristics between the placebo, atorvastatin 10mg (A10) and atorvastatin 80mg (A80) groups, except for the duration of diabetes, which was shorter in the placebo group compared to A80, respectively 8.2 ± 5.9 vs 12.2 ± 8.3 years ($p<0.05$). Mean body-mass index (BMI), glucose, HbA1c, lipid, lipoprotein plasma values and LPL activity at baseline are shown in Table 1.

Mean baseline LPL activity in the whole DALI population was not significantly different from age-matched, non-diabetic controls without hypertriglyceridemia, 139 ± 40 U/L (n=198) vs 150 ± 54 U/L (n=185), p=0.09. There was no correlation between LPL activity, age, smoking habits, alcohol use, hypertension, BMI and the Waist Hip Ratio (WHR). LPL activity was correlated significantly with total cholesterol, HDLc and apoA (respectively r=0.24, p=0.001, r=0.22, p=0.002 and r=0.26, p<0.001), but not with plasma TG or apoB (r=-0.12, p=0.08 and r=0.10, p=0.14).

Table 1 Baseline characteristics

	DALI patients
	(n=217)
Male sex (%)	53
Age (years)	59.5
BMI (kg/m2)	30.9 ± 4.5
Fasting glucose (mmol/L)	10.6 ± 3.2
HbA1c (%)	8.3 ± 1.1
Triglycerides (mmol/L)	2.62 ± 1.0
Total cholesterol (mmol/L)	5.99 ± 0.9
LDL-c (mmol/L)	3.71 ± 0.3
HDL-c (mmol/L)	1.04 ± 0.2
Apo A-I (g/L)	1.40 ± 0.2
Apo B-100 (mg/100 mL)	1.24 ± 0.2
LPL activity (U/L)	139 ± 40

Data are means ± sd.
BMI=Body mass index, LPL= Lipoprotein lipase

LPL activity and lipid profiles in carriers and noncarriers of LPL gene mutations.
The D9N mutation was detected in 11 out of 212 patients, giving an allele frequency of 0.026. The N291S mutation was present in 12 patients (0.028) and the S447Stop mutation in 38 patients (0.097). Among the 38 carriers of the LPL:S447Stop mutation, 35 were heterozygote and 3 homozygote. There was no significant difference in age or WHR, blood pressure, alcohol use and smoking habits (data not shown) or BMI (Table 2) between carriers and non-carriers of LPL mutations. There was a gender associated difference in the frequency of carriers of the N291S, most N291S carriers being males (Table 2). None of the patients carried more than one LPL variant.
The effect of the D9N and the N291S mutation on LPL activity and plasma lipids is shown in Table 2. Compared to patients without a D9N, N291S or S447Stop mutation (non-carriers), heterozygote carriers of the D9N allele showed a significantly lower LPL activity, a higher plasma TG concentration and a lower HDLc and apoA-1 concentration. The N291S allele had no effect on LPL activity, TG nor on HDLc concentrations.

Table 2 Patients characteristics according to presence of LPL D9N and LPL N291S mutations.

	Noncarriers	LPL:D9N carriers	LPL:N291S carriers
	Total (151)	Heterozygotes (11)	Heterozygotes (12)
Male/Female	81 / 70	7 / 4	11 / 1
BMI (kg/m2)	30.2 (0.4)	31.6 (1.5)	28.1 (1.1)
TC (mmol/L)	6.01 (0.9)	5.87 (0.6)	6.12 (0.9)
TG (mmol/L)	2.67 (1.0)	3.22 (1.0)#	2.64 (1.1)
HDLc (mmol/L)	1.03 (0.2)	0.87 (0.1)$	0.98 (0.1)
ApoA (g/L)	1.39 (0.1)	1.26 (0.2)#	1.36 (0.1)
LPL activity (U/L)	130 (41)	121 (44)#	136 (39)

Noncarriers are patients without a D9N, N291S, S447Stop mutation in the LPL gene.
Data are expressed as mean (sd).
#p<0.05, $p=0.01 compared to non-carriers.

In patients carrying a S447Stop allele there was a tendency for higher BMI, LPL activity, HDLc, ApoA-1 and lower TC and TG with increasing allele frequency (Table 3). Patients who were homozygote for the S447Stop mutation had a significant lower TG, higher HDLc and apoA concentration ($p<0.05$) and a higher LPL activity than the non-carriers ($p<0.01$). The LPL activity was higher in the homozygote carriers compared to the heterozygote carriers of the S447Stop mutation ($p<0.05$).

It is well known that the frequency of mutations may vary among different ethnic groups (35). To exclude other ethnic influences on LPL activity, the Caucasians carrying a LPL mutation (57 of 61 LPL mutation carriers) were analyzed separately for the effect of the LPL gene variance on lipids and lipase activities. The results of this analysis were similar to those of the whole study population.

Table 3 Patients characteristics according to presence of LPL S447Stop mutation.

	Non-carriers	LPL:S447stop	LPL:S447stop
	Total (151)	Heterozygotes (35)	Homozygotes (3)
Male/Female	81 / 70	17 / 18	1 / 2
BMI (kg/m2)	30.2 (0.4)	31.5 (0.3)	32.0 (0.4)
TC (mmol/L)	6.01 (0.9)	5.92 (0.8)	5.7 (0.6)
TG (mmol/L)	2.67 (1.0)	2.64 (1.0)	2.11 (1.1)#
HDLc (mmol/L)	1.03 (0.2)	1.09 (0.1)	1.30 (0.1)#
ApoA (g/L)	1.39 (0.1)	1.44 (0.2)	1.60 (0.2)#
LPL activity (U/L)	130 (41)	136 (61)	162 (20)$&

Noncarriers are patients without a D9N, N291S, S447Stop mutation in the LPL gene.
Data are expressed as mean (sd).
#p<0.05, $p<0.01 compared to non-carriers
&p<0.05 compared to heterozygote carriers

Lipid profiles and LPL activity in carriers of a LPL S447Stop mutation treated with atorvastatin.

We compared the effect of atorvastatin versus placebo treatment in non-carriers and carriers of LPL mutations on LPL activity and lipid profiles. For reasons of small numbers of carriers of the LPL D9N and N291S mutations treated with atorvastatin, only the patients that were carriers of the S447Stop mutation were analyzed. Patients receiving A10 or A80 were analyzed together. The single S447Stop homozygote patient that received atorvastatin was analyzed together with the heterozygote carriers as separate analysis did not reveal any significant differences (data not shown). Placebo treatment had no effect on LPL activity in non-carriers nor in carriers (126 ±50 U/L, n=42, at baseline vs 129 ±58 U/L after 30 weeks and 130 ±41 U/L, n=9, at baseline vs 133 ±43 U/L after 30 weeks, respectively). Atorvastatin tended to lower LPL in the non-carriers and to increase LPL in the carriers of the S447Stop variant (Table 4). Consequently, after 30 weeks atorvastatin, LPL activity was significantly higher in the carriers compared to the non-carriers ($p<0.05$). In the carriers and non-carriers of the S447Stop mutation, there was a beneficial change in lipid profile after atorvastatin treatment. The apo A-I concentration was significantly higher and there was a tendency towards a higher HDLc level compared with the levels in the non-carriers receiving atorvastatin.

Table 4 Effect of atorvastatin in non-carriers and carriers of the S447Stop mutation

Effect atorvastatin		Non-carriers	LPL:S447Stop
		N=109	N=29
LPL activity (U/L)	Baseline	130 (47)	120 (49)
	30 weeks	138 (58)	145 (55)*
Total cholesterol (mmol/L)	Baseline	5.96 (0.9)	5.79 (0.8)
	30 weeks	3.86 (1.0)	3.96 (0.9)
Triglycerides (mmol/L)	Baseline	2.68 (1.0)	2.39 (1.0)
	30 weeks	1.79 (1.1)	1.74 (1.1)
HDLc (mmol/L)	Baseline	1.04 (0.3)	1.14 (0.3)
	30 weeks	1.09 (0.3)	1.21 (0.3)
ApoA (g/L)	Baseline	1.39 (0.2)	1.48 (0.2)
	30 weeks	1.35 (0.2)	1.46 (0.2)*

Noncarriers are patients without a D9N, N291S, S447Stop mutation in the LPL gene.
Patients receiving atorvastatin 10mg or 80mg were analyzed together.
*$p<0.05$ compared to non-carriers at end of study, adjusted for baseline levels.

Discussion

In a previous study (30), we described the lipid-lowering effects of atorvastatin in a population of type 2 diabetes patients. Both plasma cholesterol and TG levels were significantly lowered by low (10mg) as well as high (80mg) treatment doses of atorvastatin. The TG-lowering effect of atorvastatin occured without affecting LPL activity. Although it remains controversial (36), previous studies also suggested that mechanisms other than enhancing lipolysis of TRLs by LPL contributed substantially to the TG-reduction by atorvastatin (37,38). However, in patients carrying a LPL gene variant it is still possible that the LPL gene variant affects the response to atorvastatin. The information on the presence of LPL gene mutations in patients with diabetes mellitus, is confined to some isolated cases (39) and a small group (four out of 18 patients) with functional mutations in the LPL gene resulting in lower plasma LPL activity (28). Therefore, we studied the occurrence of LPL gene mutations and the influence of a common LPL mutation (S447Stop) on the ability of atorvastatin to improve lipid profiles in patients with type 2 diabetes and mild hypertriglyceridemia. The carrier frequencies of LPL D9N, LPL N291S and LPL S447Stop mutation were 5.2%, 5.7% and 18%, respectively. This frequency of

the LPL mutations is in line with frequencies found in a meta-analysis described by Wittrup et al (20). Carriers of the LPL D9N mutation had a significant lower LPL activity and a more atherogenic lipid profile than patients without a D9N, N291S or S447Stop mutation in the LPL gene. This is in accordance with previous studies in patients without diabetes (17,22,23). The reduced LPL activity and consequently elevated TG levels and reduced HDLc levels probably affect an individual's risk of ischemic heart disease (40). Elevated TG levels result in remnant accumulation and reduced HDLc in reduced reverse cholesterol transport, both promoting atherosclerosis. Prolonged post-prandial lipemia accompanying depressed LPL activity, is also linked to increased risk for coronary artery disease.
Carriers of the LPL N291S mutation had similar LPL activity and lipid profiles as non-carriers. Homozygote carriers of the S447Stop mutation had significant higher LPL activity and a more favorable lipid profile compared with non-carriers or carriers of the LPL D9N and N291S mutations. As described earlier by Henderson and coworkers (8), the S447Stop mutation is associated with an increase in LPL activity which manifests with lower plasma TG and higher HDLc levels in carriers of this variant. Our results also point to an allele dose dependency regarding LPL activity and its effect on lipid profiles of the patients. Compared to homozygote carriers, heterozygote carriers of the S447stop mutation only had slightly higher LPL activity, HDLc and apoA-1 levels and lower TC and TG levels. However, in carriers of the S447Stop mutation the risk of ischemic heart disease is possibly decreased (20), therefore even small, favorable changes in lipid profiles in these carriers are probably of importance.
In this study we report that although atorvastatin does not significantly increase LPL activity in carriers of the S447Stop mutation that those carriers do have a significant 20% higher LPL activity after 30 weeks atorvastatin compared with the non-carriers. This is due to a tendency of atorvastatin to lower LPL activity in non-carriers and to elevate LPL activity in carriers of the LPL S447Stop mutation. So this mutation seems to modulate the effect of atorvastatin on LPL activity positively and results in a differential response to treatment. After atorvastatin treatment the less atherogenic lipid profile of the LPL S447Stop carriers is still detectable. This is important information since the S447Stop mutation is very common in different populations, with a carrier frequency of 17-22% (20). Due to a small number of carriers of the LPL D9N and N291S mutation, that were treated with atorvastatin, we were not able to study the influence of these LPL mutations on the ability of atorvastatin to improve lipid profiles in the diabetes patients. More population-based research to investigate whether these LPL mutations influence the hypolipidemic effect of statins drugs is required.

It is important to realize that our LPL data are data gathered in the fasted state. Since LPL plays an important role during the postprandial period in the plasma lipoprotein homeostasis (41), it is possible that the difference in LPL between the non-carriers and the carriers of the LPL S447Stop mutation will have an even greater impact in the postprandial state. Recently, Panarotto and coworkers (11) concluded that the regulation of adipose tissue LPL is significantly affected in insulin-resistant individuals in the postprandial period and that the impaired effect of insulin on LPL postprandially contributed to the atherogenic dyslipidemia described in the insulin resistance syndrome.
The American Diabetes Association (ADA) recommends HMG-CoA reductase inhibitors (statins) as first choice lipid regulating pharmacotherapy in patients with diabetes and a diabetic dyslipidemia in order to reach clinical targets (42). Therefore, more information about the contribution of the genetic variation in the LPL gene to the lipid profile is warranted, not only for cardiovascular risk stratification, but also to elucidate the response to hypolipidemic treatment.

Conclusions

LPL gene mutations influence lipids, lipoproteins and LPL activity in type 2 diabetes, which will affect the risk for cardiovascular disease. The S447Stop mutation modulates the response to atorvastatin resulting in a further improvement of the diabetic dyslipidemia profile.

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CHAPTER 5

Atorvastatin decreases atherogenic LpB:CIII in type 2 diabetes mellitus, a mechanism to lower plasma triglycerides.

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Submitted

Abstract

Objective

Apolipoprotein (apo)C-III is a constituent of HDL (HDL apoC -III) and apolipoproteinB-100 (apoB-100)-containing lipoproteins (LpB:CIII). It slows the clearance of triglyceride-rich lipoproteins (TRLs) by inhibiting lipoprotein lipase (LPL) activity and interfering with lipoprotein binding to cell-surface receptors. Elevated levels of LpB:CIII are an independent risk factor for cardiovascular disease.

Research design and methods

We studied the effect of 30 weeks treatment with 10mg (A10) and 80mg (A80) atorvastatin on apoC-III levels in a randomized double-blind placebo-controlled trial with 217 patients with type 2 diabetes mellitus and fasting triglycerides between 1.5 and 6.0 mmol/L.

Results

Baseline levels of total apoC-III, HDL apoC-III and LpB:CIII were respectively 41.5 (10.0) mg/L, 17.7 (5.5) mg/L and 23.8 (7.7) mg/L. ApoC-III strongly correlated with plasma triglyceride, $r=0.74$, $p<0.001$. ApoC-III (75%) (75% LpB:CIII and 25% HDL apo CIII) and LPL activity (-14%) were major determinants of plasma triglyceride levels. A10 and A80 significantly decreased apoC-III (23%), HDL apo C-III (16% and 23%) and LpB:CIII (28%). The decrease in apoC-III, mainly in LpB:CIII strongly correlated with a decrease in triglycerides ($r=0.78$, $p<0.001$).

Conclusions

Atorvastatin treatment resulted in a significant dose-dependent reduction in plasma apoC-III, HDL apoC-III, and LpB:CIII levels in patients with type 2 diabetes mellitus. These data indicate a potentially important anti-atherogenic effect of statin treatment and explain part of the triglyceride lowering effect of atorvastatin.

Introduction

Hypertriglyceridemia, low levels of HDL-cholesterol, and preponderance of small dense LDL particles are risk factors for cardiovascular disease (1,2). These factors are part of the atherogenic lipoprotein profile often found in patients with type 2 diabetes mellitus. Hypertriglyceridemia may develop as a consequence of both overproduction and delayed clearance of triglyceride-rich lipoprotein particles (TRL). Plasma concentrations of triglycerides and apolipoprotein (apo)C-III are strongly correlated in healthy controls and hyperlipidemic patients (3). ApoC-III is an important element in the metabolism of TRL. Our understanding of the function of apoC-III present on TRLs has been derived from animal studies as well as studies using cell culture systems. In vitro studies have implicated apoC-III as a noncompetitive inhibitor of lipoprotein lipase (LPL) activity (4,5). ApoC-III is also implicated in the clearance of TRL remnants. It may interfere with lipoprotein uptake by hepatic lipoprotein receptors, resulting in accumulation of large TRLs in plasma (6,7). The assumption that apoC-III is involved in the metabolism of TRLs is supported by studies with genetically modified animals. Transgenic animals overexpressing the human apoC-III gene developed severe hypertriglyceridemia (8). It is likely that the increased apoC-III content of TRLs impaired the catabolism of these particles resulting in hypertriglyceridemia (9,10). Mice lacking apoC-III, on the other hand, cleared VLDL particles very efficiently due to increased lipolysis and enhanced selective uptake of VLDL cholesteryl esters (11).
Patients with elevated levels of plasma triglycerides have increased plasma levels of apoC-III with a four-times larger proportion of apoC-III present in VLDL sized lipoprotein particles compared to normolipidemic individuals (12-15). Several clinical studies have shown that both total apoC-III and apoC-III present on TRLs (LpB:CIII) are risk indicators for cardiovascular disease (16,17). Data from the MARS study (18) suggest that apoC-III, particularly in apoB-containing lipoproteins, is a major risk factor for the severity of coronary heart disease in patients with mild and moderate atherosclerosis. In the CLAS study, in subjects treated with niacin-colestipol, the HDL apoC-III level was the major predictor of global coronary atherosclerosis progression (19).
Atorvastatin is a powerful HMG-CoA reductase inhibitor proven to reduce effectively total cholesterol and triglycerides. The mechanism leading to the decrease in plasma triglycerides is unknown. Previously, we found that atorvastatin lowers plasma triglyceride by 25 and 35% in subjects with type 2 diabetes. No effect was observed on LPL activity (20). Since in situ LPL activity may be inhibited by apoC-III, we studied whether atorvastatin affects apoC-III levels. To this end, we

determined the effect of low (10mg) and high (80mg) dose atorvastatin on plasma apoC-III levels and the distribution of apoC-III on plasma lipoprotein fractions in patients with type 2 diabetes mellitus and hyperlipidemia.

