LONG-TERM EFFECTS OF FENOFIBRATE TREATMENT ON LIPOPROTEINS AND SURROGATE MARKERS OF MACROVASCULAR AND MICROVASCULAR DISEASE IN PEOPLE WITH TYPE 2 DIABETES

Anne Hiukka
ABSTRACT

Background and aims. Diabetic dyslipidemia is a highly atherogenic triad of increased triglycerides, decreased HDL cholesterol, and small dense LDL. Fibrates have shown a beneficial effect on diabetic dyslipidemia, and they have reduced cardiovascular events in randomized placebo-controlled trials. Additionally, fenofibrate has reduced albuminuria as well as markers of low-grade inflammation and endothelial dysfunction. The present studies were undertaken to characterize the alterations of VLDL and LDL subclasses and to investigate the effects of LDL on binding to arterial wall in type 2 diabetes. Further purpose was to elucidate the effects of fenofibrate on several lipoprotein subclasses, augmentation index (AIx), carotid intima-media thickness (IMT), renal function, and albuminuria.

Subjects and methods. Two hundred and thirty-nine type 2 diabetic subjects aged 50 to 75 were recruited among participants of the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study at the Helsinki centre. Additionally, a healthy control group (N = 93) was recruited. The patients were examined at baseline, and at 2nd year and 5th year of the FIELD study. At study close-out, the number of eligible patients in this substudy was 171. An extensive panel of lipids and lipoproteins, markers of inflammation, endothelial dysfunction, renal function, and albuminuria were determined. In subsets of patients and control subjects, binding of LDL to arterial wall proteoglycan was analyzed in vitro. Carotid arteries were scanned with ultrasound to determine intima-media thickness as a surrogate marker of atherosclerosis. Pulse wave analysis (PWA) was performed to determine central arterial augmentation and augmentation index as a measure of arterial stiffness.

Results. VLDL1 triglycerides increased similarly in proportion to total triglyceride level in type 2 diabetic patients and control subjects. Despite the increase in total apoCIII levels, there was a decrease in VLDL apoCIII in diabetic patients. Enrichment of LDL with apoCIII induced a small increase in binding of LDL to proteoglycan. Further findings indicated that intrinsic characteristics of diabetic LDL, rather than levels of apoCIII, were responsible for increased proteoglycan binding of diabetic LDL with high apoCIII. Fenofibrate reduced large VLDL particle mass, increased LDL size, and produced a clear shift in HDL subclasses towards smaller particles with no change in levels of HDL cholesterol. High levels of homocysteine were associated with lower increase of HDL cholesterol and apoA-I during fenofibrate treatment. Long-term fenofibrate treatment did not improve IMT, augmentation index, inflammation, or endothelial function.
Fenofibrate decreased creatinine clearance and estimated glomerular filtration rate (eGFR). No effect on albuminuria was seen with fenofibrate. Instead, Cystatin C was increased during fenofibrate treatment.

**Conclusion** 1) Elevation of VLDL triglycerides was the major determinant of plasma triglyceride concentration in control subjects and type 2 diabetic patients. 2) LDL with high apoCIII showed multiple atherogenic properties, that were only partially mediated by apoCIII *per se* in type 2 diabetes 3) Fenofibrate demonstrated no effect on surrogate markers of atherosclerosis. 4) Fenofibrate had no effect on albuminuria and the observed decrease in markers of renal function would complicate the clinical surveillance of the patients. 5) Use of fenofibrate would be recommended to treat severe hypertriglyceridemia or in combination therapy with statins, but not to increase HDL levels.
Contents

ABSTRACT ................................................................. 3
LIST OF ORIGINAL PUBLICATIONS ................................... 9
ABBREVIATIONS .......................................................... 10
1. INTRODUCTION ...................................................... 13
2. REVIEW OF THE LITERATURE ..................................... 15
  2.1 Pathogenesis and epidemiology of type 2 diabetes .......... 15
  2.2 Lipid metabolism in type 2 diabetes .......................... 16
    2.2.1 Introduction .................................................. 16
    2.2.2 Triglyceride-rich particles and their metabolism ...... 17
    2.2.3 Low density lipoprotein particles and their metabolism . 20
    2.2.4 High-density lipoprotein particles and their metabolism. 22
    2.2.5 Diabetic dyslipidemia as a CVD risk factor ............. 23
  2.3 Inflammation and endothelial function in type 2 diabetes .. 25
    2.3.1 Inflammation in type 2 diabetes .......................... 25
    2.3.2 Endothelial dysfunction in type 2 diabetes .......... 26
  2.4 Carotid intima-media thickness in type 2 diabetes .......... 26
  2.5 Arterial stiffness and diabetes ................................ 29
  2.6 Dyslipidemia and microvascular outcomes in type 2 diabetes . 29
  2.7 Fibrates in treatment of diabetic dyslipidemia ............. 31
3. AIMS OF THE STUDY ............................................... 35
4. SUBJECTS AND STUDY DESIGN .................................... 36
  4.1 Type 2 diabetic subjects ........................................ 36
  4.2 Non-diabetic subjects ........................................... 37
5. METHODS ............................................................ 38
  5.1 Laboratory analyses ............................................ 38
    5.1.1 Glucose, insulin and C-peptide measurements .......... 38
    5.1.2 Lipid measurements ........................................ 38
    5.1.3 Apolipoprotein measurements .............................. 39
    5.1.4 Measurements of activities of lipid transfer proteins ... 41
5.1.5 Measurements of LDL function and lipid composition . . 41
5.1.6 Homocysteine and markers of inflammation and endothelial activation .......................... 42
5.2 Intima-media thickness of the carotid arteries ................. 42
5.3 Pulse wave analysis ............................................. 43
5.4 Measures of renal function and albuminuria .................. 44
5.5 Anthropometric variables ..................................... 44
5.6 Statistical analysis .............................................. 45

6. RESULTS ........................................................................ 46
6.1 Study subjects ............................................................ 46
6.2 Alterations of VLDL, LDL and HDL subclasses and apoproteins in type 2 diabetes ......................... 49
   6.2.1.1 VLDL subclasses in type 2 diabetes (Study I) ... 49
   6.2.1.2 Apoproteins CII, CIII and E (Study I) .......... 52
   6.2.1.3 Relationships between VLDL subclasses and apoCIII (Study I) .................................. 52
   6.2.1.4 Apoproteins B, CII, CIII, and E in matched-pairs (Study I) ................................. 54
   6.2.2.1 LDL subclasses in type 2 diabetes (Study II) . . 54
   6.2.2.2 In vitro modification of LDL (Study II) ......... 56
   6.2.2.3 Lipid composition of the LDL particles (Study II) ..................................................... 56
   6.2.3.1 HDL subclasses in type 2 diabetes (Study II and unpublished data) ......................... 57
6.3 Effect of fenofibrate on VLDL, LDL, and HDL subclasses in type 2 diabetes ......................................... 58
   6.3.1 Effect of fenofibrate on VLDL subclasses (Study III and unpublished data) ...................... 58
   6.3.2 Effect of fenofibrate on LDL subclasses (unpublished data) ........................................ 59
   6.3.3 Effect of fenofibrate on HDL subclasses (Study III) ...... 60
6.4 Effect of fenofibrate on homocysteine; implications to HDL (Study III) ............................................ 61
6.5 Effect of fenofibrate on markers of renal function and albuminuria (Study V and unpublished data) ............ 63
6.6 Effect of fenofibrate on markers of inflammation, endothelial activation, augmentation index, and intima-media thickness (Study IV) .................................................. 66
7. DISCUSSION ...................................................... 70
  7.1 Study subjects ............................................. 70
  7.2 VLDL particles ............................................. 71
    7.2.1 VLDL particles in type 2 diabetic patients ......... 71
    7.2.2 Effect of fenofibrate on VLDL subclasses .......... 73
  7.3 LDL particles ............................................. 73
    7.3.1 LDL particles in type 2 diabetic patients .......... 73
    7.3.2 Effect of fenofibrate on LDL particles .......... 74
  7.4 HDL particles ............................................. 75
    7.4.1 Effect of fenofibrate on HDL particles .......... 75
  7.5 Effect of fenofibrate on IMT ............................. 76
  7.6 Effect of fenofibrate on augmentation index and markers of low-grade inflammation and endothelial activation .......... 77
  7.7 Effect of fenofibrate on renal function and albuminuria .... 78
  7.8 Positioning of fibrates in the current management of type 2 diabetes ............................................. 80

8. SUMMARY OF RESULTS AND CONCLUSIONS .................. 82

9. ACKNOWLEDGEMENTS ......................................... 83

10. REFERENCES .................................................. 86
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by their Roman numerals


In addition some unpublished data are presented.

*Equal contribution in alphabetical order

The original publications are reprinted with kind permission from the following copyright holders:
Study I: Springer Science+Business Media
Study II: The American Diabetes Association
Study III: Springer Science+Business Media
Study IV: Elsevier

List of Original Publications
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-cassette binding protein G1</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>AIx</td>
<td>augmentation index</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>Apo</td>
<td>apoprotein</td>
</tr>
<tr>
<td>ARB</td>
<td>angiotensin receptor blocker</td>
</tr>
<tr>
<td>BIP</td>
<td>Bezafibrate Infarction Prevention Study</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAFE-LLA</td>
<td>Conduit Artery Function Evaluation – Lipid Lowering Arm</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>CARDS</td>
<td>Collaborative Atorvastatin Diabetes Study</td>
</tr>
<tr>
<td>CB IMT</td>
<td>the mean of maximum IMT over all scanned carotid bulb sites</td>
</tr>
<tr>
<td>CCA IMT</td>
<td>the mean of maximum IMT over all scanned common carotid artery sites</td>
</tr>
<tr>
<td>CE</td>
<td>cholesterylester</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterylester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CKP</td>
<td>creatinine phosphokinase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DAIS</td>
<td>Diabetes Atherosclerosis Intervention Study</td>
</tr>
<tr>
<td>DNL</td>
<td>de novo lipogenesis</td>
</tr>
<tr>
<td>dU-Prot</td>
<td>diurnal urinary protein excretion</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
</tr>
<tr>
<td>FC</td>
<td>free cholesterol</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>FIELD</td>
<td>Fenofibrate Intervention and Event Lowering in Diabetes</td>
</tr>
<tr>
<td>FW IMT</td>
<td>the mean of mean far wall IMT over all scanned carotid sites</td>
</tr>
<tr>
<td>GM1</td>
<td>monosialotetrahexosylganglioside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycosylated haemoglobin HbA1c</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>homeostasis model assessment for insulin resistance</td>
</tr>
<tr>
<td>HPS</td>
<td>Heart Protection Study</td>
</tr>
<tr>
<td>ICA IMT</td>
<td>the mean of maximum IMT over all scanned internal carotid artery sites</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IMT</td>
<td>intima-media thickness</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin:cholesterol-acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>Max IMT</td>
<td>the mean of maximum IMT over all scanned carotid sites</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>MDRD</td>
<td>creatinine clearance by the formula from Modification of Diet in Renal Diseases</td>
</tr>
<tr>
<td>Mean IMT</td>
<td>the mean of mean IMT over all scanned carotid sites</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>OHA</td>
<td>oral antihyperglycemic agents</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipids</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2 IIA</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
</tr>
<tr>
<td>PON-1</td>
<td>paraoxonase</td>
</tr>
<tr>
<td>PWA</td>
<td>pulse-wave analysis</td>
</tr>
<tr>
<td>SAA</td>
<td>human serum amyloid A</td>
</tr>
<tr>
<td>Sf</td>
<td>Svedberg flotation rate</td>
</tr>
<tr>
<td>SMase</td>
<td>sphingomyelinase</td>
</tr>
<tr>
<td>SR-B1</td>
<td>scavenger receptor B1</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TG</td>
<td>triglycerides</td>
</tr>
<tr>
<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
</tr>
<tr>
<td>U-ACR</td>
<td>urinary albumin/creatinine ratio</td>
</tr>
</tbody>
</table>
U-AER  urinary albumin excretion rate
UKPDS  United Kingdom Prospective Diabetes Study
VA-HIT  Veterans Affairs High-Density Lipoprotein Intervention Trial
VCAM-1  vascular cell adhesion molecule 1
VLDL  very-low-density lipoprotein
1. INTRODUCTION

Sedentary lifestyle and overflow of dietary energy has lead to a pandemic of type 2 diabetes. Although diabetes prevention programmes are set into action (Laatikainen et al. 2007, Saaristo et al. 2007, Schwarz et al. 2008, Jackson 2009), prevention of cardiovascular disease (CVD) in diabetes needs urgently intensifying to reduce the burden of the disease. Approximately 75% of type 2 diabetic patients die to cardiovascular disease (Laakso and Lehto 1998). Thus the epidemic of diabetes portends a considerable rise of CVD events at the population level.

The spectrum of problems in type 2 diabetes includes typically not only obesity and hyperglycaemia, but also hypertension, hyperlipidemia, inflammation, and coagulation disorders, which all are established CVD risk factors. Finnish Current Care Guidelines (Käypä Hoito -työryhmä 2007) agree with the international ones in recommending intensive treatment goals for these disorders in type 2 diabetic patients. Notably, the treatment of type 2 diabetes routinely includes multiple medications.

Hypertension and hyperglycemia are primary treatment targets to prevent diabetic nephropathy. The evidence is less solid for hyperlipidemia. High triglycerides are risk factor for the development and progression of nephropathy in type 2 diabetes (Colhoun et al. 2001, Cusick et al. 2004). Lipid intervention studies with statins or fibrates have however given contradictory results concerning the reduction of urinary albumin excretion in type 2 diabetes (The DAIS Investigators 2001, Douglas et al. 2006).

Solid evidence supports only the use of statins for dyslipidemia (CTT Collaborators et al. 2008). CVD risk is however considerable, even if a patient with type 2 diabetes is on statin treatment (CTT Collaborators et al. 2008). LDL-cholesterol level is characteristically only moderately elevated in diabetic dyslipidemia, but LDL particles small and dense, HDL-cholesterol is low, triglycerides high, and postprandial lipemia exaggerated (Taskinen 2003). The increased risk associated with low HDL cholesterol is well documented (Castelli et al. 1992) but more evidence is needed to define the hazards of high triglycerides.

Hypertriglyceridemia is the culprit in diabetic dyslipidemia (Taskinen 2003). It results from a disturbance of a fatty liver to process dietary and endogenous fatty acids. Increased production of triglycerides is associated with profound disturbances in the metabolism of other lipoproteins (Adiels et al. 2006). Excess triglycerides in HDL and LDL particles alter the function of these particles, increasing the atherogenicity of LDL and decreasing the atheroprotective properties of HDL. Optimal treatment of

1. Introduction
the atherogenic lipid triad should be targeted to further reduce the high CVD risk in people with type 2 diabetes.

Fibrates are peroxisome proliferator-activated receptor alpha (PPARα) agonists which reduce triglycerides and increase HDL cholesterol. Fibrates have been studied in type 2 diabetic patients for secondary prevention of CVD. Gemfibrozil reduced cardiovascular events (CHD death, stroke, or myocardial infarction) with 32% in a subgroup analysis of Veterans Affairs HDL Intervention Trial (VA-HIT) (Robins et al. 2001), whereas fenofibrate reduced progression of percentage coronary diameter stenosis with 42% in Diabetes Atherosclerosis Intervention Study (DAIS) (The DAIS Investigators 2001). The large-scale primary and secondary prevention study called Fenofibrate Intervention and Event Lowering in Type 2 Diabetes (FIELD) was launched in 1998 in Australia, New Zealand, and Finland. The study was set to randomize 8,000 patients with type 2 diabetes to fenofibrate or placebo for five years. The primary endpoint of the study was the occurrence of non-fatal myocardial infarction (MI) or coronary heart disease (CHD) death (Keech and Field Study Investigators 2004).

The present substudy has explored lipid metabolism, renal function, and early atherosclerosis using surrogate markers of carotid intima-media thickness (IMT) and augmentation index (AIx) in a cross-sectional and prospective setting in type 2 diabetic patients participating in the FIELD study.
2. REVIEW OF THE LITERATURE

2.1 Pathogenesis and epidemiology of type 2 diabetes

Type 2 diabetes is the most common form of diabetes mellitus, and it is constantly increasing due to obesity in individuals with genetic susceptibility to diabetes. The rate of increase is highest in the Third World countries (Diamond 2003), where large, genetically diabetes-prone populations are facing urbanization, obesity, and higher life expectancy.

The number of diabetic patients worldwide is estimated to increase from 175 million in year 2000 to 366 million in year 2030 (Wild et al. 2004), with India and China together accounting for 24% of the total increase in year 2030. Alarmingly, without interventions the rate of obesity is calculated to increase from 396 million at 2005 to 1.12 billion at 2030 (Kelly et al. 2008). Every 5kg/m² increase of BMI over 25 kg/m² is associated with about 30% increase of overall mortality (Prospective Studies Collaboration et al. 2009).

The number of diagnosed and undiagnosed type 2 diabetic patients in Finland is estimated to be 500 000 (Käypä Hoito -työryhmä 2007). In next 10 to 15 years, the number of diagnosed type 2 diabetes patients in Finland is expected to rise to 400 000, whereas undiagnosed cases may then reach almost one million (Käypä Hoito -työryhmä 2007). The prevalence in Finland is one of the highest in Europe, reaching almost the level of United States. The main reason for the escalating incidence of diabetes in Finland is increasing obesity.

However, both the predisposing genes and the environmental triggers are usually needed to develop manifest diabetes. Large diabetes prevention studies have shown up to 58% reduced risk of developing diabetes in overweight subjects (Tuomilehto et al. 2001, Knowler et al. 2002). The most effective intervention has been moderate weight-loss combined with exercise. Several studies have shown the effect of antihyperglycaemic medications in delaying the diagnosis of diabetes, which is quite obvious since the glucose levels are lowered (Chiasson et al. 2002, Knowler et al. 2002, Torgerson et al. 2004, DREAM Trial Investigators et al. 2006). It is doubtful whether these drugs could interfere with the pathophysiology of type 2 diabetes. Rather, the effect of the antihyperglycaemic medication lasts as long as the treatment (Knowler et al. 2005, DREAM Trial Investigators et al. 2006). On the contrary, the benefits of lifestyle intervention are sustained after the intervention period (Lindström et al. 2006).

Non-alcoholic fatty liver disease (NAFLD) predicts and precedes type
2 diabetes (Adams et al. 2009). It is characterised by accumulation of fat in the liver in the absence of alcohol abuse and other chronic liver diseases. It has been shown that plasma alanine aminotransferase (ALT), fasting plasma glucose, and liver fat increase in prediabetic state. Intra-abdominal fat mass, rather than subcutaneous fat, shows a strong association with liver fat content (Kotronen et al. 2007). This reflects both the increased inflammatory activity and direct venous drainage of intra-abdominal fat to the liver. Given the crucial role of liver in the metabolic processing of dietary and endogenous glucose and lipids, fatty liver disease can be considered as an early phenomenon in development of type 2 diabetes (Kotronen and Yki-Järvinen 2008, Adams et al. 2009).

Insulin resistance develops in genetically prone individuals and is strongly associated with abdominal obesity (Palaniappan et al. 2004). In muscle, insulin resistance decreases the glucose transport (Cline et al. 1999). In the liver, insulin fails to suppress hepatic glucose production in the fasting state, contributing to the hyperglycaemia seen in type 2 diabetic patients (DeFronzo et al. 1989).

In type 2 diabetes, insulin release by the β-cells is insufficient in compensating the increased needs during insulin resistance (Prentki and Nolan 2006). Increased levels of free fatty acids, triglycerides, and cholesterol contribute to lipotoxicity of pancreas in type 2 diabetes (Fryirs et al. 2009). These lipids and hyperglycaemic state are known to increase reactive oxygen species (ROS) (Inoguchi et al. 2000). Excessive ROS impair insulin synthesis and activate β-cell apoptosis (Evans et al. 2002). Free fatty acids (FFA) contribute to progressive β-cell failure (Lee et al. 1994), fatty acid-induced apoptosis (Shimabukuro et al. 1998), decreased insulin gene promoter activity (Gremlich et al. 1997), and decreased binding of a transcription factor to the insulin gene (Ritz-Laser et al. 1999). As discussed above, accumulating evidence shows that ROS are implicated in β-cell dysfunction (Maechler et al. 1999) and that cells exposed to fatty acids increase ROS production (Inoguchi et al. 2000).

2.2 Lipid metabolism in type 2 diabetes

2.2.1 Introduction
Diabetic dyslipidemia is a cluster of atherogenic lipid alterations affecting all lipid classes. It is characterised by raised fasting triglycerides, excessive postprandial lipemia, small dense LDL, and low levels of HDL cholesterol (Taskinen 2003). These lipid abnormalities are highly atherogenic and frequently precede diagnosis of type 2 diabetes by several years.

