Dyslipidemia beyond LDL

Dyslipidemie, meer dan alleen LDL

Reyhana Yahya

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geboren te Bagdad

Erasmus University Rotterdam

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Chapter 1

Introduction to the thesis

Dyslipidemia

Cardiovascular disease (CVD) is the leading cause of mortality worldwide. In 2012, World Health Organization estimates suggested that one third of all global deaths could be attributed to CVD (1). Although treatment with statins as a lipid-lowering drug proved its efficacy in the past decades in the prevention of CVD, still more than 60% of cardiovascular events do occur (2-8). Classical risk factors associated with CVD include non-modifiable risk factors such as age and male sex, and modifiable risk factors such as an unhealthy lifestyle (tobacco smoking, sedentary lifestyle, western type diet, and obesity), hypertension, type 2 diabetes (T2D), and dyslipidemia (9).

Dyslipidemia is one of the major CVD risk factors (10). It can be caused by a monogenic disorder as observed in subjects with familial hypercholesterolemia (FH), or by more complex conditions such as obesity and diabetes mellitus type 2 (T2D). Polygenic conditions and environmental factors can also cause or worsen dyslipidemia (9). Dyslipidemia includes all alterations in the lipoprotein profile associated with increased CVD risk, such as for example increased levels of the low density lipoprotein cholesterol (LDL-C) as observed in subjects with FH (11, 12). Hypertriglyceridemia and low levels of high density lipoprotein (HDL) cholesterol, often present in subjects with obesity or T2D, are also known to increase the CVD risk (13-15). Moreover, recently, increased levels of Lipoprotein(a) (Lp(a)) have been identified as a causal factor for atherosclerosis and CVD (16-20).

Statins are the most prescribed cholesterol-lowering treatment. They inhibit cholesterol synthesis and upregulate the LDL receptors (LDLR), thereby decreasing total cholesterol levels and mainly LDL-C plasma levels. Since their introduction in 1990, statins have been prescribed for LDL-C lowering in high risk subjects, and led to a 30% reduction in cardiovascular events (2, 21-23). However, many subjects still develop CVD despite statin treatment, and even despite achieving LDL-C levels at or below the recommended treatment target levels (24, 25). The residual CVD risk may in part be attributable to residual dyslipidemia, such as low HDL-C levels, decreased HDL function, preponderance of small dense LDL, increased triglycerides (TG) levels and increased Lp(a) levels (16, 19, 26-32). These risk factors may be determined by genetics, affected by environmental factors such as lifestyle and by drugs.

Despite the growing attention of the population and the treating clinicians for prevention of CVD and thereby the treatment of dyslipidemia, the residual CVD risk remains substantial. This is in part due to residual dyslipidemia. In this introduction, we will discuss the potential use of advanced lipoprotein profiling, which might identify several types of dyslipidemia, currently not identified by the standard lipid measurements. We will also provide a short update on Lp(a) as a CVD risk marker, and the at risk cohorts studied in this thesis (FH and T2D).

Advanced lipoprotein profiling

Lipoproteins can be divided into two types, atherogenic lipoproteins such as LDL (33), Lp(a) (16), intermediate density lipoprotein (IDL) (34), very low density lipoprotein (VLDL) and chylomicrons (35). These lipoproteins are atherogenic and thus positively associated with CVD. The other type is the atheroprotective lipoprotein such as HDL, which is negatively associated with CVD (36).

The present-day, most common lipid measurement is the standard lipid panel, which is performed in the clinical chemistry laboratories, and measures the levels of total cholesterol, HDL-C, TG and LDL-C. The latter is often calculated using the Friedewald equation (37). Unfortunately, the standard lipid panel does not give information about the subclasses of the lipoproteins, their size or density distribution. This type of information is crucial to identify specific dyslipidemias, which are missed with the standard lipid panel. Dyslipidemia can present as alterations in the levels of lipoproteins, but also by changes in the lipoproteins' composition. This is the case for small dense LDL, small dense HDL, and large buoyant VLDL, which all are associated with increased CVD risk (38-41). These subtle alterations in the relative density of the different lipoproteins could be identified using the advanced lipoprotein profiling, which separates the lipoproteins by density and identifies HDL subclasses, Lp(a), LDL subclasses, IDL and VLDL (42, 43). The advanced lipoprotein profiling might help to enhance the identification of subjects at increased risk, providing the opportunity for treatment to decrease their CVD risk (42).

In part I of this thesis, I will discuss the potential use of advanced lipoprotein profiling for improving the diagnosis of dyslipidemia, and how it is affected by increasing glucose intolerance and drug interventions.

Lipoprotein(a)

Lp(a) has recently been identified as a causal factor for CVD (16-20). Lp(a) is an LDL-like lipoprotein with an extra protein attached to it, called apolipoprotein(a) (apo(a); see figure 1.). Plasma Lp(a) levels are highly genetically determined by the number of kringle IV type 2 copies in the LPA gene encoding apo(a) (figure. 1). Subjects with a high kringle IV type 2 copy number and thus a long isoform of apo(a) have lower Lp(a) levels than subjects with a small isoform. The latter is associated with high Lp(a) levels and increased CVD risk (16, 44-47). Non-genetic factors such as lifestyle can also contribute to the variance in Lp(a) levels, which is thought to explain 25% of the levels (48).

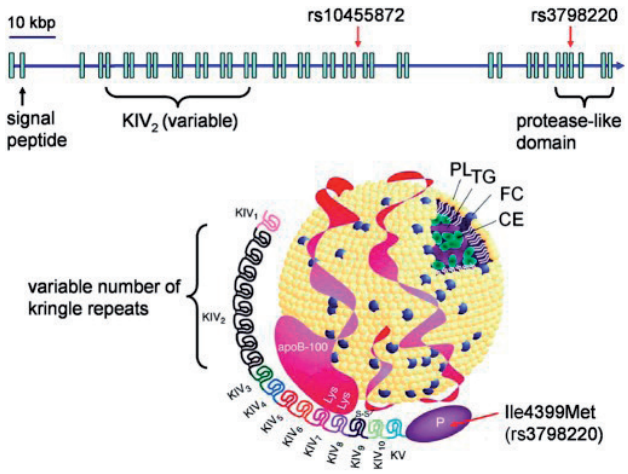

Figure 1. Lipoprotein(a) particle

Although Lp(a) is now widely accepted as a CVD risk factor, it is very difficult to treat, since there is no Lp(a)-lowering therapy available yet. This is also the reason why Lp(a) levels are still not being measured in clinical practice on a regular basis. Another issue is that those subjects with elevated Lp(a) levels often have other conditions related to increased CVD risk (49, 50). It is of importance to measure the Lp(a) levels at least once even without the possibility of specific treatment, not only for risk prediction but also for discriminating between FH and elevated Lp(a) levels. Elevated Lp(a) levels can mimic the phenotype of FH, as almost half of subjects with suspicion of FH, who do not have a mutation in one of the candidate genes, appear to have high Lp(a) levels, clearly also predicting an increased CVD risk (51). Another problem is that measurement of LDL-C by the standard lipid panel is often affected by high Lp(a) levels, and might therefore exaggerate the plasma LDL-C level.

Although no specific Lp(a)-lowering therapy is available right now, treating subjects with elevated Lp(a) levels should be possible in the nearby future, since many emerging drugs do affect Lp(a) levels (52-57), and drugs to specifically reduce Lp(a) levels are being developed. Antisense oligonucleotides targeting the apo(a) is a promising drug which has recently shown to reduce Lp(a) levels (58). Once treatment to specifically lower Lp(a) levels in subjects at risk is available and investigated in large randomized controlled trials, the effect of lowering Lp(a) levels on CVD events can be studied. Until that time, elevated Lp(a) levels can be considered a part of therapy resistant dyslipidemia, contributing to the CVD events that cannot be prevented by classical therapy.

Here, I will investigate the effect of multiple non-genetic and genetic factors on plasma Lp(a) levels. In part II, I will discuss the effect of weight loss on Lp(a) levels in obese subjects, and

the effect of statin use on Lp(a) levels in dyslipidemic subjects. I will also compare Lp(a) levels between subjects with a bi-allelic FH (also called 'homozygous FH' or 'HoFH') and subjects with heterozygous FH. In part III on treatment options, I will discuss the currently available and upcoming lipid-lowering therapies and their effect on Lp(a) levels.

High risk cohorts

Type 2 diabetes

Type 2 diabetes (T2D) is a growing healthcare problem worldwide, with an estimated 415 million persons affected worldwide in the year 2015 and the expectation that this number will increase up to 642 million in 2040 (78). This condition is the result of a relative shortcoming of the insulin production compared to the amount of insulin the body needs for maintaining euglycemia. Insulin resistance, leading to an increased amount of insulin needed for glucose uptake, often precedes T2D. Eventually exhaustion and deterioration of the beta cells occurs. Finally glucose uptake and storage is diminished, causing hyperglycemia, and overt T2D (78).

T2D is highly associated with other co-morbidities such as obesity, hypertension, dyslipidemia, and cardiovascular disease (CVD) (79). Dyslipidemia typically associated with T2D is characterized by low levels of HDL cholesterol and preponderance of small dense HDL, small dense LDL and large VLDL particles, and high levels of plasma TG (40, 41, 80-82). Although Lp(a) seems to be inversely associated with the incidence of diabetes (83), elevated Lp(a) levels are still associated with CVD risk in subjects with T2D (50). Currently, statins are the first choice of lipid-lowering drugs in T2D, because clinical trials with statins did more convincingly show substantial reductions of CVD risk than trials with fibrates and niacin (84). Although statin therapy reduce CVD risk, a substantial residual risk remains among the persons with T2D (85).

The difficulty in treating residual dyslipidemia is the fact that many features of it are not recognized by the standard lipid panel. In part I of my thesis, I will show what features of the residual dyslipidemia can be identified using the advanced lipoprotein profiling and how they change in subjects with increasing glucose intolerance.

Heterozygous Familial hypercholesterolemia

Heterozygous FH is the most common monogenic lipid disorder present in 1 in every 250-500 persons (59). In the majority of cases, this autosomal dominantly inherited condition is a mutation in the gene encoding the low density lipoprotein receptor (*LDLR*) (60). The defective LDL receptors result in reduced uptake of the circulating LDL by the liver. Subsequently, subjects with FH have increased LDL-C levels, often leading to atherosclerosis and premature CVD, mainly coronary heart disease (CHD). FH can also be caused by mutations in the genes

encoding apolipoprotein B (*APOB*) and proprotein convertase subtilisin-kexin type 9 (*PCSK9*). The diagnosis can be based on identification of the causal mutation in the gene causing FH, but also on the clinical features: elevated cholesterol levels, clinical history of premature CVD, cholesterol deposits in the skin (xanthelasmas), eyes (arcus lipoides), and/or tendons (tendon xanthomas), and family history of premature CVD or cholesterol deposits (61).

Without lipid-lowering treatment, 50% of the men with heterozygous FH die before the age of 50, and 30% of the women before the age of 60 years (62). When adequate treatment with statins is initiated early, endothelial function might be restored (63). One study in subjects with heterozygous FH identified by active screening showed that when treated with statins those subjects do not have a higher risk of myocardial infarction than the risk in the general population (12). These 2 studies are done in children and FH subjects without symptoms found by active screening, suggesting the importance of early initiation of statin treatment. Contrary to those results, evidence suggests that statin-treated FH subjects remain at increased risk for the development of CVD (64, 65). Undertreatment of FH is displayed by the fact that only 21% of the statin-treated FH subjects reach LDL-C levels at or below the recommended target levels (66). According to the current treatment guidelines, the recommended LDL-C target levels should be below 2.5 mmol/l for primary CVD prevention and below 1.8 mmol/l for secondary CVD prevention (67). Other risk factors than increased LDL-C levels in subjects with heterozygous FH include increased Lp(a) levels (68), altered HDL composition and increased triglyceride levels (69, 70). Taken together, this suggests that many FH subjects still have LDL-C levels above treatment target levels, sometimes in a combination with other dyslipidemia further increasing their risk for CVD (13, 16, 26, 27, 71).

Homozygous or compound heterozygous Familial hypercholesterolemia

Homozygous or compound heterozygous familial hypercholesterolemia (HoFH) is a rare disease, present in approximately 1:300.000 persons (59). HoFH is caused by bi-allelic mutations mostly present on the *LDLR* gene, or less frequently mutations in the *APOB* or *PCSK9* (59, 72, 73). Just like in heterozygous FH, characteristic physical signs present in HoFH include xanthelasmas, arcus lipoides, and tendon xanthomas. Untreated patients with HoFH have extremely high LDL-C levels often exceeding 13 mmol/L, rendering them susceptible to unparalleled premature atherosclerotic cardiovascular disease (CVD) and extensive aortic valve calcification and stenosis (74, 75). Without treatment the majority of patients with HoFH do not survive beyond their twenties. Early diagnosis and treatment of HoFH is therefore essential (75). However, until recently available drug therapies were not sufficient in reducing LDL-C to target levels (75). The combination of lipid-lowering medication and lipoprotein apheresis is considered the optimal treatment for these patients. However, in many countries lipoprotein apheresis is not reimbursed, and this has generated an extreme challenge in providing optimal treatment for patients with HoFH (75-77). The new emerging

therapies can, however, aggressively reduce LDL-C levels in these patients. In part III on treatment options, I will discuss these therapies and their effect on the LDL-C levels and possible side effects.

AIM AND OUTLINE

The primary aim of my thesis is to investigate the role of advanced lipoprotein profiling in identifying residual dyslipidemia to improve CVD risk classification. The secondary objectives were to investigate the effects of genetics, metabolism and interventions on plasma lipoprotein(a) (Lp(a)) levels.

In part I of this thesis (chapter 2A-B) I investigate the role of advanced lipoprotein profiling in screening of subjects at risk of developing T2D, and follow up during treatment with the new drug "lomitapide". In chapter 2.A, I investigate the effect of increasing glucose intolerance in families with type 2 diabetes on the advanced lipoprotein profile and whether this method could be used as a screening method for subjects in at risk families, for early detection of subjects at risk of developing T2D. In chapter 2.B, I investigate the effect of a new lipid-lowering drug "lomitapide" for the treatment of HoFH with a focus on HDL-C levels, HDL subclasses and HDL function as characteristics of residual dyslipidemia.

In part II (chapter 3A-C), I investigate the effect of genetics and interventions aimed at CVD prevention on plasma Lp(a) levels. In chapter 3.A, I investigate the effect of weight reduction in several cohorts on Lp(a) levels in obese subjects. In chapter 3.B, I investigate the effect of statin treatment on Lp(a) levels in dyslipidemic subjects. In chapter 3.C, I investigate whether Lp(a) levels differ between subjects with heterozygous FH and subjects with HoFH.

In part III (chapter 4A-B), I discuss treatment options. In chapter 4.A, I provide an overview of the currently available and upcoming drugs in the treatment of subjects with elevated Lp(a) levels. In chapter 4.B, I discuss treatment options of subjects with severe forms of hypercholesterolemia illustrated by the lifetime journey of two affected siblings.

Part I:

Detailed lipoprotein profiling

Chapter 2A

HDL subclass levels are associated with insulin resistance and impaired beta-cell function in South Asian families with high risk of type 2 diabetes mellitus.

R. Yahya, S. Jainandunsing, M. L. Licona, L. van der Zee, A. Touw, F.W.M. de Rooij, E.J.G. Sijbrands, A.J.M. Verhoeven, M.T. Mulder

(manuscript in preparation)

Chapter 2B

Lomitapide affects HDL composition and function

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Abstract

Background:
Lomitapide reduces low-density lipoprotein-cholesterol (LDL-C) but also high-density lipoprotein-cholesterol (HDL-C) levels. The latter may reduce the clinical efficacy of lomitapide. We investigated the effect of lomitapide on HDL-C levels and on cholesterol efflux capacity (CEC) of HDL in patients with homozygous familial hypercholesterolemia (HoFH).

Methods and results:
Four HoFH patients were treated with increasing dosages of lomitapide. Lomitapide decreased LDL-C (range -34 to -89%). Total HDL-C levels decreased (range -16 to -34%) with a shift to buoyant HDL. ABCA1-mediated CEC decreased in all patients (range -39 to -99%). The changes of total, ABCG1- and SR-BI-mediated CEC were less consistent.

Conclusion:
Lomitapide decreased LDL-C and HDL-C levels. Our report raises the hypothesis that the anti-atherogenic potential of HDL seems to be unaffected as total CEC did not seem to change consistently. Combined with the reduction of atherogenic lipoproteins, the net effect of lomitapide appears to be beneficial in HoFH patients.

Introduction

Homozygous familial hypercholesterolemia (HoFH) is a rare disease caused by mutations in the *LDLR* gene (72, 73). Untreated patients with HoFH are characterized by extremely raised low density lipoprotein-cholesterol levels (LDL-C) often exceeding 13 mmol/L, rendering them susceptible to unparalleled premature atherosclerotic cardiovascular disease (CVD) and extensive aortic valve calcification and stenosis (74, 75). Without treatment the majority of patients with HoFH do not survive beyond their twenties. Early diagnosis and treatment of HoFH is therefore essential (75).

A new treatment option for HoFH patients has become available with the microsomal triglyceride transfer protein (MTP) inhibitor, lomitapide, which resulted in 38% reduction of LDL-C levels in a phase III trial in 29 HoFH patients (54). However, high-density lipoprotein-cholesterol (HDL-C) levels were reduced by 12% (54, 115, 116). Although HDL-C levels show an inverse correlation with CVD risk, there is increasing evidence that HDL-mediated cholesterol efflux capacity (CEC) is a better predictor of CVD risk compared to HDL-C (30, 31). HDL removes cholesterol from the arterial wall by mediating cholesterol efflux via different pathways involving ABCA1, ABCG1, SR-BI, or aqueous diffusion of free cholesterol (30).

In the present study, we determined the effect of lomitapide treatment on the capacity of HDL to promote cholesterol efflux from macrophages in four HoFH patients.

Methods

Study participants

Four patients with HoFH receiving lomitapide as additional therapy in a clinical setting were included in the present study. They were amongst the first patients to be treated in a named-patients-program worldwide. The diagnosis HoFH was based on genetic analysis and clinical phenotype (LDL-C>13mmol/L) (75).

Three patients were recruited from the Erasmus Medical Center in the Netherlands and one from Palermo University Hospital in Italy, and were treated according to the prescribed protocol (117).

All patients provided written informed consent. This study was approved by the Medical Ethical Committees of the Erasmus Medical Center in the Netherlands and Palermo University in Italy.

Blood analysis and measurements

Venous blood was obtained after a 10-hour overnight fast, prior to treatment with lomitapide and every three or four weeks during the titration period. Plasma and serum obtained after centrifugation were stored at -80ºC. All samples from different timepoints were analyzed in one run.

Lipoprotein profiles were generated with density-gradient ultracentrifugation using the method described by Proudfoot et.al (118). Lipoproteins were separated according to their densities into HDL_3 (1·125-1·21 g/ml), HDL_2 (1·062-1·125 g/ml), LDL(1·019-1·063 g/ml), and IDL+VLDL (<1·019 g/ml) (101). Cholesterol and triglycerides were measured by an enzymatic method using Selectra E (DDS Diagnostic system, Istanbul, Turkey). Lipoprotein(a) [Lp(a)] plasma levels were measured using the Diasys immunoturbidimetric assay (119).

ApoB and ApoA-I levels were measured by immunoturbidimetry on a c311 automatic analyzer (Roche Diagnostics). HDL subclasses were separated by non-denaturing two dimensional (2D) electrophoresis, as previously described (120). The content of preβ-HDL was calculated as percentage of total ApoA-I signal by densitometric analysis.

Cholesterol loading capacity

Cholesterol loading capacity (CLC) was measured as previously described (121) and defined as macrophage cholesterol content after exposure of cells to serum and expressed as µg cholesterol / mg protein.