Methods

Study population

This study is part of the Diabetes Atorvastatin Lipid Intervention (DALI) study. DALI is a randomized double-blind, placebo-controlled, multi-center study conducted in the Netherlands (20). Briefly, 217 patients, aged 45 to 75 years, with type 2 diabetes mellitus of at least 1 year and an HbA1c of 10% or lower, participating in the DALI study were randomized to placebo, atorvastatin 10mg (A10) or atorvastatin 80mg (A80) during 30 weeks, to evaluate the effect of treatment on lipid metabolism. The diagnosis of type 2 diabetes was defined according to the ADA classification (21). Inclusion criteria were fasting plasma triglycerides between 1.5 and 6.0 mmol/l, total cholesterol between 4.0 and 8.0 mmol/l and no history of coronary heart disease. Patients were recruited in Leiden, Rotterdam and Utrecht. The study protocol was approved by the Ethical Committees of the participating centers and all procedures followed were in accordance with institutional guidelines. Written informed consent was obtained from all subjects.

Analytical methods

Blood samples were drawn after a fasting period of 12 h at baseline and after 30 weeks' treatment at the end of the study. Plasma was prepared by immediate centrifugation and samples were stored at –80 ° C for further analyses. Cholesterol and triglycerides were determined by enzymatic colorimetric methods on a Hitachi 911 automatic analyzer (Boehringer Mannheim, Mannheim, Germany). Plasma HDL cholesterol was measured by a direct enzymatic HDL-cholesterol method, after precipitation of apoB-containig lipoproteins with manganese chloride. LDL-cholesterol was estimated by the Friedewald formula (22). Fasting plasma glucose was determined on a Hitachi 917 analyzer using an UV-hexokinase method (Cat. Nr.18766899) from Boehringer Mannheim, Mannheim, Germany. HbA1c was determined by HPLC, using the BIO-RAD Variant TM method (Cat.Nr.270-0003.Bio-rad). Plasma apoC-III was analyzed by a commercially available electroimmuno assay (Sebia hydragel LP CIII, Issey-les-Moulineaux, France). HDL apoC-III is determined in the supernatant after precipitation of the apoB

containing lipoproteins with the use of a specific antibody. LpB:CIII is calculated from by subtracting HDL-apoC-III from total apoC-III.

Postheparin plasma LPL activity

LPL activity was measured using an immunochemical method as described previously (23) in plasma collected 20 min after contralateral intravenous administration of heparin (50 IU/kg body-weight, Leo Pharmaceutical Products, Weesp, The Netherlands). LPL was measured in a control normolipidemic population of 103 male and female volunteers, aged 45 to 75 years, without type 2 diabetes mellitus and hypertriglyceridemia.

Statistical analysis

Analyses were performed by SPSS for Windows (release 9.0). Continuous variables are presented as mean values with the standard deviation (SD). Pearson's correlation coefficients were calculated to study associations between apoC-III and other variables at baseline. Mean differences between the study groups were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels and study location. P values < 0.05 were considered statistically significant.

Results

Patient characteristics

Baseline characteristics of the patients in the DALI study were described elsewhere (20). According to the inclusion criteria, 217 patients were randomized. There were no significant differences in baseline characteristics between the placebo, A10 and A80 group, except for the duration of diabetes, which was shorter in the placebo group compared to the atorvastatin 80mg (A80) group, respectively 8.2 ± 5.9 versus 12.2 ± 8.3 years ($p<0.05$). Body–Mass Index (BMI), glucose, HbA1c, LPL activity, lipid and lipoprotein plasma values at baseline are shown in Table 1.

Table 1 Baseline characteristics

	DALI patients (n=217)
Male sex (%)	53
Age (years)	59.5
Body Mass Index (kg/m2)	30.9 ± 4.5
Fasting glucose (mmol/L)	10.6 ± 3.2
HbA1c (%)	8.3 ± 1.1
LPL (U/L)	129 ± 50
Triglycerides (mmol/L)	2.62± 1.0
Total cholesterol (mmol/L)	5.99 ± 0.9
LDL-c (mmol/L)	3.71 ± 0.3
HDL-c (mmol/L)	1.04 ± 0.2
ApoA (g/L)	1.40 ± 0.2
ApoB (mg/100mL)	1.24 ± 0.2

Data are means ±sd.
LPL= Lipoprotein lipase.

Apolipoprotein C-III at baseline.

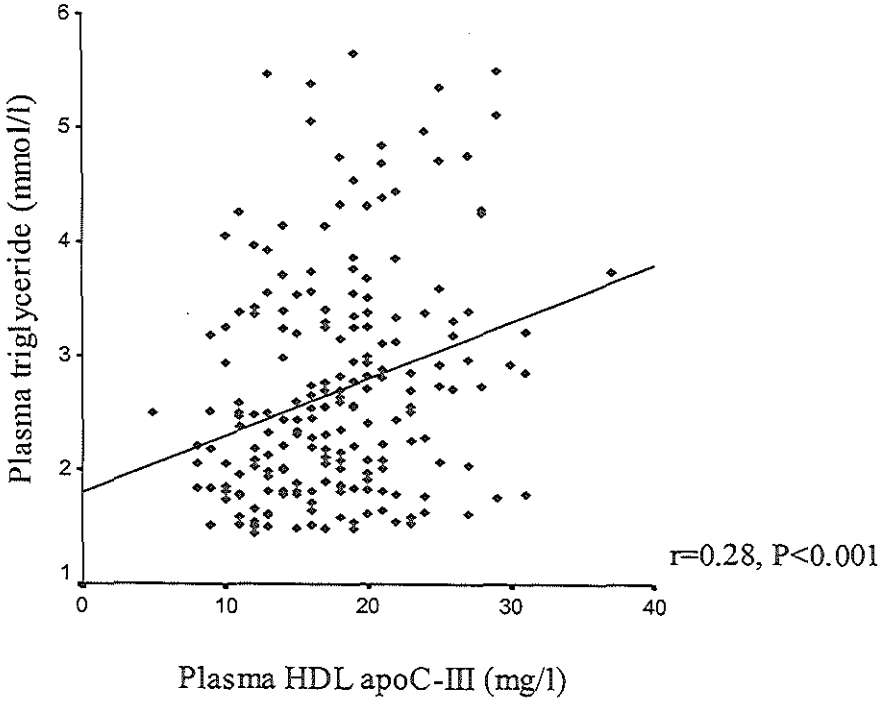
Baseline levels of apoC-III were similar in all three treatment groups (Table 2). Mean apoC-III was 41.5 ± 10.0 mg/L. ApoC-III correlated significantly with plasma triglyceride (r=0.74, p<0.001) , apoB (r=0.24, p<0.001) and TC (r=0.33, p<0.001). After division apoC-III levels into quartiles, subjects in the highest quartile (apoC-III >48.0 mg/L), compared to those in the lowest quartile (apoC-III <34 mg/L), showed an increase in plasma triglyceride levels from 1.86 to 3.79 mmol/l. ApoC-III is a constituent of HDL and triglyceride-rich apoB containing lipoproteins, (LpB:CIII). Average HDL apoC-III and LpB:CIII were respectively 17.7 ± 5.5 mg/L (12.8 – 21.8 mg/L) and 23.8 ± 7.7 mg/L (17.1 – 29.5 mg/L). HDL apoC-III and LpB:CIII both correlated with plasma triglycerides. However, the correlation between plasma triglyceride and LpB:CIII was stronger than the correlation with HDL apoC-III (r=0.76, p<0.001 and r=0.28, p<0.001, respectively) (Figure 1).
ApoC-III did not correlate with LPL activity while LPL activity showed only a weak inverse correlation with plasma triglyceride, r=-0.13, p=0.05. ApoC-III and LPL activity contributed 75% (75% LpB:CIII and 25% HDL apoC-III) and inversely 14% to the variance in plasma triglycerides.

Table 2 Apolipoproteins at baseline and after 30 weeks atorvastatin.

		Placebo (n=71)	Atorvastatin 10mg (n=71)	Atorvastatin 80mg (n=71)
Total apoC-III	Baseline	41.3 (9.0)	39.8 (9.5)	43.1 (9.9)
(mg/L)	30 weeks	41.2 (12.0)	31.8 (10.6)*	31.1 (11.3)*
HDL apoC-III	Baseline	17.6 (5.2)	16.7 (5.3)	18.4 (6.0)
(mg/L)	30 weeks	17.6 (8.0)	14.1 (5.2)†	13.2 (5.8)*‡
LpB:CIII	Baseline	23.6 (6.8)	23.1 (7.3)	24.7 (8.6)
(mg/L)	30 weeks	23.6 (7.7)	17.7 (7.8)*	17.9 (8.7)*

Data are expressed as mean (sd).
Test for difference among the three groups, adjusted for baseline value:*p<0.001, †p=0.005
Test for difference versus A10, adjusted for baseline:‡ p=0.05.

Figure 1. Association between HDL apoC-III, LpB:CIII and plasma triglycerides. r denotes correlation coefficient.

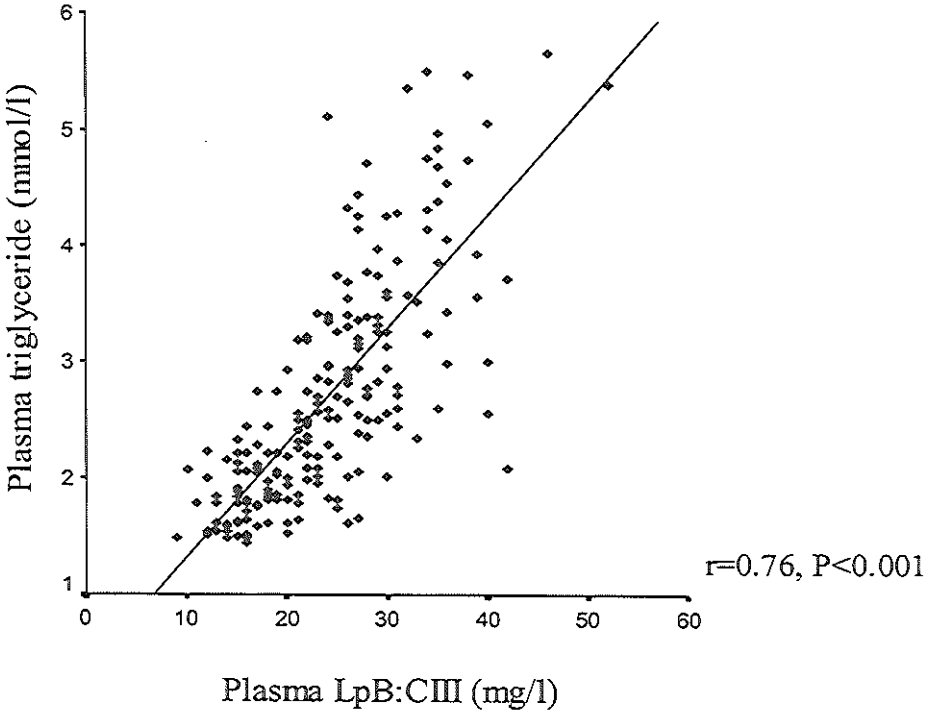


Atorvastatin effect on apolipoproteins and post-heparin LPL activity.

After 30 weeks' treatment with atorvastatin, apoC-III in the A10 and A80 groups was 23% lower than in the placebo group (Table 2). A10 and A80 decreased apoC-III in both the HDL and the non-HDL (LpB:CIII) fractions to a similar extent (16-23% and 28%). Since the LpB:C-III fraction contained the major part of the total apoC-III, atorvastatin decreased the absolute amount of apoC-III in the non-HDL fraction more than in the HDL fraction.

Earlier (20), we reported that A10 and A80 decrease plasma triglycerides in the DALI population by 25 and 35%, respectively (both $p<0.001$). The decrease in total plasma apoC-III strongly correlated with the decrease in plasma triglycerides in both A10 and A80, respectively $r=0.70$ and $r=0.78$, both $p<0.001$. Especially in the A80 group, the decrease in LpB:CIII correlated more strongly with the decrease in plasma triglyceride ($r=0.80$, $p<0.001$) (Figure 2), than the decrease in HDL apoC-III ($r=0.43$, $p<0.001$). This suggested that the apoC-III content of the non-HDL fraction was a better determinant of plasma triglyceride levels than the HDL apoC-III level.

Figure 2. Association between the reduction of plasma LpB:CIII and the reduction of plasma triglycerides after atorvastatin 80mg. r denotes correlation coefficient.



Discussion

In this study, we showed that in hypertriglyceridemic patients with type 2 diabetes, atorvastatin treatment profoundly lowered the apoC-III content of HDL and of non-HDL (apoB-100 containing) lipoproteins (LpB:CIII). This may represent an important anti-atherogenic effect of statin treatment. In several studies, a high content of apoC-III in LpB:CIII and/or in HDL was found to be associated with increased coronary risk (18,19,24,25).
The LpB:CIII concentration has been previously been sugested to be a more specific measure of coronary heart disease risk than plasma triglycerides (26).
Hypertriglyceridemia is an independent risk factor for atherosclerosis in type 2 diabetes (27). The hypertriglyceridemia may be caused by several mechanisms. The number of VLDL particles, secreted by the liver may be enhanced. Alternatively, or additionally, the clearance of triglyceride-rich VLDL may be impaired. Kissebah and coworkers (28) demonstrated that in subjects with type 2 diabetes, either normolipidemic or hypertriglyceridemic, apoB and VLDL-TG secretion is enhanced. The rate of clearance is likely, therefore, to determine whether hypertriglyceridemia develops or not. Indeed, Wilson et al (29) showed that hypertriglyceridemia only developed in type 2 diabetic subjects with genetically lowered LPL activity. In our population LPL activity was not affected (20), indicating that the hypertriglyceridemia was not due to a lowering of this enzyme. The in situ activity of LPL may however, be affected by apoC-III. ApoC-III inhibits the lipolysis of VLDL-TG by LPL (4) and interferes with the hepatic uptake of TRLs by LDL receptors (6). Consequentely, high apoC-III leads to accumulation of apoB- and apoC-III-containing particles, VLDL and IDL, and thus to hypertriglyceridemia. In our patient group, plasma apoC-III, mainly in LpB:C-III, accounted for 75% of the variance in plasma triglycerides. LPL had a much smaller effect. Batal et al (12), demonstrated that in hypertriglyceridemia the increase in VLDL apoB is the result of delayed removal rather than of increased production. The delayed removal was ascribed to increased apoC-III synthesis. Studies in rats have demonstrated that apoC-III expression is lowered by statins (30). We found, total plasma apoC-III levels reduced by 23% in respons to atorvastatin therapy. Low (A10) and high (A80) dose had similar effects. The largest reduction was established in the LpB:CIII fraction. The decrease in LpB:CIII strongly correlated with a decrease in plasma triglycerides. These results suggest that lowering of plasma triglycerides by atorvastatin is at least partly due to increased clearance of TRL because of a lowering in plasma (LpB:CIII) apoC-III content. Similar effects of atorvastatin on plasma apoC-III levels were found after 4 weeks' treatment in a

population of 27 patients with primary hypertriglyceridemia (31). In a study in 305 patients with primary hypercholesterolemia (32), atorvastatin 10mg and pravastatin 20mg also reduced LpB:CIII (26% and 33%, respectively). In contrast, a study in patients with a coronary artery bypass graft treated with lovastatin, statin therapy did affect Lp:B (LDL), but not plasma apoC-III or LpB:C-III (15).