Lipids travel in circulation in lipoprotein particles. Lipoprotein particles have a hydrophobic core consisting of cholesterylesters and triglycerides (TGs) and a hydrophilic surface of free cholesterol, phospholipids and
apoproteins. Apoproteins in the surface of the particles maintain the structure of lipoproteins and control their interactions with various receptors, hydrolytic enzymes, and lipid transport proteins. Lipoproteins can be classified based on particle size, electrophoretic mobility, apoprotein content, or hydrated density in ultracentrifugation. Commonly, five classes are separated according to the hydrated density (d) in ultracentrifugation: chylomicrons (CMs, d<0.96 g/ml), very-low density lipoproteins (VLDL, d<1.006 g/ml), intermediate-density lipoproteins (IDL, d=1.006–1.019 g/ml), low-density lipoproteins (LDL, d=1.019–1.063 mg/dl), and high-density lipoproteins (HDL, d=1.063–1.21 mg/dl) (Gotto et al. 1986). So far, limited data exist on subclasses of VLDL, LDL, and HDL in type 2 diabetes.

2.2.2 Triglyceride-rich particles and their metabolism
Triglyceride-rich particles (TRLs) are heterogeneous, being derived from both intestine (chylomicrons) and the liver (VLDL). Liver secretes a range of apoB-100-containing VLDL particles varying in size and density. VLDL particles are commonly separated into two subclasses: large buoyant VLDL1 particles (Sf 60–400) and smaller denser VLDL2 particles (Sf 20–60), that are independently regulated (Packard et al. 2000a) (Figure 1.). VLDL1 particles are the major carriers of triglycerides in circulation.

The key regulators of the normal assembly of VLDL in the liver are de novo lipogenesis (DNL), lipid oxidation, VLDL secretion, and FFA flux. FFA influx to the liver is dependent on its plasma pool and thus the increase in plasma FFA, which is usually due to adipose tissue FFA release, results to

Figure 1. Differential regulation of VLDL1 and VLDL2 by the liver.

Summary of VLDL assembly in the liver and parallel catabolic pathways of intravascular conversion of TRL into IDL and LDL (modified after Taskinen MR Diabetologia 2003). ApoB, apoprotein B; CE, cholesterylester; HL, hepatic lipase; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; TG, triglycerides.
increase in triglyceride accumulation to the liver (Tamura and Shimomura 2005). Increased delivery of fatty acids increases the secretion of VLDL-triglycerides and apoB-100 from human liver and from hepatocytes and HepG2 cells (Lewis et al. 1995, Lewis 1997). In hyperinsulinemic subjects with fatty liver, DNL accounts approximately 25% of triglyceride content in liver and VLDL compared to <5% in healthy subjects (Donnelly et al. 2005, Barrows and Parks 2006).

Accumulation of fat in the liver is associated with insulin resistance of the liver (Seppäla-Lindroos et al. 2002). Insulin resistant liver is incapable of properly handling the traffic of exo- and endogenous lipids in the liver (Adiels et al. 2006). Physiological action of insulin is to directly suppress VLDL1 synthesis in the liver and also to reduce availability of FFA flux from the adipose tissue to the liver (Malmström et al. 1997). Recent studies have shown that insulin fails to suppress the production of endogenous VLDL1 particles in people with hepatic steatosis (Adiels et al. 2007). Thus, even an acute hyperinsulinemia (as occurs in the hyperinsulinemic normoglycaemic clamp studies or after a meal) cannot shut down the production of VLDL1 particles by the liver.

Insulin-resistance of the intestine is a recently discovered phenomenon. Normal action of insulin in the intestine includes the inhibition of apoB-48 synthesis in the enterocyte, thus delaying the flow of chylomicrons in plasma (Duez et al. 2008b). Animal models and human isotope studies have recently shown that apoB-48 is produced excessively in the intestine of insulin resistant subjects (Duez et al. 2006, Hogue et al. 2007), with the FFA flux increasing also intestinal apoB-48-containing lipoprotein production (Duez et al. 2008a).

Circulating apoB-48 containing chylomicrons are removed from plasma by lipolytic action of lipoprotein lipase (LPL), followed by receptor-mediated uptake by the liver. Large chylomicrons are better substrates for LPL, competitively increasing the residence time of VLDL particles in plasma (Karpe et al. 1997). Postprandial TRLs are thus increased in type 2 diabetes. Interestingly, approximately 80% of the TRL increase in postprandial state is accounted by the VLDL particles; the major component being the VLDL1.

The concentration of VLDL1 particles in circulation is determined by both the production and the catabolic rate. VLDL production rate is exaggerated type 2 diabetic patients due to insulin resistance, liver fat, and hyperglycemia (Adiels et al. 2008). In type 2 diabetes, secretion of VLDL2 is similar to non-diabetic subjects (Gill et al. 2004, Adiels et al. 2005). The increased production of VLDL1 is more important than the decreased removal in determining the actual VLDL1 level in type 2 diabetes with mild to moderate hypertriglyceridemia. In severe hypertriglyceridemia, the impaired removal plays however a more essential role (Nagashima et al. 2005).
In circulation, LPL hydrolyzes VLDL triglycerides, releasing fatty acids and generating small triglyceride-depleted remnant lipoproteins. LPL is insulin dependent and its activity is commonly subnormal in type 2 diabetes probably due to insulin resistance (Taskinen 1987), resulting in longer residence time of TRL particles in circulation. In addition to LPL gene polymorphisms, important modulators of the LPL activity are apoproteins CII and CIII. ApoCII is a strong and specific activator of LPL (Jong et al. 1999). Patients with mutations of apoCII gene have elevated serum triglycerides (Santamarina-Fojo 1992). The balance between apoCII and apoCIII levels is critical for the LPL-mediated hydrolysis of TRLs (Jong et al. 1999).

ApoCIII is synthesized in the liver and intestine and it is a surface component of TRLs, with smaller amounts detected in LDL and HDL particles (Jong et al. 1999). ApoCIII is the most abundant apolipoprotein in VLDL particles and its concentrations are closely correlated with serum total and VLDL triglycerides (Campos et al. 2001, Marcoux et al. 2001). ApoCIII modulates catabolism of TRLs (van Dijk et al. 2004): apoCIII inhibits lipoprotein lipase activity and interferes with apolipoprotein E-mediated receptor binding. Both actions delay the removal of remnant particles. Consistently, kinetic studies have shown an increased production rate of VLDL apoCIII to be associated with increased production and delayed catabolism of VLDL (Chan et al. 2002, Cohn et al. 2004). Recently, VLDL apoCIII has been shown to inhibit insulin-induced phosphorylation of insulin-receptor substrate 1 in endothelial cells, leading to reduced nitric oxide release and endothelium-dependent relaxation (Kawakami et al. 2008).

ApoE is a primary ligand for LDL receptor and LDL receptor-related protein 1 (LRP-1) (Clavey et al. 1995, Ji et al. 1997), thus having a central role in the removal of VLDL remnants. Interestingly, LRP-1 is shown to be stimulated by insulin and can thus be partly responsible for impaired remnant clearance in insulin resistance (Laatsch et al. 2008).

Emerging evidence suggest that apoE has also a critical role in intracellular lipid metabolism (Mensenkamp et al. 1999, Heeren et al. 2003) and possibly in enhancing reverse cholesterol transport (Hasty et al. 2005). ApoE exhibits multiple genotypes that can modify lipid metabolism. ApoE4 allele is associated with higher serum cholesterol levels, increased cellular cholesterol accumulation and Alzheimer’s disease, whereas apoE2 allele is associated with higher triglyceride levels as typically evidenced in type IV dyslipoproteinemia. Since the distribution of apoE alleles is reported to be similar in type 2 diabetic subjects as in general population (Meigs et al. 2000), apoE alleles have a minor impact on lipoprotein profiles in type 2 diabetes. Despite of the central role of apoCIII and apoE in VLDL metabolism, only limited data exist on their changes in type 2 diabetes.
2.2.3 Low density lipoprotein particles and their metabolism

The perturbation of VLDL1 metabolism is not only reflected in triglycerides per se but also interferes with other lipoprotein species. The excess of VLDL1 results in increased amounts of TG transferred to LDL via cholesterylester transfer protein (CETP) (Syvänne and Taskinen 1997). TG-rich LDL particles are good substrates to hepatic lipase which hydrolyzes the TG, making the particles smaller and denser (Tan et al. 1995) (Figure 2.). In type 2 diabetes, the LDL cholesterol concentration is closely similar to the non-diabetic subjects but apoB is increased as a marker of increased number of LDL particles (Sniderman et al. 2001). Small and dense LDL is frequently present in insulin resistant states such as metabolic syndrome, obesity, and increased liver fat (Haffner et al. 1995, Toledo et al. 2006).

LDL can be separated to multiple subclasses according to the size or the density of the particles. In addition, there is considerable variation in the particle composition. In type 2 diabetic subjects, all of the LDL subclasses are enriched with triglycerides as compared with those in nondiabetic subjects. Further, the free cholesterol and protein content of the LDL particles is decreased. The low content of phospholipids and free

![Figure 2. Consequences of elevated TRLs on LDL and HDL pathways.](image)

CE, cholesterylester; CETP, cholesterylester transfer protein; HL, hepatic lipase; TG, triglycerides (modified after Syvänne M Lancet 1997).
cholesterol in small, dense LDL is associated with changes in the exposure of segments of the apoB-100 that modify its affinity for the apoB receptor (Capell et al. 1996, Olsson et al. 1997, Gustafsson and Boren 2004).

Most of the interactions of LDL are controlled by segments of apoB-100 exposed at the particle surface. LDL, IDL, and VLDL all contain one apoB per particle. Thus, apoB levels are typically elevated in type 2 diabetes due to increase in the number these particles (Figure 3.). ApoB is essential for the catabolism of these particles by the liver, since it is recognized by the LDL receptor. This binding can be abolished by apoCIII, probably by masking the receptor binding site of apoB (Clavey et al. 1995). LDL apoCIII levels are increased in type 2 diabetes, and the levels are associated with small and dense LDL phenotype (Olin-Lewis et al. 2002, Davidsson et al. 2005). It seems that the major part of apoCIII resides in the denser subclasses of LDL. Based on the available data, changes in the binding of LDL can be expected to occur in people with type 2 diabetes.

A significant reduction of LDL catabolism in parallel with a slight reduction of LDL production is observed in patients with type 2 diabetes (Duvillard et al. 2000a). Interestingly, LDL receptor has shown to be partly insulin-regulated (Mazzone et al. 1984, Wade et al. 1989). Thus, it can be partly responsible for impaired LDL catabolism in insulin resistance. One possible explanation for the reduced catabolism of LDL in type 2 diabetes is the reduced affinity of small dense LDL to LDL receptor (Galeano et al. 1994, Campos et al. 1996).

Figure 3. The changes in lipoproteins associated with insulin resistance.
2.2.4 High-density lipoprotein particles and their metabolism.

HDL particles originate from liver and intestine, or they are synthesized from excess surface components during lipolysis of chylomicrons and VLDL particles (Eisenberg 1984). Maturation of HDL particles includes esterification of cholesterol by lecithin:cholesterol-acyltransferase (LCAT) (Rye et al. 1999). Phospholipid transfer protein (PLTP) transfers surface phospholipids and unesterified cholesterol from the VLDL and chylomicron particles into HDL, thus contributing to the fusion of HDLs into large HDL2 particles and small HDL-precurors.

HDL particles vary in size and composition, and they are constantly remodelled in the circulation by various enzymes (Figure 4). The two main fractions separated in the density gradient ultracentrifugation are HDL2 and HDL3. HDL2 particles are less dense, having a higher relative amount of cholesterol and phospholipids than HDL3 particles (Gotto et al. 1986). The reduction of HDL cholesterol in diabetic dyslipidemia is mostly due to reduction of the larger HDL2 subclass (Taskinen 2001). Thus, the HDL particles are predominantly small and dense in type 2 diabetes, a feature that is associated with increased triglycerides and LDL size (Figure 2).

The mechanism between the increase of triglycerides and decrease of HDL cholesterol is similar to the decrease of LDL size. Increase of plasma triglycerides is the driving force for the exchange of TRL triglycerides and HDL cholesterylesters (Lamarche et al. 1999). Thus, HDL particles become triglyceride-enriched and further processed by hepatic lipase.
Hydrolysis by hepatic lipase is increased in hypertriglyceridemic states such as diabetic dyslipidemia. This renders HDL particles unstable and increases their catabolic rate (Rashid et al. 2003).

Most of the HDL particles contain one apoA-I, which is the most abundant structural particle. ApoA-I is crucial for the antiatherogenic function of these particles (as described later in 2.2.5). Some HDL particles also contain apoA-II. Such particles can be named by their apoA-content as LpA1-AII, while LpA-I contains only apoA-I. LpA-I predominates in HDL2 density range, while LpA1-AII predominates in HDL3 density range. ApoA-II stabilizes apoA-I and modulates the reaction of HDL with LCAT (Durbin and Jonas 1997).

Previously both of these apoproteins have been shown to be reduced in type 2 diabetes. Reduction of apoA-I is partly explained by the increased clearance rate in kinetic studies on subjects with metabolic syndrome or type 2 diabetes (Duvillard et al. 2000b, Watts et al. 2003, Verges et al. 2006). A recent study has linked the hypercatabolism of apoA-I to the overproduction of VLDL-apoB. (Chan et al. 2009) The mechanism behind this association may lie in the effects of CETP-mediated triglyceride enrichment and remodelling of HDL.

### 2.2.5 Diabetic dyslipidemia as a CVD risk factor

Diabetic dyslipidemia is an atherogenic lipid condition of elevated triglycerides, low HDL cholesterol, and small, dense LDL. The individual components of diabetic dyslipidemia are all interrelated and their independent effects are difficult to dissect. Even though overproduction of VLDL1 is clearly the dominant defect in diabetic dyslipidemia, the independence of triglycerides as a risk factor for coronary heart disease is not obvious. Recent studies suggest that both fasting and non-fasting triglycerides over 1.7 mmol/L are a cardiovascular risk factor, independent of other lipids and insulin resistance (Bansal et al. 2007, Nordestgaard et al. 2007, Freiberg et al. 2008). As the evidence is largely based on epidemiological data, more prospective studies are however needed to advice the clinicians concerning treatment of high triglycerides.

Large VLDL particles are not able to enter the arterial intima, but the smaller, cholesterylesther-rich remnants of VLDL metabolism are linked with endothelial dysfunction, oxidative stress, coagulation disorders, and also coronary heart disease (Lewis et al. 1999, Doi et al. 2000, Lundman et al. 2001, Nakamura et al. 2005) These remnants are increased in type 2 diabetes, reflecting the prolonged postprandial period. Increase of apoCIII in TRLs may also be an independent CHD risk factor (Luc et al. 1996, Gervaise et al. 2000, Sacks et al. 2000, Onat et al. 2003).

Despite appropriate statin treatment reaching the goals for LDL cholesterol, the CVD morbidity and mortality are increased in type 2 diabetic patients. Decrease of LDL size and the accompanied increase in...
particle number are both associated with elevated CHD risk (Lamarche et al. 1997, Vakkilainen et al. 2003, Williams et al. 2003, Jiang et al. 2004). Multiple factors explain the higher atherogenic potential of small dense LDL. It is more easily glycated, oxidized, and enzymatically processed (Scheffer et al. 2005). A critical step in atherosclerosis is the retention of lipoproteins by vascular wall molecules (Figure 5), particularly proteoglycans (Skalen et al. 2002). The binding of LDL to artery wall proteoglycans is ionically mediated by clusters of amino acids in apoB-100 (Williams and Tabas 1995, Boren et al. 1998a). LDL receptor-binding site in apoB-100, Site B (residues 3359–3369), is also principal proteoglycan-binding site of apoB-100 (Boren et al. 1998b).

Compositional changes modulate the proteoglycan-binding activity of LDL (Olin-Lewis et al. 2002, Flood et al. 2004). Interestingly, apoCIII increases the binding to vascular proteoglycans, but the mechanisms are unclear as apoCIII does not bind directly to biglycan (Olin-Lewis et al. 2002). ApoCIII content of LDL contributes to kinetic defects in apoB metabolism (Chan et al. 2002), and high apoCIII content in LDL increases the risk of cardiovascular disease, independent of LDL cholesterol values (Blankenhorn et al. 1990, Luc et al. 1996, Sacks et al. 2000). Epidemiological data have shown that low levels of HDL cholesterol are an independent predictor of CHD (Genest et al. 1999). This is evident even during appropriate LDL-lowering medication (Collins et al. 2003). One of the most important antiatherogenic function of HDL particles is reverse cholesterol transport, where excess cholesterol is removed from peripheral cells to the liver for excretion (Stein and Stein 1999, Assmann...

Conflicting evidence exists on the atheroprotective properties of different HDL subclasses and also the molecular mechanisms are far from resolved (Nobecourt et al. 2005, de Souza et al. 2009). In type 2 diabetes, HDL cholesterol is decreased mostly in the larger HDL2 subclass and less in the smaller HDL3 (Taskinen 2001). Type 2 diabetes is associated with several modulations of HDL that render the particles dysfunctional: enrichment with triglycerides and conformational changes or displacement of apoA-I, and oxidative modifications of the particles. (Kontush and Chapman 2008). Recently, glycosylation of HDL has been shown to reduce cholesterol efflux, destabilize ABCA1 and apoA-I, and interfere with the ability to inhibit the expression of adhesion molecules (Hoang et al. 2007, Nobecourt et al. 2007). Proteomic studies have shown altered lipid cores of small dense HDL3 in metabolic syndrome, which possibly deleteriously affect the antiatherogenic properties of that subclass (de Souza et al. 2008).

2.3 Inflammation and endothelial function in type 2 diabetes

2.3.1 Inflammation in type 2 diabetes

Chronic subclinical inflammation is increased in type 2 diabetes. Inflammation is associated with fat mass and central adiposity (Mathieu et al. 2009). Adipose tissue is actively engaged in generation of systemic inflammation, since it produces several cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) (Despres and Lemieux 2006). Visceral fat is crucial for inflammation since it is active in cytokine production and its products have a direct route to liver via portal vein (Mathieu et al. 2009). IL-6 is the major precursor of inflammation cascade and it is able to induce the production of C-reactive protein (CRP) in the liver.

CRP is by far the most used biomarker of low-grade inflammation. It has been implicated for clinical assessment of CVD risk (Pearson et al. 2003) and it has independent predictive power for cardiovascular disease such as myocardial infarction, stroke, peripheral arterial disease, sudden cardiac death and recurrent ischemic events. Inflammatory markers rise before the onset of clinical diabetes (Pickup 2004). CRP and IL-6 has been associated with the risk of developing type 2 diabetes (Duncan et al. 2003).

Systemic low-grade inflammation is involved in the initiation and
development of atherosclerosis as well as in acute coronary syndromes. CRP might even induce atherosclerosis by opsonising LDL to macrophages, reducing endothelial nitric oxide synthase, decreasing nitric oxide bioavailability (Fichtlscherer et al. 2004), activating CAM expression by endothelial cells, and inducing MCP-1 production (Yeh 2004). It is however unclear if CRP is truly an independent risk factor for CVD or simply a surrogate marker of elevated risk (Schunkert and Samani 2008).

The dyslipidemia in low-grade inflammation interestingly resembles diabetic dyslipidemia with hypertriglyceridemia, small dense LDL, and low levels of HDL cholesterol (Khovidhunkit et al. 2000, Taskinen 2003). Possible crosstalk mediators between dyslipidemia and inflammation include secretory phospholipase A2 IIA (PLA2, also known as lipoprotein-associated phospholipase A2) and human serum amyloid A (SAA).

SAA displaces apoA-I from the surface of HDL particles, attenuating the antiatherogenic function of the particles (Coetsee et al. 1986, Kontush and Chapman 2006). PLA2 depletes the phospholipid layer of LDL, making the particles smaller, denser, and thus more atherogenic (Sartipy et al. 1999). High levels of PLA2 have been shown to predict CVD events in population studies (Packard et al. 2000b, Koenig et al. 2004, Anderson 2008). PLA2 may migrate between LDL and HDL particles (Stafforini et al. 1989) and is associated with small, dense LDL and low levels of HDL (Gazi et al. 2005, Khuseyinova et al. 2005).

2.3.2 Endothelial dysfunction in type 2 diabetes

Endothelial dysfunction is a part of insulin-resistant syndrome. Endothelial dysfunction includes increased leukocyte adherence and penetration in the arterial intima, enhanced thrombosis, and a decline in endothelium-dependent vasodilatation (De Caterina 2000). The initiation of atherosclerosis requires inflammation and activation of the endothelium to express cellular adhesion molecules (CAMs).