Cholesterol efflux capacity

Serum was depleted of apoB-containing lipoproteins in order to isolate the serum HDL fraction as previously described (122). ApoB-depleted serum CEC was determined in human monocytes-derived macrophages THP-1 cultured in the presence of 100 ng/ml PMA for 72 hours to allow differentiation into macrophages. The apoB-depleted serum CEC specific for the three cholesterol efflux pathways (ABCA1, ABCG1, SR-BI) was evaluated in established cell culture models, as previously described (123, 124). Cellular cholesterol content before and after serum exposure was measured fluorimetrically as previously described (121).

Statistical analysis

We performed descriptive analyses at baseline and during lomitapide treatment values and we present data as percentage change from baseline. The number of participants did not allow statistical inference. We used Microsoft Excel and Prism Graphpad 5 for the drawing of statistical graphs and data analysis.

Results

Baseline characteristics

The baseline characteristics of the 4 patients are shown in Table 1. Patients 1, 3, and 4 had a history of CVD. All patients had some gastrointestinal-symptoms during lomitapide treatment but complaints were minimalized by a low-fat diet. Lomitapide treatment was interrupted in patient 1 because of non-adherence and in patient 4 because of persistent liver enzymes elevations >5 times upper limit of normal during treatment, which returned to normal after discontinuation of lomitapide.

Atherogenic lipoproteins

As expected the triglyceride levels (measured in intermediate density lipoprotein and very low density lipoprotein (IDL+VLDL)) decreased strongly in all 4 patients (range -78 to -30%). LDL-C and apoB levels decreased in a dose-dependent manner (range -34 to -89% and -42 to -89%, respectively). Patient 2 and 3 achieved the LDL-C treatment target levels on maximum tolerated lomitapide dose. Patient 3 was treated with LDL-apheresis once every 1-2 weeks. This frequency was reduced to once every 8 to 10 weeks during lomitapide treatment. Lp(a) decreased in patient 1-3 (-20% to -74%), but remained unchanged in patient 4.

The CLC of sera of the patients decreased by an average of 20% at maximum lomitapide dose in comparison to baseline (from 53.6 ± 18.0 to 42.8 ± 12.3 ug cholesterol/mg cell protein).

HDL, ApoA-I and cholesterol efflux capacity

Figure 1A shows the individual cholesterol levels in total HDL-C and in HDL subclasses with increasing dosages of lomitapide of the 4 patients individually. The change in HDL-C levels (range -11 to -34%) was observed during treatment with lomitapide 5 mg/day. In all patients, HDL-C levels remained stable with increasing lomitapide dosage. The reduction in HDL-C levels varied per HDL subclass, the HDL_3/HDL_2 ratio remained stable in patient 1 and decreased in the others (range -16 to -68%). Apo-AI levels and the content of Preβ-HDL decreased with lomitapide treatment (range -9 to -47% and -6 to -40%, respectively). This decrease was most pronounced with 5 mg/day lomitapide treatment.

Figures 1B, 1C, 1D and 1E show the effect of lomitapide treatment on total CEC, and on cholesterol efflux via the different pathways for the four patients individually. Changes in total, SR-BI-mediated and ABCG1-mediated cholesterol efflux were inconsistent. ABCA1-mediated cholesterol efflux decreased (-39 to -99%) in all patients.

Table 1. Baseline characteristics.

Patient	Age (years)	Sex	Tendon Xanthomas	Mutations	Total cholesterol levels without medication (mmol/L)	LDL levels without lomitapide (mmol/L)	Age of onset CVD (years)	Co-medication (mg/day)	Maximum lomitapide dose (mg/day)	Duration lomitapide treatment (wks)	Discountinuation lomitapide treatment (Yes/No)
1	29	F	+	-G352D exon 8 -2417insG exon 17	20,1	14,5	25	Atorvastatin 80 Ezetimibe 10	10	9.5	Yes
2	20	F	+	-4.4 kb, duplication exon 12 -2.5 kb deletion exon 7, 8	18,9	14,1	-	Atorvastatin 80 Cholestagel 2x 1875	30	36.5	No
3	36	M	+	-G528D exon 11 -G528D exon 11	23,8	3,9*	26	Simvastatin 60 Ezetimibe 10	20	9	No
4	62	F	+	16 kb deletion exon 7-15	16,9	12,9	58	Questran 2x 4gr Modalim 2x 100	10	9	Yes

* LDL-apheresis once every 1-2 weeks

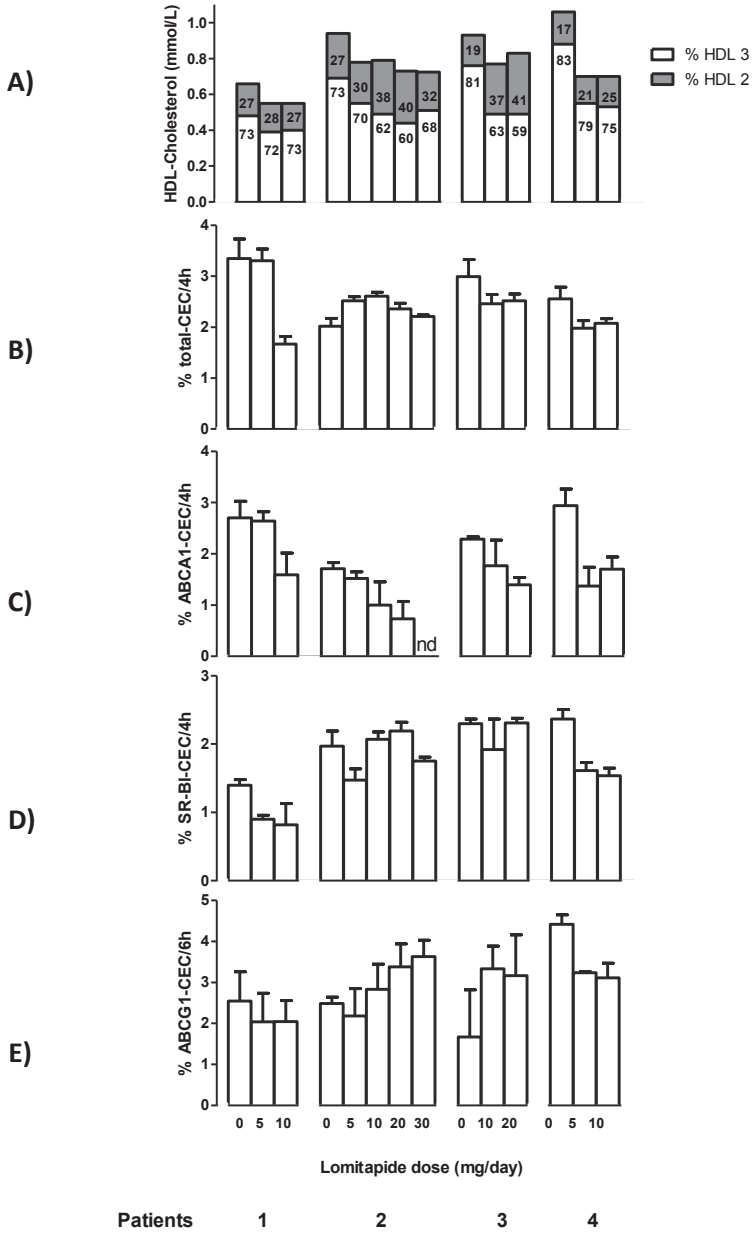

Figure 1. HDL levels and total HDL-mediated CEC and CEC pathways at baseline and during lomitapide treatment per individual

A. HDL-C levels (HDL_3+HDL_2) **B.** Total cholesterol efflux capacity (CEC) of apoB-depleted serum with increasing lomitapide daily dose. **C.** HDL-mediated cholesterol efflux via ABCA1.

D. HDL-mediated cholesterol efflux via SR-BI. **E.** HDL-mediated cholesterol efflux via ABCG1.

Discussion

Our data confirm that lomitapide treatment decreases HDL-C levels. In depth analysis show a shift in HDL subclasses to larger buoyant HDL_2. ABCA1-mediated cholesterol efflux decreased in all four HoFH patients, whereas changes in efflux via ABCG1, SR-BI and total cholesterol efflux were less consistent.

Previous studies showed that lomitapide treatment is associated with a moderate decrease of both HDL-C and ApoA-I levels during the titration period of the drug (54, 115, 116). In line, we found a decrease in the levels of HDL-C, ApoA-I, preβ-HDL, and HDL_3-C, which was most prominent on the lowest dose of lomitapide and remained stable thereafter. However, a shift of HDL to larger and more buoyant particles was observed with HDL_2-C levels remaining unchanged or increased. A reduced formation of HDL during lipolysis of predominantly postprandial triglyceride rich lipoproteins (TGRL) may underlie the reduction in HDL and ApoA-I levels and the alterations in HDL subclass levels. Additionally, lomitapide may reduce the levels of HDL derived from the intestine, since MTP-deficiency has been reported to reduce HDL-cholesterol secretion from the intestine in mice (125-127). In line with this shift in HDL subclasses, the ABCA1-mediated cholesterol efflux was decreased in all patients, whereas changes in the ABCG1- and SR-BI-mediated cholesterol efflux were less consistent.

As expected, lomitapide treatment decreased LDL-C and apoB levels substantially as well as the other atherogenic lipoproteins, i.e. IDL, VLDL, and Lp(a) (54). Consistently, we found that lomitapide reduced the macrophage CLC of serum of all patients. This reflects the improved anti-atherosclerotic potential despite the moderate decrease of HDL-C (128).

Limitations

The major limitation of this study is the small number of participants. Although two of the patients stopped lomitapide treatment this did not interfere with our analyses.

Conclusions

Lomitapide treatment substantially lowered LDL-C levels, though it moderately reduced HDL-C levels. However, HDL seemed to shift from HDL_3 to the larger and more buoyant HDL_2. In addition, the ABCA1-mediated cholesterol efflux decreased, whereas other pathways did not change consistently. Our report raises the hypothesis that the anti-atherogenic potential of HDL seems to be unaffected as total CEC did not seem to change consistently despite decreased HDL-C levels. Combined with the reduction of atherogenic lipoproteins, the net effect of lomitapide appears to be beneficial in HoFH patients.

Part II:

Lp(a)

Chapter 3A

Effect of diet-induced weight loss on Lipoprotein(a) levels in obese individuals with and without type 2 diabetes.

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Abstract

Aims/hypothesis:
Elevated levels of lipoprotein(a) [Lp(a)] are an independent cardiovascular disease (CVD) risk factor, in particular in subjects with type 2 diabetes. While weight loss improves conventional CVD risk factors in type 2 diabetes, effects on Lp(a) are unknown and possibly influencing the long term CVD outcome of diet-induced weight loss. The aim of this clinical study was to determine the effect of diet-induced weight loss on Lp(a) levels in obese individuals with type 2 diabetes.

Methods:
Plasma Lp(a) levels were determined immunoturbidimetrically in plasma obtained before and after 3-4 months of a calorie-restricted diet in four independent study cohorts. The primary cohort consisted of 131 predominantly obese patients with type 2 diabetes (cohort-1). The secondary cohorts consisted of 30 obese patients with type 2 diabetes (cohort-2) and 37 obese subjects without type 2 diabetes (cohort-3), and 26 obese subjects without type 2 diabetes who underwent bariatric surgery (cohort-4).

Results:
In the primary cohort, the calorie-restricted diet resulted in a weight loss of 9.9% (95%CI 8.9, 10.8) and improved conventional CVD risk factors such as LDL cholesterol. Lp(a) levels increased by 7.0 mg/dl (95%CI 4.8, 9.7). In univariate analysis, the change in Lp(a) correlated with baseline Lp(a) levels (r=0.38, $p<0.001$) and change in LDL cholesterol (r=0.19, $p=0.033$). In cohorts 2 and 3, the weight loss of 8.5% (95%CI 6.5, 10.6) and 6.5% (95%CI 5.7, 7.2) was accompanied by a median Lp(a) increase of 6.4 mg/dl (95%CI 1.1, 14.2) and 5.6 mg/dl (95%CI 2.7, 9.0), respectively (all $p<0.001$). When the cohorts 1-3 were combined, the diet-induced increase in Lp(a) correlated with weight loss (r=0.178, $p=0.012$). In cohort-4, no significant change in Lp(a) was found (-3.3 mg/dl (95%CI -8.9, 2.5)) despite considerable weight loss (14.0% (95%CI 12.2, 15.7)).

Conclusions/interpretation:
Diet-induced weight loss was accompanied by an increase in Lp(a) levels in obese subjects with and without type 2 diabetes while conventional CVD risk factors improved. This increase in Lp(a) levels may potentially antagonize the beneficial cardio-metabolic effects of a diet-induced weight reduction.

Introduction

Cardiovascular disease (CVD) is the main cause of morbidity and mortality in obese individuals with and without type 2 diabetes (129-131). The CVD risk of obese patients with type 2 diabetes has been attributed to age, smoking, hyperglycaemia, hypertension, and dyslipidaemia (130). Weight loss via lifestyle programs, consisting of diet and physical activity, results in improved conventional CVD risk factors and is first-line therapy to slow down the development of type 2 diabetes and progression of its' complications in overweight or obese subjects (132, 133).

Lipoprotein(a) [Lp(a)] is an independent CVD risk factor (134-140). Lp(a) consists of a low-density lipoprotein (LDL)-like particle with an additional apolipoprotein(a) [apo(a)] attached to it. Plasma Lp(a) concentrations vary highly between individuals and are largely genetically determined by the number of copies of kringle-IV type 2 (KIV-2) in the apo(a) protein (apo(a) isoform) (44-47). A low number of KIV-2 copies, associated with elevated levels of Lp(a), has been shown to increase the risk of CVD (16). A recent prospective population-based cohort of 56,367 participants showed a significantly higher contribution of Lp(a) levels to CVD and myocardial infarction risk in type 2 diabetes subjects than in non-type 2 diabetic controls (50). About 25% of the variance in Lp(a) levels has been attributed to lifestyle (48). Weight loss in obese subjects has been reported to affect Lp(a) levels, but results are controversial (48, 141-143). The effect of weight loss on plasma Lp(a) levels in type 2 diabetes has not yet been determined.

The aim of the current study was to determine the effect of diet-induced weight loss on Lp(a) levels in obese patients with type 2 diabetes. In order to confirm our findings we also examined the effect of weight loss on Lp(a) levels in three independent cohorts of obese patients with or without type 2 diabetes. As a secondary aim, we assessed the influence of apo(a) isoforms on the diet-induced changes in Lp(a) level in patients with type 2 diabetes.

Material and methods

Subjects and interventions

The effect of weight loss was examined in four independent cohorts. The primary cohort (cohort-1, n=131) consisted of overweight and obese subjects (BMI>27 kg/m^2, 93% obese) with type 2 diabetes who participated in the run-in phase of the Prevention Of Weight Regain (POWER) trial (trial registration no. NTR2264) (48). This trial aimed at studying long term weight maintenance after the run-in diet phase. The sample size of 131 patients was sufficient to find a difference of 5±55 mg/dl with a correlation of 0.95 between the measurements, an alpha of 0.05 and a power of 0.90. The diet started with 8 weeks of a

very low-calorie diet of approximately 3000 kJ (750 kcal) per day, consisting of two meal replacements (Glucerna SR®) and a small dinner daily. Thereafter, energy intake was slowly increased up to approximately 5500 kJ (1300 kcal)/day (a low-calorie diet) during 12 weeks.

Cohort-2 (n=30) also consisted of overweight and obese patients (80% obese) with type 2 diabetes, who were recruited after the POWER trial was finished, for studying the implementation of a very low-calorie diet for weight loss in type 2 diabetes. The participants underwent the same diet intervention as the patients in the primary cohort. Both cohorts 1 and 2 were recruited from the outpatient diabetes clinic of the Erasmus MC in Rotterdam, the Netherlands. To reduce risk of hypoglycaemia, doses of insulin and sulfonylurea derivates were lowered before the start of the diet but after baseline measurements. During the diet, the insulin dose was adjusted regularly to achieve optimal glycaemic control. Metformin use was continued. Only 2 participants were on GLP-1 receptor agonist treatment, which was continued during the intervention period. Statin treatment remained unchanged during the study.

Cohort-3 consisted of 37 obese individuals without type 2 diabetes, who were recruited at the 'Obesity Center CGG' of the Erasmus MC, Rotterdam, the Netherlands. They underwent a 3-month dietary intervention consisting of 2000 kJ (500 kcal)/day reduction of intake relative to baseline (low-calorie diet), with macronutrient and micronutrient content according to national dietary guidelines, while exercise was encouraged.

Cohort-4 consisted of 26 obese individuals without type 2 diabetes, who underwent a gastric banding (n=10) or gastric bypass procedure (n=16). These participants were recruited at the Leiden University Medical Center, Leiden, the Netherlands. No specific diet was recommended beyond a staged meal progression during the first 3 months after surgery. Analyses were performed at baseline and 3 months after surgery.

The dietary intervention studies and Lp(a) analysis of previously collected clinical samples were approved by the Medical Ethics Committee of the Erasmus MC in Rotterdam (reference numbers MEC-2009-143, MEC-2014-090 and MEC 2016-604). The bariatric surgery study and use of the samples was approved by the Medical Ethics Committee of the Leiden University Medical Center (reference number MEC P08.215). All investigations have been carried out in accordance with the principles of the declaration of Helsinki (2008). All participants provided written informed consent.

Measurements

Blood samples were obtained after an overnight fast and were stored at -80°C until further analysis. Demographic variables were recorded and weight, height and waist circumference (except for cohort-4) were measured. HbA_{1c}, fasting glucose, total cholesterol, low-density

lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and triacylglycerol (TG) were measured using standard laboratory techniques.

Lipoprotein(a) measurement

Plasma Lp(a) concentrations were measured using a particle-enhanced immunoturbidimetric assay, which was largely independent of apo(a) KIV repeat number (Diagnostic System #171399910930; DiaSys Diagnostic System, GmbH, Holzheim, Germany) (144). Plasma samples were stored at -80°C for 0.5-5 years and thawed for the first time prior to this analysis. Of each subject, levels at baseline and after intervention were measured in the same run. The detection limit of the assay was 3.0 mg/dl and the mean intra-assay variability was 2.8%. Interference of TG with Lp(a) measurements was minimal, as measured Lp(a) levels were affected by less than 5% by addition of plasma containing different concentrations of TG (ranging from 0 to 12 mmol/l) to plasma with a relatively high Lp(a) concentration (80 or 160 mg/dl). Repeated sampling in 27 healthy controls with an interval of 2-6 months did not reveal significant differences in Lp(a) (13.9 mg/dl (IQR 8.3-41.6) vs. 12.5 (IQR 5.9-28.6); p=0.087; day 0 and after 2-6 months, respectively).

In the primary cohort (cohort-1), the apo(a) KIV repeat number was determined by immunoblotting, as previously described (106, 144). When two distinct apo(a) isoforms were present, the smaller isoform showed the strongest band intensity in most cases and was used as a continuous variable. Apo(a) KIV repeat numbers were stratified in two groups as described earlier (106): low molecular weight (LMW) when at least one isoform with $\leq$ 22 KIV repeats was present, and high molecular weight (HMW) when only isoforms with > 22 repeats are present.

Statistical analysis

Normality of the data and homogeneity of variances were tested using the Shapiro-Wilk test and Levene's test. Variables were expressed as mean ± standard deviation or as median with inter-quartile range (IQR) and were tested for statistical significance using a two-sided paired sample t-test or a Wilcoxon ranking test, depending on normality of the data. Median differences were analysed using a related-samples Hodges-Lehman test. Due to the low numbers in cohorts 2, 3 and 4, in-depth analyses were only performed in cohort-1. We determined Spearman correlations of both baseline Lp(a) levels and Lp(a) change with different parameters of weight loss and glycaemic control.