Conclusions

Our results demonstrate that atorvastatin may lower the elevated risk of atherosclerotic disease in patients with type 2 diabetes through several mechanisms. It effectively reduces total cholesterol and triglycerides and elevates HDL cholesterol but in addition it lowers atherogenic apoC-III in LpB:CIII and HDL. Since apoC-III is a risk-indicator for cardiovascular disease and a marker for the TRL-metabolism, measurement of apoC-III in patients at risk for atherosclerotic disease may be helpful in risk assessment and treatment strategies.

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Chapter 9

Gender specific etiology of low HDL in diabetes mellitus.

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Submitted

Abstract

Objective

Quantitative and qualitative differences in lipoprotein profiles in men and women with type 2 diabetes may partly explain the increased relative risk of coronary heart disease (CHD) in women. It is possible that sex differences in hepatic lipase (HL) and lipoprotein lipase (LPL) activities contribute to a different etiology of diabetic dyslipidemia.

Research design and methods

HL and LPL activity were determined in relation to lipids and lipoproteins before and after 30 weeks treatment with placebo, atorvastatin 10mg or atorvastatin 80mg in a double-blind, randomized, placebo-controlled trail (DALI study).

Results

Baseline HL activity in Caucasian DALI males was 12% higher than in Caucasian male volunteers without diabetes or hypertriglyceridemia (452 ±155 (n=96) vs 397 ±125 (n=78), $p<0.001$).Baseline LPL activity in Caucasian DALI women was 10% lower than in Caucasian female volunteers (147 ±32 (n=72) vs 162 ±58 (n=107), $p=0.003$). In males, HL activity significantly correlated with HDL-C ($r=-0.22$, $p=0.03$), apoA-1 ($r=-0.28$, $p=0.04$) and TG ($r=0.32$, $p=0.002$). In females, LPL activity significantly correlated with HDL-C ($r=0.35$, $p=0.003$) and apoA-1 ($r=0.33$, $p=0.005$). After atorvastatin treatment HL activity was similarly reduced in males and females and LPL activity was not affected in both genders. Atorvastatin treatment abolished gender differences in plasma lipids and lipoproteins.

Conclusions

In patients with type 2 diabetes, there is a gender specific etiology of low HDL-C levels, which may contribute to the increased relative risk for CHD in women.

Introduction

Major changes in lipoprotein profiles in type 2 diabetes result in partial or complete loss of the "female survival advantage" for cardiovascular death (1,2). Whereas pre-menopausal women without diabetes have a more favourable lipoprotein profile than men and a lower risk of coronary heart disease (CHD), women with type 2 diabetes show the same combination of high triglycerides (TG) and low HDL-C as men, but the association with CHD seems to be more prominent in women (2,3). This change in CHD risk might be due to differences in levels or properties of lipoproteins and/or different dynamics of lipoprotein metabolism. Compared to men, the combination of diabetes, low levels of HDL-C, and high levels of VLDL in women seems to constitute a higher-risk metabolic profile (2). The predominance of atherogenic small dense LDL particles in diabetes may contribute to a greater relative risk of CHD in women as well. LDL size is often significantly lower in patients with diabetes of both sexes than in patients without diabetes, but there is a stronger association between LDL size and diabetes in women than in men, even after controlling for TG and HDL-C levels (4). These quantitative and qualitative differences in lipoprotein profiles in men and women partly explain the increased risk for CHD in women (5). In type 2 diabetes, gender specific differences in lipoprotein profiles are possibly related to gender specific changes in etiology of the dyslipidemia. Sex steroids may affect key factors in the regulation of plasma lipoproteins, like for instance hepatic lipase (HL) and lipoprotein lipase (LPL) activities (6,7). HL and LPL are considered to be important factors in the development of hypertriglyceridemia, low HDL-C levels and compositional abnormalities in lipoprotein fractions (8-10). HL is a lipolytic enzyme that appears to be involved in the metabolism of TG-rich and cholesterol-rich lipoproteins (11-14). HL is a major determinant of plasma HDL-C levels and of LDL-size, and is proposed to be involved in the post-prandial lipid clearance (15). In humans, HL is affected by several factors, like gender, visceral adiposity and a HL gene promoter polymorphism (denoted as *LIPC* C>T variation) (16-22). HL activity is higher in males than in females. This gender-related variation is mostly explained by sex steroid hormones and by intraabdominal fat mass (IAF) (17,23-26). Estrogen levels are negatively and IAF positively associated with HL activity.

LPL is the major enzyme responsible for the clearance of TG from the plasma and is a major determinant of HDL-C levels (27). Therefore HDL-C levels may also reflect LPL activity (28). LPL activity is also influenced by gender and genetic variation (29-32) and has been implicated in the pathogenesis of dyslipidemia in conditions associated with insulin resistance (8).

We hypothesised that possible sex differences in lipase activities contribute to gender differences in etiology of diabetic dyslipidemia. Therefore we examined in

patients with type 2 diabetes the effect of gender on HL and LPL activities in relation to differences in lipoprotein profiles. We also studied the effect of atorvastatin on lipase activities and how this affected the atherogenic lipoprotein profiles in men and women.

Patients and methods

Study population

This study is part of the Diabetes Atorvastatin Lipid Intervention (DALI) study. DALI is a randomized double-blind, placebo-controlled, multi-center study conducted in the Netherlands. The subjects and methods are described in detail earlier (33).

In short, 217 patients, aged 45 to 75 years, with type 2 diabetes mellitus participating in the DALI study were randomized to placebo, atorvastatin 10mg (A10) or atorvastatin 80mg (A80) during 30 weeks, to evaluate the effect on lipid metabolism, endothelial function, coagulation and inflammatory parameters. The main inclusion criteria were plasma TG between 1.5 and 6.0 mmol/L, total cholesterol between 4.0 and 8.0 mmol/L and no history of coronary heart disease. Post heparin lipase activity blood samples of 198 DALI-patients were available. Since ethnicity might influence lipase activities, only Caucasian patients (n=168) were evaluated in the present study. Patients were recruited in Leiden, Rotterdam and Utrecht. The study protocol was approved by the Ethical Committees of the participating centres and all procedures followed were in accordance with institutional guidelines. Written informed consent was obtained from all subjects.

Analytical methods

Blood samples were drawn after 12 h of fasting at baseline and at the end of the treatment period. Standard lipid variables, free fatty acids (FFA), plasma glucose, HbA1c, LDL size, HDL2 and HDL3 were measured. Total cholesterol (TC) and triglycerides (TG) were determined by enzymatic colorimetric methods on a Hitachi 911 automatic analyzer (Boehringer Mannheim, Mannheim, Germany). Plasma HDL cholesterol was measured by a direct enzymatic HDL-cholesterol method, after precipitation of very-low density lipoprotein and LDL by addition of manganese chloride. LDL cholesterol was estimated by the Friedewald formula (34). Apo A-1 and apo B were determined on a Hitachi 917 analyzer, using immunoturbidimetric methods (Tina-quant apo A-1 and apo B, Cat. no. 1551680 and no.1551779; Boehringer Mannheim). Fasting FFA's were determined using an enzymatic colorimetric method (Wako, NEFA C, Cat. no.994-75409 D). Fasting plasma glucose was determined on a Hitachi 917 analyzer using an UV-hexokinase method (Cat. Nr.18766899) from Boehringer Mannheim, Mannheim, Germany. HbA1c was determined by HPLC, using the BIO-RAD Variant TM method (Cat.Nr.270-0003.Bio-rad). LDL size was measured as described earlier (32). DNA

analysis for genotyping of the *LIPC* gene for the C>T variance and of the LPL gene to identify carriers for the D9N, N291S and S447Stop mutations were measured as described before (35,36).

Postheparin plasma lipase activity

HL and LPL activity was measured using an immunochemical method as described previously (37) in plasma collected 20 min after contralateral intravenous administration of 50 IU/kg body-weight heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). HL and LPL controls consisted of healthy Caucasian males (n=78) and female volunteers (n=107), aged 45 to 75 years, without type 2 diabetes mellitus or hypertriglyceridemia. 198 blood samples of DALI patients for evaluation of post heparin lipase activities were available. Eleven samples were excluded from the analysis because very low activities for HL and LPL in post heparin plasma indicated insufficient heparin delivery. Of eight patients no blood sample for post heparin lipase activity was available. Of these 198 bloodsamples, 30 bloodsamples belonged to non-Caucasian patients and were excluded from this study.

Statistical analysis

Analyses were performed by SPSS for Windows (release 9.0). Continuous variables are presented as mean values with the standard deviation. Mean differences between men and women were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels. Pearson correlation coefficients were calculated to study associations between post-heparin HL and LPL activities and other variables at baseline(SD). Multiple linear regression analysis was used to assess the relationships between gender, lipase activities, age, WHR and lipid- and lipoprotein variables simultaneously. P values < 0.05 were considered statistically significant.

Results

Baseline characteristics of males and females

The baseline characteristics of 96 male and 72 female participants of the DALI study are listed in Table 1. There were no significant gender differences in age, diabetes duration or treatment, glucose, and HbA1c. Males had a lower body mass index (BMI) than the females, but they had a significant higher waist hip ratio (WHR). Although the WHR was higher in males, they had a lower fat mass (24.2 kg), compared with the females (34,4 kg), as calculated by the formulas described by Flier et al (38). Males had 26,2 % body fat and weighted 93.1 kg, while females had 40.5 % body fat and weighted 86.1 kg. As described earlier (35,36) the frequency of the *LIPC*-T allele and the LPL S447Stop mutation was similar in men as in women. There were significantly more male (n=6) than female (n=2) carriers of the LPL:D9N mutation.

Table 1 Baseline characteristics.

	Males (n=96)	Females (n=72)	p-value
Age (years)	58.6 ± 7.6	61.2 ± 7.8	Ns
BMI (kg/m2)	29.8 ± 4.7	32.4 ± 5.0	p=0.001
Waist-to-hip ratio	1.02 ± 0.1	0.97 ± 0.1	p<0.001
Diabetes duration (years)	10.7 ± 6.6	12.8 ± 8.2	Ns
Diabetes treatment (%)			
Diet	0,92	1.1	Ns
Tablets	45.4	44.4	Ns
Insulin	31.5	25.6	Ns
Combination	22.2	28.9	Ns
Glucose (mmol/L)	10.2 ± 3.1	10.7 ± 3.4	Ns
HbA1c (%)	8.2 ± 1.2	8.3 ± 1.1	Ns

Data are expressed as mean ±sd

Baseline lipids and lipoproteins in males and females

Baseline lipids and lipoprotein variables are listed in Table 2.

Females had significantly higher total cholesterol (TC), HDL-C, LDL-C, apoA-1, apoB-100 and FFA levels. There were no gender differences between TG and LDL size. Adjustment for BMI or WHR did not change the gender differences in lipids and lipoproteins.

Table 2 Baseline lipids and lipoproteins.

	Males (n=96)	Females (n=72)	p-value
Total cholesterol (mmol/L)	5.87 ± 0.9	6.23 ± 0.8	p=0.008
HDL-C (mmol/L)	0.99 ± 0.2	1.11 ± 0.2	p=0.002
Triglyceride (mmol/L)	2.73 ± 1.1	2.59 ± 0.9	p=0.34
LDL-C (mmol/L)	3.64 ± 0.9	3.95 ± 0.8	p=0.02
Apo A-1 (g/L)	1.34 ± 0.2	1.48 ± 0.2	p<0.001
Apo B (mg/100mL)	1.21 ± 0.2	1.30 ± 0.2	p=0.006
LDL-size (nm)	25.9 ± 0.8	26.2 ± 0.7	p=0.09
FFA (mmol/L)	0.60 ± 0.2	0.76 ± 0.3	p<0.001

Data are expressed as mean ±sd

Lipase activities in males and females

The lipase activities in the DALI population and the healthy individuals are listed in Table 3. Male DALI patients had a 12% increase in HL activity (p<0.001) compared with healthy, male individuals. Female DALI patients and healthy females showed comparable HL activities. DALI females had a 10% lower LPL activity than healthy, female individuals (p=0.003). DALI males and male volunteers showed equal LPL activities.

Table 3 Gender differences in lipase activities.

	Caucasian DALI patients (n=168)	Healthy Caucasians (n=185)	p-value
Males			
HL activity (U/L)	452 ± 155* (n=96)	397 ± 125 (n=78)	p<0.001
LPL activity (U/L)	138 ± 43 (n=96)	132 ± 42 (n=78)	Ns
Females			
HL activity (U/L)	367 ± 125 (n=72)	328 ± 105 (n=107)	Ns
LPL activity (U/L)	147 ± 32 (n=72)	162 ± 58# (n=107)	p=0.003

Healthy Caucasians are subjects, matched for age but without DM or hypertriglyceridemia.
*p<0.001, compared with HL activity in DALI females,
#p<0.001, compared with LPL activity healthy Caucasian males.

Lipase activities correlations.

Caucasian DALI males showed a significant correlation between HL activity and HDL-C (r=-0.22, p=0.03), apoA-1 (r=-0.28, p=0.04), TG (r=0.32, p=0.002) or FFA (r=-0.21, p=0.04). Multiple regression analysis revealed that HDL-C and TG were independently determined by HL activity, Beta –0.240, p=0.40, r2 = 0.196 and Beta 0.344, p=0.001, r2 = 0.115, respectively. Caucasian DALI females showed no correlation between HL activity and HDL-C (r=0.09, p=0.42), apoA-1 (r=0.06, p=0.60), TG (r=-0.08, p=0.49) or FFA (r=0.14, p=0.24). In both genders, there were no significant correlations between HL activity and age, BMI, WHR, TC, LDL-C or LDL-size.

In females there was a correlation between LPL activity and HDL-C (r=0.35, p=0.003) and apo A-1 (r=0.33, p=0.005). Multiple regression analysis revealed that HDL-C and apoA-1 were independently determined by LPL activity, Beta 2.894, p=0.003, r2 = 0.127 and Beta 0.301, p<0.001, r2 = 0.200, respectively. Males showed correlations between LPL activity and TC (r=0.25, p=0.02), LDL-C (r=0.21, p=0.05) and apoA-1 (r=0.23, p=0.02). In both genders, there were no significant correlations between LPL activity and age, BMI, WHR, TC, LDL-C or LDL-size.

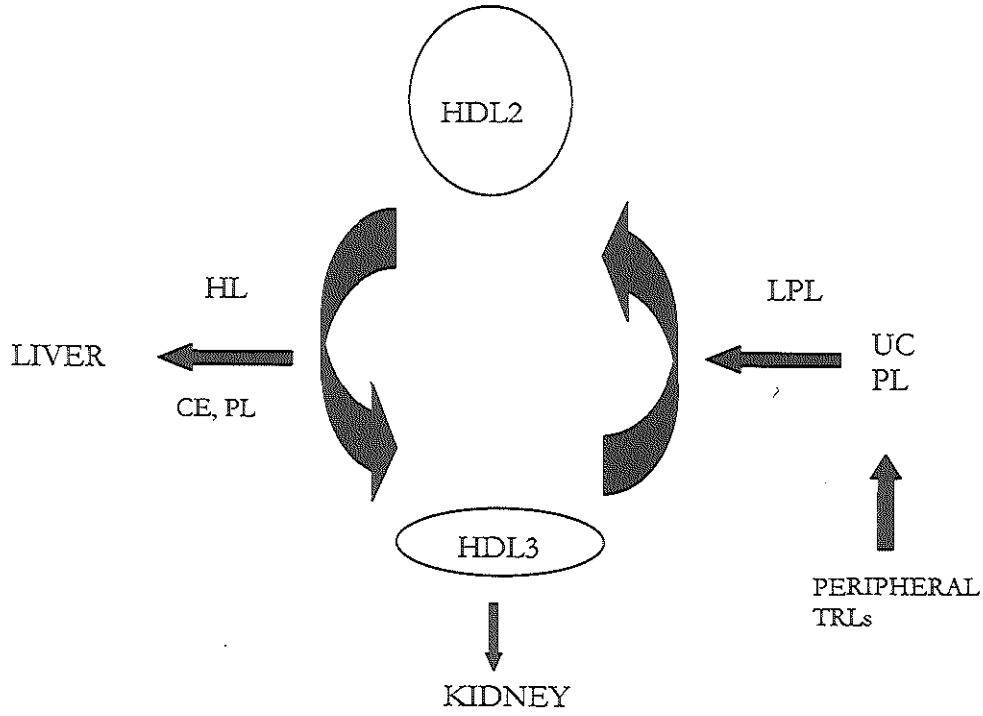
Contribution of gender differences in lipase activities to lipoprotein profiles after 30 weeks atorvastatin.