The concentrations of CAMs have correlated with traditional CVD risk factors and progression of atherosclerosis (Bonetti et al. 2003). CAMs have been elevated in diabetic patients and in conditions preceding type 2 diabetes (Blann and Lip 2000, Calles-Escandon and Cipolla 2001). The predictive value of CAMs in CVD is not obvious (Malik et al. 2001a, Bonetti et al. 2003), but some studies have suggested inflammation and endothelial dysfunction to predict CVD in type 2 diabetes (Jager et al. 2000, Saito et al. 2002, Stehouwer et al. 2002).

2.4 Carotid intima-media thickness in type 2 diabetes

Surrogate measures of atherosclerosis have proven useful in clinical trials to study the vascular response to different treatments. The B-mode
ultrasound scanning of carotid and/or femoral artery intima-media thickness (IMT) is frequently utilised as an inexpensive, non-invasive, and reproducible method (Salonen and Salonen 1993, Bots et al. 2003). The IMT measured by ultrasound corresponds well to the histological thickness of intimal and medial layers of arterial wall (Cheng et al. 2002). The slightly thinner IMT observed in histological samples may be due to post-mortem shrinkage and sample preparation. The ultrasound measures from the near wall (NW) represent 80% of the histological thickness. Inclusion of NW measurements in the IMT protocol however reduce variation of the data (Kanters et al. 1997). The reproducibility of near and far wall measurements by ultrasound has been similar (Bots et al. 2003).

Two-parallel echogenic lines can be identified by ultrasound, corresponding to the interfaces between lumen/intima and media/adventitia. Both at the near wall and at the far wall (FW), the thickness of the echogenic line next to the vascular lumen added to the thickness of the adjacent dark layer compose the IMT (Figure 6A). The ultrasound scanning is preferably performed bilaterally on the extracranial carotid artery segments (Kanters et al. 1997, Bots et al. 2003). The scanned segments are usually the distal 1 cm of the common carotid artery (CCA), the carotid bifurcation (CB), and the proximal 1 cm of the internal carotid artery (ICA) (Greenland et al. 2001) (Figure 6B).

CCA is the most readily visible part of the carotid artery and thus utilized in most studies. CB and especially ICA are more demanding to study due to anatomic reasons. However, atherosclerotic plaques are common in the carotid bifurcation and internal carotid artery, which increases the clinical relevance of IMT measurement as well as the association of IMT to clinical CVD (Simon et al. 2002).

Figure 6. Anatomical correspondence of near and far wall IMT and imaging sites of carotid arteries (modified after Ylitalo K 2001 and Soro-Paavonen A 2004).
IMT becomes thicker with age and is thicker in men compared to women (Cheng et al. 2002). The growth rate of mean IMT has been 0.12 mm in two years in a population study from Eastern Finland (Salonen and Salonen 1991). Traditional CVD risk factors predict IMT in general population: age, gender, blood pressure, total and LDL cholesterol, low HDL cholesterol, and smoking (Salonen and Salonen 1990, Salonen and Salonen 1991, Lakka et al. 1999). Also genetic and socioeconomic factors, distribution of body fat, postprandial lipemia, and hyperglycemia as well as LDL size predispose to preclinical atherosclerosis measured by carotid IMT (Yamasaki et al. 1995, Boquist et al. 1999, Skoglund-Andersson et al. 1999, Takami et al. 2001, Zannad and Benetos 2003).

Large studies have demonstrated the association of increased IMT with existing CVD and future CVD events such as myocardial infarction and stroke (O’Leary and Polak 2002, Bots et al. 2003). Recently a large meta-analysis supported the role of carotid IMT as an independent predictor of future vascular events (Lorenz et al. 2007). The correlation between carotid IMT and the angiographic status of the coronary arteries is however modest (Wofford et al. 1991, Adams et al. 1995, Graner et al. 2006, Bots et al. 2007). IMT measures also the positively (i.e outward) remodelled plaques that do not yet narrow the lumen, whereas angiography shows the lumen diameter and thus only the plaques that narrow the lumen. Notably, the angiographic approach is quite different compared to the IMT measurement, which can partly explain the modest association between these measures (Bots et al. 2007).

IMT is generally thicker and its progression faster in type 2 diabetic patients than in nondiabetic subjects (Yamasaki et al. 1995, Mykkänen et al. 1997, Temelkova-Kurktschiev et al. 1999, Bonora et al. 2000, Brohall et al. 2006). In general, the traditional CVD risk factors have shown weaker association to IMT in diabetic patients compared to the non-diabetic population (Yamasaki et al. 1995, Temelkova-Kurktschiev et al. 1999, Mohan et al. 2000). Beyond age and blood pressure, duration of diabetes is strongly associated to IMT. Microalbuminuria has been shown to correlate with IMT in both non-diabetic and diabetic patients (Mykkänen et al. 1997).

The interventions to inhibit IMT growth in type 2 diabetes have been recently summarized (Yokoyama et al. 2006). Reduction of IMT progression rate has been shown for two α-glucosidase inhibitors, repaglinide, pioglitazone, metformin, and enalapril. Of lipid-lowering agents, cerivastatin and bezafibrate have not shown any effect on IMT progression rate in comparison with placebo. However, several studies on different statins as well as one with fenofibrate have demonstrated a slower progression of carotid atherosclerosis compared to placebo (Shinohara et al. 2005, Takahashi et al. 2005, Zhu et al. 2006).
2.5 Arterial stiffness and diabetes

Arterial stiffness can be studied by various methods (Oliver and Webb 2003). Most widely used are non-invasive methods measuring pulse wave velocity, pulse pressure, blood flow waveform analysis, or arterial distensibility and diameter (Woodman and Watts 2003). Normally, arteries are elastic enough to buffer the pressure peaks caused by rapid blood flow from the left ventricle. When the arteries stiffen, the intra-arterial pressure and the speed of the pressure wave increase (Woodman and Watts 2003).


Fenofibrate has been shown to enhance endothelial function, reflected as an increase in flow-mediated vasodilatation Malik et al. 2001b, Marchesi et al. 2003, Wang et al. 2003, Koh et al. 2005. In some smaller studies, simvastatin and atorvastatin have been shown to improve arterial stiffness. In CAFE-LLA (Conduit Artery Function Evaluation –Lipid Lowering Arm) with 891 patients, atorvastatin did not improve augmentation index during a 3.5 years follow-up (Williams et al. 2006). The effect of either gemfibrozil or atorvastatin was compared to placebo in a 6-week study in patients with chronic kidney disease (Dogra et al. 2007). Approximately 20% patients had diabetes. No significant changes were seen in either endothelial function or large artery stiffness. In another small study of 16 non-diabetic obese men, 3-month treatment with fenofibrate significantly reduced the augmentation index although no changes in blood pressure were detected (Ryan et al. 2007). A significant decrease in plasma ICAM-1 and VCAM-1 were also demonstrated which may reflect the detected changes.

2.6 Dyslipidemia and microvascular outcomes in type 2 diabetes

Microvascular disease in type 2 diabetes includes nephropathy, retinopathy, and neuropathy. The number of patients facing severe complications, such as dialysis, kidney transplant, or loss of vision (Go et al. 2004), is likely to be increasing in parallel with increasing prevalence of type 2 diabetes.

Diabetic nephropathy begins with constantly increased albumin excretion in urine. Microalbuminuria (urine albumin excretion rate >20μg/ min) is present in about 20% of the patients at time of the diagnosis of type
2 diabetes, possibly due to underlying metabolic syndrome, hypertension, and endothelial dysfunction (Mogensen and Poulsen 1994). Albumin excretion can decrease to normal level with strict control of hyperglycaemia and hypertension (Araki et al. 2008). Peaks of hyperglycaemia as well as frequent hypoglycaemias have been shown to worsen the renal function. Every year 2% of the type 2 diabetic patients enter the next stage of albuminuria, i.e., from normoalbuminuria to microalbuminuria or from microalbuminuria to macroalbuminuria (Adler et al. 2003). Nowadays type 2 diabetes is more common reason for dialysis in Finland than type 1 diabetes (The Finnish Kidney and Liver Association 2007).

For treatment of hypertension in diabetic nephropathy, angiotensin converting enzyme (ACE) -inhibitors and angiotensin receptor blockers (ARBs) are essential, since they both have effects beyond blood pressure lowering. They have been shown to reduce albuminuria and slower the worsening of renal function (Brenner et al. 2001, Lewis et al. 2001, Parving et al. 2001). The UKPDS study suggested that with a 10 mmHg reduction in systolic BP in patients with diabetes, the risk of macrovascular events was lowered by 32%, the risk of microvascular complications by 37%, and the risk of death related to diabetes by 32% (UKPDS Study Group 1998b).

Diabetic nephropathy markedly increases the risk of cardiovascular disease (Watkins 2003, Weiner et al. 2004). Part of this risk has been explained by diabetic dyslipidemia. Dyslipidemia further aggravates in patients developing nephropathy, as particularly triglycerides increase and HDL cholesterol decreases (de Boer et al. 2008). In nephropathy, synthesis of apoA-I is reduced, levels of mature HDL particles are decreased, and small and dense atherogenic LDL particles are accumulated (Saland and Ginsberg 2007). The increased levels of triglyceride-rich remnant particles result from decreased clearance rate and reduced apoCIII (Deighan et al. 2000).

Hypertriglyceridemia seems to associate with development and progression of both non-diabetic (Muntner et al. 2000, Tozawa et al. 2002) and diabetic kidney disease (Colhoun et al. 2001, Cusick et al. 2004). Lipid lowering has proven some effect for treatment of diabetic nephropathy in type 1 and type 2 diabetes, shown for both reduction of triglycerides and cholesterol. For prevention of diabetic nephropathy, only lowering of triglycerides has shown effective in type 2 diabetes. There are so far no large-scale primary prevention studies that address the effects of lipid-lowering therapy on the development and progression of diabetic nephropathy, although the benefits of aggressive treatment of dyslipidemia to prevent cardiovascular disease are well-demonstrated (Collins et al. 2003, Colhoun et al. 2004).

A recent meta-analysis has explored those statin trials that have addressed albuminuria reduction (Douglas et al. 2006). The conclusion is that statins...
may reduce pathologic albuminuria (defined as excretion >30mg/d). The results of type 2 diabetic patients were however not separately reported. Fenofibrate has been shown to reduce progression of microalbuminuria in patients with type 2 diabetes (The DAIS Investigators 2001). In that study, the data on albuminuria was compared as a switch between albuminuria categories classified as normoalbuminuria, microalbuminuria, and macroalbuminuria. More patients showed regression to the lower level and fewer patients progression to the higher level of albuminuria in the fenofibrate group. The mean rates of albumin excretion remained however similar between the fenofibrate and placebo groups.

Mild retinopathy can be present at the time of diagnosis of diabetes, but more severe retinopathy occurs typically late in the course of type 2 diabetes. After 20 years of diagnosis of type 2 diabetes, 50–80% of patients develop retinopathy (Klein et al. 1984). Early diabetic retinopathy includes microaneurysms, blot hemorrhages, and cottonwool spots. Different stages of nonproliferative retinopathies comprise venous changes, capillary loss, ischemia, and extensive intraretinal hemorrhages. A proliferative retinopathy poses a threat for visual acuity with neovascularization, preretinal and vitreous hemorrhages, fibrovascular proliferation, and retinal detachments. Prevention and treatment of retinopathy is targeted to hyperglycaemia and hypertension, with less evidence of treatment of hyperlipidemia (UKPDS Study Group 1998a, Sen et al. 2002, Matthews et al. 2004).

Hypercholesterolemia and hypertriglyceridemia are associated with the development and severity of diabetic retinopathy (Ferris et al. 1996). Hyperlipidemia is linked to the development of macular oedema, hard exudates, and proliferative retinopathy (van Leiden et al. 2002, Miljanovic et al. 2004). However, statin treatment has shown unsuccessful in preventing diabetic retinopathy (Zhang and McGwin 2007). In a number of small studies, fibrates have reduced retinal and macular hard exudates (Duncan et al. 1968, Harrold et al. 1969).

2.7 Fibrates in treatment of diabetic dyslipidemia

Fibrates are peroxisome proliferator-activated receptor α (PPARα) agonists, modulating a large variety of genes in lipid metabolism (Keating and Croom 2007). Fibrates have different affinities for PPARα; for example gemfibrozil is a selective PPARα modulator (Table 1.). PPARα is a nuclear transcription factor, which forms a heterodimer with retinoid X receptor when activated. PPARα-mediated actions interfere not only in lipid metabolism, but also to inflammation and coagulation cascades (Figure 7).

The clearance rate of TRL particles is determined by LPL activity. ApoCIII is a key regulator of LPL activity and consequently a determinant of TRL clearance. As fibrates activate LPL gene but suppress apoCIII gene,
the clearance of TRL particles is enhanced (Watts et al. 2003, Bilz et al. 2004). Fibrates are reported to promote beta-oxidation and to inhibit de novo synthesis of fatty acids, thus reducing the availability of fatty acids for TG synthesis (Schoonjans et al. 1996). Whether these actions reduce the number of TRL particles or the percentage of triglycerides is unclear, since data on the effect of fenofibrate on TRL subclasses are limited. So far the data from human kinetic studies are inconsistent.

Several studies indicate that fibrates increase LDL size (Shepherd et al. 1985, Guerin et al. 1996, Ikewaki et al. 2004). The decrease in VLDL

Table 1. Features of different fibrates.

<table>
<thead>
<tr>
<th>Effect on PPARα</th>
<th>Dose</th>
<th>Use in CKD</th>
<th>Effect on creatinine</th>
<th>Use with a statin</th>
<th>Effect on HCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenofofrate*</td>
<td>agonist</td>
<td>200mg 1x1</td>
<td>Reduce dose</td>
<td>++</td>
<td>recommended choice</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>selective modulator</td>
<td>600mg 1x2</td>
<td>1x1 if GFR&gt;60</td>
<td>+</td>
<td>only with fluvastatin</td>
</tr>
<tr>
<td>Bezafrate</td>
<td>agonist</td>
<td>400mg 1x1</td>
<td>Reduce dose</td>
<td>++</td>
<td>can be used</td>
</tr>
<tr>
<td>Ciprofibrate**</td>
<td>agonist</td>
<td>100mg 1x1</td>
<td>unknown</td>
<td>++</td>
<td>can be used</td>
</tr>
</tbody>
</table>

CKD, chronic kidney disease; HCY, homocysteine.
*+, micronized fenofibrate; **, ciprofibrate is not marketed in Finland.
+, minor increase; ++, moderate increase; +++, marked increase

Figure 7. Effects of fibrates via PPARα. IL-1β, interleukin 1β; PAI-1, plasminogen activator inhibitor; PON-1, paraoxonase.
triglycerides reduces the transfer of TG to LDL and thus particles are less susceptible to the action of hepatic lipase. Fenofibrate has been shown to increase the catabolic rate of LDL without a change in the transformation of IDL to LDL (Watts et al. 2003, Bilz et al. 2004).

Fibrates seem to increase HDL-C levels more in non-diabetic than in diabetic participants (Rubins et al. 2002). However the results of different fibrates on HDL metabolism are difficult to compare. The vast majority of data shows, that fibrates raise HDL-C in the smaller and denser subclass HDL3, while the larger HDL2 remains stable or is slightly increased (Lussier-Cacan et al. 1989, Robins et al. 2001). This could be explained by decreased CETP resulting to increased HDL cholesterol levels, whereas increased hepatic lipase activity could partly explain the conversion of the larger HDL2 to smaller and denser HDL3 (Desager et al. 1996, Guerin et al. 1996, Watts et al. 2006).

Fibrates upregulate the hepatic synthesis of apoA-I and apoA-II (Keating and Croom 2007). It is unclear whether fibrates also modify the catabolic rate of apoA-I. The supposed reduced action of CETP to promote lipid transfer from HDL to VLDL (Guerin et al. 1996) could modify the changes of HDL levels observed by fenofibrate. In vitro, fenofibrate increases the reverse cholesterol transport, by increasing the transcription of ABCA1 in the macrophages and apoAI –mediated increase in lipid transport (Arakawa et al. 2005). Likewise, some effect on SR-BI has been proposed in vitro (Chinetti et al. 2000). Whether these actions also influence reverse cholesterol transport in humans is unknown. Altogether, available data is derived from small studies with short duration of treatment.

Fibrates regulate inflammation and haemostasis by inhibiting the expression of nF-kappaB, the key regulator of inflammation (Okayasu et al. 2008). In agreement, fibrates have been shown to modify markers of inflammation. CRP has been reduced, along with its upstream regulator IL-6 (Staels et al. 1998, Genest et al. 2000, Koh et al. 2005). The published studies have been mostly short-term studies and only one included patients with type 2 diabetes (McKenney et al. 2006). One large long-term study showed no effect of bezafibrate on CRP, but data for diabetes subgroup was not reported separately (Tanne et al. 2006).

Fibrates are known to increase homocysteine and creatinine levels (Keating and Croom 2007). The increase of creatinine is a class effect, but it seems to be smaller with gemfibrozil than other fibrates (Davidson et al. 2007). The exact mechanism for creatinine increase is not known, but it has been speculated that it could be due to increased production of creatinine. In a two-week study with dyslipidemic subjects (n=13) there was no effect of fenofibrate on creatinine clearance, an observation that was explained by an increased urinary excretion of creatinine, and thus no subsequent change in the creatinine clearance (Hottelart et al. 2002). Fibrates may impair the generation of vasodilatory prostaglandins,
probably because of the activation of PPARα (Tsimihodimos et al. 2002),
which might contribute even to renal function impairment. Unfortunately,
available data do not clarify the impact of fenofibrate-induced increase in
plasma creatinine.

Increased homocysteine is associated with increased risk for
cardiovascular disease (Soinio et al. 2004). All fibrates have been reported
to increase homocysteine levels, possibly by lesser extent with gemfibrozil
therapy (Davidson et al. 2007). The reason for homocysteine elevation is
unknown since the metabolic determinants of homocysteine (vitamins B6
and B12 and folate) are not reduced by fibrates (Bissonnette et al. 2001,
Westphal et al. 2001, Genest et al. 2004). Rather, increase of homocysteine
might be a direct PPARα-mediated action of fibrates. It is not evident
that administration of these vitamins would counteract the effects of
homocysteine. The trials aiming to reduce homocysteine have not reduced
CVD events although the levels of homocysteine have markedly decreased
(Bazzano et al. 2006).

In the Helsinki Heart study, treatment with gemfibrozil was shown
effective in primary prevention (Frick et al. 1987, Tenkanen et al. 2006),
although the numbers in the diabetes subgroup were small. Thereafter,
gemfibrozil has reduced CHD events in subjects with type 2 diabetes or
metabolic syndrome in subgroup analysis of VA-HIT (Rubins et al. 2002).
In the DAIS, fenofibrate slowed angiographically determined progression
of coronary atherosclerosis but did not significantly reduce cardiac end-
points (The DAIS Investigators 2001). DAIS was however underpowered
to show an effect in clinical CVD events with 418 patients. The finding that
fibrates seemed to reduce CVD risk more in subjects with type 2 diabetes
or metabolic syndrome compared to non-diabetic individuals (Rubins et
al. 2002) raised the promise that fibrates might be optimal drugs to reduce
CVD in people with type 2 diabetes. Therefore, FIELD study was designed
to answer the question concerning the efficacy of fenofibrate to reduce
CVD. The FIELD study also provided a good opportunity to explore effects
of fenofibrate on different lipoprotein subclasses, low-grade inflammation,
carotid IMT, and renal function.
3. AIMS OF THE STUDY

The first aim of the study was to characterize the alterations of VLDL and LDL subclasses and to investigate the effects of LDL on binding to arterial wall in type 2 diabetes. The second purpose was to elucidate the effects of fenofibrate on several lipoprotein subclasses, arterial stiffness, carotid intima media thickness, renal function, and albuminuria, utilizing the FIELD study cohort in Helsinki centre. This study was a pre-specified substudy for the FIELD study that is today the biggest randomized, double-blind fibrate study.

Specific aims of the study were
1) to assess the composition and concentration of TRL subclasses, specifically apo CIII concentration in TRLs in type 2 diabetic and non-diabetic subjects,

2) to investigate potential proatherogenic properties of apoCIII-containing LDL from type 2 diabetic patients,

3) to assess the effects of fenofibrate in type 2 diabetic patients on a) the concentration and composition of TRL and HDL subclasses, b) LDL size, and c) the effect of homocysteine on HDL,

4) to examine the effect of fenofibrate treatment on surrogate markers of atherosclerosis, inflammation, and endothelial activation in type 2 diabetic patients, and

5) to elucidate the effect of fenofibrate on several markers albuminuria and renal function.
4. SUBJECTS AND STUDY DESIGN

4.1 Type 2 diabetic subjects

We recruited the study subjects among type 2 diabetic patients participating to the FIELD study in the Helsinki research centre. The study was collaboration between study sites in Australia, New Zealand, and Finland, being administered in the NHMRC Clinical Trials Centre, University of Sydney, Australia. The FIELD study was primary and secondary prevention study addressing effect of fenofibrate in the prevention of cardiovascular morbidity and mortality in patients with type 2 diabetes, with or without established cardiovascular disease (Keech and Field Study Investigators 2004).