Mann-Whitney U tests were used to analyse the difference in baseline Lp(a) levels between the LMW and HMW subgroups. Repeated measurements MANOVA analysis (on Blom transformed outcome variables) was used to analyse the difference in Lp(a) change between subgroups. IBM SPSS version 21 and Graphpad Prism version 5 were used for the statistical analyses.

Results

Effect of diet in obese patients with type 2 diabetes (cohort-1)

In Table 1, the characteristics of the primary cohort (cohort-1) at baseline and after intervention are shown. The 131 subjects were predominantly obese, as 93% had a BMI >30 kg/m^2. The remainder had a BMI>27 and ≤ 30 kg/m^2. This cohort had a mixed ethnic background (56% Dutch Caucasians, and 44% South-Asians and Africans). Baseline Lp(a) levels correlated negatively with apo(a) KIV repeat number (r=-0.53, p<0.001), baseline weight (r=-0.18, p=0.046), HbA_{1c} (r=-0.20, p=0.022), fasting TG (r=-0.19, p=0.032) and ethnicity (r=-0.34, p<0.001), and positively with LDL cholesterol (r=0.18, p=0.038). We found no correlation of baseline Lp(a) levels with sex (r=0.076, p=0.389), fasting glucose (r=-0.17, p=0.057) or fasting insulin levels (r=-0.06, p=0.494). Participants of Caucasian origin had lower baseline Lp(a) levels compared to non-Caucasian participants (median 12.2 (2.7-56.9) mg/dl vs. 57.8 (16.1-101.7) mg/dl; p<0.001).

The diet resulted in weight loss of 10.5 kg (95%CI 9.4, 11.5), which was 9.9% (95%CI 8.9, 10.8) of initial body weight. Both BMI and waist circumference decreased significantly (p<0.001 for all). HbA_{1c} and fasting glucose decreased (p<0.001 for both), indicating improved glycaemic control. Lipid parameters also improved during the diet intervention (p<0.01 for all, Table 1).

Lp(a) levels increased significantly from 19.4 (IQR 6.6-75.6) mg/dl to 26.5 (IQR 10.9-95.3) mg/dl (p<0.001, Table 1). Figure 1 shows a waterfall plot of the changes in Lp(a) per individual. Of the 131 participants, 49 showed an increase of ≥ 10 mg/dl and only 6 showed a decrease of ≥ 10 mg/dl. The median increase in Lp(a) levels in cohort-1 was 7.0 mg/dl (95%CI 4.8, 9.7).

Table 1. Characteristics of the study cohorts before and after intervention

	Cohort-1 (n=131)		Cohort-2 (n=30)		Cohort-3 (n=37)		Cohort-4 (n=26)	
Variables	Before	After	Before	After	Before	After	Before	After
Age (y (range))	54 (26-74)	-	55 (34-70)	-	42 (18-63)	-	48 (34-59)	-
Sex (n (%) female)	75 (57%)	-	15 (50%)	-	29 (78%)	-	26 (100%)	-
Years after diagnosis type 2 diabetes	10.0 (3.0-15.0)	-	5.0 (2.0-10.0)	-	-	-	-	-
Weight (kg)	105.0±19.1	94.5±17.3***	103.2±23.3	94.2±21.7***	111.4±17.1	104.3±16.7***	124.0±11.8	106.6±11.2***
BMI (kg/m²)	36.8±5.6	33.1±5.2***	34.8±6.6	31.8±6.6***	38.4±4.7	35.9±4.5***	43.7±3.2	37.4±3.5***
Waist circumference (cm)	119.8±12.9	110.8±11.9***	113.1±12.3	106.0±12.3**	106.2±15.1	98.3±13.8***	-	-
HbA_{1c} (%)	7.7 (6.9-8.6)	7.0 (6.1-8.2)***	7.5 (7.0-8.2)	6.6 (6.0-8.2)**	5.5 (5.3-5.8)	5.4 (5.2-5.7)**		
HbA_{1c} (mmol/mol)	61.0 (52.0-71.0)	53.0 (43.0-66.0)***	58.0 (53.0-65.8)	49.0 (42.0-66.0)**	37.0 (34.0-39.5)	36.0 (33.0-38.5)**	-	-
Fasting glucose (mmol/l)	8.8 (6.9-10.8)	7.3 (6.1-9.3)***	8.7 (7.0-10.5)	7.4 (6.5-9.3)	5.3 (5.0-5.8)	5.1 (4.8-5.4)**	5.1 (4.7-5.2)	4.9 (4.4-5.3)
Total cholesterol (mmol/l)	4.4 (3.7-5.1)	4.1 (3.5-4.8)***	3.9 (3.6-5.1)	4.2 (3.5-5.5)	5.2 (4.3-5.7)	4.6 (4.1-5.2)***	4.7 (3.8-5.8)	4.0 (3.5-4.9)**
LDL cholesterol (mmol/l)	2.5 (2.1-3.1)	2.4 (1.8-2.9)***	2.4 (2.0-3.2)	2.2 (1.7-3.3)	3.4 (3.0-4.0)	3.1 (2.7-3.6)***	2.8 (2.3-3.6)	2.3 (1.8-3.0)**
HDL cholesterol (mmol/l)	1.1 (1.0-1.3)	1.2 (1.0-1.4)**	1.2 (1.0-1.5)	1.2 (1.1-1.5)	1.3 (1.1-1.4)	1.2 (1.1-1.4)**	1.1 (1.0-1.3)	1.0 (0.9-1.2)**
Triacylglycerol (mmol/l)	1.8 (1.2-2.6)	1.4 (1.0-2.0)***	1.5 (1.1-2.5)	1.4 (0.9-2.0)	1.3 (1.0-1.8)	1.1 (0.9-1.4)*	1.2 (0.9-1.8)	1.2 (1.0-1.4)
Lipoprotein(a) (nmol/l)	40.9 (13.9-159.5)	55.9 (23.0-201.1)***	56.9 (12.4-148.9)	61.5 (20.4-185.9)*	27.0 (2.1-75.2)	45.2 (22.7-94.5)**	36.4 (17.2-91.5)	20.6 (6.3-104.1)

Data are mean + SD, or median (IQR). *p<0.05; **p<0.01; ***p<0.001; difference before-after intervention

The change in Lp(a) correlated with baseline Lp(a) levels (r=0.38, p<0.001) and with the change in fasting glucose (r=-0.17, p=0.049) and change in LDL cholesterol (r=0.19, p=0.033). The correlations with change in fasting glucose and LDL cholesterol disappeared after correction for baseline Lp(a) levels. Change in Lp(a) did not correlate with sex (r=-0.041, p=0.543) and change in weight (r=-0.14, p=0.116). The change in Lp(a) also correlated with ethnicity (Caucasians vs. non-Caucasians: r=-0.17, p=0.048), but no longer after correction for baseline Lp(a) levels. There was no difference in the response to the diet between Caucasians and non-Caucasians in a repeated measurements MANOVA ($F_{(1;129)}$=0.199, p=0.656). In cohort-1, 95 out of the 131 (73%) participants used statins. The diet-induced change in Lp(a) levels was similar whether or not statins were used ($F_{(1;129)}$=0.669, p=0.415).

Excluding two possible outliers with ≥100 mg/dl increase in Lp(a) level did not alter the outcomes.

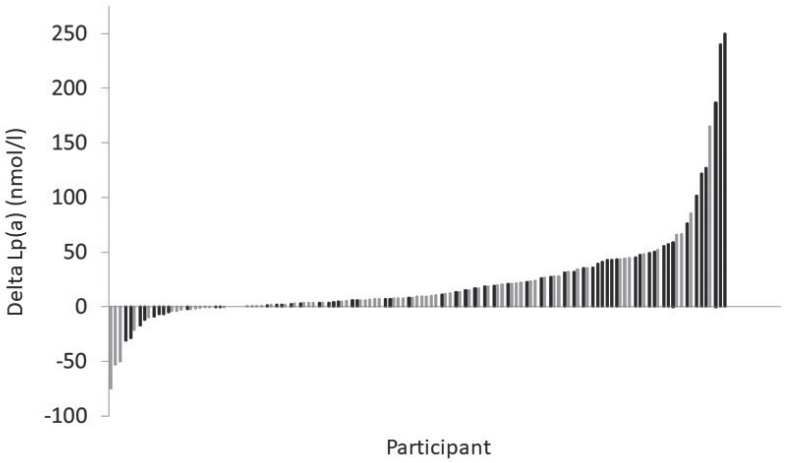

Figure 1. Diet-induced changes in Lp(a) levels per individual in cohort-1 (n=131)

Individual participants (x-axis) arranged according to the diet-induced change in Lp(a) level. Indicated in grey are the Caucasian participants and in black the non-Caucasian participants.

Effect of Apo(a) isoform on diet-induced changes in Lp(a) levels in cohort-1

Forty-three participants had a low molecular weight (LMW) and 88 a high molecular weight (HMW) apo(a) isoform. As expected, baseline Lp(a) levels were significantly higher in the LMW than in the HMW subgroup (70.5 mg/dl (IQR 12.6-141.2) vs. 14.5 mg/dl (IQR 3.1-56.6), p<0.001). Lp(a) levels increased during the diet intervention to 86.6 mg/dl (IQR 17.7-155.2; p<0.001) in the LMW subgroup and to 19.7 mg/dl (IQR 7.3-66.3; p<0.001) in the HMW subgroup, as shown in figure 2. The diet induced effect on Lp(a) in the LMW versus the HMW subgroup did not significantly differ ($F_{(1;129)}$=1.68, p=0.197). The alteration in Lp(a) levels strongly correlated with baseline Lp(a) level in the HMW subgroup (r = 0.43, p<0.001), but not in the LMW subgroup (r = 0.242, p=0.118).

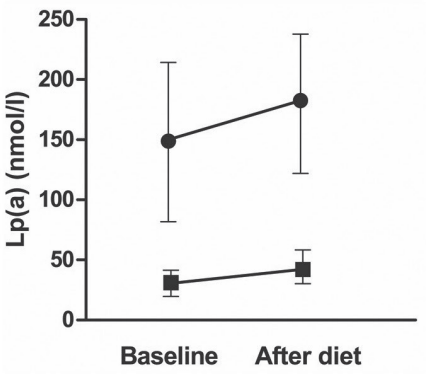

Figure 2. The effect of the diet intervention on Lp(a) levels in the Apo(a) isoform subgroups in cohort-1

Depicted are medians with 95%CI of Lp(a) levels before and after the diet intervention for the low molecular weight Apo(a) isoform group (black circles, n=43) and the high molecular weight Apo(a) isoform group (black squares, n=88).

Long term effect

Of the 131 participants of cohort-1, 69 consented to provide an additional blood sample twenty months after finishing the diet intervention. This subgroup was older (55.6 vs. 51.8 years, p=0.016), had a longer history of type 2 diabetes (12.2 vs. 8.8 years, p=0.017) and had lost more weight during the diet intervention (12.1 vs 8.6 kg, p=0.001), but did not differ from the other participants in sex distribution, ethnicity, baseline Lp(a), BMI, HbA_{1c} and LDL-cholesterol, nor in change in Lp(a) during the diet. In this subgroup, Lp(a) levels increased from 19.4 (IQR 7.4-71.9) to 26.1 (IQR 11.7-94.9) mg/dl during the diet intervention. Twenty months later, patients regained 6.8±5.5 kg of body weight and Lp(a) levels again decreased to 20.8 (IQR 5.8-74.8) mg/dl (p=0.050). Although still borderline significantly different, Lp(a) levels 20 months after the intervention clearly moved towards the baseline value and were highly correlated with baseline Lp(a) (r=0.923, p<0.001). Weight regain was not correlated with the decrease in Lp(a) levels from end of intervention to 20 months (r=-0.061, p=0.626).

Effect of weight loss on Lp(a) levels in secondary cohorts

The characteristics of the three other cohorts at baseline and after intervention are shown in Table 1. Cohort-2, consisting of predominantly obese patients with type 2 diabetes, showed effects of the diet similar to the primary cohort. Weight loss was 9.0 kg (95%CI 6.7, 11.3) or 8.5% (95%CI 6.5, 10.6) of initial bodyweight, and both BMI and waist circumference decreased significantly (p<0.01 for all). HbA_{1c} decreased as well (p=0.001), but changes in fasting glucose and lipid parameters (TC, TG, LDL, HDL) did not reach statistical significance in this small group (Table 1). During dieting, Lp(a) increased from 27.0 (IQR 5.9-70.6) mg/dl to 29.2 (IQR 9.7-88.1) mg/dl (p=0.018, Table 1). The median increase in Lp(a) was 6.4 mg/dl (95%CI 1.1, 14.2).

In cohort-3, which consisted of obese individuals without type 2 diabetes, the diet intervention led to a weight loss of 7.1 kg (95%CI 6.3, 8.0) or 6.5% (95%CI 5.7, 7.2) of initial body weight, and significant reductions in BMI and waist circumference (p<0.001 for all). Although non-type 2 diabetic, HbA_{1c} and fasting glucose improved in this group (p=0.002 and p=0.003). In addition, lipid parameters improved significantly (p<0.05 for all). Lp(a) levels increased from 12.8 (IQR 1.0-35.7) mg/dl to 21.4 (IQR 10.8-44.8) mg/dl (p=0.001, Table 1). The median increase in Lp(a) was 5.6 mg/dl (95%CI 2.7, 9.0).

Cohort-4 consisted of obese subjects without type 2 diabetes who underwent bariatric surgery and were followed for 3 months. This intervention resulted in a weight loss of 17.4 kg (95%CI 15.0, 19.8) or 14.0% (95%CI 12.2, 15.7) of initial body weight (p<0.001). During this period, most lipid parameters improved significantly (Table 1). Lp(a) levels were lower after the intervention than before (from 17.3 (IQR 8.2-43.4) mg/dl to 9.75 (IQR <3.0-49.3) mg/dl) but this result did not reach statistical significance in this small group (Table 1). The median difference in Lp(a) level was -3.3 mg/dl (95%CI -8.9, 2.5).

Figure 3 summarizes the results obtained in the four independent cohorts. The relationship between weight loss and increase in Lp(a) levels was similar for the first three cohorts. When cohorts 1-3 were taken together, the increase in Lp(a) correlated with the diet-induced weight loss (n=198, r=-0.178, p=0.012). This relationship was not observed for cohort 4, which consisted of individuals who lost weight after bariatric surgery.

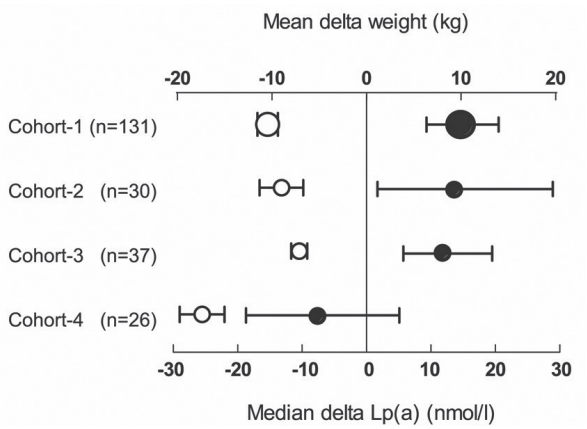

Figure 3. Delta Lp(a) and delta weight in the four independent study cohorts

Depicted are mean with 95%CI for delta weight (white circles) and median with 95%CI for delta Lp(a) (black circles) in the four cohorts. The size of the circles reflects the number of participants.

Discussion

Our data show that diet-induced weight loss increased Lp(a) levels in overweight and obese subjects irrespective of the presence or absence of type 2 diabetes. In patients with type 2 diabetes, the extent of this increase was mainly determined by baseline Lp(a) level, with the highest increase in individuals with the highest baseline levels. This effect on Lp(a) was independent of the apo(a) isoform. Such an increase in Lp(a) levels was not observed in individuals that underwent bariatric surgery, suggesting that weight loss per se does not increase Lp(a) levels.

Previous studies did not show a change in Lp(a) levels in obese adults after various diet interventions aimed at weight loss (48, 142, 143). In these studies, weight reducing drugs and diets different from ours were tested. One study reported a decrease in Lp(a) levels in obese children (141). The discrepancy with our study may be explained by different age-related hormonal states, or by differences in diet composition. The type and content of fat in the diet may be an important determinant of the dietary effect on Lp(a) levels. Increased intake of total- and saturated fat has been found to decrease Lp(a) levels, while an increased intake of monounsaturated fatty acids tend to increase Lp(a) levels in healthy and metabolically disturbed subjects (145-147). Faghihnia et al. (146) suggested that dietary fat-induced changes in LDL metabolism, notably of medium and very small LDL subclasses, may lead to altered formation, catabolism or clearance of Lp(a). The dietary interventions used in our cohorts 1-3 were all based on a low intake of total- and saturated fat, while no specific dietary restrictions were prescribed for the participants in the bariatric surgery cohort. In a subset of participants of cohort-1, Lp(a) levels had almost returned to baseline values during 20 months of follow-up. Despite weight regain, the average body weight was still lower than at baseline. Weight regain was not correlated with long-term change in Lp(a) levels. This suggests that the increase of Lp(a) levels was an acute effect of the diet that waned off after a longer period of a less strict diet. Unfortunately, we have no data on the diet during follow-up. Future studies on the effect on Lp(a) of weight reducing diets with a differential fat content in obese patients with and without type 2 diabetes are warranted.

High Lp(a) levels have consistently been associated with an increased risk of coronary heart disease (134, 137), and results from genetic studies indicate a causal association of high Lp(a) levels with cardiovascular disease (16, 17, 148). The CVD risk associated with high Lp(a) levels is notably higher in subjects with than in those without type 2 diabetes (50). The dose-response relationship of Lp(a)-levels with CVD risk has been shown to be curvilinear in shape, with no evidence of a threshold (149). This suggests that the increase in Lp(a) levels induced by weight-loss dieting observed in our study might increase CVD risk. This could potentially reduce the beneficial cardio-metabolic effects that result from the improvement of conventional CVD risk factors upon diet-induced weight loss. In the Look AHEAD study,

the incidence of CVD was not reduced by a low-calorie, low-fat diet and physical activity in type 2 diabetes patients after 10 years of follow-up, despite improved conventional CVD risk factors (133). Hypothetically, a parallel increase in Lp(a) levels could be one of the explanations why CVD events were not reduced by this lifestyle change. However, effects on Lp(a) levels were not reported in the Look AHEAD trial. Randomized clinical trials addressing the effect of alterations in Lp(a) levels, following life style changes or medication, on hard clinical endpoints or CVD risk are needed. Recently, the short-term efficacy and safety of two specific Lp(a) lowering agents has been shown (58). Long-term effects on cardiovascular endpoints are awaited.

In bariatric-surgery patients, weight loss was not accompanied by an increase in Lp(a) levels. Two previous studies showed that bariatric surgery-induced weight loss in obese individuals was accompanied by a decrease in Lp(a) levels (150, 151), whereas no significant effect was found in another study (152). Hypothetically, the effects of bariatric surgery on bile acid flow, inflammation, release of gastrointestinal hormones, the gut microbiome, plus the wound healing processes may all have had an impact on Lp(a), resulting in the absence of weight loss-induced increase in Lp(a) levels (153-157).

The baseline Lp(a) levels in our two type 2 diabetes cohorts (cohorts 1&2) were relatively high compared to the two non-type 2 diabetic cohorts (cohorts 3&4), whereas in the Women's Health Study and Copenhagen City Heart Study the Lp(a) levels of diabetes cases were significantly lower than the Lp(a) levels of controls (83, 158). Non-Caucasian patients, in particular from South-Asian ancestry, display markedly higher Lp(a) levels than Caucasians (159-161), and are overrepresented in our type 2 diabetes-cohorts. Change in Lp(a) during diet was correlated with ethnicity. However, when we accounted for baseline levels using a repeated measurements MANOVA, we did not find ethnic differences in the diet-induced effect on Lp(a) levels.