After 30 weeks treatment with atorvastatin 10mg (A10) or atorvastatin 80mg (A80), gender differences in lipids and lipoproteins disappeared. Using analysis of covariance (ANCOVA), adjusted for baseline, males and females had a comparable, significant, dose-dependent reduction in TC (-30% (A10) to -40% (A80)), LDL-C (-41% (A10) to -52% (A80) and apo B (–31% (A10) to -40% (A80)) and a significant, not dose-dependent reduction in TG (–25% (A10) to -35% (A80)) and increase in HDL-C (+6% (A10 and A80)) (data not shown). Atorvastatin therapy did not affect LDL-size in males and females. As reported before (38), there was still a significant gender difference in HL activity after treatment. HL activity was similarly reduced in males and females by 11% (A10) and 22% (A80), p<0.001. LPL activity showed still no gender difference after atorvastatin treatment.

Discussion

Diabetes is the only condition that equalizes cardiovascular risk between men and women (2,39). This implicates that diabetes is relatively more hazardous for women than for men. Gender differences in the etiology of diabetic dyslipidemia may contribute to this gender-related cardiovascular disease risk. Previously, it was demonstrated in patients without diabetes that lipase activities are important determinants of lipoprotein profiles (40-42). We investigated in patients with type 2 diabetes gender differences in HL and LPL activity, since these enzymes play a pivotal role in lipid metabolism and diabetic dyslipidemia in particular. Our results demonstrated that diabetes seems to affect lipase activities differently in men and women. There was no difference in HL activity between healthy male and female subjects, but male patients suffering from type 2 diabetes showed a significant higher HL activity than female diabetic patients. In contrast, LPL activity differed significantly between healthy male and female subjects. Diabetes equalized the gender difference in LPL activity. In agreement with the study of Goldschmid and coworkers (2), our females, all postmenopausal, showed higher plasma HDL-C, apoA-1, TC, LDL-C and apo B levels but equal TG levels compared with the males. The gender differences in lipase activities may partly explain the sex dimorphism in HDL-C levels in our population. One of the possible mechanisms underlying low HDL-C levels can be explained by the influence of HL and LPL on the HDL metabolism (Figure 1). In type 2 diabetes increased rates of secretion of VLDL into plasma appears to drive the exchange of TG from TRLs for HDL cholesteryl ester. The TG-enriched HDL is a substrate for either HL and LPL (7). When the TG in HDL is hydrolyzed, the smaller HDL3 particles affect the binding of apoA-1. Free apoA-1 and HDL3 are then more easily catabolized in the kidney.

Figure 1. A model of the role of hepatic lipase and lipoprotein lipase in HDL metabolism



HDL is a substrate for plasma lipases. HL converts large cholesterol ester (CE)-rich HDL2 to smaller HDL3 particles that are more rapidly cleared from the plasma via the kidney. HL action stimulates cholesterol uptake by the liver.
Surface unesterified cholesterol (UC) and phosholipids (PL) from triglyceride-rich lipoproteins (TRLs) are transferred to HDL during VLDL and chylomicron lipolysis by LPL.

Since removal of surface lipids from TRLs by LPL activity causes their transfer to HDL, relatively low LPL activity might lead to a decreased capacity of unesterified cholesterol uptake from peripheral tissues, reflected in a relative low HDL-C level in diabetic dyslipidemia. Indeed, in our female patients there was a significant correlation between LPL activity, HDL-C and apoA-1 levels. This relatively low HDL-C levels in the DALI females may be caused by an attenuated HDL formation due to a relatively low LPL activity. This decrease in HDL synthesis might then lead to an attenuated reverse cholesterol transport, which might promote atherosclerosis.

In the male DALI patients, LPL activity was not affected in the diabetic state. In contrast, HL activity was increased and showed an inverse correlation between plasma HDL-C and apoA-1 levels. The increased HL activity may lead to accelerated HDL apoA-1 clearance, as discussed above. Therefore low HDL levels in our DALI men may be due to increased HDL (apoA-1) clearing. At the same time, increased HL activity may induce cholesterolester uptake in the liver and therefore promote reverse cholesterol transport. As Jin and coworkers stated (43), there will probably be an optimal level of HL activity and having too much HL activity will further reduce HDL-C levels and increase atherosclerotic risk. Based on these considerations, one can expect that low HDL-C due to increased HL activity probably increases reverse cholesterol transport, while low HDL-C due to impaired LPL activity does not increase inverse cholesterol transport. Therefore it is feasible that the relatively low levels of HDL-C seen in women with type 2 diabetes, may constitute a higher-risk metabolic profile and may contribute to a greater relative risk of CHD in women compared with diabetic men.
Since gender differences in lipase activities might partly explain the sex difference in lipoprotein profiles and almost every male and female patient with type 2 diabetes will sooner or later receive hypolipidemic treatment, we also wondered whether improvement of the atherogenic lipid profile after hypolipidemic treatment would change the gender differences in lipase activities. Therefore we studied in the DALI population with regard to gender differences, the influence of lipase activities on the lipoprotein profile after 30 weeks atorvastatin. Atorvastatin treatment abolished the gender differences in TC, HDL-C, LDL-C, apo A-1, apo B and FFA, but as reported earlier gender difference in HL activity remained (35). Atorvastatin did not influence LPL activity, except for patients with a LPL:S447Stop mutation (36). These results demonstrated that women with diabetes, that are more at risk for ischaemic heart disease, may benefit at least equal to men from hypolipidemic treatment.

Conclusions

The results of the present study provide support for the notion that gender differences in lipase activities partly explain a different etiology of lowered HDL-C levels in patients with type 2 diabetes. Further prospective studies evaluating lipase activities in men and women with diabetes for developing diabetic dyslipidemia are needed to establish the validity of our study results.

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Chapter 7

Low-density lipoprotein subfractions are reduced by aggressive lipid lowering in patients with type 2 diabetes mellitus.

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Submitted

Abstract

Objective

Diabetic dyslipidemia is characterized by increased triglycerides (TG), reduced high density lipoprotein cholesterol and a predominance of smaller, more dense low density lipoprotein particles (LDL3). LDL3 is associated with increased cardiovascular risk.

Research design and methods

A randomized double-blind placebo-controlled trial to assess the effect of 30 weeks atorvastatin 10mg (A10) and 80mg (A80) on LDL subfraction distribution and concentration in 85 patients with type 2 diabetes mellitus.

Results

At baseline, 93% of the patients showed a LDL subfraction distribution with the major LDL peak within the intermediate-dense LDL2 range (d=1.030-1.040 g/ml). Atorvastatin significantly reduced cholesterol concentrations in all LDL subfractions and plasma apoB ($p<0.001$), resulting in a reduced number of particles. Compared to placebo, large, buoyant LDL1 cholesterol concentration was dose-dependently reduced from 0.93 ±0.3 to 0.58 ±0.3 mmol/l in A10 ($p<0.001$), and to 0.39 ±0.2 mmol/l in A80 ($p<0.001$) (A80 vs. A10; $p<0.005$). Like LDL1, intermediate-dense LDL2 cholesterol concentration was dose-dependently reduced from 1.64 ±0.4 to 1.06 ±0.4 mmol/l in A10 ($p<0.001$), and to 0.79 ±0.4 mmol/l in A80 ($p<0.001$) (A80 vs. A10; $p<0.005$). Both A10 and A80 significantly reduced LDL3 cholesterol concentrations to 0.38 ±0.2 (A10) and 0.39 ±0.2 mmol/l (A80) (both vs. placebo; $p<0.001$). Despite substantial fasting plasma TG reductions of 28-35% ($p<0.001$), both A10 and A80 did not shift the LDL subfraction density distribution.

Conclusions

Atorvastatin significantly reduced cholesterol concentration in all LDL subfractions but did not induce a shift in LDL subfraction density distribution towards larger and more buoyant LDL particles in patients with type 2 diabetes mellitus.

Introduction

Dyslipidemia is a common feature of the atherogenic risk profile of type 2 diabetes mellitus and is characterized by hypertriglyceridemia and reduced high density lipoprotein (HDL) cholesterol, with compositional abnormalities in the lipoprotein fractions rather than large quantitative changes in lipids (1,2). Low density lipoprotein (LDL) cholesterol concentration is not elevated, while in many patients with type 2 diabetes a preponderance of LDL particles with smaller size and increased density (LDL3) are observed (3). This small, dense LDL subfraction pattern is associated with increased levels of triglyceride (TG) (4) and the presence of insulin resistance, central obesity and hypertension (5). Increased cholesteryl ester transfer protein (CETP) concentration (6) and hepatic lipase (HL) activity (7) appear associated with small, dense LDL. The presence of a major LDL peak in the small, dense LDL fraction is associated with an increased risk of coronary artery disease (CAD) in the general population (8). As the patient with type 2 diabetes is at higher than average cardiovascular risk (9), modification of the LDL density pattern may be an additional therapeutic target (10).
Atorvastatin is a powerful HMG-CoA reductase inhibitor, proven to be safe (11-13) a nd effective in reducing total cholesterol and triglycerides in non-diabetic patients with hypercholesterolemia and primary hypertriglyceridemia (14-16). Previous studies suggest that atorvastatin modifies the LDL subfraction pattern in subjects without diabetes (17).
The aim of the present study was to examine the effect of the standard dosage atorvastatin 10mg (A10) versus the aggressive dosage 80mg (A80) versus placebo on LDL subfraction distribution and concentration in type 2 diabetes patients.

Patients and methods

Study population

This study comprised 85 patients with type 2 diabetes enrolled in the Diabetes Atorvastatin Lipid Intervention (DALI) study. DALI is a double-blind, randomized, placebo controlled, multi-center study, conducted in the Netherlands, designed to evaluate the effect of atorvastatin 10 mg versus 80 mg on lipid metabolism, endothelial function, coagulation and inflammatory factors in men and women with type 2 diabetes. The protocol and eligibility criteria have been described in detail elsewhere (18) Briefly, men and women, aged 45 to 75 years, with HbA1c £ 10% were eligible. The lipid criteria were: total cholesterol (TC) between 4.0 and 8.0 mmol/l and fasting TG between 1.5 and 6.0 mmol/l. The subjects

participating in the present study were the first 85 patients who were randomized in the DALI study, and had a baseline, 10 week, and final visit evaluation of their LDL subfraction distribution analyzed in addition to the DALI protocol. The DALI study had three recruiting centers: Leiden, Rotterdam and Utrecht. The study protocol was approved by the Ethical Committees of the participating centers and written informed consent was obtained from all subjects.

Analytical methods

After an overnight fast for a minimum of 12 hours, blood was drawn and plasma was prepared and stored at -80°C for further analysis. Lipids and apolipoproteins were quantified as described extensively (18). Plasma cholesterol ester transfer protein (CETP) mass was analyzed as described (19).

LDL subfraction distribution by density gradient centrifugation

The LDL subfraction distribution was analyzed by density gradient ultracentrifugation using a six-step discontinuous salt gradient as described by Griffin (20). Fresh plasma (3 ml) was fractionated into twenty distinct LDL subfractions after 24h (40.000 rpm at 4°C) centrifugation in a Beckman SW40 swinging bucket rotor. The LDL fractions were recovered from the tube by upward displacement, collected in 500ml aliquots, and identified by absorbance at 280 nm. The isolated fractions were frozen immediately at -80°C for subsequent duplicate measurement of cholesterol. Cholesterol in all fractions was analyzed enzymatically using a Cobas Mira S auto-analyzer (ABX Diagnostics, Montpellier, France). Major LDL subfractions were identified according to their density and divided into three LDL categories: 1.020-1.029 g/ml (LDL1: fraction 6-10), 1.030-1.040 g/ml (LDL2: fraction 11-13), or 1.041-1.066 g/ml (LDL3: fraction 14-17) (21).

Statistics

All values are expressed as mean ±standard deviation. Spearman's correlation coefficients were calculated to study associations between the LDL subfraction distribution and other variables at baseline. Mean differences between the study groups were analyzed using analysis of covariance (ANCOVA), adjusted for baseline levels and study location. Intervention effects were also further adjusted for additional potential confounders, using ANCOVA. All analyses were performed using SPSS software, version 9.0 for Windows.

Results

The baseline characteristics of the study population are given in Table 1.

Table 1. Baseline characteristics.

	Placebo	Atorvastatin 10mg	Atorvastatin 80mg
Number	26	29	30
Male gender (%)	58	69	63
Age (years)	58.0 ±7.5	60.4 ±6.7	59.8 ±7.3
Blood pressure (mmHg)	146/85	147/86	146/85
Diabetes duration (years)	10.1 ±7.0	12.5 ±7.9	12.2 ±7.5
HbA1c (%)	8.4 ±1.2	8.3 ±1.2	8.5 ±1.3
Fasting glucose (mmol/l)	10.5 ±3.0	10.1 ±3.2	10.0 ±2.9
Body-Mass Index (kg/m2)	32.3 ±5.3	29.2 ±3.3	30.2 ±4.9
Waist to Hip ratio	1.00 ±0.09	1.02 ±0.07	1.02 ±0.1
Present smoking (%)	15	28	30
Total cholesterol (mmol/l)	5.9 ±0.8	5.8 ±1.0	6.2 ±0.8
LDL cholesterol (mmol/l)	3.6 ±0.8	3.7 ±0.9	3.9 ±0.8
HDL cholesterol (mmol/l)	1.04 ±0.2	1.01 ±0.2	0.99 ±0.2
Triglycerides (mmol/l)	2.83 ±1.2	2.45 ±0.7	2.87 ±1.1
Free fatty acids (mmol/l)	0.66 ±0.2	0.59 ±0.2	0.66 ±0.2
ApoA-I (g/l)	1.41 ±0.2	1.37 ±0.2	1.35 ±0.2
ApoB (g/l)	1.23 ±0.2	1.20 ±0.2	1.26 ±0.2
CETP mass (mg/l)	2.60 ±0.6	2.31 ±0.8	2.35 ±0.5

Continuous data are expressed as mean ±standard deviation.

At baseline, the majority of the patients (80/85) showed a LDL subfraction distribution with the maximal peak within the intermediate-dense LDL2 range. Within the LDL2 range, the highest LDL cholesterol concentration was found in fraction 11 in 24% (19/80), in fraction 12 in 52% (42/80) and in fraction 13 in 24% (19/80). In 4 subjects the LDL subfraction density distribution was within the small, dense LDL3 range, whereas in one subject it was within the large LDL1 range. The LDL subfraction density distribution was positively associated with TG ($r = 0.493$; $p<0.001$), CETP mass ($r = 0.330$; $p<0.005$), fasting glucose ($r = 0.219$; $p<0.05$)

and inversely associated with HDL cholesterol (r = -0.201; p=0.07). No associations were found with other lipid variables, lipolytic enzymes, other diabetes parameters and lifestyle variables.

Atorvastatin treatment effectively improved all lipid variables. After 30 weeks, LDL cholesterol (SD) was reduced to 2.2 (0.8) mmol/l in A10 (-42%; p<0.001) and 1.6 (0.8) mmol/l in A80 (-59%; p<0.001, A80 vs. A10; p<0.005). Plasma apoB was reduced to 0.82 (0.19) g/l in A10 (-32%; p<0001) and 0.71 (0.18) g/l in A80 (-43%;p<0.001, A80 vs. A10; p<0.001). Fasting TG were reduced to 1.63 (0.78) mmol/l in A10 (-33%; p<0.001) and 1.97 (1.58) mmol/l in A80 (-28%; p<0.001). CETP mass was reduced to 1.76 (0.47) mg/l in A10 (-21%; p<0.001) and 1.58 (0.32) mg/l in A80 (-31%; p<0.001, A80 vs. A10; p<0.05). The effects of atorvastatin treatment on lipid variables have been described extensively elsewhere (18). Atorvastatin significantly reduced the absolute cholesterol concentrations in all LDL subfractions (Table 2).