Type 2 diabetic men and women aged 50 to 75 years were eligible to the FIELD study using the following lipid criteria: serum (S-) total cholesterol 3.0 to 6.5 mmol/L, plus either S-TG 1.0 to 5.0 mmol/L or S-cholesterol/HDL cholesterol ratio over 4. In Finland a lower limit of total cholesterol was used (upper limit from 5.5 mmol/l to 6.0 mmol/l, based on clinician’s judgement) instead of 6.5mmol/l due to local treatment practice. Subjects with hepatic or renal dysfunction, gallstones, lipid-lowering medication, cyclosporin, alcohol overuse, or other severe mental or physical illness were excluded.

A total of 9795 type 2 diabetic patients were recruited in the study globally (Keech and Field Study Investigators 2004). In Finland, 2068 patients were screened and 1393 recruited. At Helsinki, study subjects were recruited by newspaper advertisements and from several outpatient clinics in the capital area of Finland. The patients were randomly assigned to receive either placebo or micronised fenofibrate (200mg/d) for 5 years in a double-blind design. Statin use was allowed after randomization.

Two hundred and seventy patients were recruited in the FIELD main study in the Helsinki Centre. Of these, 239 (76 female) participants volunteered for this substudy. The main FIELD study recruited patients at Helsinki Centre from January 1998 to June 2000, whereas our substudy started at June 1998. Thus, some patients had entered the blinded treatment phase at June 1998 and were ineligible for this substudy. Some patients had tight daily schedules preventing the participation to substudy. During the study there were 2 deaths and 12 serious adverse events (SAEs) in the placebo group and 5 deaths and 15 SAEs in the fenofibrate group (Figure 8.). We excluded from prospective analysis the patients who had statin added to their medication during the study course (15 in placebo vs. 8 in the...
fenofibrate group). The main FIELD study had its last close-out visits at April 2005 and our last substudy visit was at March 2005.

4.2 Non-diabetic subjects

We recruited 93 healthy non-diabetic control subjects (44 female) by letters to the spouses of the study patients, by advertisements in the hospital magazine and intranet, and among retired pilots. The subjects had to be 50–75 years old, with no signs or history of major chronic diseases except mild treated hypertension. We performed a 75g oral glucose tolerance test (OGTT) to ensure normal glucose tolerance according to the WHO criteria (World Health Organisation 1999). Thus, fasting plasma glucose was required to be <6.1mmol/L, with 2hr glucose value <7.8mmol/L. Subjects with impaired glucose tolerance or impaired fasting glucose, total chol >6.0 mmol/l, LDL chol >4.0mmol/l, S-triglycerides >2.5 mmol/l, transaminase levels over 2 x upper limit normal, or abnormal thyroid stimulating hormone concentration were ineligible. Non-diabetic control subjects were not followed after the baseline substudy visit.

All patients and control subjects signed informed consent forms. The Ethics Committee of the Helsinki University Central Hospital approved the protocol. In addition we acquired approval by the Ethics committees of Helsinki City health care, Vantaa City health care, Peijas hospital, and Jorvi hospital, as we also recruited patients from these sites.
5. METHODS

5.1 Laboratory analyses

Baseline examinations of the type 2 diabetic patients were performed during the placebo run-in period of the FIELD study whereas the 2nd and 5th year examinations were performed during the allocated study treatment. All blood samples were obtained after a 12-hour overnight fast. Serum and EDTA plasma were separated by centrifugation and stored at -80° if not analyzed immediately.

5.1.1 Glucose, insulin and C-peptide measurements

In OGTT performed to non-diabetic control subjects, blood samples were drawn at 0, 30, 60 and 120 minutes for the determination of plasma glucose, serum insulin, and serum C-peptide. Plasma glucose during OGTT was measured instantly by the hexokinase method (Roche Diagnostic Gluco-quant, Basel, Switzerland) using a Hitachi 917 (Hitachi Ltd, Tokyo, Japan). For diabetic patients, we measured only fasting levels of glucose, insulin, and c-peptide. Plasma glucose concentration were analysed by a glucose dehydrogenase method (Precision-G Blood Glucose Testing System, Medisense, Abbott, Illinois, USA). HbA1c was measured by a nephelometric inhibition of agglutination (CV 2.6 %) using a commercially available kit (DCA 2000 Analyzer, Bayer Diagnostics, NY, USA). Serum free insulin concentrations were determined by radioimmunoassay using the Phadeseph Insulin RIA kit (Pharmacia, Uppsala, Sweden, CV 6.5 %). C-peptide was determined by RIA (RIA-coat®C-peptid, Byk-Sangtec Diagnostica, Dietzenbach, Germany, CV 10 %).

5.1.2 Lipid measurements

The lipid measurements were performed in the research laboratory of the Helsinki University Central Hospital, Division of Cardiology, Helsinki, Finland, unless otherwise specified. Fasting serum lipoproteins, including HDL 2 and HDL3 subclasses, were isolated from fresh serum by sequential ultracentrifugation (Taskinen et al. 1988). TRL subclasses VLDL1 (Sf 60–400), VLDL2 (Sf 20–60), and IDL (Sf 12–20) were isolated by density gradient ultracentrifugation (Taskinen et al. 1990). The IDL+LDL fraction (d=1.019–1.063 g/mL) was separated and divided to two similar tubes from all the non-diabetic control subjects and 114 consecutive type 2 diabetic patients. Due to loss of samples during LDL subclass separation, data is available from 101–107 patients as denoted in the Tables.
Followingly, the LDL subclasses LDL1, LDL2, and LDL3 were separated by density gradient ultracentrifugation, modified from Griffin et al. (Griffin et al. 1990), in a Beckman SW40 TI rotor using Ultraclear 9/16 × 33/4 tubes (Beckman Coulter, Inc, Fullerton, CA, USA). The discontinuous NaBr gradient was layered with 0.5 mL \( d = 1.190 \text{ g/mL} \); 2.5 mL sample (1 mL sample of LDL+IDL in 1.5 mL NaBr \( d = 1.100 \text{ g/mL} \)); 1.5 mL \( d = 1.060 \text{ g/mL} \); 1.5 mL \( d = 1.055 \text{ g/mL} \); 1.5 mL \( d = 1.045 \text{ g/mL} \); 2.0 mL \( d = 1.034 \text{ g/mL} \); 2.0 mL \( d = 1.023 \text{ g/mL} \); and 1.0 mL \( d = 1.019 \text{ g/mL} \). The tubes were placed in a Beckman Optima TL ultracentrifuge at 202,000g (ie. 40,000rpm) for 23 h 45 min at 23°C. The tubes were discharged from the top, using Beckman Recovery System and infusing Maxidens solvent (Nyegaard&C A/S, Oslo, Norway) to the bottom of the tubes. The protein absorbance in the samples was monitored with an absorbance meter (Pharmacia, Uppsala, Sweden) and the density gradient was controlled with a DMA 46 density meter (Anton-Paar GmbH, Graz, Austria). Three LDL subclasses LDL1 (\( d = 1.024–1.033 \text{ g/mL} \)), LDL2 (\( d = 1.033–1.042 \text{ g/mL} \)) and LDL3 (\( d = 1.042–1.055 \text{ g/mL} \)) were each collected in a volume of 2.1 mL.

Enzymatic colorimetric assays were used to measure cholesterol (Unimate 7 CHOL, Hoffman-La Roche, Basel, Switzerland for baseline samples and later ABX Diagnostics Cholesterol and ABX Pentra Cholesterol, HORIBA ABX, Montpellier, France), triglyceride (Unimate 7 TRIG, Hoffman-La Roche, Basel, Switzerland for baseline samples and later ABX Diagnostics Triglycerides and ABX Pentra Triglycerides, HORIBA ABX, Montpellier, France), free cholesterol (Boehringer Mannheim, Mannheim, Germany), and phospholipid (Wako Chemicals, Neuss, Germany) concentrations in whole sera and in lipoprotein fractions using a Cobas Mira automatic analyzer (Hoffman-La Roche, Basel, Switzerland). Protein concentrations in the lipoprotein subclasses were measured by a modification of the method of Lowry (Lowry et al. 1951) (DC protein assay, BIO-RAD, California, USA) with Kone/OLL1C848 (Kone, Helsinki, Finland) for baseline samples and later with Perkin-Elmer (Waltham, Massachusetts, USA). Particle mass concentrations of the lipoprotein subclasses were calculated as the sum of concentrations (in mg/dL) of triglycerides, free cholesterol, cholesterylester, phospholipids, and protein. LDL particle diameter i.e. particle size was measured with non-denaturing gradient gel electrophoresis (Vakkilainen et al. 2002). As for lipid measurements, the laboratory participates in an international quality assessment scheme organised by Labquality (Helsinki, Finland).

5.1.3 Apolipoprotein measurements

Apolipoproteins were measured in three research laboratories for the purposes of Studies I-III. Immunoturbidimetric methods were used to measure apolipoprotein B (apoB; Orion Diagnostica, Espoo, Finland), apolipoprotein AI (apoA-I; Wako Chemicals GmbH, Neuss, Germany).
and apolipoprotein AII (apoA-II; own polyclonal antibody produced in rabbits against human apoA-II). LpA-I particle number was measured by differential immunoassay (Sebia, Issy-les-Moulienaux, France) (Parra et al. 1990) and LpA-I apoAII particle number was calculated by subtracting the concentration of LpA-I from total apoA-I concentration, assuming that there is one apoA-I per particle. Serum apoCII and apoCIII were measured using commercially available kits using a single radial immunodiffusion method (Daichi Pure Chemicals, Tokyo, Japan). Due to discontinued production of the apoCII and CIII kits, the levels were determined of 77 control subjects and of the baseline patient samples only. ApoE phenotype was determined by a method based on isoelectric focusing of delipidated serum followed by immunoblotting (Havekes et al. 1987). Our control phenotype samples were genotyped in the central laboratory of Helsinki University Central Hospital to ascertain the method.

For Study I, plasma samples and fractions of VLDL1, VLDL2, and IDL were frozen to -80°C and sent to the laboratory of Prof. Fruchart (Department of Atherosclerosis, Pasteur Institute of Lille). Plasma apoCIII and apoE and apoCIII in TRL fractions were measured by nephelometry, a method allowing small concentrations of apolipoproteins to be determined. The polyclonal antibodies against apoCIII and apoE were generated, using total synthetic apoCIII and purified apoE, in rabbit. An ELISA system was used to search antibody activity from the antisera. The pooled antisera were precipitated with PEG and dialysed. The antibody specificity was tested with Western-Blot against VLDL and HDL. For each antibody, only one band in VLDL and HDL lanes was detected as expected. The antisera was tested by nephelometry against the purified apoproteins to confirm that no signal was detected except for the corresponding purified apoprotein. The quantitation assays of apoCIII and apoE were performed on the Beckman immunochrometry system (IMMAGE) which measures the rate of change in light scatter induced by the formation of the immunoprecipitin complex between the polyclonal antibody and the corresponding apoproteins. The control and the standard used for this study were purchased from Behring. 15μl and 40μl of sample was used for the quantitation of apoCIII and apoE respectively, 200μl of potassium phosphate polymer-enhancing buffer (buffer1, ref: 447650, Beckman coulter) and 20μl of antisera were mixed in a reaction cell. The maximum rate of change of light scatter was determined at an optimized gain setting. The inter-assays CV were 5.2% and 4.2% respectively for apo E and apo CIII measurements. The recovery of apoCIII in lipoprotein fractions (VLDL, IDL, LDL, HDL, and the bottom) was measured in 8 samples and averaged 82 ± 17%. The amount of apoCIII found in the density of > 1.21 g/ml averaged 1.7 ± 1.3 μg/dl, (i.e 0.06% ± 0.05).

For Study II, serum samples and one tube of the separated IDL+LDL fraction were frozen to -80°C and sent to the laboratory of Prof. Jan
Borén (Wallenberg Laboratory and Department of Molecular and Clinical Medicine, Sahlgrenska Academy at Göteborg University, Göteborg, Sweden). From these samples, apo B was determined by an immunoprecipitation method (Thermo Electron Corporation, Vantaa, Finland, CV% 3.5%) and apoCIII by an immunoturbidimetric method (Kamiya Biomedical Company, Seattle, USA, CV 5.2%). All analyses were performed on a Konelab 20 autoanalyser (Thermo Electron Corporation). Again, the concentrations of apolipoproteins in LDL samples were too low to detect in our own laboratory. Plasma apoCIII levels measured by these three methods were closely correlated (from $r=0.772$ to $r=0.918$, pairwise comparisons with Spearman) although the absolute values differed.

5.1.4 Measurements of activities of lipid transfer proteins
Serum samples were frozen to -80°C and analyzed in the laboratory of Prof. Christian Ehnholm (Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland). Serum CETP activity was analyzed after removal of VLDL and LDL by phosphotungstate-magnesium chloride precipitation (Groener et al. 1986). For the PLTP activity assay, phosphatidylcholine small unilamellar vesicles were prepared. Each assay contained HDL3 acceptor, [14C]phosphatidylcholine-vesicles, sample (4μl or 10μl of 1:10 diluted serum), and sample buffer in a final assay volume of 400 μl. Assay tubes were incubated for 45 min at +37°C after which the excess of vesicles and plasma-derived apoprotein B-containing lipoproteins were precipitated (Jauhiainen and Ehnholm 2005). LCAT was analyzed as described previously (Jauhiainen and Dolphin 1986).

5.1.5 Measurements of LDL function and lipid composition
The LDL samples of our patients were further analyzed regarding function, lipid composition, and in vitro modelling. The binding of human plasma LDL to biglycan was determined with solid-phase assay using Maxisorp immunoplates coated with biglycan (Skalen et al. 2002). Enrichment of LDL with human purified apoCIII (Chemicon, Temecula, CA, USA) was performed as described for apoE (Skalen et al. 2002).

Lipid classes were detected and quantified using a PL-ELS 1000 detector (Polymer Laboratories, Amherst, MA, USA) (Ekroos et al. 2002). The ceramide levels were low, and were collected using a Gilson FC 204 fraction collector (Gilson Inc., Middleton, WI, USA). The lipids were determined from a total lipid extract using a QSTAR XL QqTOF mass spectrometer (MDS Sciex, Concord, Canada) and normalized against the apoB protein value. The GM1 ganglioside was measured using a microtiter well binding assay (Wang and Gustafson 1995).
5.1.6 Homocysteine and markers of inflammation and endothelial activation

Circulating ICAM-1, VCAM-1, E-selectin 1, ultra-sensitive CRP, SAA, IL-6, and PLA2 concentrations were determined in the laboratory of Dr. Sven-Olof Olofsson (Wallenberg Laboratory and Department of Molecular and Clinical Medicine, Sahlgrenska Academy at Göteborg University, Göteborg, Sweden) by commercially available ELISA; the ICAM-1 (CV 7.4%), VCAM-1 (CV 9.2%), E-selectin (CV 7.3%), and IL-6 (CV 15.1%) by R&D Systems, MN, USA; the ultra-sensitive CRP kit (CV 12.8%) by Medix Biochemica, Kauniainen, Finland; the SAA kit (CV 21.9%) by Biosource International, Camarillo, CA, USA; and the sPLA2 kit (CV 22.1%) by Cayman Chemical Company, Ann Arbor, USA. Plasma homocysteine was determined by a fluorescence polarization immunoassay (Abbott Laboratories, Illinois, USA) in the laboratory of Prof. Jouko Sundvall (Department of Chronic Disease Prevention, National Institute of Health and Welfare, Helsinki, Finland).

5.2 Intima-media thickness of the carotid arteries

Our non-diabetic control subjects visited the carotid ultrasound once, and the type 2 diabetic patients were studied at baseline, at 2nd year, and at study close at 5th year (Leinonen et al. 2002). Ultrasound scans were performed with a Hewlett Packard Image Point M2410A equipped with a high frequency 10 MHz linear array transducer and videotaped with a Panasonic AG-MD830E PAL S-VHS VCR. Both carotid arteries were scanned from three projections for the distal 1 cm of CCA and the entire CB, whereas for the proximal 1 cm of ICA the best visualized view was selected by the sonographer. Both far wall and near wall were measured. Ultrasound images were analyzed with a computer interfaced to a PAL S-VHS VCR, at Oy Jurilab Ltd (www.jurilab.com) in Kuopio. 100 measurements per 1 cm were measured with Prosound software (Caltech, Pasadena, CA, USA) (Selzer et al. 1994).

The mean of maximal IMT (Max IMT) was chosen for the primary outcome variable. Secondary outcome variables were 1) the mean of mean IMTs (Mean IMT) over all scanned carotid sites, 2) the mean of mean far wall IMT over all scanned carotid far wall sites (FW), 3) the mean of maximum over all scanned CCA sites, 4) the mean of maximum over all scanned CB sites, 5) the mean of the maximum for all scanned ICA sites, and 6) the plaque height difference between site-specific maximums and minimums averaged for all scanned carotid sites (Plaque).

Eeva Leinonen performed the ultrasounds at baseline and 2nd year, and Anne Hiukka at 5th year. The intra-observer repeatability (R) for Max IMT was 0.994 with a standard error of measurement errors (SE) 0.0152 for
E.L and R was 0.971 with a SE of 0.029 for A.H. The inter-observer R was 0.950 and SE 0.035. The IMT scans of the patients were read by ultrasound technicians Arja Malkki (R=0.996, SE 0.0082) and Jarmo Tiikkainen (R=0.986, SE 0.032), their inter-reader R was 0.977 and SE 0.040. Both readers and sonographers were blinded to the treatment group, but not to time sequence.

5.3 Pulse wave analysis

When the arterial pressure wave generated in systole travels through the arteries, at different points of discontinuity the pressure wave is reflected back (Safar et al. 2003). These two waves and the timing between them generate the amplitude and shape of the pulse wave (Safar et al. 2003). As arterial stiffness increases, the velocity of both waves increase. Therefore the backward reflected wave arrives earlier in the central aorta and increases (ie. augments) the arterial pressure during late systole, causing an increase in ventricular afterload (O’Rourke and Mancia 1999). The augmentation index (AIx) is the central pressure divided by the pulse pressure, and it is a measure of arterial stiffness (Kelly et al. 1989) (Figure 9.).

Pulse wave analysis (PWA) was used in this study to measure central aortic pressure, augmentation, and the AIx (O’Rourke and Gallagher 1996, Westerbacka et al. 1999). Pressure waves were recorded from the radial artery by applanation tonometry (SPC-301; Millar Instruments, Houston, TX, USA). A validated and generalised transfer function (Wilkinson et
al. 1998) was then used to construct the corresponding central arterial waveform. The aortic waveform was further analysed to calculate aortic pressure augmentation, the AIx, central blood pressure, ejection duration, and Buckberg’s subendocardial viability ratio (SEVR, area of diastole divided by area of systole during one cardiac cycle in the aorta).

The measurement of AIx is highly reproducible (Wilkinson et al. 1998). A sole investigator (Jukka Westerbacka) performed all measurements. Data were collected directly into computer and processed with SphygmoCor Blood Pressure Analysis System (BPAS-1; PWV Medical, Sydney, Australia), which allows continuous recording of the waveform. An integral system software was used to calculate an average radial artery waveform, and to generate the corresponding aortic pressure waveform with a validated transfer factor (Pauca et al. 2001). Blood pressure was measured with a calibrated mercury sphygmomanometer after a 10 minutes rest in the supine position. The average of three measurements was used for analysis.

5.4 Measures of renal function and albuminuria

Serum/plasma/urine creatinine was measured using the Jaffe’s method and later using an enzymatic method. Due to 15% lower levels of creatinine with the enzymatic method, a conversion factor of 0.85 was used for values measured with Jaffe’s method. The values from the two methods were highly correlated with R²=0.977. Albumin concentration from the timed overnight urine samples were analyzed by an immunoturbidimetric method. At baseline, AER was measured from three consecutive overnight samples, and the median was used in the analysis. The results of these 3 samples were highly significantly correlated (r=0.904–0.966, p<0.001). Thus, at the 2nd year and at the 5th year one AER was measured. The patients collected a 24h urine sample at each substudy visit, and urinary protein excretion rate (measured by a turbidimetric benzetoniumchloride method) and creatinine clearance was calculated from that sample. Estimated GFR was calculated both by the Cockroft-Gault equation and MDRD-4 (Modification of Diet in Renal Diseases). The estimates of GFR and creatinine clearance were normalized to body surface area by DuBois formula. Main FIELD study data on ACR, determined from a spot sample, was also used. Cystatin C was measured by an immunoprecipitation method (Thermo Fisher Scientific, Vantaa, Finland).

5.5 Anthropometric variables

We measured waist circumference (midway between lowest rib and iliac crest) and hip circumference (maximum of buttocks). Height was
measured at baseline and at 5th year visits. Body mass index (BMI, kg/m²) was calculated by dividing weight in kilograms by height in meters squared. We used the waist circumference as a measure of body fat distribution.