Strengths of this study are its prospective design and the use of four independent cohorts for investigating the effect of weight loss on Lp(a), which more than doubled the total number of participants studied on this topic so far. Our study is descriptive in nature. Future studies should clarify the mechanisms underlying the increase in Lp(a) levels upon diet-induced weight loss as well as the consequence of weight loss on the functionality of Lp(a). As all participants were referred to a tertiary center, our findings may not be generalizable to the entire population of overweight and obese patients with or without type 2 diabetes. We found that the effect of diet-induced weight loss on Lp(a) levels was irrespective of the presence or absence of type 2 diabetes. However, some of the individuals in cohorts 3 and 4 may have been pre-diabetic, since classification was based on fasting glucose and not on the oral glucose tolerance test. Finally, a long-term follow-up study is required to determine

whether elevated Lp(a) levels after weight loss dieting affects the incidence of CVD in obese patients with and without type 2 diabetes.

In conclusion, Lp(a) levels increased significantly in obese subjects with and without type 2 diabetes during diet-induced weight loss, but not in subjects who underwent bariatric surgery. This may hypothetically reduce the beneficial cardio-metabolic effects of a diet-induced weight loss. Therefore, Lp(a) may be an additional target in overweight and obese subjects on a calorie-restricted diet to reduce the risk of CVD. Long term follow-up studies are required to establish whether adding a specific Lp(a) lowering agent to a diet intervention will improve long term CVD outcome in obese subjects with and without type 2 diabetes.

Chapter 3B

Statin treatment increases lipoprotein(a) levels in subjects with low molecular weight apolipoprotein(a) phenotype.

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(Submitted)

Chapter 3C

Plasma lipoprotein(a) levels in patients with homozygous autosomal dominant hypercholesterolemia.

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Abstract

Background:
Patients with familial hypercholesterolemia (FH), caused by mutations in either *LDLR, APOB or PCSK9* are characterized by high low-density lipoprotein cholesterol (LDL-C) levels and in some studies also high lipoprotein(a) (Lp(a)) levels were observed. The question remains whether this effect on Lp(a) levels is gene-dose-dependent in individuals with either 0, 1 or 2 *LDLR* or *APOB* mutations.

Objective:
We set out to study whether Lp(a) levels differ among bi-allelic FH mutation carriers, and their relatives, in the Netherlands.

Methods:
Bi-allelic FH mutation carriers were identified in the database of the national referral laboratory for DNA diagnostics of inherited dyslipidaemias. Family members were invited by the index cases to participate. Clinical parameters and Lp(a) levels were measured in bi-allelic FH mutation carriers and their heterozygous and unaffected relatives.

Results:
We included 119 individuals; 34 bi-allelic FH mutation carriers (20 homozygous/compound heterozygous *LDLR* mutation carriers (HoFH), 2 homozygous *APOB* mutation carriers (HoFDB), and 12 double heterozygotes for an *LDLR* and an *APOB* mutation), 63 mono-allelic ADH mutation carriers (50 heterozygous *LDLR* (HeFH), and 13 heterozygous *APOB* (HeFDB) mutation carriers), and 22 unaffected family members. Median Lp(a) levels in unaffected relatives, HeFH, and HoFH patients were 19.9 [11.1; 41.5], 24.4 [5.9; 70.6], and 47.3 [14.9; 111.7] mg/dL, respectively (P = 0.150 for gene-dose-dependency). Median Lp(a) levels in HeFDB and HoFDB patients were 50.3 [18.7; 120.9] and 205.5 [no IQR calculated], respectively (P = 0.012 for gene-dose-dependency). Double heterozygous carriers of *LDLR* and *APOB* mutations had median Lp(a) levels of 27.0 [23.5; 45.0], which did not significantly differ from HoFH and HoFDB patients (P = 0.730 and 0.340, respectively).

Conclusion:
A (trend towards) increased plasma Lp(a) levels in homozygous FH patients compared to both, heterozygous FH as well as unaffected relatives was observed. Whether increased Lp(a) levels in homozygous FH patients add to the increased CVD risk and whether this risk can be reduced by therapies that lower both LDL-C and Lp(a) levels, remains to be elucidated.

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in either the gene encoding for the low-density lipoprotein receptor (*LDLR*; 86-88% of genetically diagnosed FH patients), apolipoprotein B (*APOB;* ~12%) or proprotein convertase subtilisin-kexin type 9 (*PCSK9*; 0.1-2%) (199, 200). FH patients are at increased risk for premature cardiovascular disease (CVD) due to high low-density lipoprotein cholesterol (LDL-C) levels. It has been shown that lipoprotein(a) (Lp(a)) levels are also increased in FH patients (201-203). High Lp(a) levels are independently associated with both an increased risk of CVD as well as aortic valve calcification in the general population (204-206) and in FH patients (144, 203). Plasma Lp(a) levels are predominantly determined by genetic factors and the Lp(a) synthetic rate by the liver (48). The catabolic pathway of Lp(a) is largely unknown but it has been proposed that the Lp(a) particle could be removed from the circulation by the LDLR (48, 207). This would be a plausible explanation for the increased Lp(a) levels observed in FH patients, as loss-of-function mutations in *LDLR* and *APOB* would lead to diminished clearance and, as a consequence, increased plasma Lp(a) levels. Although cell culture and *in vivo* turnover studies did not support a causal link between the LDLR pathway and increased Lp(a) levels in patients with *LDLR* mutations (208), Kraft and co-workers described a clear gene-dosage effect; Lp(a) levels were lower in heterozygous compared to homozygous FH patients (209). The latter has, however, only been studied in a South African population with a relatively small number of *LDLR* mutations. Moreover, the observation that treatment with HMG-co-A reductase inhibitors that increase hepatic expression of LDLRs do not reduce Lp(a) levels, does not support a role of the LDLR in Lp(a) clearance (19, 187). To the best of our knowledge, the gene-dosage-effect of Lp(a) levels in *APOB* mutation carriers has not previously been studied. The presence of a gene-dosage-effect would be of direct clinical relevance in light of recently approved LDL-C lowering therapies for homozygous FH patients that also reduce Lp(a) levels (eg. mipomersen, evolocumab, and lomitapide) and in light of currently investigated therapies that specifically lower Lp(a).

We set out to study this effect among the population of bi-allelic FH mutation carriers and their family members, in the Netherlands.

Methods

Patients, Laboratory Analyses, and Molecular Diagnostic Procedures.
Patients were identified as previously described (210). In summary, the database of the national referral laboratory for DNA diagnostics of inherited dyslipidemias at the Academic Medical Center in Amsterdam, the Netherlands, comprising almost all molecular diagnostic results of FH patients in our country, was queried to identify all bi-allelic FH mutation

carriers. Carriers of non-pathogenic mutations as well as patients who were deceased were excluded. All identified individuals were contacted for participation. After informed consent, blood samples were obtained for measurement of lipids and lipoproteins after an overnight fast. Data on the use of lipid-lowering therapy was collected. Patients who used lipid lowering drug therapy with a known effect on plasma Lp(a) levels (ie. Lomitapide (54)) were excluded from the analyses. When patients were treated with lipoprotein apheresis blood samples were drawn immediately prior to the apheresis procedure and the time to the apheresis procedure prior to the blood withdrawal was at least 14 days (76). Blood was centrifuged for 10 minutes at 3,000 rates per minute at 4ºC. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured by a commercially available enzymatic colorimetric assay (Roche, Basel, Switzerland). LDL-C levels were calculated by the Friedewald formula (211). Lp(a) levels were measured as previously reported (144). Briefly, plasma Lp(a) concentrations were measured using a particle enhanced immunoturbidimetric assay, independently of apo(a) KIV repeats (Diagnostic System #171399910930; DiaSys Diagnostic System, GmBH, Germany). In samples with a low Lp(a) concentration, Lp(a) was quantified using an enzyme-linked immunosorbent assay with a low detection limit (212). The number of apo(a) KIV repeats was determined by immunoblotting as previously published by Vongpromek and co-workers (144). Normal values for Lp(a) levels can be defined according to the FH treatment guidelines (213).

Molecular diagnostic procedures (including DNA diagnostics as well as criteria for functionality of mutations) were performed as previously described (210).

Family members were invited to participate by the index cases and upon consent the same laboratory analyses and data collection procedures were performed in them as well. This study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam and the Erasmus Medical Center in Rotterdam, the Netherlands.

Statistical Analyses

Due to a skewed distribution and unequal variances, Lp(a) levels were normalized using empirical normal quantile transformation. Differences in Lp(a) levels between homozygous/compound heterozygous FH patients, heterozygous patients and their relatives were analysed using linear regression assuming an allele dose effect with correction for family relations (lmekin function, coxme package) (214). Kringle IV type 2 repeats, age and gender were used as covariates, but correction for age and gender did not change the outcome of the study. Based on the Akaike Information Criterion (AIC), the best fitting model for kringle IV type 2 sizes used as covariate was an ordinal classification of group 1: kringle IV repeats < 20, group 2: 21-25, group 3: 26-30, group 4: 31-35, and group 5: > 35, of the lower kringle size. (Log transformed) data are presented as mean ± SD, median [interquartile range (IQR)] or number (%), where appropriate. A *P*-value of 0.05 was considered to be statistically

significant. All statistical analyses were performed in R-Statistical package version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Hundred-and-nineteen individuals were included in this study of whom 34 bi-allelic FH mutation carriers (20 were homozygous/compound heterozygous for *LDLR* mutations (HoFH), 2 were homozygous *APOB* mutation carriers (HoFDB), and 12 were double heterozygous for both, an *LDLR* and an *APOB* mutation), 63 mono-allelic FH mutation carriers (50 heterozygous *LDLR* (HeFH), and 13 heterozygous *APOB* mutation carriers (HeFDB)), and 22 unaffected family members. All diagnoses were based on identification of DNA mutations. Seven out of 20 homozygous/compound heterozygous *LDLR* mutation carriers had either one (n = 4) or two (n = 3) null alleles (210, 215). The total study population comprised individuals from 26 different families (mean number [IQR] of individuals per family: 3.5 [3; 6]). Eighty-nine percent (n = 23) of families were of Caucasian origin.

The clinical characteristics of the bi-allelic FH mutation carriers and their heterozygous and unaffected relatives are presented in *Table 1*. The median age of study participants was 41.8 [28.2; 59.2] years. Forty-five percent (n = 54) of individuals were male and 55% (n = 65) were female. Sixty-one percent (n = 73) of study participants used lipid lowering therapy (LLT).

A dose-dependent effect of plasma Lp(a) levels between unaffected relatives, mono-allelic and bi-allelic FH mutation carriers was observed. This effect remained statistically significant after correction for age, gender and categories of kringle IV type 2 size (*P* = 0.037; *Figure 1*). The effect remained also statistically significant after additional correction for the affected gene (*LDLR* or *APOB*; *P* = 0.002).

Median Lp(a) levels in unaffected relatives, HeFH, and HoFH patients were 19.9 [11.1; 41.5], 24.4 [5.9; 70.6], and 47.3 [14.9; 111.7] mg/dL, respectively. A trend towards a dose dependent effect of Lp(a) levels for the number of *LDLR* mutations (zero in unaffected relatives, one in HeFH and two in HoFH patients; *Figure 2*) was observed, after correction for age, gender and categories of kringle IV type 2 size. This effect was, however, not statistically significant (*P* = 0.150).

Median Lp(a) levels in HeFDB and HoFDB patients were 50.3 [18.7; 120.9] and 205.5 [no IQR calculated], respectively. A dose dependent effect of plasma Lp(a) levels for the number of *APOB* mutations was observed, after correction for the covariates age, gender and categories of kringle IV type 2 size (*P* = 0.012; *Figure 3*).

Table 1. Characteristics of Included Individuals.

	Homozygous/Compound Heterozygous		Double Heterozygous	Relatives		
				Heterozygotes		Unaffected
	LDLR	*APOB*[†]	*LDLR/APOB*	*LDLR*	*APOB*	
Number of individuals	20	2	12	50	13	22
Age, median [IQR]	34.2 [27.3; 38.9]	55; 79	42.5 [27.7; 60.4]	46.7 [28.9; 63.4]	50.0 [23.6; 54.1]	51.6 [32.7; 68.0]
Female sex, n (%)	10 (50)	1 (50)	5 (42)	32 (64)	6 (46)	11 (50)
Lipid levels, median [IQR]						
TC	8.5 [6.3; 12.8]	5.6; 6.3	5.7 [5.0; 7.6]	5.5 [4.3; 6.5]	5.2 [4.9; 6.1]	5.1 [4.4; 5.9]
LDL-C	6.8 [4.2; 10.3]	3.6; 4.4	4.1 [3.0; 5.7]	3.4 [2.5; 4.5]	3.6 [2.9; 4.1]	3.1 [2.5; 3.6]
HDL-C	1.4 [1.1; 1.5]	1.3; 1.6	1.1 [1.0; 1.6]	1.4 [1.2; 1.7][*]	1.3 [1.0; 1.6]	1.5 [1.3; 1.7]
TG	1.11 [0.72; 1.47]	0.79; 1.59	1.16 [0.91; 1.64]	1.06 [0.71; 1.72][*]	0.85 [0.78; 1.62]	1.14 [0.80; 1.84]
Lp(a)	47.3 [14.9; 111.7]	20.0; 390.9	27.0 [23.5; 45.0]	24.4 [5.9; 70.6]	50.3 [18.7; 120.9]	19.9 [11.1; 41.5]
Lower kringle IV, type 2 size median [range]	26 [19; 34]	19; 38	27 [24; 31]	27 [19; 33]	21 [21; 34]	26 [20; 28]
LLT, n (%)	20 (100)	2 (100)	10 (83)	31 (62)	7 (54)	3 (14)
Statin monotherapy	1 (5)	-	3 (25)	20 (40)	5 (39)	3 (14)
Statin and cholesterol absorption inhibitor	13 (65)	2 (100)	7 (58)	11 (22)	2 (15)	-
Other combination of LLT	2 (10)	-	-	-	-	-
Lipoprotein apheresis	4 (20)	-	-	-	-	-

Abbreviations: TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides; Lp(a), lipoprotein(a). [*] HDL-C and TG levels were available from 48 heterozygous LDLR mutation carriers. [†] Data of continuous variables are absolute values of both hoFDB patients. TC, LDL-C, HDL-C and TG levels are in mmol/L. Lp(a) levels are in mg/dL.

Double heterozygous carriers of *LDLR* and *APOB* mutations had median Lp(a) levels of 27.0 [23.5; 45.0], which did not statistically significantly differ from HoFH and HoFDB patients (*P* = 0.730 and 0.340, respectively).

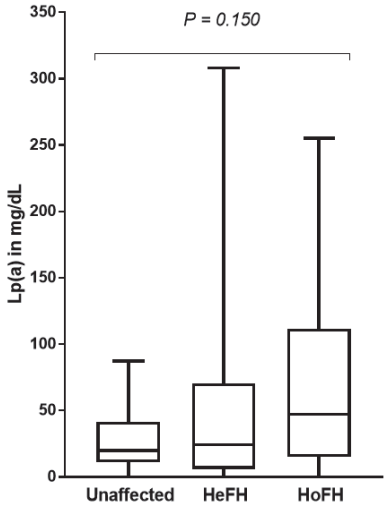

Figure 1. Box and whisker plot for the association between the plasma Lp(a) levels and FH mutation status

Abbreviations: mono-allelic = heterozygous familial hypercholesterolemia (either 1 LDLR or APOB mutation), bi-allelic includes patients with homozygous or compound familial hypercholesterolemia or patient with double heterozygous familial hypercholesterolemia (2 mutations in 2 different alleles of the LDLR and/or APOB gene).

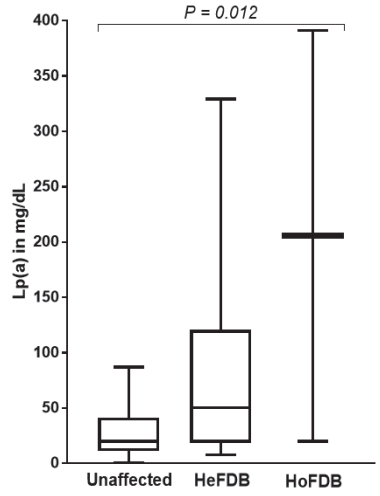

Figure 2. Box and whisker plot for the association between the plasma Lp(a) levels and LDLR mutation status

Abbreviations: HeFH = heterozygous familial hypercholesterolemia (1 LDLR mutation), HoFH = homozygous familial hypercholesterolemia or compound heterozygous familial hypercholesterolemia (2 mutations in 2 different alleles of the LDLR gene).

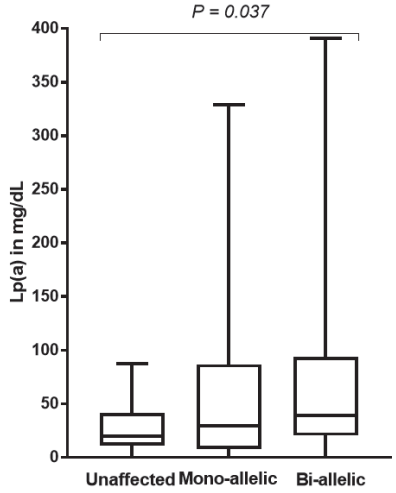

Figure 3. Box and whisker plot for the association between plasma Lp(a) levels and APOB mutation status

HeFDB = heterozygous familial defective apolipoprotein B (1 APOB mutation), HoFDB = homozygous familial defective apolipoprotein (2 mutations in 2 different alleles of the APOB gene).

Discussion

In the present study we investigated the consequences of *LDLR* and *APOB* mutations for plasma Lp(a) levels in individuals from FH families. While we only observed a trend towards a gene-dose-dependent effect on plasma Lp(a) levels among unaffected relatives and individuals with either one (heterozygotes) or two (homozygotes) *LDLR* mutations, a significant gene-dosage effect was observed among unaffected relatives and individuals with either one or two loss-of-function mutations in *APOB*. To the best of our knowledge, plasma Lp(a) levels among homozygous/compound heterozygous *LDLR* mutation carriers was examined in only one study (209), while plasma Lp(a) levels have not been studied in homozygous *APOB* mutation carriers, to date.

Lp(a) is a liver-synthesized LDL-like particle containing a glycoprotein, apolipoprotein(a) (apo(a)), that is covalently bound to apolipoprotein B (apoB). High plasma Lp(a) levels are associated with coronary artery disease (CAD), peripheral artery disease and stroke (204, 205). Although the role of plasma Lp(a) levels as risk factor for CVD in heFH has been controversial, some retrospective and cross-sectional studies have shown that Lp(a) is indeed an independent risk factor for CVD in these patients (216, 217). The absence of an association between Lp(a) and CVD risk in other studies (218, 219) has been ascribed to several factors such as study size and method of Lp(a) measurement (202, 216). The definitions of FH (genetic or clinical diagnosis and variety of FH causing genes and FH mutations included) and (surrogate markers of) cardiovascular events may have also played a role (202, 217, 220). Alonso and co-workers recently described that molecularly defined FH patients (all carriers of *LDLR* mutations), especially those with CVD, have significantly higher Lp(a) levels compared to their non-FH relatives and confirmed the observation that Lp(a) is an independent predictor of CVD in heterozygous *LDLR* mutation carriers (203).