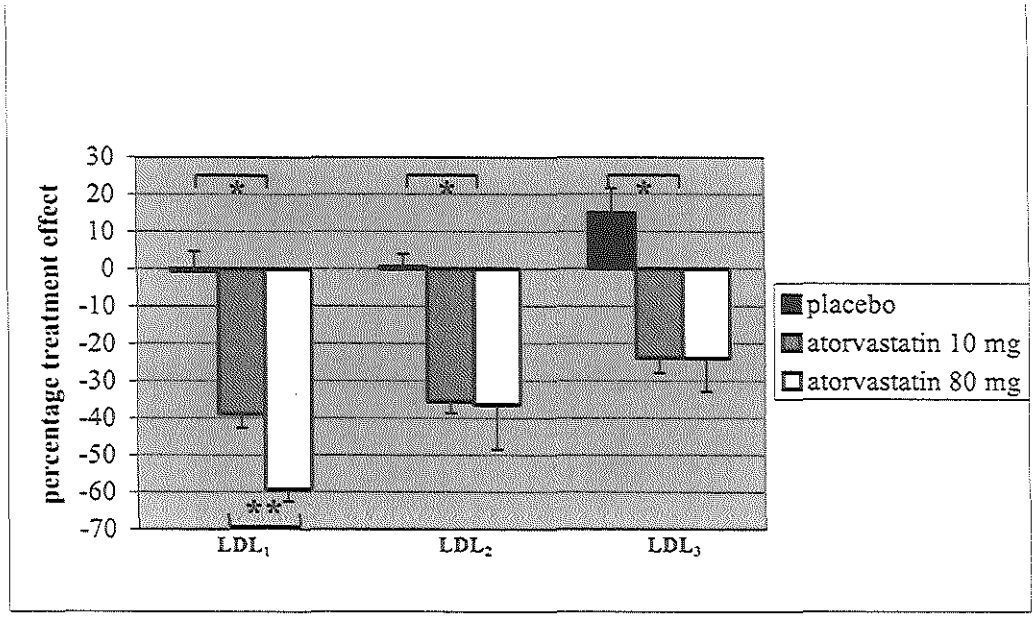
Table 2. Concentration of cholesterol (mmol/l) in LDL subfractions at baseline and after 30 weeks atorvastatin treatment.

Baseline	Placebo	Atorvastatin 10mg	Atorvastatin 80mg
Large, buoyant LDL1 (mmol/l)	0.96 ±0.3 (30%)	0.93 ±0.3 (30%)	0.98 ±0.4 (31%)
Intermediate-dense LDL2 (mmol/l)	1.65 ±0.5 (51%)	1.64 ±0.4 (53%)	1.59 ±0.6 (51%)
Small, dense LDL3 (mmol/l)	0.60 ±0.3 (19%)	0.52 ±0.2 (17%)	0.58 ±0.2 (18%)
After 30 weeks of treatment			
Large, buoyant LDL1 (mmol/l)	0.92 ±0.3 (29%)	0.58 ±0.3 * (29%)	0.39 ±0.2 * § (25%)
Intermediate-dense LDL2 (mmol/l)	1.61 ±0.5 (51%)	1.06 ±0.4 * (53%)	0.79 ±0.4 * § (50%)
Small, dense LDL3 (mmol/l)	0.65 ±0.2 (20%)	0.38 ±0.2 * (19%)	0.39 ±0.2 * (25%)

Continuous data are expressed as mean ±standard deviation.
The number between brackets indicates the percentage cholesterol content.
Test for difference among the 3 study groups, adjusted for baseline value and study location: * p<0.001
Test for difference versus atorvastatin 10 mg, adjusted for baseline value and study location: § p<0.005

The substantial plasma apoB reduction after atorvastatin therapy indicates that the total number of LDL particles is reduced. Compared with placebo, large, buoyant LDL1 cholesterol concentration was dose-dependently reduced to 0.58 ±0.3 mmol/l in A10 ($p<0.001$), and to 0.39 ±0.2 mmol/l in A80 ($p<0.001$) (A80 vs. A10; $p<0.005$). Atorvastatin also reduced intermediate-dense LDL2 cholesterol concentration dose-dependently. Both A10 and A80 significantly reduced small, dense LDL3 to a similar concentration (0.38 ±0.2 (A10) vs. 0.39 ±0.2 mmol/l (A80), both vs placebo; $p<0.001$). The relative proportion of cholesterol present in LDL1, LDL2 and LDL3 did not change after 30 weeks of treatment. Figure 1 shows the treatment efficacy of atorvastatin to reduce LDL1, LDL2 and LDL3.

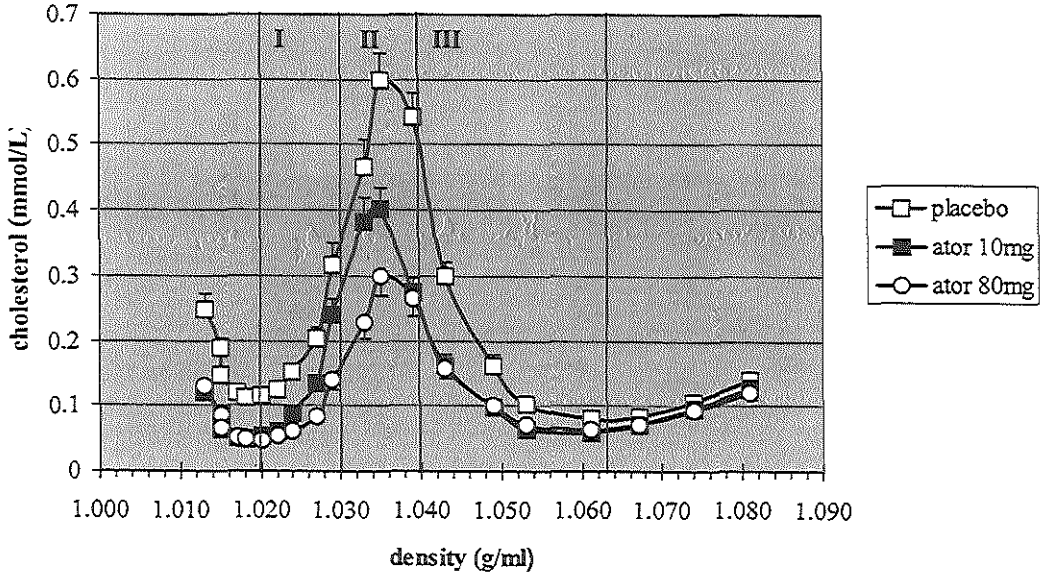
Figure 1. Percentage reduction in cholesterol concentration in each LDL subclass after 30 weeks of treatment in the DALI study.



Test for difference among the 3 study groups, adjusted for baseline value and study location: * $p<0.001$
Test for difference versus atorvastatin 10 mg, adjusted for baseline value and study location: ** $p<0.001$

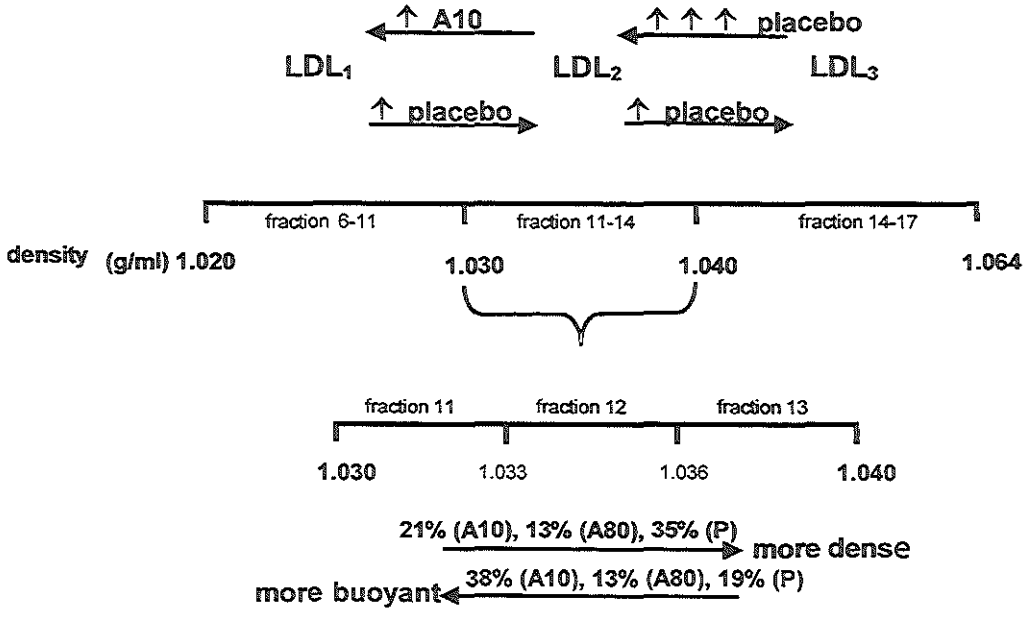
Atorvastatin did not shift the LDL subfraction density distribution (Figure 2). In the majority of the patients (n=79; 93%) the LDL peak remained within the LDL2 density. In only 6 subjects (7%) the LDL peak shifted towards another LDL subclass. In the upper part of figure 3 these 6 subjects are depicted by the 6 symbols on the arrows. A closer look at the individual LDL fractions (1-20) revealed that, within the LDL2 density range, 21% (A10), 13% (A80) and 35% (placebo) of the patients showed a shift to the right (towards fraction 13; a more dense LDL profile) after 30 weeks of treatment. On the other hand, 38% (A10), 13% (A80) and 19% (placebo) showed a shift to the left (towards fraction 11; a more buoyant LDL profile) (lower part of figure 3). In the majority of the patients (41% (A10), 70% (A80) and 42% (placebo)) the major LDL peak remained unchanged during the study (not shown in figure 3). These results after 30 weeks of treatment are similar to the results after 10 weeks treatment (data not shown).

Figure 2. Cholesterol concentration in LDL subfractions after 30 weeks of treatment in the DALI study.



Compared with placebo, the peak cholesterol concentration was dose-dependently reduced to 0.40 ±0.17 mmol/l ($p<0.001$) by A10 and to 0.30 ±0.16 mmol/l by A80 ($p<0.001$, A80 vs. A10; $p<0.05$) (Figure 2).
Because the LDL subfraction distribution was associated with fasting TG and CETP mass at baseline we performed additional analyses to investigate whether the baseline levels of TG and CETP mass did influence the effect of atorvastatin. The first group included all patients with baseline plasma TG levels >2.5 (median) mmol/l (n=43), whereas the second group included 42 patients with baseline plasma TG levels <2.5 mmol/l. In both groups similar effects were observed. When the results were analyzed in strata of CETP mass, again similar results were obtained. Further adjustment for BMI did not affect the results either.

Figure 3. Changes in density distribution of LDL after 30 weeks of treatment in the DALI study.



In only six subjects, who are depicted by the 6 symbols (≠) on the arrows in the upper part of the figure, the LDL peak shifted (e.g. LDL_1, LDL_2 or LDL_3). The majority (n=79) remained in the LDL_2 subfraction density range. In these patients the percentage of shifts within the LDL_2 density range are shown in the lower part of the figure.

Discussion

Atorvastatin therapy resulted in a dose-dependent reduction of cholesterol concentrations in large, buoyant LDL1 and intermediate-dense LDL2. Cholesterol in small, dense LDL3 was significantly reduced to a similar extent by both the standard dosage atorvastatin 10 mg and the aggressive dosage 80 mg. Despite substantial reductions in fasting plasma TG of 28-35% towards levels of 1.63-1.97 mmol/l, both A10 and A80 did not shift the LDL subfraction density distribution towards a more buoyant LDL pattern. In 93% of the subjects the LDL peak remained in the LDL2 subfraction density range after 30 weeks of treatment.
An increased level of plasma LDL cholesterol is a major risk factor for CHD (22). There is considerable heterogeneity in the size, density and composition of LDL particles. Classification according to size by gel electrophoresis (pattern A, B) does not take into account the concentration of cholesterol in LDL subfractions. Recent data from the CARE study suggest that the concentration of cholesterol in the LDL subfractions may be more relevant than LDL size concerning CHD risk (23). We measured LDL subfraction by a density gradient ultracentrifugation method (20) that provides both a distribution and a quantitative measurement of cholesterol in individual LDL subfractions. Using this method, we found that 94% of the patients had an LDL subfraction distribution with the peak in the intermediate-dense LDL2 density range at baseline. These results are in accordance with our observation that the majority of the total DALI population (n=217) had large, buoyant LDL particles (pattern A or AB) when LDL size was measured by gel electrophoresis (18). These observations are remarkable because our population expressed a typical diabetic dyslipidemia with mean fasting TG of 2.71 mmol/l (Table 1). Fasting TG levels above 1.7 mmol/l are associated with an increased prevalence of small, dense LDL (24). Furthermore, patients with type 2 diabetes frequently have an abnormal composition of LDL particles and a predominance of small, dense LDL (3). Indeed, compared to data in non-diabetic controls, which mainly show an LDL1 density pattern, the patients in the DALI study have a more dense LDL2 density distribution.
We observed significant associations between the LDL subfraction density distribution and TG, CETP mass and fasting glucose levels. The association between the presence of small, dense LDL and TG or fasting glucose levels is well known (4,25,26). The observed association between CETP mass and LDL subfraction distribution is interesting. Recently, Guérin et al. reported a significant contribution of CETP to the formation of small, dense LDL particles by a preferential CE transfer from HDL particles to small, dense LDL particles, as well as through an

indirect mechanism involving an enhanced CE transfer from HDL to VLDL1, the specific precursor of small, dense LDL in plasma (6). We observed a significant decrease in CETP mass after atorvastatin treatment, that may have contributed to the reduction in LDL3. Atorvastatin reduced the cholesterol content in all three LDL subfractions. Statins may enhance removal of LDL1 and LDL2 by LDL receptor upregulation and may reduce the formation of LDL3 from large, triglyceride-rich VLDL1, by stimulating clearance of remnants in VLDL2 and intermediate density lipoprotein (IDL) density ranges (27). In addition, VLDL concentration is reduced by inhibition of VLDL synthesis in the liver.

Small, dense LDL particles are atherogenic because they bind to the arterial wall with greater affinity than native LDL (28). Furthermore, they are more susceptible to glycation and oxidation (29). In non-diabetic populations the presence of small, dense LDL is associated with increased CHD risk (8,30-32). One may debate whether the presence of small, dense LDL is an independent risk factor for CHD in patients with type 2 diabetes (10,33,34) because their presence is related to elevated plasma TG level that is a characteristic feature of diabetic dyslipidemia. In our study we found a strong association between plasma TG levels and LDL subfraction density distribution as well.

In a recent study in non-diabetic patients with an atherogenic lipid profile, a reduction in the concentration of small LDL particles after atorvastatin was observed (35). McKenney et al. suggested that small, dense LDL particles are reduced to a larger extent with a higher dose of atorvastatin than with a lower dose (35). However, in our study both atorvastatin 10 and 80 mg significantly reduced small, dense LDL cholesterol concentration similarly by 24%. On the other hand, this study shows that cholesterol concentrations in LDL1 and LDL2 are dose-dependently reduced by atorvastatin, which suggests that a higher dose of atorvastatin is more effective in reducing LDL-mediated risk for atherosclerosis.

Two reports suggested that atorvastatin not only reduces overall concentrations of TG and LDL cholesterol but induces a shift in LDL subfraction distribution as well (36,37). These were open-labeled, uncontrolled studies in small numbers of non-diabetic patients. Data from double-blind, randomized, placebo-controlled studies in type 2 diabetes are lacking. As far as we know, there is one valid-designed study addressing this issue. Frost et al. compared the effect of atorvastatin 10 mg versus fenofibrate on LDL subfraction pattern and composition in patients with type 2 diabetes and observed results, regarding atorvastatin 10 mg, comparable to our data (21). They hypothesized that a higher dosage of atorvastatin might further decrease plasma TG concentration resulting in a shift in LDL subfraction distribution towards more buoyant, larger LDL (21). We observed a substantial reduction of

plasma TG levels induced by statin therapy that was similar for both dosages (18), without a shift of LDL subfraction distribution towards a large, buoyant LDL pattern. In addition, the proportion of cholesterol in LDL1, LDL2 and LDL3 did not change after 30 weeks of treatment, which underlines the absence of a shift. In the same study of Frost et al. fenofibrate induced a shift in LDL subfraction distribution from small, dense LDL (-31%) to intermediate-dense LDL (+36%) (21). The effects of fibrates are different from statins because they decrease TG concentration by activation of peroxisome proliferator-activated receptors a. Fibrates reduce the proportion of LDL3 by increasing LDL2 concentration and therefore induce a shift in LDL subfraction distribution without reducing total LDL concentration (38,39). The mechanism by which fibrates reduce concentrations of small, dense LDL particles is thought to be due to a specific reduction in hepatic TG levels relative to apoB, thereby promoting the secretion of smaller very low density lipoprotein (VLDL2) precursor particles and decreased neutral lipid exchange (40). Furthermore, fibrates may stimulate lipoprotein lipase activity, thereby increasing VLDL clearance (40). This suggests that only combination therapy of a statin and a fibrate will lead to the desired effect of total LDL cholesterol reduction together with a shift in LDL subfraction distribution towards less atherogenic, larger and more buoyant LDL particles in patients with type 2 diabetes. Indeed, recently Niemeijer-Kanters et al. showed that a strategy of intensive lipid-lowering by combination therapy leads to LDL cholesterol lowering and a shift towards more large, buoyant particles in patients with type 2 diabetes (19).
We studied the role of TG and CETP mass on the effect of atorvastatin on LDL subfraction distribution. Triglycerides may influence LDL particles through a cycle of lipid exchange via the action of CETP (41). LDL becomes enriched in TG and a population of small, dense LDL is formed by the action of hepatic lipase (7). However, we did not find evidence for a modulating effect of these parameters on the effect of atorvastatin on LDL subfraction density distribution. This could be due to the fact that the majority of the subjects in this study expressed an LDL2 subfraction profile. This does not exclude that the observed reduction in CETP mass after atorvastatin therapy may add favorably to the reduction in LDL3 cholesterol and to the reduction of the total number of LDL particles present.