5.6 Statistical analysis

The statistical analysis was performed using SPSS 11.0–14.0 for Windows (SPSS Inc., Chicago, USA). Results are shown as mean and standard deviation or median and interquartile range (IQR) in case of skewed distribution of the variable. Qualitative variables are presented as N (%). Factors that were not normally distributed were log transformed. During treatment, changes in qualitative variables were compared using Fishers’ exact test or 2x2 likelihood ratio test for transition probability matrixes (http://www.kttl.helsinki.fi/sarna/Stats/LRtest2x2.xls). Continuous variables during treatment were compared using Repeated Measures ANOVA if possible or the Mann–Whitney U test for non-normally distributed variables: we calculated the percentage changes from baseline to study close and compared these changes between the treatment groups. Treatment effects are shown with median difference of the percentage changes (±95% confidence interval) from baseline between the groups, calculated with CIA 2.1.2 (www.som.soton.ac.uk/cia/). We used repeated measures ANOVA and Wilcoxon Signed Rank test matched pairs to compare the changes within the groups. Significant correlations were studied using two-tailed Pearson correlation for normally distributed variables and two-tailed Spearman correlation for non-normally distributed variables.

In study I and II, where appropriate, samples were compared using the general linear model, univariate analysis of variance, adjusted for age, BMI, and gender. In matched-pairs settings in Studies I and II, samples were compared using the paired samples t-test. In study I, the frequency distribution of apoE phenotype between the groups was compared with the Fisher's exact test and the means of plasma apoE concentration in the groups of apoE phenotype were compared with the F-test and further with the Scheffe's method. To compare the baseline data divided to quartiles by homocysteine in Study III, we used the Jonckheere–Terpstra trend-test for several samples. In study II, One Way Analysis of Variance with All Pairwise Multiple Comparison Procedures (Tukey Test) was used and binding parameters and their standard errors were determined using the non-linear regression (curve-fitting). Linear regression slopes for patients and controls were compared using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). We used linear regression analysis to calculate the individual slopes of IMT progression or regression in Study IV. A p-value of <0.05 was considered significant in all analyses.

5. Methods
6. RESULTS

6.1 Study subjects

The characteristics of the study subjects are shown in Table 2. and 3. There were more males than females in our diabetic cohort, whereas the gender distribution was almost equal among the control subjects. The control subjects were slightly younger than the patients, and they had lower waist circumference and BMI. Mean values for total and LDL cholesterol were similar between these two groups, but type 2 diabetic patients had smaller LDL size than non-diabetic control subjects. Consistent with diabetic dyslipidemia, the patients had lower HDL cholesterol and higher levels of TG than control subjects. The type 2 diabetic patients were in good glycemic control with HbA1c of 7.1% at the baseline. Systolic and diastolic blood pressures were higher in type 2 diabetic patients.

<table>
<thead>
<tr>
<th>Table 2. Characteristics at baseline.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control (n=93)</th>
<th>T2DM (n=239)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>49 / 44</td>
<td>161 / 78</td>
<td>0.010</td>
</tr>
<tr>
<td>Age</td>
<td>59 (54–63)</td>
<td>62 (56–67)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist</td>
<td>88 (78–97)</td>
<td>100 (93–109)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>24.9 (23.3–27.4)</td>
<td>29.6 (26.8–33.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>52 (56%)</td>
<td>Never 100 (42%)</td>
<td>&lt;0.043</td>
</tr>
<tr>
<td>Ex</td>
<td>33 (35%)</td>
<td>Ex 106 (44%)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>7 (8%)</td>
<td>Current 33 (14%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose mmol/L</td>
<td>5.0 (4.6–5.5)</td>
<td>7.6 (6.5–9.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1C %</td>
<td>5.2 (5.0–5.4)</td>
<td>7.1 (6.3–7.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.3 (1.0–1.8)</td>
<td>4.6 (2.7–6.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Syst BP mmHg</td>
<td>134 (124–143)</td>
<td>142 (133–152)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diast BP mmHg</td>
<td>82 (78–87)</td>
<td>88 (80–93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-Chol mmol/L</td>
<td>5.1 (4.6–5.5)</td>
<td>5.0 (4.6–5.6)</td>
<td>0.748</td>
</tr>
<tr>
<td>LDL Chol mmol/L</td>
<td>3.1 (2.7–3.5)</td>
<td>3.1 (2.8–3.6)</td>
<td>0.219</td>
</tr>
<tr>
<td>TG mmol/L</td>
<td>1.0 (0.8–1.4)</td>
<td>1.6 (1.2–2.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL Chol mmol/L</td>
<td>1.6 (1.3–1.9)</td>
<td>1.1 (1.3–1.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Notably, Finnish study subjects in general had slightly higher median HbA1c (7.6%) compared to the subjects from Australia and New Zealand (HbA1c 6.8% for both). This difference might be due to longer duration of diabetes in Finnish study subjects, median of 7 (IQR 3–11) years at the baseline as compared to the 5 (IQR 2–10) years of the whole FIELD study.

Concerning the modes of diabetes treatment, there were no differences between the fenofibrate and the placebo groups (Table 4). During the 5-year surveillance of the eligible 171 patients, treatment of hyperglycemia intensified similarly in both groups. Notably, compared to the whole study population, the Finnish FIELD study patients had less single-agent oral therapy or diet-alone and more combination therapy of oral agents and insulin. This can be due to the more progressed state of diabetes or different guidelines of care.

Systolic and diastolic blood pressures were higher in Finland with 147/86 mmHg compared to 139/81 mmHg in Australia. In the whole FIELD study group, as well as in our patients, the systolic and diastolic blood pressures decreased during the study. This was achieved by increased

6. Results
use of antihypertensive medication. The use of angiotensin-receptor blockers emerged to the market during the study period, and the use of other antihypertensive agents also increased. In our study group, the use of betablockers and nitrates increased more in the fenofibrate treatment group (Table 4.).

<table>
<thead>
<tr>
<th>Table 4. Medication at baseline and at 5th year.</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=239)</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>Hypertension treatment</td>
</tr>
<tr>
<td>ACE-inh.</td>
</tr>
<tr>
<td>ATR-blockers</td>
</tr>
<tr>
<td>Beta-blockers</td>
</tr>
<tr>
<td>Ca-blockers</td>
</tr>
<tr>
<td>Diuretics</td>
</tr>
<tr>
<td>Nitrates</td>
</tr>
<tr>
<td>ASA</td>
</tr>
</tbody>
</table>

Data are N (%) and p-value from 2X2 likelihood ratio –test for transition probability matrixes.

Seventy-one type 2 diabetic patients were diagnosed with previous CVD (defined as one or more of the following: angina pectoris, myocardial infarction, previous coronary artery bypass grafting (CABG) or percutaneous coronary intervention, stroke, TIA, carotid endarterectomy, claudication, leg amputation, peripheral arterial reconstruction or balloon dilatation as determined on the basis of clinical history and examination, including resting electrocardiography, and review of all available patient records). Of the patients who were eligible for the 5th year substudy visit, 51 had CVD at baseline, 59 at the 2nd year substudy visit, and 63 at the 5th year substudy visit. There were no significant differences between the two treatment arms in the prevalence of CVD during the study.

Statin therapy was allowed after the randomisation. During the FIELD study, several landmark studies were published, including Heart Protection Study. However, the use of statins increased differentially in the FIELD study countries (Figure 10.). In Finland 18% of the FIELD study patients used statins, compared to the about 30% in Australia and New Zealand. The factors behind this difference were partly economical, since in New
Zealand the statin drugs were 100% reimbursed, whereas in Finland the 72% reimbursement required a diagnosis of coronary heart disease. In Finland the Current Care guidelines for treatment of dyslipidemia were published in August 2004, recommending statin use for all type 2 diabetic patients with LDL cholesterol >2.5 mmol/L.

6.2 Alterations of VLDL, LDL and HDL subclasses and apoproteins in type 2 diabetes

6.2.1.1 VLDL subclasses in type 2 diabetes (Study I)
Type 2 diabetic patients had an increase in VLDL1 and VLDL2 triglycerides, reflecting the increase of total triglycerides (Table 5.). Analysis of the percentage composition of VLDL1, VLDL2 and IDL particles revealed, that the percentage of TG in these particles was not increased in type 2 diabetic patients (Figure 11A-C). Rather, their VLDL1 contained relatively more CE and phospholipids but less TG and free cholesterol than that of non-diabetic subjects. VLDL2 in type 2 diabetic patients was relatively enriched with CE and poor in free cholesterol and protein compared to control subjects. The composition of IDL particles was similar in the two groups. The mass concentrations of VLDL1 and VLDL2 were increased in type 2 diabetic compared to non-diabetic subjects by 125 and 82% (p<0.001) (Table 5.). Thus, the increased number of TRL particles rather than compositional changes seem to account for the hypertriglyceridemia in type 2 diabetes.
Table 5. TG and mass concentration of TRLs in control subjects and T2DM patients.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 93)</th>
<th>T2DM (n = 239)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL1 triglyceride (mmol/l)</td>
<td>0.31 (0.16–0.49)</td>
<td>0.65 (0.37–1.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL1 mass concentration (mg/dL)</td>
<td>40 (20–62)</td>
<td>87 (49–142)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL2 triglyceride (mmol/l)</td>
<td>0.17 (0.1–0.24)</td>
<td>0.31 (0.21–0.41)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL2 mass concentration (mg/dL)</td>
<td>33 (22–45)</td>
<td>58 (40–80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IDL triglyceride (mmol/l)</td>
<td>0.08 (0.06–0.10)</td>
<td>0.10 (0.07–0.13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IDL mass concentration (mg/dL)</td>
<td>35 (26–44)</td>
<td>39 (30–52)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median + interquartile range. * Adjusted for age, gender, BM with ANOVA.

Figure 11A-C. Composition of TRL particles at baseline.
VLD1 particles were the most abundant TRLs both in the type 2 diabetic patients and in non-diabetic controls. Triglycerides in VLDL1 and VLDL2 correlated strongly with total triglyceride levels in both diabetic subjects and in control subjects (Figure 12a-b). However, in both groups the slope for VLDL1 was much steeper than that for VLDL2 (p<0.001). Thus, for each increment of serum triglycerides, triglycerides in VLDL1 increased more than in VLDL2 particles. The regression lines of VLDL1 and VLDL2 TG and total TG were similar between diabetic and the control group (Figure 12c-d).

Figure 12 A-D. The relationship of VLDL 1 and VLDL 2 triglyceride and serum triglyceride concentrations in type 2 diabetic patients and control subjects.

In A and B, (•) for VLDL1 TG and (+) for VLDL 2 TG. In A, r=0.879, p<0.001 for VLDL 1 TG and r=0.870, p<0.001 for VLDL 2 TG in the control group. In B, r=0.895, p<0.001 for VLDL1 TG and r=0.771, p<0.001 for VLDL 2 TG in the patient group. Difference between the slopes p<0.001 in A and B. In C and D, (•) for type 2 DM patients and (+) for control subjects. Difference between the slopes p=0.27 for C and p=0.85 for D.
6.2.1.2 Apoproteins CII, CIII and E (Study I)

The concentrations of plasma total apoCIII and apoCII were significantly elevated in diabetic patients (Table 6). The ratio of serum apoCII/apoCIII was elevated in type 2 diabetic patients (0.50±0.33 vs. 0.40±0.10, p<0.001). Plasma apoE levels were reduced, resulting in higher ratio of apoCIII/apoE in diabetic patients compared to control subjects (1.35±0.42 vs. 0.73±0.21, p<0.001). The distribution of apoE phenotype did not differ between diabetic patients and control subjects (data not shown). Plasma apoE concentrations were similar in the groups of different apoE phenotypes in type 2 diabetic patients (data not shown). Likewise apoE phenotype did not have significant effects on triglyceride and cholesterol contents in VLDL subclasses (data not shown). ApoCIII in VLDL1 particles was slightly higher in diabetic patients than in control subject. In contrast type 2 diabetic subjects had significantly less apoCIII in VLDL 2 and IDL particles than non-diabetic subjects.

Table 6. Apo CIII at baseline in plasma and TRL subclasses.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 93)</th>
<th>T2DM (n = 239)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total apo CII (mg/dL)</td>
<td>4.3 (3.4–5.0)</td>
<td>3.12 (2.35–4.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total apo CIII (mg/dl)</td>
<td>2.75 (2.2–3.4)</td>
<td>4.66 (3.93–5.40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total apo E (mg/dl)</td>
<td>3.80 (3.18–4.83)</td>
<td>3.57 (3.00–4.16)</td>
<td>0.020</td>
</tr>
<tr>
<td>VLDL1 apo CIII (mg/dl)</td>
<td>0.40 (0.19–0.61)</td>
<td>0.43 (0.33–0.60)</td>
<td>0.039</td>
</tr>
<tr>
<td>VLDL 2 apo CIII (mg/dl)</td>
<td>0.24 (0.17–0.39)</td>
<td>0.19 (0.15–0.24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IDL Apo CIII (mg/dl)</td>
<td>0.09 (0.06–0.12)</td>
<td>0.06 (0.03–0.09)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR). p values are based on T-test with log-transformed values or Mann-Whitney U-test for non-normally distributed values.

6.2.1.3 Relationships between VLDL subclasses and apoCIII (Study I)

There was a close correlation between plasma apoCIII and triglycerides in non-diabetic subjects but the correlation was much weaker in type 2 diabetic subjects (r=0.762, p<0.001 vs. r=0.473, p<0.001, p=0.001 for the difference between the slopes). In contrast the correlation between serum apoCII and triglycerides was stronger in type 2 diabetic subjects compared to control subjects (r=0.641, p<0.001 vs. r=0.431, p<0.001). Plasma apoE correlated positively with triglycerides in non-diabetic subjects but negatively in diabetic patients (r=0.513, p<0.001 vs. r= -0.278, p<0.001, p<0.001 for the difference between the slopes). The levels of serum apoCII and apoCIII were closely correlated in control subjects (r=0.727, p<0.001)
as well as in type 2 diabetic patients \((r=0.601, p<0.001)\). Plasma apoCIII and apoE were strongly related in non-diabetic subjects \((r=0.502, p<0.001)\) but this relationship was only marginal in the diabetic patients \((r=0.197, p<0.005)\).

Strong correlations between apoCIII and triglycerides were observed in both VLDL1 and in VLDL2 in control subjects and in type 2 diabetic patients. The slope for apoCIII in VLDL1 in non-diabetic subjects was considerably steeper than in type 2 diabetic subjects (Figure 13a, \(p<0.001\)). Likewise the slope for apoCIII in VLDL2 was considerably steeper in control subjects (Fig. 13b, \(p<0.001\)). Thus, for the increment of TG there was smaller increase of apoCIII in VLDL1 and VLDL2 in diabetic subjects than in control subjects. The association between TG and apoCIII in IDL was significant in diabetic subjects \((r=0.616, p<0.001)\) but not in control subjects \((r=0.204, p=0.051)\).

Figure 13 A, B. The relationship of apo CIII and triglycerides in VLDL 1 (A) and VLDL 2 (B).

In A and B, (•) for type 2 DM patients and (○) for control subjects. In A, \(r=0.900, p<0.001\) for control group and \(r=0.756, p<0.001\) in type 2 diabetic patients. In B, \(r=0.803, p<0.001\) for control group and \(r=0.575, p<0.001\) for type 2 diabetic patients. Difference between the slopes \(p<0.001\) in panel A and B.
6.2.1.4 Apoproteins B, CII, CIII, and E in matched-pairs (Study I)
The diabetic patients and control subjects were matched for gender, BMI (mean intra-pair difference 1.14 kg/m²) and serum TG levels (mean intra-pair difference 0.09 mmol/l). Altogether 52 matched pairs (31 male and 21 female pairs) were found. Despite of similar serum TG level (1.2 mmol/l in both groups) diabetic subjects had lower HDL cholesterol (1.5±0.4 vs. 1.2±0.5 mmol/l, p=0.004). The concentrations of plasma apoB (96.0±13 vs. 90.5±15 mg/dl, p=0.005) and apoCIII (4.37±0.8 vs. 3.12±0.8 mg/dl, p<0.001) were higher in diabetic patients than in control subjects. The concentrations of plasma apoCII and apoE did not differ between the pairs. The concentration of apoCIII in VLDL1 (0.34±0.18 vs. 0.57±0.42 mg/dl, p=0.001), VLDL2 (0.17±0.08 vs. 0.35±0.25 mg/dl, p<0.001) and IDL (0.05±0.02 vs. 0.12±0.08 mg/dl, p<0.001) was significantly lower in diabetic patients than in normal subjects.

6.2.2.1 LDL subclasses in type 2 diabetes (Study II)
Despite similar levels of LDL cholesterol, LDL-apoB was higher and LDL particle size smaller in patients with diabetes (Table 7). This was clearly reflected in the cholesterol levels of LDL subclasses, with higher cholesterol concentrations in subclasses LDL2 and LDL3 and lower cholesterol concentration in LDL1 in the patients with diabetes. The LDL-apoCIII concentration and LDL-apoCIII/apoB molar ratio were increased in the patients with diabetes. In addition, there was a relative enrichment of

Table 7. Characteristics of LDL in control subjects and patients with T2DM at the baseline.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=93)</th>
<th>T2DM (n=107)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL size, nm</td>
<td>26.5 (25.9–27.1)</td>
<td>25.9 (25.1–26.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-apoCIII (mg/dL)</td>
<td>0.47 (0.33–0.77)</td>
<td>0.73 (0.37–1.07)</td>
<td>0.009</td>
</tr>
<tr>
<td>LDL1 cholesterol, mmol/L</td>
<td>1.61 (1.31–2.00)</td>
<td>1.34 (1.13–1.67)</td>
<td>0.007</td>
</tr>
<tr>
<td>LDL2 cholesterol, mmol/L</td>
<td>1.28 (1.06–1.53)</td>
<td>1.50 (1.29–1.83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL3 cholesterol, mmol/L</td>
<td>0.26 (0.22–0.32)</td>
<td>0.30 (0.25–0.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL1 particle mass, mg/dL</td>
<td>193 (155–226)</td>
<td>164 (140–206)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL2 particle mass, mg/dL</td>
<td>149 (123–180)</td>
<td>177 (156–220)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL3 particle mass, mg/dL</td>
<td>34 (28–40)</td>
<td>39 (33–50)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data presented as median (IQR). For LDL size, n=239 at baseline. * adjusted for age, BMI and gender with ANOVA.
triglyceride percentage and depletion of protein in all LDL subclasses in the patients with diabetes (Figure 14A-C).

To study the importance of apoCIII on LDL−proteoglycan binding, the patients with diabetes were divided into two groups according to median value of LDL-apoCIII. The patients with high LDL-apoCIII were paired with patients having low LDL-apoCIII based on age, HbA1c, LDL size, and LDL cholesterol levels, resulting to 12 matched pairs. The matched pairs had similar particle composition in all three LDL subclasses. The LDL-apoCIII/apoB molar ratios in the low and high LDL-apoCIII groups were 0.67±0.27 and 2.64±0.86 (mean±SD), respectively ($P<0.001$). LDL particles in A, LDL2 particles in B, and LDL3 particles in C. P-values from Mann-Whitney U-test comparing the percentage values between type 2 diabetic patients and control subjects.

with high apoCIII displayed higher binding to biglycan than LDL with low apoCIII in every pair. The dissociation constants ($K_d$) of LDL in the low and high apoCIII groups were 14.1±0.92 and 11.9±1.15 (mean±SD), respectively ($P<0.001$).
6.2.2.2 In vitro modification of LDL (Study II)
The low LDL-apoCIII samples were enriched with apoCIII \textit{in vitro} so that the particles on average contained as much apoCIII as the high LDL-apoCIII samples. The aim was to clarify whether the increased binding of high LDL-apoCIII to proteoglycans is mediated by apoCIII or other intrinsic properties of the LDL. ApoCIII-enriched LDL displayed higher binding to biglycan than LDL with low apoCIII, but significantly lower binding than LDL with endogenously high apoCIII (K_d=11.0±1.0 nmol/L). Identical binding study was performed with non-diabetic control LDL. ApoCIII content of control LDL did not significantly increase the binding to biglycan. Taken together, these data indicate that apoCIII \textit{per se} slightly increases the binding of diabetic LDL to arterial wall proteoglycans. However, other characteristics of diabetic LDL seem to be more essential for the increased proteoglycan binding of apoCIII-containing LDL.