To date, the question remains whether the increased Lp(a) levels in FH patients are due to the presence of *LDLR* mutations and as a consequence, a decreased clearance of Lp(a) particles or due to other (largely unknown) pathophysiological mechanisms. In contrast to the results of the present study, Kraft and co-workers observed that homozygous *LDLR* mutation carriers had statistically significantly higher Lp(a) levels compared to both, heterozygous and unaffected individuals (209). While comparing the data derived by Kraft and co-workers with our study, it is of note that the genetic background of hoFH patients in our population is more heterogeneous since our patients were affected by more than 25 different pathogenic *LDLR* mutations, compared to only 7 in the previous study. Moreover, we included unaffected relatives in our study, while in the previous study unaffected individuals were not family members of the HoFH patients per se (209). Although we did not perform any kinetic studies in our population and therefore apo(a) production rates are unknown, the absence of a significant gene-dosage effect observed in our study is in

line with the observation that absence of functional LDLRs did not result in delayed Lp(a) clearance in five unrelated hoFH patients compared to their heFH relatives and unrelated unaffected individuals (208). The latter is also in line with the fact that treatment with HMG-co-reductase inhibitors ('statins'), known to increase hepatic LDLR expression, does not result in significant reductions of plasma Lp(a) levels, but was found to increase plasma Lp(a) levels in some studies (19, 187). In contrast to statins, monoclonal antibodies against PCSK9 have shown to significantly lower Lp(a) levels in heterozygous (185) and homozygous FH patients with either one or two defective *LDLR* alleles (186). This, together with the observation that Lp(a) levels were equally increased in heterozygous FH patients with *LDLR* and *PCSK9* gain-of-function mutations (221), suggests a role of PCSK9 in Lp(a) metabolism. Indeed, *in vivo* experiments have recently demonstrated that the LDLR may be involved in Lp(a) internalization, mediated by PCSK9 (207). Since LDL may compete with Lp(a) for binding to LDLR (207), we cannot exclude the possibility that the large heterogeneity of *LDLR* mutations and, as a consequence, differential numbers of LDL particles competing with Lp(a) particles may have resulted in the absence of a statistically significant gene-dosage-effect between Lp(a) levels and *LDLR* mutation status (0, 1 or 2 affected alleles), in our study.

Some other limitations should be taken into account while interpreting the results of this study. First, we observed a wide variety of plasma Lp(a) levels among heterozygous *LDLR* mutation carriers. This heterogeneity may have influenced the absence of a significant gene-dosage effect in our population. Second, although plasma Lp(a) levels have been shown to be mainly genetically determined and to remain relatively stable over time, non-genetic factors including kidney and liver disease, alcohol abuse and some (non-primarily lipid altering) therapies may influence plasma Lp(a) levels.

Although it is not very likely, we cannot completely exclude the possibility that the presence of these influencing factors may have resulted in biased outcomes. Lastly, since we could only include 2 homozygous *APOB* mutation carriers, we should be cautious with drawing conclusions about the presence of a gene-dosage-effect of plasma Lp(a) levels among unaffected relatives, heterozygous and homozygous *APOB* mutation carriers. This observation is, however, in line with the previous observation that plasma Lp(a) levels are elevated in apo(a) matched siblings with an *APOB* mutation compared to their sibling without an *APOB* mutation (201). It is of note that the gene-dosage-effect among unaffected relatives, heterozygous and homozygous *APOB* mutation carriers remained significant after excluding the hoFDB patient with a very high Lp(a) level (390.9 mg/dL). This excludes the possibility that the gene-dosage-effect is only caused by the very high Lp(a) level in this patient.

The pathophysiological mechanism behind the increased plasma Lp(a) levels in both heterozygous as well as homozygous *APOB* mutation carriers remains to be elucidated, but

might be related to the observations that apoB, rather than apo(a) in Lp(a) is the ligand for LDLR (222). The latter might also be relevant in light of the observation that plasma Lp(a) levels in double heterozygous patients for both an *LDLR* as well as *APOB* mutation were equally elevated to hoFH and hoFDB patients.

From a clinical perspective, the question remains whether increased Lp(a) levels in homozygous FH patients would add to the increased CVD risk and whether this risk will be reduced by therapies that lower both LDL-C and Lp(a) levels (ie. mipomersen, lomitapide, PCSK9 monoclonal antibodies) (223, 224) or by apo(a) lowering therapies (58).

In conclusion, we observed a (trend towards) increased plasma Lp(a) levels in homozygous FH patients compared to both heterozygous FH and unaffected relatives. The results of our study are relevant from both a pathophysiological as well as clinical perspective.

Part III:

Treatment options

Chapter 4A

Latest developments in the treatment of lipoprotein (a).

Sven Bos, Reyhana Yayha, Jeanine E Roeters van Lennep

Current Opinion Lipidolology. 2014 Dec;25(6):452-60. doi: 10.1097/MOL.0000000000000126.

Abstract

Purpose of review

Lipoprotein (a) (Lp(a)) is an independent risk factor for cardiovascular disease (CVD). The aim of this review is to provide an overview of treatment options for Lp(a) lowering.

Recent Findings

Recent studies confirmed that lifestyle intervention and statins do not affect Lp(a) levels, whereas Lp(a) is lowered by estrogens, niacin, and lipoprotein apheresis. CETP inhibitors and PCSK9 antibodies, currently studied in phase 3 trials, also lower Lp(a) concentrations by 30-50%. However, all of these compounds have modifying effects on multiple lipoprotein classes. An antisense oligonucleotide directed to apolipoprotein (a) has recently been developed to specifically lower circulating Lp(a) levels. This compound inhibited Lp(a) mRNA up to 90%, and Lp(a) levels up to 82% in human volunteers independent of Lp(a) levels at baseline.

Summary

Multiple agents, including the next generation RNA based antisense therapeutics have Lp(a) lowering properties . However, it remains to be established whether lowering Lp(a) reduces CVD events with specific Lp(a) lowering therapies.

Keypoints

- Lp(a) is a risk factor for CVD.
- It is not clear if lowering Lp(a) lowers CVD risk.
- First-line treatment of Lp(a)-hyperlipoproteinemia should be statin therapy to decrease CVD risk.
- Currently, the only Lp(a) lowering agents are estrogens, niacin, mipomersen, and lomitapide with either no effect on CVD outcome (estrogens and niacin), or an unknown effect on CVD outcome (mipomersen and lomitapide)
- New drugs [ISIS apo(a) Rx] are being developed that will be more commonly available and more specific for Lp(a) lowering.

Introduction

Lipoprotein (a) (Lp(a)) is a low density lipoprotein (LDL) like particle with an apolipoprotein (apo(a)) moiety attached to it (figure 1 (225-226)). Multiple isoforms of apo(a) exist because the length of this protein is genetically determined by variations in the number of Kringle IV type 2 repeats encoded by the *LPA gene* (48). The size of the apo(a) is inversely related with plasma Lp(a) levels (48). In addition elevated plasma Lp(a) levels are causally related to cardiovascular disease (CVD), and the development of aortic valve calcification and aortic valve stenosis (16, 227-229). However, it is not known if reducing Lp(a) levels will also reduce the risk of CVD, because the first specific Lp(a) lowering compound has only recently been developed and outcome data is not yet available. The aim of this review is to give an overview of the current knowledge of Lp(a) modifying agents and interventions.

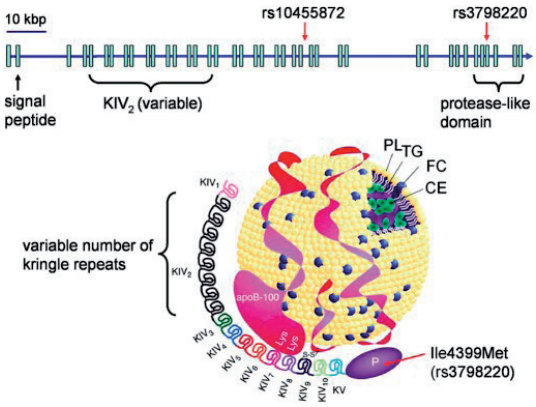

Figure 1: Lipoprotein(a) particle.

Adapted from J E Roeters van Lennep and M T Mulder(225).

Life style and diet

Healthy lifestyle and a prudent diet are cornerstones of CVD prevention. Recently two studies have addressed the effect of lifestyle intervention on Lp(a) levels. Both showed that Lp(a) levels are not influenced by rigorous exercise (230, 231).

Studies on the influence of diet on Lp(a) have produced conflicting results for a long time, and it remains to be established if diet indeed modifies Lp(a) or not (231, 232). The main shortcomings of most of these studies include the small sample size, the use of firmly isoform dependent assays for measuring Lp(a), and improper use of statistics. Recently, the Copenhagen Heart study established that Lp(a) levels are not directly influenced by food intake: No difference in Lp(a) levels was observed between fasting and non-fasting blood

samples (233). In conclusion, these studies reinforce that the influence of exercise and food intake on Lp(a) levels is limited at best.

Drug Treatment

Next to life style, weight control and dietary hygiene, pharmacological treatment plays a crucial role in CVD prevention. The remainder of this review focusses on the effect of different compounds on circulating Lp(a) levels.

Estrogens

Hormone replacement therapy containing estrogens favourably influences Lp(a), LDL-cholesterol (LDL-C), and high dense lipoprotein-cholesterol (HDL-C) levels in postmenopausal women. Recently, Howard et al. (234), provided an excellent overview of all cardiovascular effects of hormone replacement therapy, including Lp(a). These authors concluded that despite the Lp(a) lowering effect of estrogens, there is no place for hormone replacement therapy in CVD prevention because it did not lead to a decrease in CVD events. Reversely, Lp(a) levels increase when the action of estrogens is blocked (235). A recent double blinded randomized controlled trial (RCT) investigated the effect of Letrozole (Novartis, Basel, Switzerland), an aromatase inhibitor which inhibits the conversion of testosterone to estrogens, on lipoprotein levels. After 60 months of follow-up Lp(a) was measured in 103 postmenopausal women with breast cancer, showing that Lp(a) levels where 106% higher compared to baseline in those randomized to Letrozole treatment (235). Although, the mechanism is uncertain Hoover-Plow and Menggui Huang proposed influence of estrogen on the LPA promoter(236). This is highly suggestive of an association between estrogens and Lp(a) levels. Given the outcome of the hormone replacement therapy trials on CVD endpoints it is unlikely that estrogens will even be used as Lp(a) lowering medication.

Thyroid hormone analogues

Abnormal thyroid function has serious consequences for lipoprotein levels and body composition (237). These effects can be explained by the interaction of thyroid hormone with the thyroid hormone receptor. This receptor has two major isoforms, the α and the β isoform. The α isoform is predominantly present in heart and bone, whereas the β isoform is predominantly present in the liver. The thyroid hormone β-receptor analogue eprotirome (Karo Bio, Huddinge, Sweden) has been studied in two RCTs (237). Eprotirome was found to lower Lp(a) levels by 43% from baseline, without any change in body weight, heart rate, blood pressure, or bone turnover (237). This effect seems to be synergistic to either statins or ezetimibe because administration of eprotirome as monotherapy does not influence Lp(a) levels (238). The proposed mechanism of Lp(a) lowering is that activation of the β isoform leads to a decreased apo-B synthesis. However, because of cartilage damage in toxicology studies in dogs and recent reports that elevation in liver function tests were observed in

patients randomized to eprotirome, the trials were prematurely terminated (239). To our knowledge there are no new thyroid analogues under development.

Statins

Statins are prescribed for over 20 years for treating dyslipidaemia to prevent CVD. Their effect is mainly due to lowering of LDL-C. Previous studies have reported either a lowering, no effect, or an increase in Lp(a) levels after statin treatment (19, 172). It seems clear that Lp(a) cannot be cleared by the LDL-receptor. The mechanisms by which statins may affect Lp(a) levels, if they do, remain to be clarified. Two recent studies evaluated the effect of statins on Lp(a) levels (240, 241). In the first study patients who were receiving a standard statin dose were switched to the maximum dosage of rosuvastatin, i.e. 40mg (240). In this study, optimizing statin dose led to a decrease of LDL-C (23%), but did not show an effect on Lp(a) (240). In the second study the effect of morning and evening dosages of simvastatin were compared, in previously untreated patients (241). In this study, the use of simvastatin led to a decrease in LDL-C (36-38%), but to no changes in Lp(a). In addition, there was no difference in morning or evening dosages on any lipoprotein (241). In conclusion, the effect of statins on Lp(a) levels, if present, is most likely not clinically significant.

Lipoprotein apheresis

Lipoprotein apheresis can lower LDL-C 60-70% by removal of lipoproteins from the circulation. It is used in patients with severe hypercholesterolemia such as homozygous familial hypercholesterolemia (FH) (242). Another indication for lipoprotein apheresis is Lp(a)-hyperlipoproteinemia (Lp(a) > 0,6g/L) with progressive CVD (243). In these patients, who are adequately treated with statins, lipoprotein apheresis reduces Lp(a) by 70% directly post-treatment (242, 243), this led to a decrease of major adverse coronary events by 78% (242). However, it is uncertain whether the reduced event rate is due to Lp(a) lowering per se, because lipoprotein apheresis also lowers other lipoproteins, and may as well reduce other unknown risk factors. Disadvantages of lipoprotein apheresis include it's time expenditure and costs. Furthermore apheresis is not reimbursed in all countries. Despite the limited indication and availability, lipoprotein apheresis is a sound method to reduce CVD events in Lp(a)-hyperlipoproteinemia patients who have progressive CVD, although it is unknown if this effect is due to Lp(a) lowering per se.

Niacin

Niacin (Vitamin B3 or nicotinic acid) has multiple effects on different lipoproteins; it lowers LDL-C and triglycerides (TG), and it increases HDL-C. Since 1990 it is being reported that niacin can also lower Lp(a) although the mechanism is unclear (244). In the AIM-High (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides) trial patients were treated with high dose extended release niacin (1,5-2,0 g/day) or placebo, on top of statins. Baseline Lp(a) and on-study Lp(a) predicted CVD events in both arms (245).

This suggest that Lp(a) still contributes to residual risk. In the extended release niacin group Lp(a) was 19% lower than in the placebo group. Despite this reduction in Lp(a), extended release niacin did not lead to a reduction in CVD events (245). The criticism regards this trial include the fact that patients were at low LDL-C levels (1.97 mmol/L), and critical differences in terms of LDL-C, HDL-C and TG levels were very small between treatment arms. The observed event rate was lower than expected, and the overall study was seriously underpowered (245, 246). In addition, the recent HPS-2-THRIVE (Heart Protection Study-2-Treatment of HDL to Reduce the Incidence of Vascular Events) trial also failed to show benefit on CVD outcome, despite an Lp(a) reduction of 24% (247). In this trial Tredaptive (niacin 2g/laropiprant 40mg, MSD, Whitehouse Station, NJ, USA) was compared to placebo, on top of statin therapy. LDL-C , HDL-C and TG levels were optimal and it is questionable whether 2g of nicotinic acid is the correct therapy in that situation. It is also possible that the addition of lapopiprant, a prostaglandin D2 antagonist, had influence on outcome and safety. Neither the AIMHIGH or the HPS-Thrive analysed whether the subgroup of patients with high Lp(a) at baseline did have a particular benefit of niacin therapy. In 2010 the European Atherosclerosis Society Consensus Panel recommended the use of niacin in high risk patients with elevated Lp(a) (>0,5g/L) (226). However given the outcome of the recent RCT's it is questionable if this recommendation is correct (245, 248). In conclusion, niacin can significantly reduce Lp(a) and effects on the lipoprotein profile are beneficial, but RCT's have not shown a decrease in CVD outcome when added to statins, although specific subgroup analysis of patients with high Lp(a) has not been performed.

Ezetimibe

Previously it was shown that ezetimibe (MSD, Whitehouse Station, NJ, USA) does not influence Lp(a) levels, which is not surprising since the mechanism of inhibiting intestinal cholesterol uptake by blocking Nieman-Pick C1-like protein, is not involved in Lp(a) metabolism as far as we know. In the recent PROBE (Prospective, Randomized, Open-label, Blinded Endpoint) study Lp(a) was not reduced in dyslipidaemic patients after addition of ezetimibe to statins (249).

Anti-sense Apo-B

Mipomersen (Carlsbad, CA, USA) is an antisense nucleotide that binds to the mRNA encoding the Apo-B protein and thereby inhibit its synthesis. Apo-B synthesis is essential for the formation of lipoprotein particles, and its inhibition reduces TG levels (25-33%), very low dense lipoprotein-cholesterol (VLDL-C) (33-37%), LDL-C (28-37%) as well as Lp(a) (21-28%) (250, 251). Although mipomersen reduces plasma levels of these atherogenic lipoproteins, no outcome study has been performed. Mipomersen is not very well tolerated. It was discontinued in 43% of patients after 26 weeks follow up, due to side effects such as injection site reactions (up to 92%), flu-like symptoms, and elevated liver enzymes (250, 251). In January 2013, the FDA approved mipomersen for the treatment of homozygous

FH. However the EMA did not follow, and mipomersen is therefore not approved in Europe [http://www.medscape.com/viewarticle/781317]. Due to the approval for an orphan disease, the Lp(a) lowering will merely be a beneficial side effect. It is improbable that mipomersen will be used specifically to lower Lp(a).

Microsomal triglyceride transport protein (MTP) inhibition

MTP is an enzyme that facilitates the transport of TG into VLDL in the liver, and the secretion of chylomicrons from the intestine. Inhibiting the activity of this protein prevents the formation of chylomicrons and lipoproteins including Lp(a). The effect of the MTP inhibitor, Lomitapide (Aegerion Pharmaceuticals, Cambridge, Massachusetts, USA) in combination with a low-fat diet and maximum statin therapy, was studied in patients with homozygous FH. Following a 26 week open label study, a long-term extension study showed that 56 weeks of treatment led to a reduction of LDL-C (44%), and a reduction in Lp(a) of 19%. However, after 78 weeks Lp(a) had returned to baseline levels (252). The most frequent encountered side effects were gastrointestinal complaints (93%), and elevated liver enzymes >3x upper limit normal (34%) and >5x upper limit normal (14%) (252). In 2013, Lomotapide was approved by the FDA and EMA, for the treatment of homozygous FH patients. The safety profile makes it likely that lomitapide will remain solely registered for this indication. As with mipomersen, this implies that the decrease in Lp(a) will remain an additional beneficial effect for those homozygous FH patients who use the drug for LDL-C lowering. Furthermore, the long-term extension study showed that the effect of lomitapide on Lp(a) is temporary so it is questionable whether this effect is clinically relevant.

CETP inhibition

Cholesterol ester transfer protein (CETP) transfers cholesterol esters and TG between HDL-C and Apo-B containing lipoproteins. CETP inhibition decreases Apo-B containing lipoproteins and increases cholesterol enrichment in HDL-C. The first two CETP inhibitors were terminated because of respectively safety concerns (ILLUMINATE (Investigation of Lipid Level Management to Understand its Impact in Atherosclerosis Events) with torcetrapib) and futility (dal-OUTCOMES with dalcetrapib) (55). Currently a third CETP inhibitor, anacetrapib was investigated in two phase 3 safety trials. The DEFINE (Determining the EFficacy and tolerability of CETP INhibition with AnacEtrapib) showed a reduction in LDL-C (45%), TG (7%), an increase in HDL-C (169%), but no data on Lp(a) was available (253). Furthermore, a CVD outcome trial with anacetrapib (REVEAL (Randomized EValuation of the Effects of Anacetrapib Through Lipid-modification)) is underway, results are expected in 2017. A phase 2 trial of anacetrapib in Japanese dyslipidaemic patients showed an increases in HDL-C of 160%, a decrease in LDL-C of 32%, and a decrease in Lp(a) cholesterol of 50% (55). Furthermore a phase 3 trial in heterozygous FH patients (REALIZE (Study to Assess the Tolerability and Efficacy of Anacetrapib Co-administered With Statin in Participants With Heterozygous Familial Hypercholesterolemia)) was completed in February 2014. However,

the data have not been published yet. Evacetrapib, (Eli Lilly, Indianapolis, Indiana, USA) , another CETP currently under investigation, reduces LDL-C (22%), increases HDL-C (136%) and TG (7%), but Lp(a) levels were not investigated (254). Recently Dezima Pharma , announced a phase 1 trial to investigate the effect of their CETP inhibitor TA-8995 (Dezima Pharma, Naarden, The Netherlands) on Lp(a) levels [http://www.dezimapharma.com/dezima-pharma-extends-clinical-development]. The mechanism of the Lp(a) lowering effect of the CETP inhibitors is not clear, and if CETP inhibition will prove to lower CVD risk it will be a challenge to determine to which extent Lp(a) will contribute to the reduction of CVD outcome, given its other beneficial effect on other lipoproteins.