Conclusions

Compared with placebo, atorvastatin 10 and 80 mg dose-dependently reduced the cholesterol content in LDL1 and LDL2. The cholesterol concentration in small, dense LDL3 was significantly reduced to a similar extent. However, both dosages of atorvastatin did not induce a shift towards a larger, more buoyant LDL pattern although the total number of LDL particles present was substantially reduced. These observations suggest that probably combination therapy of statins and fibrates is needed to achieve the desired effect of LDL1, LDL2 and LDL3 cholesterol reduction together with a shift in LDL subfraction density distribution towards less atherogenic, larger and more buoyant LDL particles in patients with type 2 diabetes.

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Effects of atorvastatin on cognitive functioning in hyperlipidemic patients with type 2 Diabetes Mellitus.

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Letter to the editor

Cognitive functioning is reduced in patients with type 2 diabetes mellitus (DM) as compared to age-matched patients without DM (1). Especially verbal memory and complex information processing are affected in patients with DM, which will have an impact on daily functioning (2). The severity of cognitive dysfunction in patients with DM presumably results from an interaction between risk factors for macro- and microvascular disease (3). Previous studies suggest a positive association between indices of cognitive impairment and elevation of plasma triglyceride level (4,5). The effect of lowering serum triglyceride levels by gemfibrozil on cognitive functioning has been investigated in elderly hypertriglyceridemic patients, 11 out of 44 patients had DM. Lowering triglyceride levels appeared beneficial to cerebral perfusion and cognitive performance after four to six months (6). Therefore we studied in the Diabetes Atorvastatin Lipid Intervention (DALI) study (7) the effect of atorvastatin on diabetic dyslipidemia and cognitive functioning. Thirty patients with DM, aged 45 to 75 years with fasting triglycerides between 1.5 and 6.0 mmol/L and total cholesterol levels between 4,0 and 8.0 mmol/l, without ischemic heart and cerebrovascular disease were included. Patients received placebo (n=8), 10mg atorvastatin (n=7) or 80mg atorvastatin (n=11) during 30 weeks. Two patients withdrew before the end of the study for personal reasons and two patients because of protocol violation. Fasting lipids and neuropsychological tests were assessed at baseline and after 30 weeks. The neuropsychological test-battery was composed in line with the findings of previous studies with comparable groups (1). Orientation and auditory-verbal memory were tested, as well as attention, psychomotor speed and executive functioning. Furthermore, we estimated premorbid intelligence with the Dutch version of the National Adult Reading Test (NLV). Baseline characteristics, lipids and neuropsychological tests results did not differ between the intervention groups. The mean HbA1c was 8.1 ± 1.0 % and the diabetes duration was 8.9 ± 5.9 years. Atorvastatin 10mg and 80mg respectively reduced plasma triglyceride by 19% and 39% and total cholesterol by 27% and 42%. The baseline auditory-verbal test results were lowered in 71% of the study population, as compared to a population without DM. The baseline results on the other neuropsychological tests did not differ from a non-diabetic

population. The verbal memory test (CVLT) improved 24% (a mean of 7 extra words) after 50 weeks of treatment with atorvastatin 80mg. In the atorvastatin 10mg group the CVLT improved only 8% (a mean of 2 extra words) and in the placebogroup no effect was observed. Verbal memory improvement correlated with an increase in HDLc ($r=0.67$, $p<0.05$), a reduction in LDLc ($r=-0.34$, $p<0.05$), and a reduction in TG ($r=-0.34$, $p=0.07$) after adjustment for age, baseline HDLc, LDLc, TG and verbal memory. Atorvastatin did not affect psychomotor speed, attention and executive functioning.

To summarize, in this small cohort of hyperlipidemic patients with type 2 diabetes mellitus treated with atorvastatin, verbal memory improvement was associated with improvement of the diabetic dyslipidemia profile. Low and high dose atorvastatin had no significant effect on cognitive functioning.

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Introduction

Clinical studies have shown that cognitive functioning is reduced in type 2 diabetic patients, particularly in patients older than 60 years of age, compared with an age-matched nondiabetic group (1-4). Especially verbal memory and complex information processing are affected in type 2 diabetes mellitus patients which will have an impact on daily functioning.(5-7). With the increase of the prevalence of type 2 diabetes, the prevention and treatment of possible cognitive dysfunction attracts more attention. The severity of cognitive dysfunction in patients with type 2 diabetes mellitus presumably results from an interaction between risk factors for macrovascular and microvascular disease, like elevated serum triglyceride (TG) levels and hypertension (8,9), the duration of the diabetes and the presence of peripheral neuropathy (1,10), and metabolic changes like fasting plasma glucose and HbA1c (11,12). A critical review of published studies (3) on cognitive impairment in patients with type 2 diabetes mellitus strongly suggests a correlation between indices of cognitive impairment and elevation of plasma TG (8,13,14). To our knowledge, only one study with 44 elderly hypertriglyceridemic patients, among them 11 patients with type 2 diabetes, investigated the effect of lowering serum TG levels on cognitive functioning. In this study lowering TG levels, as part of the diabetic dyslipidemia, by gemfibrozil appeared beneficial to cerebral perfusion and cognitive performance after four to six months (15).
The Diabetes Atorvastatin Lipid Intervention (DALI) study (16) is a double-blind, placebo-controlled, randomized study which investigates the effect of atorvastatin 10mg (A10) and 80mg (A80) on plasma TG levels in 217 patients with diabetes mellitus type 2 without coronary heart disease. In this study, as part of the DALI study, we investigated the dose effect of atorvastatin on diabetic dyslipidemia and cognitive functioning.

Patients and methods

Patients
The DALI study is a 30 week, double blind, randomized, placebo-controlled multicenter clinical trial, designed to assess the effect of aggressive lipid lowering by atorvastatin 80mg versus low dose atorvastatin 10mg versus placebo on TG level in type 2 diabetes mellitus patients without a history of manifest ischaemic heart disease. The design, methodology and baseline characteristics of this study are earlier described (16). The main inclusion criteria were total cholesterol between 4.0 and 8.0 mmol/l and a fasting TG between 1.5 and 6.0 mmol/l. Of the 80 patients

recruited for the DALI study in the study center Rotterdam, 30 patients fulfilled the in- and exclusion criteria and were willing to participate in the present study.
We excluded patients with a history of stroke, dementia, cerebral tumor, use of centrally acting drugs, severe psychiatric symptoms and hypothyroidism. Furthermore, known alcohol or drug abuse and insufficient command of the Dutch language lead to exclusion. All patients gave written informed consent and the study was approved and performed according to the rules of the hospital medical ethics committee.

Study Design
At baseline we performed a full medical history and physical examination. Routine hematology, blood chemistry, fasting lipid profiles and diabetes parameters were determined at baseline and at the end of the study. Neuropsychological tests were assessed at baseline and after 30 weeks treatment. Before neuropsychological testing, the bloodpressure was taken and the plasma glucose was measured. The plasma glucose had to be above 4.5 mmol/l.

Neuropsychological tests
Neuropsychological tests were performed by a trained physician or a neuropsychologist. The test battery consists of six neuropsychological tests, composed in line with the findings of previous studies in comparabel groups (1,6,9,11,13,17). These tests were always administrated in the same order: the orientation items of the Mini-Mental State Examination (MMSE) (18), the Dutch version of the California Verbal Learning Test (CVLT) (19), the Trail Making Test part A and B (TMT) (20), two subtests of the Wechsler Adult Intelligence Scale (WAIS) (21): the Digit Symbol Substitution Test (DSST) and the digit span forward (DSF) and digit span backward (DSB) and the Verbal and Category Fluency test (VCF) (22).
The NLV (23), the Dutch version of the National Adult Reading Test (NART) was administered at the end of the neuropsychological assesment at baseline. The orientation items consists of orientation to time and place, each 5 items. The score was calculated as the total number of correct responses. The CVLT is an auditory verbal memory test, which consists of five presentations with recall of a 16-word list, followed by one presentation of a different 16-word list. Then, the free and cued recall of the initial list are measured. After a 20 minutes delay, the long-term free and cued recall condition are administrated. Finally, recognition is tested by requiring the patient to identify correctly the words that were originally presented.
The total CVLT score was obtained by summing the total number of words correctly

recalled in the first five trials The TMT is used as a measure for psychomotor speed, attention and executive functioning and given in two-parts. The subject must first draw lines to connect consecutively numbered circles (part A), and then connect the same number of consecutively numbered and lettered circles by alternating between the two sequences (part B). The score is expressed in seconds needed to finish the test. The DSST is a test in which subjects are allowed 90 seconds to replace each symbol in a series with a digit, with which it has been paired. This test indexes the psychomotor speed and focus of attention. The independent measure was the number of symbols correctly transcribed. The DSF and DSB are both tests for measuring span of immediate verbal recall and auditory attention. A string of three to nine single-number digits were presented orally and immediately asked to recall the string aloud in the same order (DSF) or the reversed order (DSB). The dependent measure in each case was the longest string of digits correctly recalled without error. The VCF test was used mainly as a measure of executive functioning. The participants were asked to recall during one minute as many words as possible in the category given by the examiner (animals and professions) or starting with a specific letter (letter B). The score of the category fluency (animals and professions) and the letter fluency, was the sum of the number of words generated. The NLV comprises 50 phonetically irregular words which have to be read out loud. The NLV provides an estimate of premorbid intelligence. The NLV-score is based on the amount of right pronounced words.

Statistical methods

Statistical analyses were carried out by SPSS for Windows release 9.0. Mean differences between the study groups were analysed using analysis of covariance (ANCOVA), adjusted for baseline levels. Intervention effects were also further adjusted for additional potential confounders, using ANCOVA. The association between lipids and cognitive function was assessed by multiple linear regression analysis, adjusted for baseline lipid levels and other potential confounding factors.

Results

Baseline Characteristics

Thirty patients with type 2 diabetes mellitus and mild dyslipidemia, who met the in- and exclusion criteria were randomized. Two patients withdrew before the end of the study for personal reasons and two patients because of protocol violation. The baseline characteristics of remaining 26 patients are described in Table 1. There were no significant differences between the three groups prior to the treatment.

Table 1. Baseline characteristics

	Placebo	Atorvastatin 10mg	Atorvastatin 80 mg
Number of patients	8	7	11
Male gender (%)	3 (37.5)	4 (57.1)	6 (54.5)
Age (yrs)	59.1 ± 7.6	63.3 ± 9.8	59.8 ± 8.6
Duration diabetes (yrs)	10.4 ± 5.2	9.6 ± 7.6	8.7 ± 4.9
Current smoking (%)	75.0	85.7	63.6
Neuropathy (%)	50.0	28.6	36.4
Hypertension (%)	62.5	71.4	72.7
Fasting glucose (mmol/l)	10.5 ± 2.8	9.9 ± 3.3	9.0 ± 2.4
HbA1c (%)	8.2 ± 1.3	8.2 ± 0.9	7.9 ± 0.8

Values are mean± standard deviation

Effects on serum lipids

The baseline and on-treatment values of total cholesterol, HDL cholesterol (HDL-C), plasma TG, LDL cholesterol (LDL-C), Apo A1 and Apo B-100 are summarized in Table 2. The results were similar as those earlier described in the DALI study (16).

Table 2. Lipids and lipoproteins at baseline and percentage change after 30 weeks treatment..

		Placebo	Atorvastatin 10 mg	Atorvastatin 80 mg
Total cholesterol	Baseline	6.14 ± 0.9	6.02 ± 1.4	6.02 ± 0.7
(mmol/l)	% Change (IQR)	-3.4 (0.1)	-26.6 (0.3)*	-41.9 (0.2)**$
HDL-C	Baseline	1.12 ± 0.2	0.92 ± 0.2	0.81 ± 0.2
(mmol/l)	% Change (IQR)	-0.07 (0.1)	+0.2 (0.07)	+3.4(0.2)
Triglycerides	Baseline	2.51 ± 0.72	2.51 ± 0.66	3.26 ± 1.18
(mmol/l)	% Change (IQR)	+8.3 (0.6)	-18.9 (0.8)	-39.4 (0.6)*
LDL-C	Baseline	3.94 ± 0.7	4.02 ± 1.4	3.50 ± 0.6
(mmol/l)	% Change (IQR)	-5.3 (0.2)	-35.5 (0.8)*	-55.7 (0.2)***$

Values are median * standard deviation or median percentage change with interquartile range (IQR).
Test for difference versus placebo:*p<0.05, ** p<0.005, ***p<0.001.
Test for difference versus atorvastatin 10 mg:$ p<0.05

Effects on cognitive functioning

Cognitive tests results at baseline and after 30 weeks treatment are shown in Table 3. Intelligence, as estimated with the NLV at baseline, was not significantly different between the three groups, but patients receiving A10 had the lowest NLV score. Orientation in place and time was completely normal in all patients. The baseline auditory-verbal memory (CVLT) test results were lowered in 71% of the study population, as compared to an age-matched population without diabetes mellitus. The baseline results on the other neuropsychological tests did not differ from a non-diabetic, age-matched population. The CVLT showed a trend to improve with the atorvastatin dose. The CVLT test results improved 24% (a mean of 7 extra words) after 30 weeks treatment with A80. In the A10 group the CVLT improved only 8% (a mean of two extra words) and in the placebo group no effect was observed. All three treatment groups scored better in immediate and delayed memory testing as well as in correctly recalled words. There was no change in performance at baseline and after 30 weeks treatment between the groups in the other tests of psychomotor speed, attention, and executive functioning.

An inverse association between verbal memory and LDL-C reduction (Beta-0.34, p=0.03) and a positive association with HDL-C improvement (Beta +0.67,p=0.02) was observed, after adjustment for age, respectively baseline LDL-C and HDL-C and verbal memory at baseline. Increase in verbal memory correlated also, but not statistically significantly with reduction in total cholesterol and TG (Beta –0.27, p=0.08 and Beta -0.34, p=0.07), after adjustment for age, baseline total cholesterol, TG and verbal memory). Changes in TG, HDL-C and LDL-C are stronger correlated with verbal memory improvement then total cholesterol. These data suggests that changes in the profile of diabetic dyslipidemia was associated with verbal memory changes. Further adjustments for plasma glucose, HbA1c, polyneuropathy, smoking, duration of the diabetes, hypertension, BMI or intelligence, did not alter these associations.

Table 3. Cognitive function test at baseline and percentage change after 30 weeks treatment.