6.2.2.3 Lipid composition of the LDL particles (Study II)
On the next step, alterations in the lipid composition of the LDL particles and their relation to the apoCIII content were examined by a mass-spectrometric approach. Phosphatidylcholine, the major membrane lipid on LDL, did not correlate with the apoCIII/apoB molar ratio in controls (n=20) or patients with type 2 diabetes (n=20). In contrast, a significant negative correlation existed between the apoCIII/apoB molar ratio and the content of unesterified cholesterol, sphingomyelin, ceramide and the ceramide-containing ganglioside GM1 in LDL isolated from subjects with type 2 diabetes, but not in LDL isolated from controls. Furthermore, LDL isolated from subjects with type 2 diabetes contained significantly less GM1 than LDL isolated from controls (26.5 vs. 42.3 μmol/mg apoB, respectively, P<0.001).

Notably, apoCIII is not present in every LDL particle. In this study, 29±5% of the LDL particles contained apoCIII, and the apoCIII-containing LDL had significantly less unesterified cholesterol, sphingomyelin and ceramide, and significantly more triacylglycerol than apoCIII-free LDL (Table 8.). Analysis of the LDL-apoCIII/apoB molar ratio vs. the LDL diameter in all available samples showed a significant negative correlation in both controls (n=101; P<0.05) and subjects with type 2 diabetes (n=93; P<0.0001).

| Table 8. Lipid composition of apoCIII-containing LDL and apoCIII-free LDL. |
|-----------------|-----------------|-----------------|-----------------|
|                 | ApoCIII-containing LDL | ApoCIII-free LDL | P-value |
| TG/PC (ratio weight) | 0.21±0.04 | 0.15±0.03 | 0.008 |
| FC/PC (ratio weight) | 0.44±0.05 | 0.55±0.09 | 0.007 |
| SM/PC (signal ratio) | 15.0±2.7 | 18.0±3.0 | 0.007 |
| Cer (pmol/μg PC) | 3.5±1.0 | 4.7±1.1 | 0.037 |

FC, free cholesterol; SM, sphingomyelin; Cer, ceramide; PC, phosphatidylcholine (mean±SD, n=8).
6. Results

6.2.3.1 HDL subclasses in type 2 diabetes (Study II and unpublished data)

HDL cholesterol was reduced in type 2 diabetic patients. However, the reduction was more apparent in the larger and more buoyant HDL 2 particles (Table 9.). The composition of the particles also differed, as type 2 diabetic patients had relatively more TG and less free cholesterol in both HDL subclasses. (Figure 15). The particle mass concentrations of HDL2 and HDL3 were lower in type 2 diabetic patients. Levels of apoAI as well as apoAII were lower in type 2 diabetic patients. Altogether, these results imply that both the number of the HDL particles and the compositional changes are relevant for the decrease of HDL cholesterol in type 2 diabetes.

Table 9. Cholesterol concentration, particle mass of HDL2 and HDL3, and concentrations of apolipoproteins and apoA-containing lipoproteins in control subjects and T2DM patients at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=93)</th>
<th>T2DM (n=239)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL2-C (mmol/l)</td>
<td>0.71 (0.49–0.95)</td>
<td>0.41 (0.30–0.56)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL3-C (mmol/l)</td>
<td>0.86 (0.75–0.97)</td>
<td>0.71 (0.64–0.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL2 particle mass (mg/dl)</td>
<td>153 (107–195)</td>
<td>91 (72–126)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL3 particle mass (mg/dl)</td>
<td>242 (217–260)</td>
<td>213 (193–233)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoA-I (mg/dl)</td>
<td>148 (130–166)</td>
<td>134 (122–146)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoA-II (mg/dl)</td>
<td>36 (31–40)</td>
<td>33 (30–37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LpA-I (mg/dl)</td>
<td>57 (47–73)</td>
<td>49 (41–61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LpA-I-AII (mg/dl)</td>
<td>86 (79–97)</td>
<td>83 (76–91)</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR). P-values are based on T-test with log-transformed values or Mann-Whitney U-test for non-normally distributed values.
6.3 Effect of fenofibrate on VLDL, LDL, and HDL subclasses in type 2 diabetes

6.3.1 Effect of fenofibrate on VLDL subclasses (Study III and unpublished data)

The decrease in serum triglycerides during fenofibrate treatment reflected mainly the reduction of VLDL1 TG, with a smaller but significant change of VLDL2 TG (Table 10.). TG and particle mass of VLDL1 and VLDL2 were decreased in similar proportions. Minor increases in the percentage of TG and decreases in the percentage of cholesterylesther were detected in all TRL subclasses in the fenofibrate treatment group (data not shown). Activities of lipid transfer proteins (CETP, PLTP and LCAT) remained unaffected by fenofibrate (data not shown). In an interim 2nd year substudy published as an abstract (Hiukka et al. 2004), we showed that fenofibrate decreased plasma apoCIII levels compared to the placebo group (4.18 ± 2.03 mg/dL vs. 2.93 ± 1.30 mg/dL, p<0.001). Accordingly, apoCIII was decreased in VLDL subclasses. This was however not in proportion to the reduction of TRL TG, since the apoCIII/TG –ratio was lower in all TRL subclasses in the fenofibrate treatment group (data not shown).
Table 10. TG and mass concentration of TRLs in T2DM patients during the study.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=84)</th>
<th>Fenofibrate (n=87)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5th year</td>
<td>Baseline</td>
</tr>
<tr>
<td>Total apo CIII (mg/dl)</td>
<td>4.6 (3.85–5.19)</td>
<td>3.74 (2.83–5.20)</td>
<td>4.67 (3.95–5.66)</td>
</tr>
<tr>
<td>Total apo E (mg/dl)</td>
<td>3.54 (3.00–4.22)</td>
<td>4.03 (3.31–4.99)</td>
<td>3.76 (3.00–4.25)</td>
</tr>
<tr>
<td>VLD1 triglyceride (mmol/l)</td>
<td>0.65 (0.38–1.05)</td>
<td>0.67 (0.40–1.03)</td>
<td>0.62 (0.36–1.06)</td>
</tr>
<tr>
<td>VLDL1 mass concentration (mg/dL)</td>
<td>88 (50–144)</td>
<td>81 (51–126)</td>
<td>85 (49–142)</td>
</tr>
<tr>
<td>VLDL2 triglyceride (mmol/l)</td>
<td>0.31 (0.21–0.39)</td>
<td>0.30 (0.22–0.41)</td>
<td>0.30 (0.18–0.41)</td>
</tr>
<tr>
<td>VLDL2 mass concentration (mg/dL)</td>
<td>60 (42–78)</td>
<td>54 (42–80)</td>
<td>58 (39–78)</td>
</tr>
<tr>
<td>IDL triglyceride (mmol/l)</td>
<td>0.09 (0.08–0.12)</td>
<td>0.09 (0.08–0.11)</td>
<td>0.10 (0.07–0.12)</td>
</tr>
<tr>
<td>IDL mass concentration (mg/dL)</td>
<td>39 (30–50)</td>
<td>40 (32–47)</td>
<td>38 (30–49)</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR). p values are based on comparing the percentage changes from baseline between the groups with Mann–Whitney U test.

6.3.2 Effect of fenofibrate on LDL subclasses (unpublished data)

LDL cholesterol was expectedly reduced during fenofibrate treatment. This was associated with changes in both LDL2 and LDL3 cholesterol, without change in LDL1 cholesterol (Table 11.). LDL2 and LDL3 particle mass concentrations were similarly reduced, which was reflected in a significant increase in LDL particle size in the fenofibrate-treated group. In fenofibrate group, cholesterylester was decreased and TG increased in LDL1–2, corresponding to the similar changes in TRL subclasses (data not shown).

In the fenofibrate treatment subgroup compared to the placebo group, there were significant reductions in LDL-apoCIII and LDL-apoB. These results indicate that fenofibrate treatment improved the lipoprotein profile. However, neither the total apoCIII/apoB nor the LDL-apoCIII/apoB ratios changed significantly with fenofibrate treatment (data not shown).
6. Results

6.3.3 Effect of fenofibrate on HDL subclasses (Study III)

Fenofibrate treatment resulted in changes in the HDL2 and HDL3 subclasses (Table 12.), with no change in total HDL cholesterol. HDL2 cholesterol decreased and HDL3 cholesterol increased in the fenofibrate group relative to the placebo group. The observed changes in the cholesterol levels were similar to the changes in the particle mass of HDL2 and HDL3. During the study, levels of apoA-I remained similar, whereas apoA-II increased in the fenofibrate group. This difference was reflected in the apoA-I-containing lipoprotein particles in the fenofibrate group, with a marked decrease in LpA-I and an increase in LpAI-AII particles. HDL particle composition was slightly changed in the fenofibrate group, with lower cholesterylester in HDL2 and higher free cholesterol in HDL3 (data not shown).

Table 11. Characteristics of LDL in patients with T2DM during the study.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=37)</th>
<th>Fenofibrate (n=34)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5th year</td>
<td>Baseline</td>
</tr>
<tr>
<td>LDL size, nm</td>
<td>25.9 (25.2–26.7)</td>
<td>25.8 (24.8–26.5)</td>
<td>25.8 (25.2–26.7)</td>
</tr>
<tr>
<td>LDL-apoB, mg/dL</td>
<td>62 (55–74)</td>
<td>73 (64–83)</td>
<td>71 (64–80)</td>
</tr>
<tr>
<td>LDLi cholesterol, mmol/L</td>
<td>1.33 (1.08–1.52)</td>
<td>1.20 (0.87–1.57)</td>
<td>1.48 (1.04–1.98)</td>
</tr>
<tr>
<td>LDLii cholesterol, mmol/L</td>
<td>1.44 (1.13–1.72)</td>
<td>1.43 (1.19–1.77)</td>
<td>1.57 (1.33–1.83)</td>
</tr>
<tr>
<td>LDLi cholesterol, mmol/L</td>
<td>0.27 (0.24–0.36–9)</td>
<td>0.33 (0.28–0.4)</td>
<td>0.30 (0.26–0.39)</td>
</tr>
<tr>
<td>LDLi particle mass, mg/dL</td>
<td>162 (138–192)</td>
<td>140 (102–180)</td>
<td>174 (131–223)</td>
</tr>
<tr>
<td>LDLii particle mass, mg/dL</td>
<td>170 (150–210)</td>
<td>138 (117–174)</td>
<td>181 (164–219)</td>
</tr>
<tr>
<td>LDLii particle mass, mg/dL</td>
<td>38 (32–46)</td>
<td>38 (31–48)</td>
<td>37 (34–51)</td>
</tr>
</tbody>
</table>

Data presented as median (IQR). For LDL size, n=84 for placebo, and n=87 for fenofibrate.

p values are based on comparing the percentage changes from baseline between the groups with Mann–Whitney U test.
6.4 Effect of fenofibrate on homocysteine; implications to HDL (Study III)

As expected, fenofibrate treatment increased serum homocysteine levels. The rise was substantial (58.8%) and resulted in a median homocysteine level of 19.9μmol/L in the fenofibrate group. To study whether homocysteine levels would associate with HDL variables, the patients were divided into quartiles according to their baseline homocysteine levels. Patients in the highest quartile of homocysteine had lower HDL-C, HDL3-C and apoA-II. Thus, higher homocysteine was associated with lower levels of small HDL. (Table 13).

Next step was to investigate if the fenofibrate-induced increase of homocysteine would associate with changes in HDL particles. We divided the fenofibrate treatment group by its median homocysteine level at study close (Figure 16.). In the fenofibrate group, low vs high homocysteine (medians 15.1 vs 23.5 μmol/l, respectively) resulted in parallel changes in HDL-C and apoA-I. In patients with low homocysteine levels (n=42), HDL-C and apoA-I levels were slightly increased by fenofibrate, whereas in patients with high homocysteine levels (n=42) HDL-C and apoA-I levels decreased.

Table 12. Cholesterol concentration, particle mass of HDL2 and HDL3, and concentrations of apolipoproteins and apoA-containing lipoproteins in T2DM patients during the study

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=84)</th>
<th>Fenofibrate (n=87)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5th year</td>
<td>Baseline</td>
</tr>
<tr>
<td>HDL2-C (mmol/l)</td>
<td>0.39 (0.29–0.53)</td>
<td>0.43 (0.32–0.70)</td>
<td>0.42 (0.30–0.58)</td>
</tr>
<tr>
<td>HDL3-C (mmol/l)</td>
<td>0.72 (0.63–0.81)</td>
<td>0.74 (0.65–0.83)</td>
<td>0.71 (0.63–0.81)</td>
</tr>
<tr>
<td>HDL2 particle mass (mg/dl)</td>
<td>89 (72–120)</td>
<td>97 (71–141)</td>
<td>95 (72–129)</td>
</tr>
<tr>
<td>HDL3 particle mass (mg/dl)</td>
<td>213 (193–235)</td>
<td>241 (226–265)</td>
<td>214 (192–228)</td>
</tr>
<tr>
<td>ApoA-I (mg/dl)</td>
<td>134 (121–151)</td>
<td>134 (123–151)</td>
<td>136 (125–145)</td>
</tr>
<tr>
<td>ApoA-II (mg/dl)</td>
<td>34 (31–37)</td>
<td>31 (27–37)</td>
<td>33 (30–37)</td>
</tr>
<tr>
<td>LpA-I (mg/dl)</td>
<td>48 (41–48)</td>
<td>48 (39–65)</td>
<td>49 (41–59)</td>
</tr>
<tr>
<td>LpAI-AII (mg/dl)</td>
<td>83 (75–90)</td>
<td>83 (77–96)</td>
<td>83 (77–92)</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR). *p* values are based on comparing the percentage changes from baseline between the groups with Mann–Whitney U test.
Table 13. The baseline HDL panel in the groups of T2DM patients with low and high homocysteine levels

<table>
<thead>
<tr>
<th></th>
<th>Lowest quartile (n=56)</th>
<th>Highest quartile (n=60)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>7.9 (7.1–8.5)</td>
<td>14.4 (13.3–17.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.17 (1.05–1.44)</td>
<td>1.05 (0.94–1.32)</td>
<td>0.036</td>
</tr>
<tr>
<td>HDL2-C (mmol/l)</td>
<td>0.46 (0.30–0.61)</td>
<td>0.38 (0.27–0.54)</td>
<td>0.191</td>
</tr>
<tr>
<td>HDL3-C (mmol/l)</td>
<td>0.75 (0.67–0.85)</td>
<td>0.70 (0.63–0.77)</td>
<td>0.018</td>
</tr>
<tr>
<td>apoA-I (mg/dl)</td>
<td>137 (121–155)</td>
<td>129 (119–143)</td>
<td>0.131</td>
</tr>
<tr>
<td>apoA-II (mg/dl)</td>
<td>34 (30–38)</td>
<td>32 (31–06)</td>
<td>0.040</td>
</tr>
<tr>
<td>LpA-I (mg/dl)</td>
<td>51 (41–65)</td>
<td>48 (41–61)</td>
<td>0.904</td>
</tr>
<tr>
<td>LpA1-AII (mg/dl)</td>
<td>85 (79–93)</td>
<td>81 (74–87)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR). P values for the trends were calculated with the Jonckheere–Terpstra test for several independent samples.
Patients on fenofibrate treatment were divided into two groups by median homocysteine level (20umol/L) at 5th year. White bars represent baseline and black bars 5th year data (mean). Absolute changes of apoA-I and HDL cholesterol were compared with Mann-Whitney U-test between the groups.

6.5 Effect of fenofibrate on markers of renal function and albuminuria (Study V and unpublished data)

Plasma creatinine increased during fenofibrate treatment (Figure 17.), as in the main FIELD study. Urine creatinine levels remained similar between the treatment groups (Figure 17.). This resulted in a decreased creatinine clearance and eGFR (Figure 17.) in the fenofibrate treatment group. The annual decreases were smaller than expected in both groups. There were no differences in 24h urine protein excretion (dU-Prot), AER, or ACR between the treatment groups (Table 14). Of the albuminuria markers, AER decreased in both groups whereas ACR remained stable. Cystatin C increased in the fenofibrate treatment group.

Increase of serum creatinine and decrease of renal function is frequently accompanied by an increase of homocysteine levels. To investigate this
Figure 17. Creatinine levels in plasma and urine, and markers of renal function during the study.

White bars present baseline, grey bars 2nd year and black bars 5th year data (median). The change during study is expressed as total change for plasma and urine creatinine and as annual change for the markers of renal function. These values have been compared by the Mann-Whitney U-test.
association, the treatment groups were divided by their median decrease of eGFR. In the fenofibrate treatment group, the relative increase of homocysteine was significantly higher in the patients who had greater decrease in eGFR compared to the patients with smaller eGFR decrease (Figure 18A.). The difference was not significant in the placebo group.

Similarly, Cystatin C increase was also associated with the increase of homocysteine. Again, the treatment groups were divided by their median increase of Cystatin C. In the fenofibrate group, homocysteine was increased significantly more in the patients with higher increase of Cystatin C. (Figure 18B.).
6. Results

Stratified by a) median decrease of GFR and b) median increase of cystatin C. White bars representing patients on placebo and black bars representing patients on fenofibrate.

6.6 Effect of fenofibrate on markers of inflammation, endothelial activation, augmentation index, and intima-media thickness (Study IV)

Fenofibrate treatment did not change plasma levels of CRP and IL-6 (Table 15.) PLA2 values were similar for fenofibrate and placebo, despite a decrease in levels after 5 years in the placebo group. Decrease in SAA in the fenofibrate group was not significantly different to the decrease in placebo group. Fenofibrate had no effect on plasma VCAM-1, ICAM-1 and E-selectin compared to the placebo after 5 years.

The augmentation index increased in both groups (fenofibrate: 27.4±0.9 vs. 30.4±1.0 %, baseline vs. 5 yrs, p<0.001, placebo: 25.5±0.9 vs. 29.3±1.0 %, p<0.005) with no significant effect of fenofibrate (Figure 19). Heart rate and central pressure augmentation remained unchanged in both
Table 15. Biomarkers of inflammation and endothelial function in T2DM patients at baseline and at 5 years.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Placebo Baseline</th>
<th>Placebo 5th year</th>
<th>Fenofibrate Baseline</th>
<th>Fenofibrate 5th year</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1 ng/mL</td>
<td>561 (434–668)</td>
<td>627*** (534–786)</td>
<td>534 (432–641)</td>
<td>679*** (568–874)</td>
<td>0.271</td>
</tr>
<tr>
<td>ICAM-1 ng/mL</td>
<td>264 (222–308)</td>
<td>256* (216–290)</td>
<td>256 (224–306)</td>
<td>244* (208–299)</td>
<td>0.761</td>
</tr>
<tr>
<td>E-selectin ng/mL</td>
<td>56 (42–75)</td>
<td>49*** (35–59)</td>
<td>54 (43–72)</td>
<td>43*** (33–53)</td>
<td>0.325</td>
</tr>
<tr>
<td>CRP mg/L</td>
<td>1.7 (1.0–3.6)</td>
<td>1.6 (0.8–3.5)</td>
<td>1.8 (1.0–4.0)</td>
<td>2.5 (0.8–4.8)</td>
<td>0.101</td>
</tr>
<tr>
<td>PLA ng/mL</td>
<td>3.1 (2.1–5.3)</td>
<td>2.4*** (1.7–4.0)</td>
<td>2.7 (1.8–4.5)</td>
<td>2.9 (1.8–3.9)</td>
<td>0.064</td>
</tr>
<tr>
<td>IL6 pg/mL</td>
<td>2.6 (1.7–3.8)</td>
<td>2.5 (1.6–3.9)</td>
<td>2.3 (1.7–3.6)</td>
<td>2.7 (1.9–4.1)</td>
<td>0.946</td>
</tr>
<tr>
<td>SAA μg/mL</td>
<td>21 (13–35)</td>
<td>20* (14–28)</td>
<td>25 (15–38)</td>
<td>17*** (13–23)</td>
<td>0.123</td>
</tr>
</tbody>
</table>

Data are median (IQR) and treatment effect as median difference of change between the groups (95%CI). P-values are from the Mann-Whitney U-test, comparing the relative changes from baseline to 5th year between the groups. P-values between baseline and 5th yr in each group from Wilcoxon Signed Rank test for 2 related variables. ***, p<0.001; **, p<0.01; *, p<0.05. N=73 for placebo and N=77 for fenofibrate, since patients with CRP>10mg/L were excluded.

groups after 5 years. Aortic systolic and diastolic blood pressure decreased significantly in fenofibrate group (Table 16.). However, when treatment effect was analyzed using two-way ANOVA for repeated measures, there were no significant effects of fenofibrate on aortic blood pressures.