PCSK9-inhibitors

Proprotein convertase subtilisin/kexin type 9 (PCKS-9) is secreted by the liver and regulates expression of the LDL-receptor by targeting it for lysosomal degradation (255). To inhibit PCSK-9 activity, monoclonal antibodies have been developed that specifically target the PCSK-9 protein (255-257). In recent phase 2 trials (**AMGEN:** MENDEL (Monoclonal Antibody Against PCSK9 to Reduce Elevated LDL-C in Patients Currently Not Receiving Drug Therapy for Easing Lipid Levels) / LAPLACE-TIMI 57 (LDL-C Assessment With PCSK9 Monoclonal Antibody Inhibition Combined With Statin Therapy) / RUTHERFORD (Reduction of LDL-C With PCSK9 Inhibition in Heterozygous Familial Hypercholesterolaemia Disorder) /GAUSS (Goal Achievement After Utilizing an Anti-PCSK9 Antibody in Statin-Intolerant Subjects), **REGENERON/SANOFI:** ODYSSEY (Evaluation of Cardiovascular Outcomes After an Acute Coronary Syndrome During Treatment With SAR236553) programs) the compounds of Amgen and Regeneron/Sanofi showed that PCSK-9 inhibition on top of statin therapy reduces LDL-C by 55-65% , and Lp(a) by 30-40% (255-257). This is also confirmed in a recently published phase III trial were after 52 weeks, there was a decrease in TG (4-23%), VLDL-C (20-79%), LDL-C (48-61%), Lp(a) (23-33%), and in increase in HDL-C (4-11%) (258). As with CETP inhibition the question how PCSK9 influences Lp(a) levels remains to be answered. It is hypothesized that PCSK-9 inhibition improves clearance either through an unknown receptor, directly from the circulation, or reduces synthesis by a decrease in substrate availability (256). Although the phase 3 outcome trials are ongoing, PCSK-9 inhibition can be potentially important for Lp(a) reduction. However, because of the multiple actions of PCSK-9 inhibition, the contribution of the direct effect of reduced Lp(a) on lowering CVD incidence will be a challenge to investigate.

Anti-sense apo-(a)

Recently, the results of a phase 1 study with an anti-sense compound was presented (ISIS APO(a)Rx, Gazelle Court Carlsbad, CA, USA) which acts specifically against the mRNA of apo(a), and lowers apo(a) mRNA by 90%, and Lp(a) levels up to 82% (259)[http://ir.isispharm.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=1877550&highlight]. The phase I trials of ISIS APO(a)Rx have been completed, and a phase II trial will soon commence. This trial will

assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of ISIS APO(a)Rx administered subcutaneously to patients with high Lipoprotein(a) levels (0,50-1,75 g/L) and very high Lp(a) levels. (>1,75 g/L). It is the first agent which specifically targets Lp(a) and will cast the final verdict whether Lp(a) lowering will lower CVD event rates.

Conclusion

Multiple agents have shown to have Lp(a) lowering properties. However statins, the most effective drugs in reducing CVD risk, do not modify Lp(a) to a clinical relevant degree. The drugs that do decrease Lp(a) have either no overall effect on CVD risk (estrogens and niacin), are currently investigated in phase 3 trials (CETP inhibitors and PCSK9 inhibitors) or are registered for an orphan population (homozygous FH patients for lomitapide and mipomersen). An overview of all drugs discussed in this study is shown in table 1. The mechanism by which Lp(a) is modified is mostly, as in case of niacin, CETP inhibitors and PCSK9 inhibition, unknown, which may be not surprising since insight into the metabolism of Lp(a) is limited. We created an overview of known and proposed mechanisms by which different drugs lower Lp(a) (figure 2). None of Lp(a) modifying agents which were reviewed, with the exception of antisense Lp(a), solely reduced Lp(a) without the modification of other lipoproteins. To establish whether Lp(a) reduction is a relevant target for CVD prevention this will be an essential piece of the puzzle to be determined in the future.

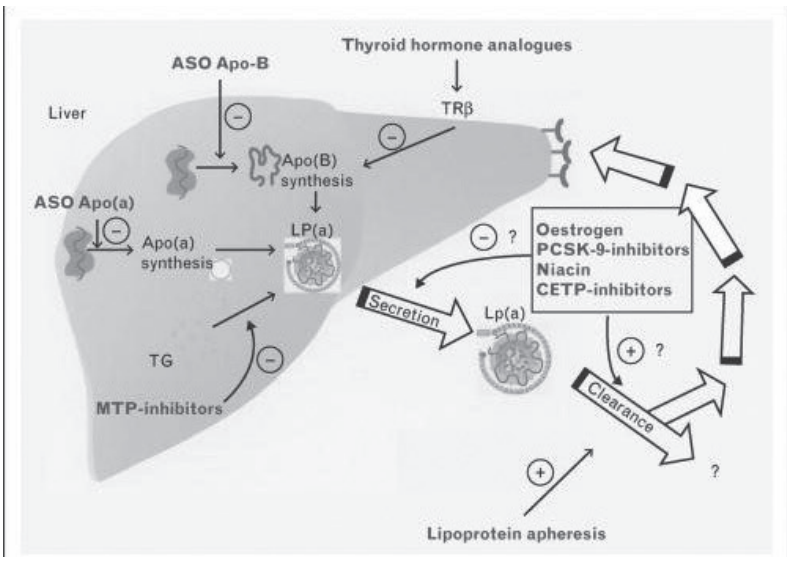

Figure 2: Known and proposed mechanisms of compounds that lower Lp(a).

ASO Apo-B, anti-sense oligonucleotide for apolipoprotein B (mipomersen); ASO apo(a), anti-sense oligonucleotide for apolipoprotein (a) (ISIS APO(a)Rx); MTP-inhibitors, microsomal triglyceride transport protein inhibitor

(Lomitapide); CETP, cholesterol ester transfer protein; PCSK-9, proprotein convertase subtilisin/kexin type 9; Lp(a), lipoprotein (a); TRβ, the β isoform of the thyroid hormone receptor

Because the length of the kringle IV repeat can interfere with Lp(a) measurements, it is difficult to compare studies using different assays for Lp(a) measurement, and this may explain some of the contradictory results between studies. For reliable reproducible studies a gold standard for measuring Lp(a) is needed as is recently discussed by Jacobson (260).

Presently the most evidence based strategy for CVD prevention in patients with increased Lp(a) levels is to lower LDL-C by statin therapy, and for patients with progressive CVD combined with Lp(a)-hyperlipoproteinemia lipoprotein apheresis has proven to reduce CVD events.

Table 1: Overview of different drugs and their effects on Lp(a) levels.

Compound	Effect Lp(a)*	Effect other lipoproteins	Discussed study on effect Lp(a)	Assay used in discussed study	Effect cardiovascular events	Conclusion
Lifestyle	None	Decrease TG variable Decrease LDL-C variable Increase HDL-C variable	Langsted et al. [11]	Diasys and Denka Seiken	Decrease	No effect on Lp(a) Improves lipoprotein profile Decreases cardiovascular events (observational studies)
Oestrogen	Decrease 23-25% (Increase 106% when blocking Oestrogen with Letrozole)	Decrease LDL-C 14% Increase TG 10% Increase HDL-C 8%	Howard and Rossouw [12] (The Woman's Health Initiative)	Unknown	No effect	Decreases Lp(a) Improves lipoprotein profile No effect on cardiovascular events Studied in women only
Eprotirome	Decrease 43%	Decrease LDL-C 32% Decrease TG 33%	Shoemaker et al. [15]	Tinaquant (Roche)	Unknown	Decreases Lp(a) Improves lipoprotein profile Program terminated due to safety issues
Statins	If present at all not clinically significant	Decrease LDL-C 36-38% Decrease TG 7-12% Increase HDL-C 11-12%	Kim et al. [21]	Tinaquant (Roche)	Decrease	No effect on Lp(a) Improves lipoprotein profile Decreases cardiovascular events Recommended for treatment of Lp(a)-Hyperlipoproteinemia

Table 1: continued

Lipoprotein Apheresis	Decrease 70% (directly post treatment) Decrease 26% (one week average)	Decrease LDL-C 67% (directly post treatment)	Leebman et al.[22]	Unknown	Decrease	Decreases Lp(a) Decreases cardiovascular events in Lp(a)-Hyperlipoproteinemia Registered for specific patient population High burden for patients Time consuming Expensive Not reimbursed in every country
Niacin	Decrease 19-24%	Decrease TG 20-50% Decrease LDL-C 5-25% Increase HDL-C 15-35%	Albers et al.[25] (AIM-HIGH) Boden et al.[27] (HPS-2-THRIVE)	Northwest Lipid Research Clinic Protocol	No effect on top of statins Possible decrease monotherapy	Decreases in Lp(a) Improves lipid profile No effect on cardiovascular events on top of statins possible decrease in cardiovascular events with monotherapy Problematic safety profile
Ezetimibe	No effect	Decrease LDL-C 10%	Moutzouri et al. [29]	Randox	Unknown	No effect on Lp(a) Decreases LDL-C Unknown effect on cardiovascular events

Mipomersen	Decrease 21-28%	Decrease TG 25-33% Decrease VLDL-C 33-37% Decrease LDL-C 28-37%	Stein et al. [30] Thomas et al. [31]	Northwest Lipid Research Clinic Protocol	Unknown	Decreases Lp(a) Improves lipoprotein profile Unknown effect on cardiovascular events Registered for Homozygous FH only
Lomitapide + fat restricted diet	No effect (after 78 weeks)	Decrease TG 31% Decrease VLDL 31% Decrease LDL-C 38%	Cuchel et al. [32]	Unknown	Unknown	No effect on Lp(a) Unknown effect on cardiovascular events Registered for Homozygous FH only
CETP-inhibitors	Decrease 50%	Decrease TG 6% Decrease LDL-C 32% Increase HDL-C 160%	Teramoto et al. [33]	Unknown	Unknown	Decreases Lp(a) Improves Lipoprotein profile Unknown effect on cardiovascular events
PCSK-9 monoclonal antibodies	Decrease 30-40%	Decrease LDL-C 55-65%	Davidson et al. [36] Desai et al. [37] Raal et al. [38] Blom at al. [39]	AMGEN: Denka Seiken REG/SAN: Randox.	Unknown	Decreases Lp(a) Improves lipoprotein profile Unknown effect on cardiovascular events
Anti-sense Apo(a)	Decrease 82%	Unknown	Presented by Tsimikas at the American heart Association 2014. [http://ir.isispharm.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=1877550&highlight]	Unknown	Unknown	Decreases Lp(a) The only Lp(a) specific treatment

Chapter 4B

LDL-receptor negative compound heterozygous familial hypercholesterolemia: Two lifetime journeys of lipid lowering therapy.

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Introduction

Patients with homozygous FH (HoFH) have extremely high low-density lipoprotein cholesterol (LDL-C) levels, rendering them susceptible to a very high risk of premature cardiovascular disease (CVD) and extensive aortic valve calcification and stenosis. Therefore effective treatment is required starting in early childhood. However, until recently available drug therapies for these patients render insufficient lipid-lowering capacity (75). The combination of lipid-lowering medication and lipoprotein apheresis is considered the optimal treatment for these patients. However, in the Netherlands lipoprotein apheresis is not reimbursed, and this has generated an extreme challenge in providing optimal treatment for patients with HoFH. Here, we present two adult patients with compound heterozygous FH without clinical CVD events, who have been treated with a wide array of lipid-lowering medication but not with lipoprotein apheresis. We describe the effects of these lipid-lowering treatment regimes during their life course in the light of the available medical managment of HoFH in past and present. These case histories provide a unique insight in the effects and side effects of these treatment options, and can aid clinicians who treat patients with HoFH.

Case series

Two patients, a 25 year old man (patient 1) and his 23 year old sister (patient 2) with compound heterozygous FH (HeFH), have two mutated alleles encoding the LDL receptor (*LDLR*), from their mother the Leiden-3 mutation 4.4 kb duplication exon 12, and from their father the Capetown-2, 2.5 kb deletion exon 7,8 (figure 1). Both mutations are considered as LDLR negative mutations and no LDLR-protein activity is expected. They were treated from the age of 5 and 3 years, respectively, at the outpatient clinic of the Sophia Children's Hospital and subsequently at the cardiovascular genetics clinic of the Erasmus medical Center, Rotterdam, the Netherlands. In 2010, they were offered to be referred to the only center in the Netherlands performing lipoprotein apheresis, but they declined because of the travel distance and the intensity of this treatment.

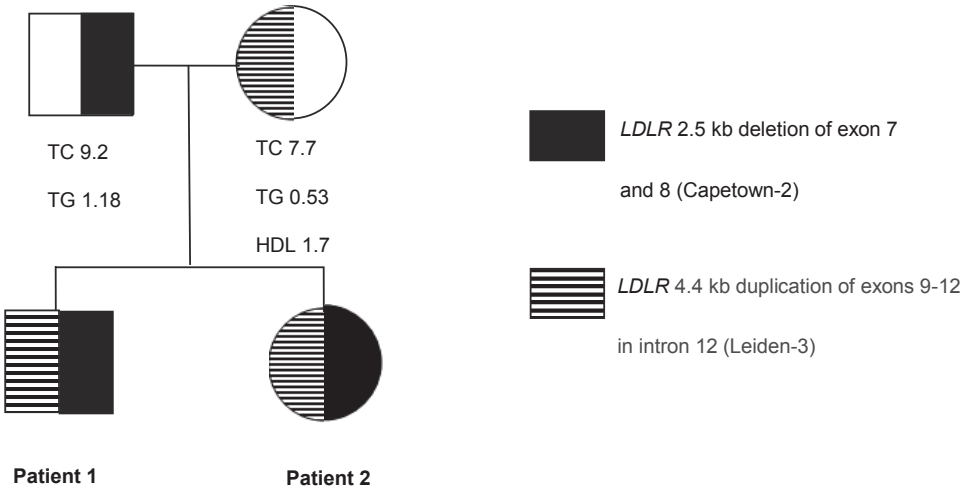


Figure 1. Pedigree of the affected patients, with untreated lipid levels in parents.

Patient 1 is a 25-year old man with compound HeFH. At the age of 5 years he was referred to the pediatrician on the suspicion of HoFH with painful tendon xanthomas at his Achilles tendon and hands and eruptive xanthomas on his elbow. Lipid measurements at baseline showed a total cholesterol (TC) of 21.0 mmol/L (812 mg/dL) and LDL-C level of 19.6 mmol/L (758 mg/dL). He started with a cholesterol-lowering diet and lipid-lowering medication: simvastatin (the first available statin in the Netherlands) 20mg/day in combination with niacin 3x 100mg/day up to 3x 200mg/day. This therapy resulted in an LDL-C level of 16.4 mmol/L (634 mg/dL) (maximum decrease of 16%). Subsequently, at the age of 6 years he switched to increasing dosages of pravastatin 10-40mg/day (lowest LDL-C 15.6 mmol/L (603 mg/dL), -20%) for a few months,. Because LDL-C levels were still very high, pravastatin was switched to increasing dosages of atorvastatin 20 mg to 80 mg/day (lowest LDL-C 11.2 mmol/L (433 mg/dL), -43%). At the age of 13 years ezetimibe was added to this treatment regimen, resulting in 20% additional reduction of the LDL-C level to 9.0 mmol/L (348 mg/dL). In 2011 at the age of 20 years, twice daily nicotinic acid/laripoprant 1000/20 mg was added to the treatment without side effects, leading to 16% additional reduction of the LDL-C level to 7.6 mmol/L (294 mg/dL). However, in January 2013 he had to stop this medication as it was withdrawn from the market.

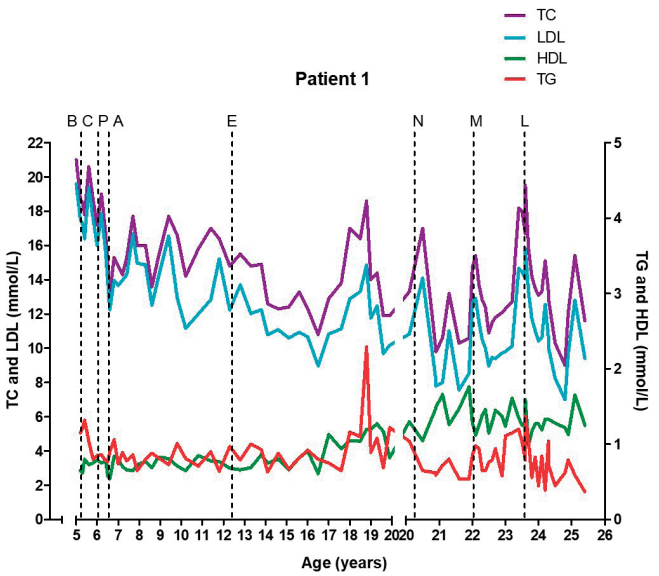
He went back on treatment with atorvastatin and ezetimibe and his LDL-C level was 12.7 mmol/L (491 mg/dL). In February 2013, mipomersen 200 mg once weekly subcutaneously was added to this treatment regimen. Mipomersen was prescribed as usual care in a named patient program and resulted in a maximum additional 29% reduction of the LDL-C level to

9.0 mmol/L (348 mg/dL). He had mild injection site reactions and no liver test elevations. However, after 14 months of treatment, he was hospitalized with petechiae and epistaxis. Laboratory measurements showed a thrombocyte count < 3*10E9/L. He was diagnosed with idiopathic thrombocytopenic purpura (ITP). Despite thorough analysis at the hematology department, it was not possible to establish whether the ITP was caused by mipomersen treatment or not. This episode of ITP was reported as a possible side effect of mipomersen to Genzyme as part of pharmacovigilance. Prednisone treatment was initiated and tapered slowly when his thrombocytes normalized and withdrawn 3,5 months later. Mipomersen was not restarted. In September 2014 he began with lomitapide in a named patient program. He started with lomitapide 5 mg/day, slowly uptitrated to 20 mg/day, resulting in a maximum additional 45% reduction of LDL-C level to 7.0 mmol/L (271 mg/dL). Prior to the start of lomitapide an ultrasonography of the liver in combination with a fibroscan showed no abnormalities especially no hepatosteatosis. He adhered to a low-fat diet with supplementation of vitamin E and omega 3 and 6. He occasionally has stomach complaints and diarrhea if he does not adhere to the diet. However, his liver tests did not increase above the upper limit of normal during follow-up. His most recent LDL-C level was 9.4 mmol/L (363 mg/dL). The intention is to increase the lomitapide dose to 30 mg. As yet he has not experienced CVD events. Yearly cardiac ultrasound showed stable moderate aortic valve stenosis and mild to moderate insufficiency. He does not have other comorbidities.