Cognitive function test		Placebo	Atorvastatin 10 mg	Atorvastatin 80 mg
NLV (score)	Baseline	104.5 ± 22.7	84.0 ± 19.1	92.0 ± 22.2
California Verbal	Baseline	40.5 ± 6.4	34.0 ± 13.6	41.0 ± 10.5
Learning Test (score)	% Change (IQR)	+1.7 ± 0.4	+7.7 ± 0.3	+23.5 ± 0.01
Trail making test A	Baseline	43.5 ± 17.0	45.0 ± 22.2	50.0 ± 15.9
(sec)	% Change (IQR)	-12.9 ± 0.4	-11.7 ± 0.5	-9.1 ± 0.3
Trail making test B	Baseline	75.5 ± 43.7	95.0 ± 49.6	100.0 ± 28.2
(sec)	% Change (IQR)	-1.8 ± 0.4	+17.9 ± 0.3	+2.5 ± 0.3
Digit symbol substitution	Baseline	42.0 ± 9.8	40.0 ± 17.4	49.0 ± 10.2
test (score)	% Change (IQR)	2.5 ± 0.4	-2.4 ± 0.3	-4.4 ± 0.3
Digit span forward	Baseline	5.0 ± 0.5	6.0 ± 1.1	5.0 ± 1.2
(score)	% Change (IQR)	+0.0 ± 0.2	+0.0 ± 0.3	+0.0 ± 0.4
Digit span backward	Baseline	3.5 ± 1.0	3.0 ± 1.3	5.0 ± 1.0
(score)	% change (IQR)	+0.0 ± 0.5	+0.0 ± 0.0	+0.0 ± 0.2
VCF animal	Baseline	21.0 ± 8.5	22.0 ± 3.5	23.0 ± 4.6
(score)	% Change (IQR)	+0.0 ± 0.2	+5.8 ± 0.5	+0.0 ± 0.3
VCF profession	Baseline	19.0 ± 8.0	15.0 ± 5.5	19.0 ± 3.8
(score)	% Change (IQR)	-25.5 ± 0.3	+21.0 ± 0.2	-17.3 ± 0.2
VCF letter B	Baseline	17.0 ± 8.7	8.0 ± 6.8	13.0 ± 8.3
(score)	% Change (IQR)	-4.5 ± 0.4	+33.3 ± 0.6	-16.7 ± 0.2

Values are median ± standard deviation or median percentage change with interquartile range (IQR).
NLV (Dutch version of the National Adult reading Test), VCF (Verbal and Category Fluency test).
For the CVLT, DSF, DSB and VCF tests higher scores indicate better performance.
For the TMT and DSST tests lower scores indicate better performance

Discussion

Our study in patients with type 2 diabetes mellitus indicates that improvement of verbal memory is associated with normalization of the lipid profile by atorvastatin. To our knowledge this is the first study that shows that both treatment of hypertriglyceridemia (and associated low HDL-C levels) as well as treatment of elevated LDL-C levels may enhance cognitive function on patients with type 2 diabetes mellitus. Although a relatively small group of patients was studied and interpretation and extrapolation of data to larger group of patients should be done with care, the results of our study are promising for carrying out larger studies. The patients in this study were relatively young, between 55 and 65 years of age, compared to other cognitive function studies. It is known that older adults have an increased risk of diabetes-associated memory dysfunction (24). As a result our patients had no or only slightly impaired cognitive function and although no further improvement can be expected in this group, they did show positive changes in verbal memory. A previous investigation used gemfibrozil instead of a statin to study the effect on cognitive functioning in patients with type 2 diabetes mellitus. Gemfibrozil has its main effect in lowering triglycerides and increasing HDL-C levels while statins have proven also to reduce LDL-C, next to its reduction of TG and increase of HDL-C. HDL-C levels are inversely related to plasma TG levels. In this study HDL-C correlated stronger with verbal memory improvement than TG levels. From literature it is known that hypertriglyceridemia in patients with type 2 diabetes mellitus is associated with a decline in verbal memory, in reaction time and backward digit span (25). The specific pathophysiology of cognitive disturbances associated with elevated TG levels is unknown. Probably, the induction of atherosclerosis by dyslipidemia, reducing cerebral blood flow and the acute effect of hypertriglyceridemia on the cognitive function (26), contribute to cognitive dysfunction. Lowering serum TG levels decreases blood viscosity, resulting in increased blood flow and a better hemorheology (27).

There are speculations about the psychological and behavioral effects of changes in serum lipid levels in humans. Two studies (28,29) found some correlation between cholesterol levels and cognitive function. A higher cholesterol level was associated with a better cognitive function. To the extent that cholesterol lowering is a necessary treatment for the prevention of atherosclerotic disease in type 2 diabetic patients, it is important to look for changes in cognitive function and quality of life. A few studies evaluated the effect of pharmalogical cholesterol lowering on cognitive function and psychological well being. In order to investigate the effect of statins on the CNS, Cutler et al (30) described the effect of simvastatin and pravastatin on

cognitive function in patients with hypercholesterolemia. There was no change in the cognitive function during a 4 weeks period of active treatment. Another recent study showed no difference in psychological distress and cognitive function after reducing LDL-C by lovastatin during a 6-month treatment period (31). To our knowledge there is no further information about the effect of atorvastatin on cognitive performance. Our study showed no reduction of cognitive functioning after cholesterol reduction by low and high dose atorvastatin but suggested an improvement in verbal memory after reduction of total and LDL cholesterol.
Statins reduce ischaemic stroke by the anti-thrombotic and anti-atherosclerotic effects and are neuroprotective. Vaughan et al (32) reviewed the effect of statins on the cerebral circulation and brain parenchyma during ischaemic stroke and reperfusion. Statins upregulate endothelial nitric oxide synthetase and inhibit inducible nitric oxide synthetase, both neuroprotective effects. They also influence the inflammatory cytokine responses and reduce ischemic oxidative stress in the brain through antioxidant properties. An other recent study (33) showed the lowered risk of developing dementia by statins, independent of hyperlipidemia. It is clear that additional studies on the neuroprotective effects of statins are necessary.

Conclusions

In this small cohort of hyperlipidemic patients with type 2 diabetes mellitus treated with atorvastatin, verbal memory improvement was associated with improvement of the diabetic dyslipidemia profile. Low and high dose atorvastatin showed no decline in cognitive functioning. The results encourage further investigation of the effect of adequate lipid lowering on cognitive functioning during a longer period and in a larger type 2 diabetic population.

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Chapter 5

Discussion

Discussion

The main results of the studies described in this thesis can be outlined as follows:

1 Administration of atorvastatin at doses of either 10 or 80 mg is effective in lowering plasma cholesterol and triglycerides in patients with type 2 diabetes mellitus. Both doses are well tolerated in our patient population.

2 Gender, *LIPC* promoter variant and ethnicity significantly contribute to the baseline variance in HL activity. Atorvastatin treatment in diabetic dyslipidemia results in a significant dose-dependent decrease in HL activity, regardless of gender or the *LIPC* promoter variant.

3 LPL gene mutations influence lipids, lipoproteins and LPL activity in type 2 diabetes mellitus, which may affect the risk for cardiovascular disease. The S447Stop mutation modulates the response to atorvastatin which may result in a further improvement of the diabetic dyslipidemia profile.

4 Atorvastatin treatment results in a significant dose-dependent reduction in plasma apoC-III, HDL apoC-III, and LpB:CIII levels in patients with type 2 diabetes mellitus. These data indicate a potentially important anti-atherogenic effect of statin treatment and explain part of the triglyceride lowering effect of atorvastatin.

5 In patients with type 2 diabetes, there is a gender specific etiology of low HDL-C levels. Gender differences in lipase activities may partly explain this sex difference in etiology. Especially in women this may contribute to the increase in relative risk of CHD.

6 Atorvastatin significantly reduces cholesterol concentration in all LDL subfractions but does not induce a shift in LDL subfraction density distribution towards larger and more buoyant LDL particles in patients with type 2 diabetes mellitus.

7 Apart from effects on lipids and CHD, improvement of the diabetic dyslipidemia profile with atorvastatin associates with verbal memory improvement.

Triglyceride lowering in the DALI population, efficacy and mechanisms

Diabetic dyslipidemia is characterized by increased plasma TG and decreased HDL-C levels together with compositional changes in VLDL, LDL and HDL particles. Improvement of clinical outcome in type 2 diabetes may be expected when the atherogenic lipid profile is changed into a less atherogenic lipid profile.

Statins are efficacious for LDL-lowering and disease prevention in diabetic patients. High dose statins also reduce elevated levels of plasma TG. In the DALI study we

used a standard dose of 10mg atorvastatin and an aggressive dose of 80mg atorvastatin, in order to endeavour normalisation of lipoprotein profiles in patients with type 2 diabetes. In Chapter 2 we reported a reduction of 25% in plasma TG after 30 weeks treatment with low dose atorvastatin and a reduction of 35% with high dose atorvastatin. Although there is no statistical significance between both dosages of atorvastatin, the 10% further reduction in plasma TG levels with high dose atorvastatin might indicate a possible dose-response reaction. Identification in our study of subjects that benefited most of the high dose atorvastatin therapy is not possible. It would be very interesting to identify those patients, since further reductions of plasma TG levels may lead to a further reduction in cardiovascular risk. Moreover, a study by co-investigators of the DALI study, favours the titration to a higher dose of atorvastatin (1). They studied the effect of high versus low dose atorvastatin on postprandial hyperlipidemia, since dyslipidemic patients with type 2 diabetes usually have both increased fasting and postprandial TG levels. Increased postprandial TG levels are associated with increased intima media thickness in type 2 diabetes mellitus (2). Both dosages of atorvastatin resulted in a reduced postprandial TG and TRL response with a significant greater improvement of the higher dose. Also the dose-dependent effect of atorvastatin on cholesterol–related parameters **(Chapter 2)** and the dose-dependent reduction of the cholesterol-content in the LDL subfractions **(Chapter 7)**, may favour high dose statin therapy in order to reach ADA treatment goals.

What is the mechanism involved in plasma TG reduction by atorvastatin in our population? In **Chapter 2 and 4**, we described the influence of atorvastatin on LPL activity, since LPL is the major enzyme responsible for clearance of plasma TG. The results demonstrating the effect of atorvastatin on LPL activity, pointed out that the reduction of plasma TG by atorvastatin is not due to enhanced LPL expression.

In **Chapter 4** we studied the effect of atorvastatin on post-heparin LPL activity in relation to lipids and lipoproteins in non-carriers and carriers of LPL gene mutations. The post-heparin LPL activity seemed to be in the normal range, suggesting that the hypertriglyceridemia is mainly caused by an increased secretion of TG into the blood. We don't know why LPL activity is "normal" in our population, but it is possible that this relatively " normal" LPL activity represents a situation in which there is already an insufficient compensation of insulin-mediated stimulation of LPL activity for the enhanced TG secretion. In type 2 diabetes a relatively increase in LPL activity is probably necessary, since substrate deliverance (VLDL-TG) is often increased and plasma TG levels may rise if lipolysis by LPL is impaired. It is therefore likely in patients with type 2 diabetes that especially in the postprandial state LPL activity may be affected and may contribute significantly to the atherogenic lipid profile.

This may be even more the case in carriers of the LPL D9N allele, since this allele appears to be associated with a trend towards a decrease in LPL activity. In contrast, the LPL S447Stop mutation seems to be associated with an allele-dose dependent increase in LPL activity and it is therefore likely that the difference in LPL activity between carriers and non-carriers of the S447Stop mutation will have an even greater impact in the postprandial state.
Atorvastatin did not affect LPL activity in non-carriers of LPL mutations. The LPL S447Stop mutation seemed to modulate the effect of atorvastatin on LPL activity. Carriers of the S447Stop mutation had a significant 20% higher LPL activity after 30 weeks atorvastatin compared with non-carriers. Since the S447Stop mutation is very common in different populations, more population-based research seems to be necessary for studying the effect of statin therapy on lipid profiles and CHD risk.
In addition to LPL activity, it is likely that the hypertriglyceridemia is due to an increased apoC-III content of TRLs, impairing the catabolism of these particles. ApoC-III is a risk indicator for atherosclerotic disease and in **Chapter 5** we suggested that atorvastatin may reduce plasma TG through a modulation in apoC-III expression. Atorvastatin treatment profoundly lowered the apoC-III content of HDL and of non-HDL (apoB-100 containing) lipoproteins (LpB:CIII). This reduction of apoC-III probably partly restored altered LPL activity and increased lipoprotein uptake.
One could argue that the reduction of apoC-III is due to the TG reduction and not the other way around. ApoC-III partitions to VLDL, the more VLDL in the circulation, either from increased secretion or decreased fractional removal, the more apoC-III in LpB (VLDL).
Therefore lowering TG will very likely lower apoC-III levels. But, Schoonjans et al (3) demonstrated in rats that statins decreased mRNA as well as plasma levels of apoC-III.
Although this information favours our results, more studies unravelling mechanisms involved in apoC-III lowering are necessary.

Implication of HL activity lowering by statins
Besides LPL activity and apoC-III levels, HL activity is also a major factor in the maintenance of normal lipid profiles. Atorvastatin decreased HL activity in type 2 diabetes dose-dependently regardless of gender or the *LIPC* promoter variant, as described in Chapter 3. In most studies, elevated HL activity in patients with diabetes is strongly associated with reduced HDL cholesterol and increased small, dense LDL. Zambon and Brunzell identified HL as focal point for the development and treatment of CHD. They proposed a new pathway of regression of

atherosclerosis through HL-mediated improvement in LDL buoyancy (4). In our DALI population increased HL activity is associated with decreased HDL and increased plasma TG, but not with an excess of small dense LDL particles. Atorvastatin therapy reduced HL activity significantly without abolishing the association between HL and TG or HDL-C. Changes in HL activity showed no correlation with changes in LDL-size.

Remarkably, most of the DALI patients had already large, buoyant LDL particles before treatment, as described in **Chapter 2**. One should expect that patients, that are often obese, inactive and hypertriglyceridemic, have increased levels of small dense LDL. Our patients had no overt ischemic heart disease and this may explain the low levels of small dense LDL particles, since most patients with CHD will have an excess of small dense LDL particles.

Since HL lowering by atorvastatin did not affect LDL size in our DALI population, the question remains whether HL lowering is beneficial for treatment of diabetic dyslipidemia. Jansen et al pointed out in an excellent review (5) that HL lowering may have pro- as well as anti-atherogenic consequences dependent on the conditions, which makes its difficult to predict whether HL is a suitable target for intervention to lower CHD risk. As stated before, high HL activity is most likely associated with low HDL-C, increased reverse cholesterol transport and clearing of remnant particles, but also with an excess of small dense LDL particles. HL lowering will probably reverse these processes. If HL lowering by statin therapy does not affect LDL size, the clinical significance of our findings is hard to assess. However, this does not exclude the clinical significance of our findings and following mechanism can be proposed to demonstrate whether HL lowering by statins may be beneficial in patients with type 2 diabetes.

HL activity increases with the degree of insulin resistance in type 2 diabetes (6). The impact of HL on lipoprotein metabolism will vary depending on the amount of potential substrate. Longterm insulin resistance is associated with an excess of VLDL-TG production. This probably stimulates HL expression in order to increase hydrolysis of HDL-TG and maintain intracellular lipid levels in the liver. Eventually, the TG deliverance exceeds the HL capacity which may subsequently lead to the atherogenic lipid profile seen in diabetic dyslipidemia. Lowering HL activity during this hypertriglyceridemic state may decrease the atherogenic risk through the improvement of the lipid profile. Atorvastatin decreases hepatic production of VLDL and stimulates LDL receptor activity. This might possibly prevent impairment of the reverse cholesterol transport during HL activity lowering and direct the lipid metabolism in type 2 diabetes into a less atherogenic direction. However, atorvastatin reduced the CETP mass in the DALI population as well (7).

Reductions of HL and CETP are both associated with increased HDL. Of course raising HDL is one of the main targets to reduce atherosclerosis, since the antiatherogenic properties of HDL include promotion of cellular cholesterol efflux and reverse cholesterol transport, as well as antioxidant, anti-inflammatory and anticoagulant properties. High HDL-C levels may represent enhanced reverse cholesterol transport, but what will happen if both pathways of facilitating the process of reverse cholesterol transport are influenced by statin therapy? Since HL stimulates HDL cholesterol ester uptake by hepatocytes and CETP facilitates reverse cholesterol transport by transferring cholesterol from HDL to LDL, HDL particles are probably not able to loose their cholesterol ester-content, which leaves an atherogenic situation. At this moment, already randomized phase II dose-reponse studies are performed to study the effect of novel CETP inhibitors in humans (8). Four weeks treatment with a CETP inhibitor in healthy patients with mild hyperlipidemia led to 37% decrease in CETP activity and to 34% increase in HDL-C. Although these results are promising, further studies are needed to investigate whether the observed increase in HDL cholesterol translates into a concomitant reduction in CHD and whether concomitant CETP mass reduction affects the response of other hypolipidemic treatment.

Gender differences in etiology of diabetic dyslipidemia; implications for therapy?

Elizabeth Barrett-Connor wrote an review article describing the sex differences in CHD (9). Women with type 2 diabetes loose their "female survival advantage" for cardiovascular death. This loss seemed partially due to major changes in lipoprotein profiles. Besides gender differences in the quantity and quality of lipoproteins, we postulated in **Chapter 6**, that gender differences in lipase activities participated in the gender specific etiology of HDL-C levels. As mentioned above, HDL probably has many antiatherogenic properties and is therefore recognised as a potential target for therapeutic intervention of atherosclerotic vascular diseases. The relatively low HDL-C levels in the DALI women are probably caused by an attenuated HDL formation due to a relatively low LPL activity. In contrast, low HDL-C levels in the DALI males are caused by accelerated HDL or apoA-1 clearance due to increased HL activity. Increased HL activity may induce cholesterolester uptake in the liver and therefore promote reverse cholesterol transport, it is therefore possible that low HDL-C levels in women with diabetes may constitute a higher-risk metabolic profile than in men with diabetes. Since HL and LPL may play a major role in determining the HDL-C levels as well as influencing the function of HDL particles, they may have important effects on atherosclerosis and are viable targets for (new) drug therapy.