The pre-selected primary measure of IMT in our study was the mean of maximum IMT (Max IMT) (Figure 20.). In both groups max IMT was thicker at closeout than at baseline. All of the IMT measures were similar between treatment groups during the study. (Table 17.). The annual increase of mean IMT was rather modest and not statistically significant between the treatment groups (0.0069 vs. 0.0054 mm).
Table 16. Results of pulse wave analysis in T2DM patients at baseline and at 5th year.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>Fenofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5th year</td>
</tr>
<tr>
<td>Aortic systolic BP (mmHg)</td>
<td>136 ± 18</td>
<td>132 ± 16</td>
</tr>
<tr>
<td>Aortic diastolic BP (mmHg)</td>
<td>81 ± 11</td>
<td>81 ± 9</td>
</tr>
<tr>
<td>Aortic mean arterial BP (mmHg)</td>
<td>105 ± 13</td>
<td>102 ± 10</td>
</tr>
<tr>
<td>Aortic pulse pressure (mmHg)</td>
<td>55 ± 14</td>
<td>51 ± 14</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>67 ± 9</td>
<td>67 ± 9</td>
</tr>
</tbody>
</table>

**p<0.01, ***p<0.001 for 5th year vs. baseline within each treatment group. There were no significant treatment effects when two-way ANOVA for repeated measured was used. Data are mean ± STD. N=75 for placebo and N=76 for fenofibrate.

Figure 19. Results of the augmentation index.
Results

Black circles and solid line represents the fenofibrate group; open circles and dotted line represents the placebo group. Data are mean±SD.

Table 17. Results of carotid IMT in T2DM patients at baseline and at 5th year.

<table>
<thead>
<tr>
<th>IMT</th>
<th>Baseline</th>
<th>2nd year</th>
<th>5th year</th>
<th>Rate of change (mm/y)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Far wall, mm</td>
<td>Placebo</td>
<td>1.01 (0.87–1.11)</td>
<td>1.04** (0.91–1.16)</td>
<td>1.01* (0.88–1.14)</td>
<td>-0.0093 to 0.0148</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>1.02 (0.90–1.17)</td>
<td>1.03 (0.90–1.22)</td>
<td>1.04 (0.91–1.22)</td>
<td>-0.0099 to 0.0170</td>
</tr>
<tr>
<td>Plaque, mm</td>
<td>Placebo</td>
<td>0.54 (0.47–0.63)</td>
<td>0.56* (0.48–0.66)</td>
<td>0.58*** (0.51–0.67)</td>
<td>-0.0031 to 0.0283</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>0.55 (0.49–0.65)</td>
<td>0.57* (0.49–0.67)</td>
<td>0.61*** (0.57–0.66)</td>
<td>-0.0032 to 0.0281</td>
</tr>
<tr>
<td>CCA, mm</td>
<td>Placebo</td>
<td>1.14 (1.09–1.31)</td>
<td>1.17 (1.06–1.29)</td>
<td>1.19* (1.03–1.29)</td>
<td>-0.0087 to 0.0185</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>1.21 (1.09–1.31)</td>
<td>1.19 (1.06–1.29)</td>
<td>1.22** (1.03–1.32)</td>
<td>-0.0051 to 0.0177</td>
</tr>
<tr>
<td>CB, mm</td>
<td>Placebo</td>
<td>1.45 (1.26–1.67)</td>
<td>1.51*** (1.30–1.74)</td>
<td>1.51*** (1.32–1.73)</td>
<td>-0.0064 to 0.0419</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>1.45 (1.27–1.68)</td>
<td>1.51* (1.26–1.73)</td>
<td>1.51*** (1.37–1.74)</td>
<td>-0.0039 to 0.0378</td>
</tr>
<tr>
<td>ICA, mm</td>
<td>Placebo</td>
<td>1.12 (1.01–1.32)</td>
<td>1.13 (1.01–1.35)</td>
<td>1.18** (1.05–1.48)</td>
<td>-0.0171 to 0.0587</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>1.12 (1.01–1.32)</td>
<td>1.13 (1.01–1.41)</td>
<td>1.22** (1.02–1.45)</td>
<td>-0.0154 to 0.0601</td>
</tr>
</tbody>
</table>

Data are median (IQR), for annual change median (95%CI). P-values are from the Mann-Whitney U-test, comparing the annual change between the groups. P-values between baseline and 2nd year or 5th yr in each group from Wilcoxon Signed Rank test for 2 related variables. ***, p<0.001; **, p<0.01; *, p<0.05.
7. DISCUSSION

7.1 Study subjects

The patients in this study were middle-aged to elderly type 2 diabetic subjects with variable duration of diabetes and different treatments. This study cohort was relatively representative of type 2 diabetic patients in primary health care clinics. The lipid inclusion criteria were defined to allow inclusion of subjects with mild diabetic dyslipidaemia, but not patients requiring open-label hypolipidaemic therapy at the run-in phase of the study. The upper lipid inclusion criteria of total cholesterol in the Finnish FIELD study patients was 5.5 to 6.0mmol/L, compared to 6.5mmol/L in Australia and New Zealand. The difference was due to different guidelines of care at the time of the study beginning 1998.

The mean cholesterol level in Finland was about 5.5 mmol/l in late 1990’s in both genders (Harald et al. 2008). Total and LDL cholesterol in this study were similar in diabetic subjects and healthy controls and somewhat lower than in the general population. None of the participants had hypolipidaemic treatment at baseline, which excludes any effects by statins on lipids, inflammation and IMT. The lipid values of our diabetic subjects were slightly better than those reported in the UKPDS (Turner et al. 1998) and DAIS (The DAIS Investigators 2001). The imbalance in gender distribution was adjusted for in the statistical analyses or the genders were analysed separately to detect possible differences.

The presence of CVD was determined by clinical history at every main study visit. All available hospital records were reviewed. The electrocardiograms will be analysed separately in the FIELD main study to detect silent ischaemia (manuscript under preparation).

The inclusion criteria of healthy controls were designed to include subjects representing healthy population. Our control group was allowed to have treated mild hypertension only. We excluded subjects with abnormal fasting (≤6.1 mmol/L) or 2hr OGTT glucose (≤7.8 mmol/L) values as well as high total cholesterol, LDL cholesterol, and triglyceride values. The control subjects had less metabolic syndrome than general population, and the anthropometric measurements were more favourable than in the diabetic patients of this study. Additionally, the subjects in the control group were significantly younger and smoked less compared to the group of type 2 diabetic patients. To find subjects matched for age, gender, and anthropometric measurements, but free of glucose derangement and dyslipidemia, would have been impossible. Therefore different statistical approaches were used.
to compensate for the different confounders. Obviously, the results obtained from the healthy control group cannot as such be extrapolated to the general population, as the “healthy” subjects in all probability are healthier than their age-cohort in the general population.

The type 2 diabetic patients were accurately treated, having good and stable HbA1c during the study. The FIELD study showed that glycaemic control can be maintained in type 2 diabetes with standard therapy but without overall weight gain (Best et al. 2008). The clinicians of the FIELD study managed in reducing the systolic and diastolic blood pressures. As the weight of the patients remained stable, this obviously resulted from increased hypoglycaemic and antihypertensive treatment. These facts, together with only moderate diabetic dyslipidemia, may have affected our results concerning vascular imaging, albuminuria, and renal function.

7.2 VLDL particles

7.2.1 VLDL particles in type 2 diabetic patients

Study I demonstrated that the elevation of VLDL1 triglycerides was the major determinant of serum triglyceride concentration in healthy normolipidemic subjects and patients with type 2 diabetes. In agreement with these results, the NMR profile of VLDL subclasses indicated that increase of VLDL in type 2 diabetes was primarily due to increased number of large VLDL particles (Garvey et al. 2003). The slope for VLDL1 TG over a wide range of serum triglycerides was much steeper than that for VLDL2. Thus, VLDL1 triglycerides increasingly contribute to serum triglyceride concentration when the triglyceride level rises. This relationship is probably not due to diabetes per se but linked to factors behind the elevation of VLDL1 triglycerides.

The data on lipid composition of VLDL particles in diabetic dyslipidemia is controversial. VLDL particles, as a single entity, have been reported to be triglyceride enriched (Taskinen et al. 1986, McEneny et al. 2000). In this study, the percentage of triglycerides in VLDL1 was slightly reduced in diabetic subjects compared to control subjects. This is consistent with a previous study, that reported a depletion of VLDL1 triglycerides percentage in diabetic subjects (James and Pometta 1991). These results agree with previous data showing that in both normolipidemic and hypertriglyceridemic subjects, the elevation of serum triglycerides reflect merely an increase in the number but not the size of the VLDL particles (Steiner et al. 1998).

Consistent with one previous study, plasma apoCIII was elevated in type 2 diabetic patients (Briones et al. 1984). Interestingly a deficiency of apoCIII in all TRL species in type 2 diabetic subjects compared to control subjects was detected. The novel observation is that the concentration of apoCIII
did not increase in concert with core lipids in VLDL subclasses. Since apoCIII has a pivotal role of in the hepatic clearance of VLDL remnants, lack of apoCIII may disturb the removal of TRL remnant particles and contribute to a prolonged residence time in circulation (Zheng et al. 2007). Theoretically, this would increase the atherogenicity of the remnants and the conversion of IDL to LDL particles. Thus, apoCIII is expected to be increased in LDL particles of the diabetic patients.

In a small previous study, diabetes status was associated with lower concentration of VLDL particles containing apoCIII, whereas hypertriglyceridemia was linked to increase of apoCIII-containing VLDL particles (Lee et al. 2003). A lower ratio of apo CIII/triglycerides in VLDL has been reported in type 2 diabetic patients (Pan et al. 1986). Despite elevation of plasma apo CIII, diabetes status could thus be linked with a relative deficiency of apo CIII in TRL species.

Interestingly, apoCIII might have different actions in insulin resistance syndrome. The promoter region of the apoCIII gene has an insulin response element and its mutations are associated with increased risk of myocardial infarction (Olivieri et al. 2002). These mutations inhibit the insulin-induced down-regulation of apoCIII production (Ruiz-Narvaez et al. 2008). Thus, in insulin-resistant states, this mechanism might be insufficient to decrease postprandial TRL particle load.

A potential limitation of this study is the separation of TRL fractions using density gradient ultracentrifugation, which may have resulted to loss of some apoCIII into the d > 1.20 g/ml lipid-free fraction. Only trivial amount of apoCIII was however detected in the lipid free fraction analyzed from 10 diabetic patients. These results agree with the finding that ultracentrifugation causes <5 % of total apoCIII to be stripped off in the density > 1.20 g/ml fraction (Schonfeld et al. 1979).

ApoE seems to be more easily detached and about 20 -40 % of apoE is lost into the non-lipid fraction (Havel et al. 1980). The lack of apoE measurements in VLDL subclasses prohibits the conclusions on the relevance of the observed plasma apoE reduction in type 2 diabetes. However, in type 2 diabetes the inverse correlation between apo E and triglycerides, oppositely to the strong positive correlation observed in non-diabetic subjects, suggest a derangement of apoE recycling between lipoprotein particles. As recently discovered, HDL and/or apoA-I is needed to mobilize the internalized apoE from cellular endosomes, where apoE resides after degradation of TRL particles (Hasty et al. 2005, Heeren et al. 2006). It would thus be interesting to study apoE recycling in a population with HDL/apoA-I deficiency, such as type 2 diabetic patients.

As suggested by these results, the associations of apoCIII and apoE with TRL metabolism are disturbed in type 2 diabetes. Further studies are requested to establish if there are abnormal shifts of apoproteins CIII, CII and E between VLDL and HDL particles in type 2 diabetes.
7.2.2 Effect of fenofibrate on VLDL subclasses
Fenofibrate decreased plasma triglycerides in patients with type 2 diabetes. This was mostly due to a reduction of VLDL1 TG, and to a lesser extent of VLDL2 TG. The reductions were proportional to the decreases in particle mass concentration. Thus, fenofibrate seems to correct a dominant defect in diabetic dyslipidaemia. Interestingly, the percentage of triglycerides increased and that of cholesterylester decreased in all TRL subclasses, which theoretically could result from a decreased CETP-mediated core lipid transfer (Guerin et al. 1996, Watts et al. 2006). However, the measured CETP activity did not change in this study and furthermore, compositions of LDL and HDL subclasses did not demonstrate a corresponding increase of cholesterylester and decrease of TG.

Fenofibrate is known to increase the catabolism of VLDL1 particles and also the fractional transfer rate to VLDL2 (Watts et al. 2003). This increase of catabolism occurs via PPARα activation, with an increase in LPL activity accompanied by a decrease in apoCIII synthesis. The concentration of VLDL apoCIII is a strong determinator of the catabolic rate of VLDL (Chan et al. 2006). In the interim analysis of the substudy population, fenofibrate treatment was associated with a decrease of apoCIII in all TRL subclasses (Hiukka et al. 2004). Interestingly, the ratio of apoCIII/TG decreased in TRL subclasses. It is obvious, that the decrease in apoC-III by fenofibrate is a major factor in the lowering of plasma TG (Lemieux et al. 2003). It would be would expected that the increased LPL activity together with the reduction of apoCIII would further increase TRL catabolism. Considering VLDL production, available data on the effects of fenofibrate is less conductive, as fenofibrate has shown only a non-significant tendency to reduce the production rates of VLDL1 TG and apoB in patients with metabolic syndrome (Watts et al. 2006).

7.3 LDL particles

7.3.1 LDL particles in type 2 diabetic patients
LDL-apoCIII was increased in type 2 diabetic patients. ApoCIII induced a small increase in proteoglycan binding of LDL from patients with type 2 diabetes. However, intrinsic characteristics other than apoCIII seemed to be more essential for increased proteoglycan binding of apoCIII-containing LDL of diabetic patients.

As increase of total apoCIII and a decrease of TRL apoCIII were noted this study, the increased amount of apoCIII in LDL particles of type 2 diabetic patients was expected. The diabetic patients and control subjects seemed quite ideal for studying this, since the LDL-apoB was quite similar between the groups, i.e. the number of LDL particles was similar. The diabetic patients with low LDL-apoCIII were also matched to the patients with high...
LDL-apoCIII, to study the effect of apoCIII-increase in the context of LDL particles with similar composition. Interestingly, as discussed below, the LDL particles with different amount of apoCIII showed distinct differences in a more detailed mass spectrometry analysis.

The amount of LDL apoCIII was associated with a profoundly altered lipid composition of LDL from type 2 diabetic patients, such as a reduction in unesterified cholesterol, sphingomyelin, ceramide, and the ceramide-containing ganglioside GM1. Interestingly, cholesterol has been demonstrated to enhance a closer lateral packing in LDL, whereas ceramide can induce a less fluid monolayer membrane (Ibdah et al. 1989, Sola et al. 1990). Therefore, the observed changes could be associated with increased membrane fluidity and increased freedom in lateral moving, which would allow such conformational changes in apoB that facilitate proteoglycan binding.

It is not known whether the increase of apoCIII alters the lipid composition of LDL or if the increased membrane fluidity merely allows the binding of more apoCIII to LDL particles. Study II showed that apoCIII enrichment in vitro increased the susceptibility of LDL to hydrolysis and aggregation by SMase. This suggests that diabetic LDL with high apoCIII content is especially vulnerable to the proatherosclerotic modifications by SMase. In addition, depletion of cell surface sphingomyelin with SMase results in increased efflux of unesterified cholesterol (Gorska et al. 2003), which could explain the parallel decrease of sphingomyelin and unesterified cholesterol in diabetic LDL with high apoCIII. The detected lipid alterations in our study could be induced by previously demonstrated increased SMase activity (Gorska et al. 2003) and increased susceptibility of LDL to SMase.

GM1 is known to be an inhibitor of SMase (Fanani and Maggio 1997). Our lipid composition studies showed that LDL of type 2 diabetic patients contained significantly less GM1 than LDL of control subjects, and that high apoCIII was associated with lower GM1 content. Thus, apoCIII-containing LDL had reduced levels of a SMase inhibitor, rendering the particle further susceptible for atherosclerotic modifications.

7.3.2 Effect of fenofibrate on LDL particles
The above-demonstrated features of diabetic LDL with high apoCIII could explain some of the proatherogenic role of apoCIII. Further, this study showed that treatment with fenofibrate reduces the LDL-apoCIII. However, the molar ratio of apoCIII/apoB in LDL remained similar. In the cross-sectional data, the “high LDL-apoCIII” was defined as a high molar ratio of apoCIII/apoB in LDL. Thus, we cannot speculate whether the reduction of LDL-apoCIII by fenofibrate would have an effect on the lipid composition data or proteoglycan binding results. On the other hand, fenofibrate may also alter the arterial proteoglycans. One in vitro study...
has examined the effect of fenofibrate on cultured human vascular smooth muscle cells, revealing a reduction in binding of human LDL (Nigro et al. 2004).

Three pairs selected to the proteoglycan binding analysis dropped out of the FIELD study. Furthermore, of the original pairs only five were randomized to different treatment groups (eg, another patient to fenofibrate group and another to placebo group). Thus, proteoglycan binding analysis was chosen not to be performed from the samples taken during the fenofibrate treatment period.

7.4 HDL particles

7.4.1 Effect of fenofibrate on HDL particles

The FIELD main study was the first long-term fenofibrate study without a sustained effect on HDL-C and apoA-I (Keech et al. 2005). No changes in HDL-C levels were shown in this study, but marked changes were noted in the distribution of HDL subclasses 2 and 3. A significant increase in small, dense HDL3 and a marked decrease in large HDL2 were observed. As in the main FIELD study, the serum levels of apoA-I remained unaltered by fenofibrate. As apoA-I has potent antiatherosclerotic actions, this is clearly a disadvantage for fenofibrate.

By what mechanisms is the expected effect of fenofibrate on HDL attenuated? Fenofibrate has been reported to increase both the production as well as catabolism of apoA-I (Watts et al. 2003). Recent animal models have revealed a role for homocysteine in apoA-I metabolism. Homocysteine has been reported to decrease apoA-I expression, which may result from a loss of transcription mediated by PPARα (Mikael et al. 2006). Likewise, homocysteine levels in humans show negative correlations with apoA-I and HDL-C levels (Mikael et al. 2006). These results are in agreement with the baseline findings of this study.

To the best of knowledge, this is the first study to demonstrate the homocysteine-associated reduction of HDL-C and apoA-I during fenofibrate treatment. The increase of homocysteine is probably only a part of the explanation for the lack of durable effect by fenofibrate on HDL-C and apoA-I. Recently published analysis of the FIELD main study confirms this substudy data, showing a highly significant inverse relationship between homocysteine and HDL-C and apoA-I during fenofibrate treatment (Taskinen et al. 2009). The data further suggests that there might be a threshold level (>16.7 μmol/L) for the harmful effects of homocysteine. Interestingly, increase of homocysteine may have affected the main results of the FIELD study. A post-hoc analysis of the FIELD study showed proportionately less reduction in CVD events in patients with the highest tertile increase (>4 μmol/L) in homocysteine (Keech 2006).
Increased catabolism of HDL apoA-I is the main cause for lowering of HDL in type 2 diabetic subjects (Frenais et al. 1997). Hypercatabolism of apoA-I is linked to triglyceride enrichment of HDL particles. In type 2 diabetes, HDL particles are rich in triglycerides and depleted in cholesterol (Frenais et al. 1997). This core lipid imbalance results to the decrease in the delivery of cholesterylesters to liver and a diminished stability of apoA-I, impairing the efflux process (Kontush and Chapman 2006). In this study fenofibrate was unable to correct the imbalance of core lipids in HDL (data not shown). The TG-enriched HDL particles are good substrates to the lipolytic action of hepatic lipase that is frequently elevated in Type 2 diabetes and might be further raised by fenofibrate (Desager et al. 1996).

HDL particles are remodelled by LCAT, PLTP, CETP, and lipases (Huuskonen et al. 2001) The effects of these proteins on lipoprotein metabolism as well as on atherosclerosis are rather complex and still partly poorly understood. In the present study we observed no significant changes in PLTP, CETP, or LCAT activities during fenofibrate treatment. Thus these proteins may not contribute to the observed changes of HDL subclasses. We did not measure postheparin plasma LPL and hepatic lipase activities. The postulated increase of hepatic lipase by fenofibrate (Desager et al. 1996) would favour increased catabolism of HDL particles.

Fenofibrate treatment increased plasma apoA-II levels. This is consistent with the effect of fenofibrate on APOA2 gene expression (Keating and Croom 2007). This study agrees with the concept of increased apoA-II production resulting to a shift towards smaller LpAI-AII particles. Gemfibrozil has been reported to increase the number of small HDL particles (Otvos et al. 2006). In that study HDL particle number measured by NMR was predictive of CVD benefit. The NMR lipid profile substudy of the FIELD study will further elucidate the effects of fenofibrate on NMR lipid classes.

7.5 Effect of fenofibrate on IMT

The study patients presented with relatively high baseline IMT levels and no previous use of statins, and can thus be regarded as an ideal population to study the progression of atherosclerosis (Baldassarre et al. 2007). In this population, long-term fenofibrate treatment had no effect on carotid IMT in patients with type 2 diabetes. At the time of its publication, study IV was the longest RCT with measured IMT (Stein 2008).