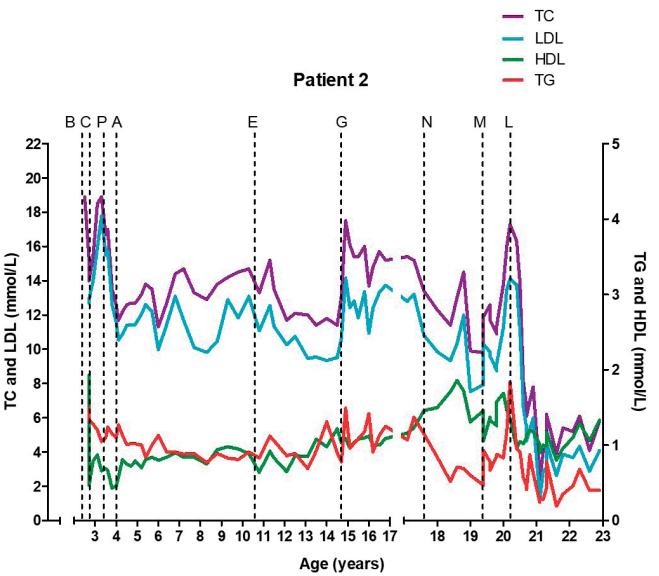
Patient 2 is a 23-year old woman with compound HeFH. She was referred to the pediatrician at the age of 2.7 years together with her older brother (patient 1) because of possible HoFH. She had elevated cholesterol levels (total cholesterol 18.9 mmol/L (731 mg/dL)) and no xanthomas. Untreated LDL-C level has not been documented. Similar to her brother, she also immediately started with a cholesterol-lowering diet in combination with lipid-lowering medication: statins were not administered because of her young age, instead questran 4 x 1 gr/day and niacin 4 x 50 mg/day up to 3 x 200 mg/day were started. This therapy resulted in a maximal decrease in total cholesterol of 26% (14.0 mmol/L (541 mg/dL)). The first measurement of LDL-C level was 12.7 mmol/L (491 mg/dL) during the combination therapy described earlier, and increased up to 17.8 mmol/L (688 mg/dL) in the following months. The latter measurement was taken as baseline LDL-C level. She switched to pravastatin at the age of 3.5 years (lowest LDL-C 12.6 mmol/L (487 mg/dL), -29%). At the age of 4 years, pravastatin was replaced by increasing dosages of atorvastatin 20 mg/day to 80 mg daily (lowest LDL-C 9.8 mmol/L (379 mg/dL), -45%). At the age of 11 years, ezetimibe was added to this treatment regimen, which resulted in an additional decrease of -5% of LDL-C level to 9.4 mmol/L (363 mg/dL). Four years after the start of ezetimibe, she experienced severe angioedema. Ezetimibe was stopped, because the lipid-lowering effect was negligible and it could not be fully ruled out that this episode was triggered by ezetimibe. In 2008, she was treated with atorvastatin monotherapy (LDL-C level of 14.2 mmol/L (549 mg/dL)). At the age of 15 years colesevelam became available, which was prescribed in a dose of 2 x 1250 mg

up to 2 x 1875 mg/day (lowest LDL-C 10.9 mmol/L (422 mg/dL), -23%). In 2011 at the age of 18 years, nicotinic acid/laripoprant twice 1000/20 mg/day was added to the treatment, resulting in a maximum additional reduction of 31% in LDL-C level to 7.5 mmol/L (290 mg/dL), without side effects, until it was withdrawn from the market.

At the age of 20 years, she was treated with atorvastatin and colesevelam resulting in an LDL-C level of 10.3 mmol/L (398 mg/dL). In February 2013, mipomersen 200 mg once weekly subcutaneously was added in a named patient program. This treatment resulted in a 15% reduction of the LDL-C level (8.8 mmol/L (340 mg/dL)). Mipomersen was stopped after 5 months of treatment due to painful injection site reactions, which could not be prevented by alleviating measures such as pre- and post-injection icing of the injection site. In January 2014, she started with lomitapide treatment in a named patient program with the same low-fat diet as her brother. Colsevelam was stopped before the start of lomitapide, because of its minimal effect. The experience with lomitapide in this patient was previously described (117). In brief, the dosage was titrated from 5 mg to 30 mg/day with remarkable reductions in LDL-C levels of 87% with levels as low as 1.3 mmol/L (50 mg/dL). However, due to gastro-intestinal symptoms, lomitapide dose was lowered to 20 mg/day, minimalizing the symptoms. The most recent LDL-C level was 4.1 mmol/L (159 mg/dL). She has currently not experienced CVD events. Yearly cardiac ultrasound showed stable moderate aortic valve stenosis and mild insufficiency. She does not have other comorbidities. Figure 2 shows the course of total cholesterol, LDL-C, triglyceride and HDL-C levels over the years of both patients.



B: Baseline
C: Combination of diet, simvastatin 20mg/day, niacin (300-600) mg/day
P: Pravastatin (10-40) mg/day
A: Atorvastatin (20-80) mg/day
E: Addition of ezetimibe 10 mg/day
N: Addition of niacin/laripoprant 2000/40 mg/day
M: Atorvastatin 80mg, ezetimibe 10 mg, mipomersen 200 mg/week
L: Atorvastatin 80mg, ezetimibe 10 mg, lomitapide (5-20) mg/day



B: Baseline
C: Combination of diet + Questran 4x 1 gr/day
+ Niacin (200-600) mg/day
P: Pravastatin (10-40) mg/day
A: Atorvastatin (20-80) mg/day
E: addition of Ezetimibe 10 mg/day
G: atorvastatin 80 mg, Colesevelam (2500-3750) mg/day
N: addition of Niacin/Laripoprant 2000/40 mg/day
M: atorvastatin 80mg, Colesevelam 3750 mg Mipomersen 200 mg/week
L: atorvastatin 80 mg, Lomitapide (5-20) mg/day

Figure 2. Total cholesterol (TC), LDL-C, TG and HDL-C levels in patient 1 and 2. The dotted lines indicate key events over the course of treatment. Letters indicate the different medication used, these are further described under the figure

Discussion

Long-term treatment of HoFH patients is extremely challenging, with very high baseline LDL-C levels, which remain far above target levels when treated with combinations of the conventional lipid-lowering drugs. These case histories tell the lifelong journey of the lipid-lowering treatment of two siblings affected with the most severe form of HoFH, namely HoFH without LDLR activity. HoFH Patients without LDLR activity have higher baseline LDL-C levels and a poorer prognosis and life-expectancy compared to HoFH patients with residual LDLR activity (75). In the recent position paper of the clinical consensus panel on Familial Hypercholesterolemia of the European Atherosclerosis Panel it is mentioned that "Untreated HoFH patients who are LDLR-negative rarely survive beyond the second decade" (75). These two patients have not developed clinical CVD events at the age of 25 and 23 years old. However, it is certainly possible that they have subclinical atherosclerosis. CT angiography (CTA) as proposed by recent consensus statements (75, 261) has been considered but not been performed in these patients for several reasons, mainly because CTA results will not change clinical management as currently patients are already treated with most intense lipid-lowering medication available and the cardiologist reasoned that being asymptomatic they will not undergo prophylactic revascularization. Lastly, we would burden these patients with the knowledge about their probably already diseased vessels without an option to alleviate their risk other than their current maximum treatment which posed the treatment team for an ethical dilemma.

In our opinion the main factors contributing to this favorable clinical course are that they were diagnosed at a very young age and that their treating physicians despite not having access to lipoprotein apheresis have been keen on offering them almost all available lipid lowering medications as soon as they became available.

Although statin monotherapy will not be sufficient in HoFH patients to lower LDL-C levels to target levels, it still leads to an average 26% reduction of LDL-C levels and more importantly to a decrease in CVD events and all-cause mortality (262). Despite the absence of LDLR in our patients, maximum statin therapy still led to a 43% reduction of LDL-C level. The proposed mechanism being the decreased production of LDL-C. Until recently, a combination of statins, ezetimibe and lipoprotein apheresis was considered the most effective lipid-lowering therapy available for patients with HoFH (75). Main disadvantages of lipoprotein apheresis are the large fluctuations in LDL-C levels, the frequency of weekly or biweekly treatment, the high costs and the lack of randomized, sham-controlled trials. As a consequence, lipoprotein apheresis is not fully if at all reimbursed in many countries, raising barriers for use as a standard treatment option for HoFH (76, 77).

Emerging therapies in the treatment of HoFH include mipomersen and lomitapide. Both drugs are promising for the treatment of HoFH as they showed their pharmacological efficacy in lowering the LDL-C levels. However, both still have to be evaluated for long-term safety and clinical endpoints (75, 175). Mipomersen inhibits apoB100 synthesis, resulting in 25% reduction of LDL-C levels in HoFH, as well as lowering of all other apoB containing lipoproteins (263). Common side effects are injection site reactions, flu-like symptoms and elevations in transaminases and liver fat (52, 53). Although decrease of platelet count was described in the Phase 3 studies in <3% of the treated subjects, ITP was not previously reported as side effect (264). Mipomersen has been approved by the Food and Drug Administration (FDA) for treatment of HoFH in the USA but was disapproved by the European Medical Agency (EMA), because of potential cutaneous, hepatic and cardiovascular side effects. Our patients were the first patients worldwide to use mipomersen outside a clinical study in a named-patient program, and confirmed the shown reductions in LDL-C levels. However, due to side effects mipomersen was stopped in both patients. Lomitapide was approved by both the FDA and EMA for treatment of HoFH. Lomitapide inhibits the microsomal triglyceride transfer protein and thereby reduces the chylomicron and VLDL production resulting in reduction of LDL-C levels. In a pivotal open label phase 3 study in HoFH patients, lomitapide treatment in addition to maximum conventional lipid-lowering treatment led to an LDL-C reduction of 50% at 26 weeks and a long term effect of 38% reduction at 78 weeks. Reported side effects consisted of gastro-intestinal symptoms which can be minimized by a low-fat diet, and liver test elevations (54). In our patients a maximum additional decrease of LDL-C levels of 45% was reached in patient 1 on 20 mg, and 87% in patient 2 on 30 mg/day, which is a larger effect than would have been expected from the phase 3 trial. It has been observed previously in real world clinical practice that the LDL decrease is higher than observed in the phase 3 trial (117, 265), a proposed reason could be that in the phase 3 trial patients were forcibly uptitrated every four weeks, while in clinical practice the optimal effect of each dose is awaited and the time period of dose increases is longer. It is important to acknowledge that treatment with mipomersen and especially lomitapide require a high intensity of care with continuous evaluation of side effects and safety monitoring. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors lower LDL-C by preventing degradation of the LDL receptors, increasing LDL uptake. The TESLA study showed that PCSK9 inhibition would not be effective in our patients as they do not have any residual LDLR function (266). Therefore, PCSK9 inhibitors were not prescribed in our patients.

Although we achieved substantial LDL-C reduction at very young age and continued to improve that during the past years in both patients, the treatment goal of LDL-C<2.6 mmol/L has been far out of reach. Therefore, new therapies for LDLR negative patients are urgently needed. LDLR gene therapy is one of these promising therapies, which is currently under development and could even be the ultimate cure for these patients (267).

Conclusion

This report shows the eternal crusade of finding optimal treatment for very severely affected HoFH, LDLR negative patients, and illustrates the progress in the development of lipid-lowering medication in the last decades, but also indicate that additional therapy is still needed. Notably, close monitoring of side effects, adverse events and the effect of combinations of drugs in childhood as well as early access to novel drugs in adolescence and adulthood was feasible and enabled to improve the treatment of these patients as they remain free of CVD until now.

Chapter 5

Discussion

The primary aim of my thesis was to investigate the role for advanced analyses of lipoproteins beyond the conventional lipid panel that is used for cardiovascular disease (CVD) risk classification. The secondary objectives were to investigate the effects of genetics, dietary and medical interventions on plasma lipoprotein(a) [Lp(a)] levels, and to review current and upcoming treatment options.

In this chapter, I discuss the main findings of this thesis, their effect on clinical practice, and I present directions for future research.

Interpretation of main findings

Is there a role for advanced lipoprotein profiling in screening of subjects at high risk of developing type 2 diabetes (T2D)?

Most physicians are familiar with the standard lipid panel that is used for CVD risk estimation (268). The standard lipid panel is used to identify subjects with increased levels of pro-atherogenic lipoproteins and decreased levels of anti-atherogenic lipoproteins. However, with that approach and despite statin-treatment aimed at cardiovascular (CV) prevention a residual risk of approximately 60% of CVD events still occurs and about half of the individuals without abnormalities in the standard lipid panel still experience a CV event (2-8, 269). Previous studies have shown that advanced lipoprotein analyses might improve risk estimation especially in subjects with normal standard lipids, with low density lipoprotein (LDL) particle number being a better predictor for CVD than LDL-cholesterol (LDL-C) level (270-272). Increased number of small dense LDL particles, as determined by nuclear magnetic resonance (NMR) spectroscopy, is also related to CVD even after adjusting for the Framingham risk score (273).

Diabetic dyslipidemia is characterized by low levels of high density lipoprotein (HDL) cholesterol, a preponderance of small dense HDL, small dense LDL and large very low density lipoprotein (VLDL) particles, and high levels of plasma triglycerides (TG), and is present well before the diagnosis of type 2 diabetes (T2D) (40, 41, 80-82). In **chapter 2.A** I used advanced lipoprotein profiling to identify parameters of diabetic dyslipidemia in normoglycemic first-degree relatives of T2D patients. Total HDL-C and its subclasses (HDL_2-C HDL_3-C) levels were lower in normoglycemic individuals from T2D families than in controls from non-T2D families. A low HDL-C level in family members of T2D may indicate the risk of developing T2D. The lower HDL-C levels, especially lower levels of the small dense HDL, as reflected by the lower HDL_2-C levels I show here, might contribute to the increasing glucose intolerance in those families. Lower HDL_2-C levels during progression of glucose intolerance may be linked to reduced cholesterol efflux and higher cholesterol levels in the pancreatic beta-cells (112). Accumulation of cholesterol in beta-cells has been reported to decrease insulin secretion in

animal models (113, 114). A link between HDL metabolism and glucose homeostasis is also illustrated by the lowering of plasma glucose levels in T2D patients upon treatment with cholesterol ester transfer protein (CETP) inhibitors (274) which increases HDL-C levels and shifts HDL toward more buoyant particles. There is growing evidence for the role of HDL in glucose metabolism via different mechanisms (275). One of these mechanisms is by affecting insulin secretion via the pancreatic beta-cells possibly mediated by sphingosine-1-phosphate (S1P) (a bioactive lipid carried within HDL particles). Glucose increases S1P via activation of sphingosine kinase 2, and increased S1P correlates with increased glucose-dependent insulin secretion. Decreased S1P by sphingosine kinase inhibitor leads to reduced glucose-dependent insulin secretion (276). HDL can affect direct glucose uptake, as apolipoprotein A-I (apoA-I) (a major protein component of HDL) stimulates the phosphorylation of AMP-activated protein kinase (AMPK), which is related to increased glucose uptake (277). These results suggest that HDL itself or proteins attached to its surface such as apolipoprotein M (apoM) has a protective effect against the development of diabetes. In this chapter I found that the HDL_2-C and HDL_3-C levels are more strongly linked to beta cell function and fractional insulin synthesis rate than to insulin sensitivity, suggesting a link between lower HDL-C levels and deteriorating beta-cell function. Whether alterations in HDL concentration, size or density have a causal role in the development of insulin resistance and beta cell dysfunction remains to be clarified. However, the fact that some characteristics of diabetic dyslipidemia such as low HDL-C levels are already present in family members of T2D patients while they are still normoglycemic, promotes the development of a screening strategy to identify those subjects at risk for T2D. Screening these individuals offers the opportunity for early prevention. In subjects with prediabetes, metformin use not only reduces insulin resistance but also diabetic dyslipidemia (92). Diabetic dyslipidemia in normoglycemic subjects is associated with increased risk of developing T2D. Normoglycemic relatives of T2D patients are more susceptible to having characteristics of diabetic dyslipidemia than normoglycemic individuals in families without T2D. Treatment with metformin and statins of early detected signs of diabetic dyslipidemia as an indicator of a high T2D risk in normoglycemic relatives of T2D patients, may lead to the prevention of T2D.

What is the role of advanced lipoprotein profiling in addition to the standard lipid panel in subjects with homozygous familial hypercholesterolemia (HoFH)?

Untreated patients with HoFH have very high LDL-C levels that often exceed 13 mmol/L, rendering them susceptible to premature atherosclerotic CVD and extensive aortic valve calcification and stenosis (74, 75). Without treatment the majority of patients with HoFH do not survive beyond their twenties. Early diagnosis and treatment of FH is therefore essential (75).

The risk of CVD in subjects with FH is mostly determined by their LDL-C levels, especially in HoFH, because of the lifelong exposure to elevated LDL-C levels. Low HDL-C levels, preponderance of small dense HDL and small dense LDL, high TG levels and elevated

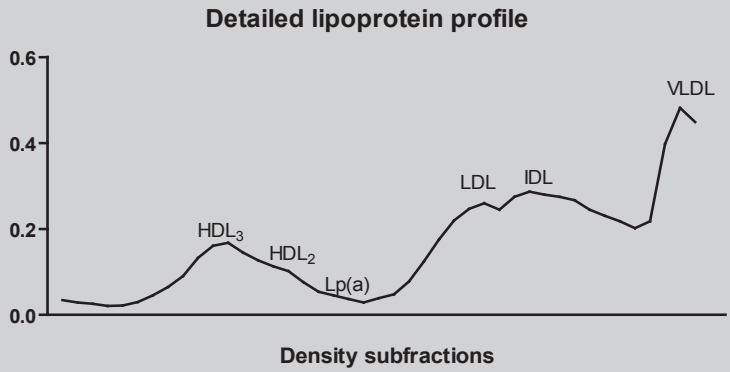
Lp(a) levels could further increase the risk (38, 278-284). The use of advanced lipoprotein profiling can help understand the effects of treatment on dyslipidemia characteristics which are missed by the standard lipid panel. In **chapter 2.B** I used advanced lipoprotein profiling and cholesterol efflux pathways to investigate the effect of the microsomal triglyceride transfer protein (MTP) inhibitor lomitapide in HoFH patients on their lipoprotein profile and HDL function in reverse cholesterol transport. Previous studies showed that lomitapide treatment in addition to lowering LDL-C levels, is associated with moderate decrease of both HDL-C and ApoA-I levels (54, 115, 116). In line, I found a decrease in the levels of HDL-C, ApoA-I, preβ-HDL, and HDL_3-C. However, lower HDL_3-C levels and more buoyant particles were observed with HDL_2-C levels remaining unchanged or increased. A reduced formation of HDL during lipolysis of predominantly postprandial triglyceride rich lipoproteins (TGRL) may underlie the reduction in HDL and ApoA-I levels and the alterations in HDL subclass levels. Additionally, lomitapide may reduce the levels of HDL-C derived from the intestine, since MTP-deficiency has been reported to reduce HDL-C secretion from the intestine in mice (125-127). In parallel with this shift in HDL subclasses, the ATP binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux was decreased in all patients, whereas changes in the ATP binding cassette transporter G1 (ABCG1)- and scavenger receptor BI (SR-BI)-mediated cholesterol efflux were less consistent.

Lomitapide treatment not only decreased LDL-C and apoB levels but also the other atherogenic lipoproteins, i.e. intermediate density lipoprotein (IDL), VLDL, Lp(a) (54) and chylomicron (remnants). This reflects the reduced pro-atherosclerotic potential. Despite the moderate decrease of HDL-C, unaffected total cholesterol efflux capacity (CEC) suggests a stable anti-atherogenic potential of HDL. Although a very small study, it illustrates the benefit of using advanced lipoprotein profiling in understanding the effects of a new drug in subjects at extremely high CVD risk. The use of advanced lipoprotein profiling could also improve personalized treatment. While the Dutch Cardio Vascular Risk Management (CVRM) guidelines take TC/HDL ratio into account for risk classification, during treatment goals are based on LDL-C target levels. However, this 'one-size fits all' approach does not always agree with 'real life' practice, as illustrated by the case report about a patient with familial dysbetalipoproteinmemia in case report A.