These interesting results point out that a differentiation in treatment strategy towards men an women might be necessary, but more studies on sex differences in etiology of diabetic dyslipidemia are necessary.

Quality of life

In **Chapter 2** we concluded that low (10mg) and high (80mg) dose atorvastatin are effective and safe in the treatment of diabetic dyslipidemia and that there are no differences in side effects between placebo, atorvastatin 10mg and atorvastatin 80mg. Since lipid-lowering drugs are used within a framework of systematic approach for effective treatment of patients with type 2 diabetes mellitus, one single drug that lowers plasma TG and cholesterol levels will certainly increase patient compliance and probably improves quality of life.

Finally in **Chapter 8**, we found an association between improvement of verbal memory and improvement of diabetic dyslipidemia by atorvastatin. Our study was based on the intriguing hypothesis that cognitive functioning is reduced in patients with diabetic dyslipidemia. As described in the article, the study was not without limitations. One could argue about the small number of patients involved or the chance on finding associations between lipid improvement and the large battery of cognitive function tests in this study. Of course, the small number of patients restricted us from making any conclusions of note, but the interesting results are promising for carrying out further studies. To the extent that lipid improvement reduces the risk for atherosclerotic disease and may lead to changes in cognitive functioning, our study may serve as a stimulus to look more closely at the hypothesis that statins affect cognitive functioning in hyperlipidemic patients with type 2 diabetes. In order to obtain this information, a larger study with a demographically-similar nondiabetic, non-hyperlipidemic control group to compare the baseline neuropsychological test results and a longer duration of therapy is necessary. Clinical, epidemiologic, and pathologic observations suggest that vascular risk factors are associated with impaired cognition. For example, recently the VLDL receptor polymorphism, that constitute a genetic susceptibility factor for dementia, is suggested to be a vascular risk factor in the occurrence of dementia (10).

Since type 2 diabetes is such a complex metabolic disorder, the etiology of any associated cerebral dysfunction is likely to be multifactorial. Therefore more studies of the nature of cognitive impairment in type 2 diabetes must be carefully designed.

Conclusions

Standard and aggressive lipid lowering with atorvastatin is effective, safe and well tolerated in patients with type 2 diabetes and diabetic dyslipidemia.

Lowering of hepatic lipase activity by statins may have pro- as well as anti-atherogenic consequences.

Reduction of plasma triglycerides by atorvastatin is not due to enhanced LPL expression, but is possibly due through modulation of apoC-III expression.

Since gender and genetic variance may influence key factors in the pathogenesis of lipoprotein abnormalities in type 2 diabetes and genetic factors may interact with the efficacy of statin therapy, differentiation in treatment strategy seems useful.

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Chapter 10

Summary

Diabetic dyslipidemia is an established risk factor for coronary heart disease (CHD) and further elucidation of the lipoprotein metabolism and the effect of statin therapy seems necessary in order to prevent atherosclerotic disease. As discussed in **Chapter 1**, studying the role of lipolytic enzymes and apolipoproteins in diabetic dyslipidemia, may lead to more information of mechanisms involved and may reveal possible targets for therapeutic intervention. Results of the Diabetes Atorvastatin Lipid Intervention (DALI) study revealed some of this information. The DALI study is a double blind, placebo controlled, randomized multi-center study, conducted in Leiden, Rotterdam and Utrecht, The Netherlands, between 1998 and 2000. The participants in this study are male and female patients, aged 45-75 years, with type 2 diabetes. The main inclusion criteria are fasting total cholesterol (TC) levels between 4.0 and 8.0 mmol/L and fasting triglyceride (TG) levels between 1.5 and 6.0 mmol/L. Patients were randomized to receive with placebo, atorvastatin 10mg or atorvastatin 80mg for a period of 30 weeks. The HMG-CoA reductase inhibitor atorvastatin was chosen for its hypothesized extra effect on TG reduction.

In **Chapter 2** the effect of low and high dose atorvastatin on fasting plasma TG levels, the primary endpoint of the study, is described. Both dosages provided significant reductions in fasting TG levels. Atorvastatin 10mg reduced plasma TG with 25% and atorvastatin 80mg with 35%, not statistically significantly different. Baseline TG levels did not have any effect on the outcome. Total cholesterol and LDL-C levels are both dose-dependently reduced. More patients reached the ADA treatment goals with atorvastatin 80mg. Both dosages seemed safe and well tolerated.

In **Chapter 3** the role of hepatic lipase (HL), the influence of gender, *LIPC* promoter variant and ethnicity on HL activity, as well as the influence of atorvastatin on HL activity in diabetic dyslipidemia is described. Gender, *LIPC* promoter variant and ethnicity significantly contributed to the baseline variance in HL activity. Atorvastatin treatment resulted in a dose-dependent significant reduction of HL activity, regardless of gender or the *LIPC* promoter variant.

In **Chapter 4** the influence of lipoprotein lipase (LPL) gene mutations on LPL activity, lipids and lipoproteins is described. Carriers of the D9N LPL mutation had a significantly lower LPL activity and a more atherogenic lipid profile than patients without a mutation in the LPL gene. Carriers of the LPL N291S mutation had similar LPL activity and lipid profiles as non-carriers. The S447Stop variant showed an allele-dose dependent increase in LPL activity, apoA-1, HDL-C and decrease in plasma TG. Since genetic variants may modulate the efficacy of drugs, the effect of

atorvastatin treatment on plasma lipids in carriers of a LPL gene variant is evaluated. Only the S447Stop mutation modulated the response of LPL activity to atorvastatin. LPL activity after atorvastatin treatment was significantly higher in patients carrying the LPL S447Stop mutation than in non-carriers.

In **Chapter 5** the effect of atorvastatin on apolipoprotein (apo)C-III is studied. ApoC-III is a risk indicator for atherosclerotic disease. Increased apoC-III content of triglyceride-rich lipoproteins (TRLs) may impair the catabolism of these TRLs, resulting in hypertriglyceridemia. Atorvastatin therapy profoundly lowered the apoC-III content of HDL and of non-HDL (apoB-100 containing) lipoproteins. These results suggest a possible mechanism of TG reduction by atorvastatin. The TG reduction by atorvastatin is possibly due mediated through a modulation in apoC-III expression. Our data may indicate a potentially important anti-atherogenic effect of statin treatment.

In **Chapter 6** the gender specific etiology of low HDL-C levels in patients with type 2 diabetes is discussed. Female patients with diabetes had relatively low LPL activity and male patients with diabetes had increased HL activity compared with non-diabetic patients. The relatively low HDL-C levels in females are possibly caused by an attenuated HDL formation due to low LPL activity. Low HDL-C levels in males are possibly caused by increased HDL (apoA-1) clearance due to increased HL activity. Although atorvastatin therapy did not affect LPL activity and reduced HL activity equally in males and females. Lipids and lipoproteins in males and females are influenced similar.

In **Chapter 7** the effect of atorvastatin therapy on LDL subfractions is discussed. In patients with type 2 diabetes LDL-C levels are often not elevated, but there is a preponderance of LDL particles with smaller size and increased density that are associated with increased cardiovascular risk. Atorvastatin significantly reduced cholesterol concentration in all LDL subfractions but did not induce a shift in LDL subfraction density distribution towards larger and more buoyant particles.

In **Chapter 8** the effect of improvement of the lipid profile by atorvastatin on cognitive functioning is studied. Improvement of verbal memory correlated with improvement of the diabetic dyslipidemia by atorvastatin. These results may serve as a stimulus to conduct further studies in this area, since improvement of cognitive functioning in patients with type 2 diabetes will certainly improve the quality of life. Finally in Chapter 9 the implications of the DALI study results for treatment of diabetic dyslipidemia are discussed.

Chapter 7

Samenvatting

Diabetische dyslipidemie is een bewezen risicofactor voor hart- en vaatziekten. Voor de preventie van atherosclerose is verdere opheldering van het lipiden metabolisme en het effect van statine behandeling daarop noodzakelijk. Zoals besproken is in **Hoofdstuk 1**, zal het bestuderen van de rol van lipolytische enzymen en apolipoproteïnen in diabetische dyslipidemie leiden tot beter inzicht in het mechanisme. Dit kan weer leiden tot herkenning van mogelijke nieuwe therapeutische aangrijpingspunten. De resultaten van de Diabetes Atorvastatin Lipid Intervention (DALI) studie leverden aanvullende informatie op over het mechanisme en de therapeutische opties ten aanzien van diabetische dyslipidemie. De DALI studie is een dubbel-geblindeerde, placebo-gecontroleerde, gerandomiseerde studie, welke in Leiden, Rotterdam en Utrecht is uitgevoerd van 1998 tot 2000. De deelnemers zijn mannen en vrouwen met type 2 diabetes, met een leeftijd tussen de 45 en 75 jaar. Het belangrijkste inclusiecriteria is een totaal cholesterol gehalte tussen 4.0 en 8.0 mmol/L en een nuchtere triglyceride gehalte tussen de 1.5 en 6.0 mmol/L. Patiënten zijn gerandomiseerd voor placebo, atorvastatine 10mg of atorvastatine 80mg en behandeld gedurende 30 weken. De HMG CoA reductase remmer atorvastatine is gebruikt in de studie vanwege het veronderstelde extra therapeutisch effect op de triglyceriden.

In **Hoofdstuk 2** is het effect van een lage en hoge dosering atorvastatine op de nuchtere plasma triglyceriden, het primaire eindpunt van de studie, beschreven. Beide doseringen zorgden voor een significante verlaging van de plasma triglyceriden. Atorvastatine 10mg reduceerde plasma triglyceriden met 25% en atorvastatine 80mg met 35%. Er is geen significant verschil tussen beide doseringen. De plasma triglyceriden spiegel bij aanvang van de studie beïnvloedde het resultaat niet. Zowel het totale cholesterol als het LDL-cholesterol is dosisafhankelijk verlaagd met atorvastatine. Met name bij de patiënten die behandeld zijn met atorvastatine 80mg zijn de streefwaarden, volgens de ADA criteria, gehaald. Beide doseringen zijn veilig en goed verdragen.

In **Hoofdstuk 3** is de rol van hepatisch lipase (HL) in diabetische dyslipidemie en het effect van atorvastatine behandeling beschreven. Geslachtsverschillen, de *LIPC* promotor variant en verschillen in etniciteit, beïnvloeden de HL activiteit bij aanvang van de studie. Atorvastatine therapie resulteerde in een dosisafhankelijke reductie van de HL activiteit, onafhankelijk van geslacht of dragerschap van een promotor variant.

In **Hoofdstuk 4** is de invloed van lipoproteine lipase (LPL) genmutaties op LPL

activiteit, lipiden en lipoproteïnen beschreven. Dragers van de D9N LPL mutatie hebben een significant lagere LPL activiteit en een meer atherogeen lipiden profiel in vergelijking met patiënten die geen drager waren van een LPL genmutatie. Dragers van de N291S mutatie hebben een vergelijkbare LPL activiteit en lipiden profiel als patiënten zonder genmutaties. De patiënten met de S447Stop mutatie toonden een allel-dosis afhankelijke toename in LPL activiteit, apoA-1, HDL-C en daling in plasma triglyceriden. Aangezien genvarianten the effect van statine therapie kunnen beïnvloeden, is tevens het effect van atorvastatine in dragers van genmutaties bestudeerd. Alleen de S447Stop mutatie veranderde het effect van atorvastatine therapie. De LPL activiteit na atorvastatine therapie is significant hoger in dragers van de S447Stop mutatie dan in patiënten zonder genmutaties.

In **Hoofdstuk 5** is het effect van atorvastatine op apolipoproteine (apo)C-III beschreven. Een verhoogd apoC-III gehalte is een risico-indicator voor atherosclerose, met name vanwege de invloed op het catabolisme van triglycerideriјke deeltjes. Atorvastatine therapie verlaagd het apoC-III gehalte van HDL en non-HDL (de apoB-100 bevattende) lipoproteïnen. De resultaten suggereren een mogelijk mechanisme van triglyceriden verlaging door atorvastatine. De triglyceriden reductie die gevonden wordt tijdens atorvastatine behandeling zou mogelijk verklaard kunnen worden door verandering van apoC-III expressie. Deze resultaten tonen een in potentie belangrijk anti-atherogeen effect van statine therapie aan.

In **Hoofdstuk 6** is de mogelijke verklaring van het verschil in het lage HDL gehalte tussen mannen en vrouwen met type 2 diabetes beschreven. Vrouwelijke diabetes patiënten hebben een relatief lage LPL activiteit ten opzichte van vrouwen zonder diabetes. Mannen met type 2 diabetes daarentegen hebben een verhoogde HL activiteit ten opzichte van mannen zonder diabetes. Het relatief lage HDL-C gehalte bij vrouwen wordt zeer wel mogelijk veroorzaakt door een afgenomen HDL synthese, veroorzaakt door de lage LPL activiteit. Bij de DALI mannen lijkt er een toegenomen klaring van HDL en/of apoA-1 te zijn, vermoedelijk veroorzaakt door de hoge HL activiteit. Er lijkt dus een verschil in etiologie mbt het HDL gehalte bij vrouwen en mannen te bestaan. Ondanks het feit dat atorvastatine geen invloed heeft op de LPL activiteit en een vergelijkbare afname in HL activiteit veroorzaakt bij mannen en vrouwen, is er geen verschil in het lipiden profiel na behandeling.

In **Hoofdstuk 7** is het effect van atorvastatine op de LDL subfracties beschreven. In patiënten met type 2 diabetes is het LDL-C gehalte vaak niet verhoogd, maar zijn de

LDL deeltjes wel kleiner en bevatten ze relatief meer cholesterol ("small dense LDL"), waardoor ze beduidend atherogener zijn. Atorvastine behandeling zorgt voor een vermindering van het cholesterol gehalte in alle LDL subfracties, maar veranderde de deeltjes niet qua grootte.

In **Hoofdstuk 8** is het effect van verbetering van het lipiden profiel door atorvastatine op het cognitief functioneren in patiënten met type 2 diabetes beschreven. Er is met name een verbetering van het verbale geheugen te zien na een verbetering van het lipiden profiel. Ondanks het feit dat er slechts een gering aantal patiënten bestudeerd zijn, kunnen deze resultaten dienen voor het opzetten van grotere studies, aangezien het verbeteren van de cognitieve functie bij patiënten met type 2 diabetes zou kunnen bijdragen aan een belangrijke verbetering van de kwaliteit van leven.

In **Hoofdstuk 9** zijn de implicaties van de bovengenoemde DALI studie resultaten voor de behandeling van diabetische dyslipidemie beschreven.

Chapter 12

Dankwoord

Dankwoord

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Curriculum Vitae

Ingrid Ingeborg Louise Planken werd geboren op 11 februari 1964 te Brielle. Na het behalen van het Atheneum-B diploma aan de Libanon Scholen Gemeenschap te Rotterdam en 2 jaar onderwijs aan het van 't Hoff instituut voor Hoger Laboratorium Onderwijs ivm uitloting voor de studie geneeskunde, werd er in 1985 aangevangen met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In 1991 werd het artsexamen cum laude afgelegd. In de periode 1992-1993 was zij werkzaam als AGNIO op de afdeling Inwendige Geneeskunde van het Sint Franciscus Gasthuis te Rotterdam. Op 1 januari 1994 startte de opleiding tot internist in het Havenziekenhuis te Rotterdam (opleider dr. A.G.C. Bauer), gevolgd door 2-jaar opleiding in Sint Franciscus Gasthuis te Rotterdam (opleider dr. H.S.L M. Tjen). De laatste opleidingsjaren werden gevolgd in het Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider Prof.dr. M.A.D.H. Schalekamp en Prof.dr. S.W.J. Lamberts (plv opleider). Tijdens het laatste jaar van de opleiding werd tevens aangevangen met het opzetten van de DALI studie en was zij werkzaam op de lipidenpolikliniek. Op 1 januari 2000 werd zij als internist ingeschreven in het specialisten register. 1 juli 2000 startte zij met het aandachtsgebied Endocrinologie in het Dijkzigt ziekenhuis te Rotterdam (opleider Dr A.J. van der Lely), welke op 1 juli 2002 werd afgerond. Vanaf 1 november 2001 is zij werkzaam als internist-endocrinoloog in het Vlietland ziekenhuis te Vlaardingen.