Our study was limited by the relatively small size of the study cohort. However, two surrogate markers of atherosclerosis showed similar kind of progression in both groups, while no significant effect of fenofibrate was demonstrated. The progression of mean IMT was surprisingly slow in this study (0.0069 vs. 0.0054 mm/y). A recent meta-analysis has calculated an IMT progression rate of 0.034mm/y for type 2 diabetic patients (Yokoyama...
et al. 2006). However, the progression rate was shown to be dependent on mean HbA1c, which was 7.86% in the meta-analysis. Thus, the rather aggressive treatment of CVD risk factors may have affected the low IMT progression rate in this study. Correspondingly, the 13-year surveillance data from the Steno-2 study showed drastic reductions in cardiovascular morbidity and mortality in the group of intensified medical treatment (Gaede et al. 2008).

There are only a few IMT studies with fenofibrate in type 2 diabetic patients. In a study of 594 hypertensive non-diabetic subjects, fenofibrate treatment slowed the progression of IMT/arterial diameter –ratio, but the mean IMT did not change (Zhu et al. 2006). A non-randomized study observed a lipid-independent effect towards greater IMT and steeper IMT progression in patients on different fibrates (n=82) compared to those on statins (n=291) (Chironi et al. 2005). The DAIS study demonstrated a significant reduction of focal angiographic coronary artery lesions after 3 years of fenofibrate treatment in 418 men with type 2 diabetes (The DAIS Investigators 2001). The reduction in the progression of mean segment diameter in coronary arteries was not significant. Moreover, the correlation between coronary angiography and carotid IMT is only moderate (Graner et al. 2006, Bots et al. 2007).

7.6 Effect of fenofibrate on augmentation index and markers of low-grade inflammation and endothelial activation

This was the first study to examine the long-term effect of a fibrate on augmentation index with sufficient number of patients. In the present study, neither a reduction in endothelial markers nor a decrease in augmentation index was detected in type 2 diabetic patients. Rather, a similar progression of augmentation index was shown in both the fenofibrate and placebo groups, validating the natural progression of atherosclerosis in our study group.

This study did not replicate the previous results showing reduction in markers of low-grade inflammation and endothelial activation. In these studies, the biomarkers of endothelial dysfunction, ICAM-1, VCAM-1, and E-selectin, decreased with fenofibrate intervention (Marchesi et al. 2003, Koh et al. 2005). Of the other markers of inflammation, PLA-2 has recently been shown to decrease 6.6% during gemfibrozil treatment (Robins et al. 2008). Interestingly, reduction of high levels of PLA-2 was associated with reduction of CVD events. In contrast, we demonstrated a non-significant tendency to 13.6% higher PLA-2 levels in the fenofibrate group. Inhibition of PLA-2 has been recently studied in the context of reduction of CVD risk factors, with promising surrogate results (Mohler et al. 2008, Serruys et al. 2008, Rosenson et al. 2009).
Fenofibrate treatment has in previous studies reduced plasma CRP levels, along with its up-stream regulator interleukin 6 (IL-6) (Malik et al. 2001b, Wang et al. 2003, Koh et al. 2005, McKenney et al. 2006, Muhlestein et al. 2006, Zhu et al. 2006). However, these studies have involved small number of patients in short-term design and only one of them included type 2 diabetic patients. McKenney et al. reported results from 576 patients with mixed hyperlipidemia who were on fenofibrate for over one year (12+48 weeks). The reduction in CRP from baseline was slightly over 20%. Data from type 2 diabetic patients (n=93) was not reported separately (McKenney et al. 2006). Interestingly, all fibrates have reduced inflammatory markers in short-term studies but the long-term data is mostly missing. Some common variants of the CRP gene polymorphism may also affect the response to fenofibrate (Shen et al. 2008). Long-term results have been reported only for bezafibrate, which did not reduce CRP levels in the 6-year BIP study (Tanne et al. 2006). Furthermore, the lack of CRP-lowering effect of fenofibrate agrees with the unpublished results from the DAIS study (Taskinen MR, personal communication).

### 7.7 Effect of fenofibrate on renal function and albuminuria

Fenofibrate reduced measures of renal function to a greater extent than placebo. Additionally, long-term fenofibrate treatment had no effect on albumin excretion rate. This is in agreement with the lack of changes in the mean values of ACR and AER by fenofibrate in the FIELD and the DAIS studies, respectively (The DAIS Investigators 2001, Keech et al. 2005). In the FIELD study, the reported beneficial renal outcome was based on 2.6% more patients allocated to fenofibrate than placebo regressing or not progressing in a categorized albuminuria variable (p=0.002). This benefit is rather modest and its clinical relevance should be clarified in a long-term outcome study focusing on renal function. Further, analysis of a categorized variable is sensitive to changes in variance of the data, even if the mean values of the group would not change.

The reduction of renal function during fenofibrate treatment has been observed only once previously as a decrease of para-aminohippurate clearance and increase of Cystatin C (Ansquer et al. 2008). In that study, the reduction in creatinine clearance resulted from an increase in plasma creatinine accompanied with no change in urinary creatinine levels. Importantly, MDRD and Cockroft-Gault estimates of renal function are unreliable during fenofibrate treatment due to increase of creatinine, and the estimates tend to vary significantly in subjects with relatively normal renal function. Therefore Cystatin C was used in this study as a creatinine-
independent marker of renal dysfunction during fenofibrate treatment (Roos et al. 2007, Stevens et al. 2008).

The reason for the fenofibrate-induced increase in plasma creatinine is unclear. This study wasn't designed to examine the hypothesis of increased production of creatinine. This seems unlikely however, since an accompanying increase of creatinine excretion has not been observed (Ansquer et al. 2008), as confirmed in this study. An option remains, that the increase of creatinine is caused by the decrease in creatinine clearance. It has been speculated, that fenofibrate might have an inhibitory effect on the excretion of creatinine via the kidneys, and that it might increase the flow of creatinine from the muscle (Hottelart et al. 2002). In this study, urinary creatinine did not change during fenofibrate treatment. If fenofibrate increases creatinine outflow from the muscle, muscle damage is possible. In our study creatine phosphokinase levels were however lower in the fenofibrate group. It has been suggested that fibrates, probably via PPARα, may impair the production of vasodilatory prostaglandins, which might contribute to renal function impairment (Tsimihodimos et al. 2002). Interestingly, tesaglitazar, a PPARα and PPARγ agonist, was withdrawn from phase III trials because of renal function impairment.

In this study, direct measures of GFR could not be used due to demanding study protocol. Notably, all used parameters of albuminuria and renal function showed consistent results in the fenofibrate group.

The observed decrease in AER in both groups can be explained by the decrease in arterial blood pressure. Further, the increased use of renin-angiotensin system blockers in both groups may have had additional nephroprotective effects. It's also recognizable that HbA1c remained at a good level of 7.1% during the five years of the study. These factors may explain the rather modest annual decrease of estimated renal function seen in placebo group. There were minor differences in blood pressure and glycemia between the treatment groups during the study. These together with lipid variables were found not to account for the changes in renal function in statistical analysis.

Altogether, these results do not support the benefits of fenofibrate on regression of albuminuria. However, indirect evidence links hypertriglyceridemia to the progression of renal disease (UKPDS Study Group 1993, Retnakaran et al. 2006). Hypertriglyceridemia is linked to endothelial dysfunction and thus it may have a significant role in the microvascular circulation. Accordingly, long-term reduction of triglycerides may still prove to have beneficial effects on microvascular complications such as nephropathy, retinopathy, and neuropathy.

Increase of creatinine has been associated with high mortality and worse prognosis of cardiovascular disease (Damsgaard et al. 1990, Cullerton et al. 1999, Shlipak et al. 2001, Shlipak et al. 2002). This study does not allow to conclude whether the fenofibrate-induced increase in creatinine impairs...
the prognosis of these patients. Obviously the follow-up of renal function in clinical practice will be complicated, as the values of commonly used markers are changed. As shown in the FIELD main study, the creatinine levels in the fenofibrate treatment group were 10–12 μmol/L higher than in the placebo group, but returned back to baseline level after 6–8 weeks from the study close-out. As fenofibrate treatment is accompanied by an increase of Cystatin C, there are currently no methods to accurately detect minor changes in renal function during fenofibrate treatment.

7.8 Positioning of fibrates in the current management of type 2 diabetes

The outcomes of fibrate trials have been conflicting. The FIELD study, the largest fibrate study so far, reported a non-significant 11% reduction in primary endpoint (non-fatal and fatal CHD events) (Keech et al. 2005). This was less than expected compared to other fibrate studies such as VA-HIT and BIP (Rubins et al. 1999, The BIP Investigators 2000). In the FIELD study, a significant 19% reduction in total cardiovascular disease events was noted in patients with no previous cardiovascular disease. It has also been speculated, whether the high drop-in rate to statins would have diluted the results. A recent statistical approach of the FIELD study adjusted for the drop-outs and additional therapies suggest a 15% reduction in CVD events and a 16% reduction in CHD events for fenofibrate (manuscript under preparation). So far, fibrates have not reduced mortality in randomized clinical trials.

The effect of fenofibrate on HDL cholesterol faded from +5.1% to +1.2% at FIELD study close. Still, an approach to raise HDL in type 2 diabetes would be needed due to high residual risk of CVD events despite appropriate LDL lowering treatment (Barter et al. 2007). Of fibrates, only gemfibrozil and bezafibrate have reduced CVD events and raised HDL in large-scale randomized clinical trials (Rubins et al. 1999, The BIP Investigators 2000). Interestingly, the BIP investigators recently published 16-year mortality follow-up, showing that the bezafibrate-treated patients with upper tertile HDL response (>0.2mmol/L increase) had a 22% reduction in mortality whereas the patients with lower tertile HDL response had similar mortality than the placebo control group (Goldenberg et al. 2009). Data was however based on small number of endpoints.

Both novel and older HDL-raising agents are currently studied in large-scale trials. Unfortunately, both CETP-inhibitor torcetrapib and ACAT-inhibitor pactimibe have increased carotid IMT and CVD risk in patients with familial hypercholesterolemia (Bots et al. 2007, Meuwese et al. 2009). Unlike torcetrapib, a new CETP-inhibitor anacetrab did not increase blood pressure and aldosterone in a 8-week trial (Bloomfield et al. 2009).
Niacin is currently investigated in endpoint study of 25,000 patients and is already available as an extended-release form with a prostaglandin D2 receptor antagonist combined to decrease the skin-related side effects.

According to this study and the main FIELD study, fenofibrate can no longer be considered as a robust HDL-raising agent. It can be speculated that the antiatherogenic function of HDL is compromised during fenofibrate treatment, since reduction of apoA-I is considered to affect the antiatherogenic potential of HDL particles. Altogether, data from this substudy and the main FIELD study suggest a link between fenofibrate-induced homocysteine increase and apoA-I decrease (Taskinen et al. 2009). This might explain also the attenuation of the HDL increase during the study.

Further, results of this study do not support the use of fenofibrate to reduce albuminuria progression. Rather, a decrease is reported in Cystatin C, a creatinine-independent marker of renal function. This data does not allow concluding whether increase of Cystatin C has prognostic value during fenofibrate treatment. Despite positive signals from the main FIELD substudies concerning retinopathy and peripheral amputations (Keech et al. 2007, Rajamani et al. 2009), potential benefits of fenofibrate on microvascular disease remain unresolved and request further studies specifically addressing these issues.

In a recent analysis of the FIELD study, CHD event rate was highest in those patients who had at baseline high triglycerides and low HDL cholesterol (Scott et al. 2009). In this subgroup, the reduction of CHD events by fenofibrate was highest, similarly with other fibrate studies. The evidence concerning CVD reduction with fibrates remains however modest in comparison with statin therapy. Thus, use of fenofibrate should only be considered in combination with statin or in the case of moderate or severe hypertriglyceridemia. Of the fibrates to be used with a statin, fenofibrate seems to be the safest choice (Davidson et al. 2007). Whether combination therapy of fenofibrate and statin beneficially influences the CVD endpoints, will be answered by the lipid-lowering arm of the ACCORD study in early 2010 (Ginsberg et al. 2007).
8. SUMMARY OF RESULTS AND CONCLUSIONS

The results of Studies I-V can be summarized as follows:

I Elevation of VLDL triglycerides was the major determinant of plasma triglyceride concentration in normal subjects and in type 2 diabetic individuals. Both apoCIII and apoE metabolism seemed disturbed in type 2 diabetes. Despite elevated serum apoCIII, type 2 diabetic subjects had a relative deficiency of apoCIII in all TRL subclasses suggesting profound disturbances in apo CIII metabolism.

II Enrichment of LDL with apoCIII induced a small increase in LDL–proteoglycan binding in type 2 diabetes. Further findings indicated that intrinsic characteristics of diabetic LDL other than apoCIII per se were responsible for further increased proteoglycan binding of diabetic LDL with high endogenous apoCIII, and alterations in the lipid composition of diabetic LDL with high apoCIII were shown.

III Fenofibrate markedly reduced large VLDL particle mass, increased LDL size and produced a clear shift in HDL subclasses towards smaller particles. Interestingly, fenofibrate-induced high levels of homocysteine were associated with lower increase of HDL cholesterol and apoA-I.

IV Fenofibrate treatment was not associated with beneficial changes in IMT, augmentation index, or biomarkers of inflammation and endothelial function. Rather, IMT and the augmentation index increased similarly in both treatment groups during the study. Plasma levels of CRP, IL-6, SPLA2, SAA, VCAM-1, ICAM-1, and E-selectin remained similar in both groups.

V Fenofibrate decreased creatinine clearance and eGFR. Such changes would complicate the clinical surveillance of renal function during fenofibrate treatment. In contrast to the main FIELD study, no beneficial effect on albuminuria was demonstrated with fenofibrate. Cystatin C increased in type 2 diabetic patients during fenofibrate treatment, which may even indicate renal dysfunction.
9. ACKNOWLEDGEMENTS

I am deeply grateful for Professor Marja-Riitta Taskinen for her guidance and encouragement during these years. Her wide experience, research facilities, and excellent international network of collaboration have been vital for the success of this substudy. I am proud of being part of her research group and thankful for her understanding with issues of private life. Her good-humoured support, trust, and patience were essential in the final year of this project.

Professor Pirjo Nuutila and Docent Katriina Aalto-Setälä are gratefully acknowledged for sharing their summertime with the manuscript of this thesis. I am thankful for their kind comments, thorough review, and valuable suggestions.

This work would not have been possible without Dr. Eeva Leinonen, who was the driving force for this substudy. I also want to thank Eeva for introducing me to the IMT measurements and medical research in general. I am grateful for Docent Jukka Westerbacka for the performance of pulse wave analysis and for delightful company at the laboratory and abroad. I thank Professor Hannele Yki-Järvinen for scientific collaboration and for the research facilities for pulse wave analysis.

We have had the privilege to collaborate with true experts in many fields. I am grateful to Professor Jukka T. Salonen, Dr. Tomi-Pekka Tuomainen, and Dr. Riitta Salonen for their expertise and facilities concerning IMT measurements. Warmest thanks to Professor Seppo Sarna for sharing his statistical knowledge. I have truly enjoyed the afternoon discussions with Dr. Carol Forsblom and Docent Per-Henrik Groop. Their precise comments and scientific knowledge in diabetic nephropathy have been invaluable for our mutual project. I thank Professor Jean-Charles Fruchart and Jamila Fruchart for their efforts and knowledge in apoCIII.

I wish to thank many of the personnel in National Institute of Health and Welfare. The expertise and laboratory facilities of Docent Matti Jauhiainen and Professor Christian Ehnholm are gratefully acknowledged. I thank Jouko Sundvall for collaboration that has been prompt and essential.

I am grateful for the cheerful and prompt collaboration with colleagues from Sahlgrenska Academy at Gothenburg University. Professor Jan Borén has driven our mutual project with admirable energy. Prof. Borén and MSc Marcus Ståhlberg, among others in their group, are warmly thanked for their contribution. I am thankful and proud of our collaboration with such mass spectrometry specialists as Kim Ekroos and Matej Orešič are.

I thank Hannele Hildén, Helinä Perttunen-Nio, Virve Naatti, Ritva
Marjanen, and Tomi Silvennoinen for good company and superior laboratory excellence. I am thankful to Arja Malkki and Jarmo Tiikkainen for their competence in reading of the IMT scans and to Kimmo Ronkainen for the IMT data management.

I am grateful for the FIELD study coordinator Anne Salo and site coordinators Iiris Suonranta, Nina Hyvärinen, Tuija Mård and Virve Naatti for their efforts in the FIELD main study as well as in this substudy. I wish to thank the FIELD study doctors Eeva Leinonen, Satu Vehkavaara, Pia Pajunen, and Maritta Lindström-Karjalainen for their expertise concerning the clinical care of type 2 diabetic patients. I thank the secretarial assistance and delightful company of Helena Laakkonen, Anne-Mari Syrjänen, Anne Kokkonen, Päivi Närävä, and Maaria Puupponen.

These studies were financially supported by the Helsinki University Central Hospital Research Foundation, the Aarne Koskelo Foundation, the Aarne and Aili Turunen Foundation, the Biomedicum Helsinki Foundation, and the Finnish Medical Foundation.

I express my deepest gratitude to all diabetic patients and healthy subjects who volunteered to this study.

As I hanged around Marsu’s laboratory for ten years, I have learned to know many excellent researchers and skillful clinicians. Dr. Sanni Söderlund is gratefully acknowledged for her company and help in laboratory as well as outside and for letting me rent their apartment. I thank Dr. Aino Soro-Paavonen for concretely showing me the basics of SPSS. Aino and Docent Heikki Koistinen are acknowledged for encouraging me with clinical work. For the cheerful and helpful atmosphere in our researcher corner, I would also like to thank doctors Kati Ylitalo, Juha Vakkilainen, Sakari Mänttäri, Ming-Lin Liu, Hiroshi Watanabe, and Shuhei Nakanishi. Of the colleagues in neighbouring groups, I wish to thank Ksenia Sebastianova and Lena Thorn for good company.

Also outside Biomedicum, I have had the privilege to work in excellent workplaces and with delightful people. I thank the colleagues and nurses at the Maria Hospital for their clinical experience and team work during the nights of 2004–2007. I stretch my sincerest thanks to the personnel of the Linnainmaa Health Centre for the most pleasant year I had in primary health care. My study nurses in Tampere, Arja Putila and Johanna Hietala, are acknowledged for the efficient and cheerful teamwork. And last, I’d like to thank my current colleagues in Tampere University Hospital for warmly welcoming me to the fascinating world of Oncology.

I am still nostalgicizing over the Girls’ Evenings during and after the Medical School. I wish that I’ll have time to catch up with you all, dear friends. I am grateful to Piia Pohja for being nearby and for your earnest help, whether I needed a delicious pie (as a recipe or served) or the transit abilities of your car. I am glad that our friendship has extended over the period of stepmotherhood.

84 9. Acknowledgements
I am fortunate to have such dearest friends as Katja Heikkinen and Sari Kilpamäki for over twenty years. Our discussions continue from where we left last time, no matter how many months or miles between us. I sincerely enjoy the cheerful moments with your families, and the days babysitting Mikael. Of the very special children in my life, I will always remember Visa and Venla. I loved you more than I ever told and I wish you every best in your life.

Last, I would like to thank my parents Sirkka and Heino. I am indebted to you for your guidance in my life, and for your sensible understanding when not to interfere. I am grateful for your caring even if I wasn't always approachable. Your continuous support was invaluable during 2008.

Tampere August 2009
Anne
10. REFERENCES


Best J, Drury P, Kesäniemi YA, Colman P, Scott R, Taskinen MR, Pardy C, Keech A (2008) Maintenance of Glycemic Control in over 4,000 People with Type 2 Diabetes in 3 Countries for 5 Years with Metformin, Sulfonylurea and Insulin Therapy. *Presented at the 68th Scientific Meeting of the American Diabetes Association*


Cohn JS, Patterson BW, Uffelman KD, Davignon J, Steiner G (2004) Rate of production of plasma and very-low-density lipoprotein (VLDL) apolipoprotein C-III is strongly related to the concentration and level of production of VLDL triglyceride in male subjects with different body weights and levels of insulin sensitivity. J.Clin.Endocrinol.Metab. 89:3949-3955


Desager JP, Horsmans Y, Vandenplas C, Harvengt C (1996) Pharmacodynamic activity of lipoprotein lipase and hepatic lipase, and pharmacokinetic parameters measured in normolipidaemic subjects receiving ciproadipate (100 or 200 mg/day) or micronised fenofibrate (200 mg/day) therapy for 23 days. Atherosclerosis 124 Suppl:S65-73


10. References


92 10. References


94 10. References


10. References


10. References


10. References 101


Otros JD, Collins D, Freedman DS, Shalaurova I, Schaefer EJ, McNamara JR, Bloomfield HE, Robins SJ (2006) Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the Veterans Affairs High-Density Lipoprotein Intervention Trial. Circulation 113:1556-1563


104 10. References


10. References 105


10. References 107


Stein O and Stein Y (1999) Atheroprotective mechanisms of HDL. Atherosclerosis 144:285-301


108 10. References


110 10. References


10. References