The use of advanced lipoprotein profile in this patient is a good example of personalized medicine. Advanced lipoprotein profiling in this subjects was a better diagnstic tool to identify the type of dyslipidemia and led to a better personalized treatment choice, resulting in an improved lipoprotein profile, with less side effects. Physicians should be aware of the possibility that the standard lipid panel does not identify all types of dyslipidemia, especially in general practice when it does not include measurements of apoB and Lp(a). When lipid abnormalities do not respond to statins, it is not always a case of non-compliance. Further analysis via advanced lipoprotein profiling is then recommended.

Case report A

A 62 years old Caucasian woman was referred to the cardiovascular genetics outpatient clinic at the Erasmus MC because of statin-induced myalgia . She was known with hypercholesterolemia for the past 4 years and was treated with several lipid lowering drugs (Simvastatin, Fluvastatin, Rosuvastatin, and ezetimibe). Treatment adjustment did not affect lipid abnormalities nor the side effects. The standard lipid panel showed elevated levels of LDL (6.7 mmol/L) and total cholesterol (9.5 mmol/L). Advanced lipoprotein profiling, however, showed normal LDL level, extremely increased VLDL and increased IDL levels. Statin then was switched to a fibrate (Gemfibrozil 600 mg/day). A few months later, the lipid profile normalized (LDL=2.6 mmol/L and total cholesterol= 5.0), and the patient had for the first time in years no myalgia.



DNA analysis later on confirmed the presence of APO E2/E2, which has a reduced affinity for hepatic lipoprotein receptors, resulting in impaired removal of remnants, chylomicrons and VLDL. This condition is called familial dysbetalipoproteinemia (FD) and is associated with premature CVD.

Is the use of Lp(a) measurement in subjects with FH recommended?

Although the role of plasma Lp(a) levels as a risk factor for CVD in FH was controversial, since multiple retrospective and cross-sectional studies have shown that Lp(a) is indeed an independent risk factor for CVD in FH, interest in Lp(a) has been reignited. (216, 217, 284). A recent study showed that subjects with genetically confirmed FH, especially those with CVD, have significantly higher Lp(a) levels than their non-FH relatives (203). In **chapter 3.C** I investigated the effect of *LDLR* and *APOB* mutations on plasma Lp(a) levels in FH families. I found a trend towards increased plasma concentrations of Lp(a) in HoFH patients in comparison with heterozygous FH (HeFH) family members and unaffected relatives. I therefore recommend the use of Lp(a) measurement for risk classification in HoFH in

the clinical setting. For non-FH patients, a recent paper showed that high Lp(a)-levels are predictive of the progression of atherosclerosis despite intensive lipid-lowering therapy (285).

The measurement of Lp(a) is of importance especially in families with a high prevalence of premature CVD, which is not explained by other classical risk factors (environmental factors, LDL-C, metabolic syndrome), as illustrated by **case report B**. This case illustrates the value of Lp(a) measurement in risk prediction.

In **Chapter 3.B** I show that statin treatment in FH subjects increases Lp(a) levels in subjects with the low molecular weight isoform of apolipoprotein(a) [apo(a)]. The mechanism underlying increased Lp(a) levels in FH patients is unknown. One of the proposed mechanisms is due to decreased LDL-receptor mediated clearance of Lp(a) particles. This hypothesis fits with the increased Lp(a) levels in hoFH patients compared to their heFH and unaffected relatives. However, on the other hand statins are known to increase hepatic LDLR expression, but do not significantly reduce plasma Lp(a) levels, and in some studies including ours plasma Lp(a) levels even increase (19, 187). Moreover, the increase in Lp(a) levels I show in **chapter 3.B.** was not associated with changes in LDL-C levels. Overall, this suggests clearance of Lp(a) via receptors other than the LDL-receptor. In contrast to statins, monoclonal antibodies against PCSK9 have been shown to significantly lower Lp(a) levels in HeFH (185) and HoFH patients with one or two defective *LDLR* alleles (186), respectively. The observation that Lp(a) levels were above average in individuals with *PCSK9* gain-of-function mutations (221) as well as in HeFH patients, indicates a role of PCSK9 in Lp(a) metabolism. Indeed, *in vivo* experiments have recently demonstrated that PCSK9 plays a role in the internalization of Lp(a)(207). Although it has been proposed that the decrease in Lp(a) levels with PCSK9 inhibitors can be explained by overexpression of LDL receptors in combination with markedly reduced circulating LDL particles, increasing Lp(a) clearance via its binding to the LDL receptor (188, 189, 207), I consider this unlikely. This is supported by the fact that Lp(a) levels were also decreased by PCSK9 inhibitors in HoFH patients with null mutations, without changes in LDL-C levels (190). This suggests that there are other pathways of Lp(a) clearance than via LDLR, such as for example SORT1 (sortilin) a high affinity sorting receptor for PCSK9 (192). It has been shown recently that overexpression of SORT1 increases Lp(a) internalization even in fibroblasts with defective LDLR, and may also increase the secretion of apo(a). Therefore, the decrease in Lp(a) levels by PCSK9 inhibitors, might be mediated by SORT1 (193). This suggests that PCSK9 inhibitors are not only promising for the wide use in terms of LDL-lowering, but also a possible treatment option for Lp(a) lowering.

Case report B
A 20yr old healthy woman was referred to the cardiovascular genetics outpatient clinic at the Erasmus medical centre for cardiovascular risk classification. Her father had suffered from a myocardial infarction at the age of 45 years without having traditional cardiovascular risk factors. Investigation of the family for classical risk factors (high cholesterol, FH, smoking, low HDL-C, metabolic syndrome) did not reveal any abnormalities. Subsequent measurement of Lp(a) showed very high levels for both father and mother (105 and 120 mg/dL, respectively). The index patient herself had an extremely high Lp(a) level (279 mg/dL). Therefore elevated Lp(a) levels could be the pathogenesis for the risk of premature CVD in this family. We assessed cardiovascular risk in our patient higher than the general population on which cardiovascular risk charts are based. Currently no specific treatment is available for isolated hyperlipoproteinemia(a). However, we will lower the threshold to treat modifiable risk factors such as hypertension and dyslipidemia.

Statin treatment might also affect inflammatory processes, influencing apo(a) synthesis and increase Lp(a) levels via increased production, as recently shown by Muller et al (286). An increased risk awareness and initiation of a healthier lifestyle in parallel with the start of statin treatment possibly resulting in less fat-intake, may have resulted in an increase of Lp(a) levels. Decreased fat-intake, saturated fat in particular, is associated with increased Lp(a) levels (145, 146). The mechanisms underlying increased Lp(a) levels in FH versus non-FH subjects, and the statin-induced increase in Lp(a) levels in FH patients, remains to be clarified.

What is the effect of widely used interventions aimed at CVD prevention on Lp(a) levels?
Lp(a) as a CVD risk factor contributes to CV events in particular in patients with T2D compared to those without T2D (50). However, plasma Lp(a) levels are often not measured in these patients in clinical practice. The main reason is that Lp(a) is not a treatable risk factor yet. However, Lp(a)-lowering drugs are currently being tested in phase 3 studies (58). The plasma Lp(a) concentration is highly genetically determined by the number of kringle IV (KIV) type 2 repeats in the LPA gene encoding apo(a) (16, 44-47). However, about 25% of the variance in Lp(a) levels has been attributed to lifestyle (48). Changes in lifestyle aimed at weight loss in obese patients have been reported to affect Lp(a) levels, but results are controversial (48, 141-143). In **chapter 3.A**. I have shown that diet aimed at weight reduction increases Lp(a) levels in obese T2D patients. The type and content of fat in the diet may be an important determinant of the dietary effect on Lp(a) levels. Increased intake of total- and saturated fat has been found to decrease Lp(a) levels, while an increased intake of monounsaturated fatty acids tends to increase Lp(a) levels in healthy and metabolically disturbed subjects (145-147). Faghihnia et al. (146) suggested that dietary fat-induced changes in LDL metabolism,

notably of medium and very small LDL subclasses, may lead to altered formation, catabolism or clearance of Lp(a). The dietary interventions used in our cohorts were based on a balanced low calorie diet with low carbohydrate and low total fat intake. In a subset of participants, After an initial increase, Lp(a) levels had almost returned to baseline values at 20 months of follow-up. Despite weight regain, the average body weight was still lower than at baseline. Weight regain was not correlated with long-term change in Lp(a) levels. This suggests that the increase of Lp(a) levels was an acute effect of the diet that waned off after a longer period of a less strict diet. The mechanism underlying the short term increase in Lp(a) levels following diet aimed at weight reduction remains to be elucidated.

Although statins have been prescribed since the early 1990's, the literature is still controversial about its effect on Lp(a) levels. In **chapter 3.B**. I have shown that short-term statin treatment increases Lp(a) levels in FH patients carrying the lower apo(a) isoform. The question remains whether this short-term increase in plasma Lp(a) levels has a significant influence on the CVD risk for the long term.

Both common interventions (weight loss and statin treatment) increase Lp(a) levels especially in those subjects who already have high Lp(a) level at baseline. I recommend the measurement of Lp(a) level for risk prediction before an intervention in FH and T2D patients is initiated, to be able to select those with high Lp(a) level at baseline. When the Lp(a) level is increased at baseline in an individual, based on my data I recommend that the treating clinician should be aware of the possibility of further increase following a diet aimed at weight reduction or statin treatment, and prescribe specific Lp(a)-lowering drugs once they become available and have proven their efficacy in lowering CVD risk.

What are treatment options for dyslipidemias identified by advanced lipoprotein profiling?

It is well known that dyslipidemias (including preponderance of small dense HDL and small dense LDL, VLDL) do increase CVD risk. Many studies have shown that these dyslipidemias contribute to the risk in particular of patients with T2D (40, 41), and with FH (70). However, since these dyslipidemias are missed by the standard lipid panel, effects of the different cholesterol-lowering drugs on the different lipoprotein subclasses in patients with dyslipidemia have not been studied very well yet. Statins do not only decrease LDL-C levels, but also affect VLDL, Lp(a) and inflammatory pathways. Recent data of the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial suggest that statin-induced reductions in small dense VLDL further decrease CVD risk (287). So why take only the LDL-C treatment target level into account during treatment with a statin? A recent study showed a positive correlation between PCSK9 level and different atherogenic lipoproteins such as small LDL, large VLDL and IDL(288). Theoretically this could

mean that PCSK9 inhibition could improve a large part of the lipoprotein profile, and not only lower LDL-C and Lp(a) levels.

Although there are no available therapies to exclusively lower Lp(a) levels yet, as discussed in **chapter 4.A**, PCSK9 inhibitors, do lower Lp(a) levels about 20-30% (255-258) in addition to lowering LDL-C. Taking the idea of 'the lower, the better' into account, major reductions in LDL-C levels by PCSK9 inhibitors further reduce CVD events (289). A drug which specifically lowers Lp(a) levels, which might be more suitable for subjects with only increased Lp(a) levels as a CVD risk factor is an antisense oligonucleotide targeted to the APOA gene. Results from a phase II study showed a major reduction in Lp(a) levels, and also decreased LDL-C levels by 20% (58). This drug could eventually become the perfect treatment for lowering this otherwise persistent risk factor (58). However, studies will still have to show whether Lp(a) lowering drugs affect hard endpoints, such as CV events, CVD mortality and all-cause mortality.

Implications for clinical practice

I have shown in part I of this thesis that normoglycemic family members of T2D have characteristics of diabetic dyslipidemia (low HDL-C levels) compared to controls. Previous studies have shown that diabetic dyslipidemia is present well before the diagnosis of T2D, and even before any signs of glucose intolerance (40, 41). Therefore, normoglycemic family members with low HDL-C level might be at risk for T2D, even when they do not yet show signs of glucose intolerance. Family screening is more feasible than screening on population base and might help identify those subjects at risk based on characteristics of diabetic dyslipidemia, well before the development of glucose intolerance. I showed in part I that first degree relatives of T2D patients might be the right population to screen for diabetic dyslipidemia. So why wait until T2D is developed and offer treatment for T2D and diabetic dyslipidemia? Why not start with primary prevention by prescribing metformin and statins to delay the onset of T2D and thereby CVD before the development of glucose intolerance? This would be ideal if we have a simple measure with clear cut-off points to discriminate subjects with from those without diabetic dyslipidemia.

Studying the effect of the current and upcoming lipid-lowering drugs at detailed lipoprotein profile assessment is needed to understand which dyslipidemias are associated with residual CVD risk. The use of advanced lipoprotein profiling is also instrumental for improvement of personalized treatment. In part II of this thesis I show that Lp(a) is not only increased in HeFH but even more in HoFH compared to non-FH, and increases upon widely used interventions aimed at CVD prevention. Measurement of Lp(a) and the detailed lipoprotein profile might improve risk prediction and thereby the possibility of early prevention for

CVD. Taken together I support extending the standard lipid panel with measurements of lipoprotein subclasses and Lp(a).

There is a variety of methodologies for the analysis of the distribution of lipoprotein particles based on their density or size (88, 290). The density gradient ultracentrifugation is the one used in the first part of this thesis (42, 291), which is a well-known and long established lipoprotein subfractionation technology. This time consuming and expensive method is not suitable for wide use in clinical practice, but is highly suitable for research purposes. After separation, lipoprotein subfractions can be analyzed with respect to their composition and functionality. Another method is the NMR spectroscopy technology providing a simultaneous quantification of the size and concentration of all lipoprotein particles is fully automated and more suitable for clinical practice. With NMR the lipoprotein particle sizes and numbers are derived by a convolution program from the total lipoprotein proton NMR signal from the methyl and methylene groups in fatty acid residues (292, 293). However, these 2 methods describe and measure the lipoprotein distribution differently (290). NMR identifies different subclasses of HDL, LDL, IDL and VLDL, but cannot distinguish Lp(a) from LDL or IDL. The reproducibility of the measurement of the lipoprotein profile by NMR has been reported to be very good (293). NMR has been used in large studies, and has proven its efficacy in identifying lipoprotein alterations correlated with CVD risk (38, 40, 294). NMR in combination with Lp(a) measurement could be the best option for improving risk prediction on a large scale when the standard lipid panel fails to identify residual-dyslipidemia.

Future research

Although much progress has been made in the past decades, CVD risk estimation especially in T2D patients is still relatively poor (75, 88, 267, 295, 296). Unfortunately, the standard lipid panel fails to identify important characteristics of diabetic dyslipidemia, and treatment guidelines still mainly focus on LDL-C levels, which are usually not elevated in individuals with T2D (268). Many large studies have identified characteristics of diabetic dyslipidemia associated with increased CVD risk (40, 41, 297). The next step is to implement all these new findings in clinical practice. First, future research should determine whether advanced lipoprotein profiling is suitable for implementation in clinical practice, in terms of risk estimation, robustness and cost-effectiveness. A multicenter randomized controlled trial initiated by the Erasmus MC to test this has started recently, which includes patients with T2D and randomizes them into two groups, either treatment following the guidelines based on the standard lipid panel, or personalized treatment based on a combination of the advanced lipoprotein profiling and standard lipid panel. Major end points are CV events, CVD death and all-cause mortality. The use of advanced lipoprotein profiling as a potential screening tool has been demonstrated in part I of this thesis. Future research should aim

at improving the use of the advanced lipoprotein profile, and establish cut-off levels for diabetic dyslipidemia. Studies on the efficacy of new lipid-lowering drugs should include the detailed lipoprotein profiling.

Many newly emerging drugs to treat dyslipidemia and thereby aiming at CVD prevention are being studied, either in trials or in clinical practice. Some drugs aimed at lowering LDL-C levels, also reduce Lp(a) levels, such as PCSK9 inhibitors (298, 299). Others have been designed to specifically lower Lp(a) levels such as the antisense oligonucleotides targeting the apo(a) (58). Research on Lp(a) lowering therapies is hampered by the lack of knowledge on its production and clearance. On the other hand, specifically lowering Lp(a) is very important for understanding the role of this particular lipoprotein in the development of CVD. Although, Lp(a) is an independent CVD risk factor, it remains to be demonstrated that Lp(a) lowering will eventually lead to improved CVD prevention. Future follow-up research comparing CVD events in subjects with elevated Lp(a) levels in 2 groups (1: conventional treatment and 2: conventional treatment and antisense apo(a)), could provide insight into whether Lp(a) lowering is beneficial in reducing CVD events over time.

WHAT WAS ALREADY KNOWN?

- Diabetic dyslipidemia is present well before the onset of T2D
- Statin treatment lowers LDL-C levels, but might increase Lp(a) levels
- Lp(a) is increased in heterozygous FH compared to non-FH subject, and is associated with increased CVD risk
- High saturated fat diet reduces Lp(a) levels

WHAT IS NEW?

- Normoglycemic first degree relatives of T2D patients have lower HDL levels
- Lomitapide decreases all atherogenic lipoproteins, but does not affect HDL function
- Statin treatment increases Lp(a) levels in carriers of the low apo(a) isoform independently of changes in LDL-C level
- Lp(a) is increased in heterozygous FH and even more in homozygous FH compared to non-FH subjects
- Low caloric diet interventions aimed at weight reduction increase Lp(a) levels in obese T2D patients for the short-term

WHAT IS NEXT?

- What are cut-off points for defining diabetic dyslipidemia using advanced lipoprotein profiling?
- What is the effect of the different lipid- lowering drugs on the detailed lipoprotein profile?
- Does CVD risk increase due to short-term increase in Lp(a) levels following conventional treatment by weight loss or statin?
- Does Lp(a) lowering result in fewer CVD events?

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Appendix

SUMMARY

In part I of this thesis, I investigated the potential application of advanced lipoprotein profiling for improving the diagnosis of dyslipidemia. Besides I investigated the effect of increasing glucose intolerance and drug interventions on the lipoprotein profile. In chapter 2.A I questioned whether diabetic dyslipidemia is already present in normoglycemic individuals who are family members of type 2 diabetes (T2D) patients and therefore at increased risk of developing T2D. It is known that features of diabetic dyslipidemia are present several years before the development of T2D, but not if they are already present before the onset of any signs of glucose intolerance. I also investigated whether present features of diabetic dyslipidemia were associated with insulin sensitivity (insulin sensitivity index=ISI) and beta cell function (disposition index=DI) and (fractional synthesis rate=FSR). The glucose intolerance state was determined using an extended oral glucose tolerance test and was defined as normoglycemic, pre-diabetic or having T2D. Compared to the controls from families without T2D, the normoglycemic T2D family members displayed lower plasma levels of HDL_3-C and HDL_2-C. In South Asian, but not in Caucasian families, total HDL-C correlated with both ISI and DI, whereas, HDL subclasses (HDL_3-C and HDL_2-C) correlated only with DI. HDL_2-C and TG correlated also with FSR. In South Asian families the HDL_2-C and HDL_3-C levels were more strongly linked to beta cell function and FSR than to insulin sensitivity. I conclude that the changes in HDL subclasses may contribute to the deterioration of beta cell function in these families. In this chapter I show that low HDL-C levels as a feature of diabetic dyslipidemia are already present in normoglycemic first-degree relatives of T2D patients. These features of diabetic dyslipidemia progress in parallel with glucose intolerance and precede not only the onset of T2D but also of prediabetes in high risk families. These results indicate the need for a screening strategy of those subjects at increased risk of T2D. The use of advanced lipoprotein profiling might help identify those subjects before they develop glucose intolerance. Chapter 2.B, reports the effect of the novel lipid-lowering drug “Lomitapide” on HDL composition and function, because there was evidence that Lomitapide not only reduces LDL-C but also HDL-C levels. I investigated the effects of this drug on plasma HDL-C levels, on HDL subclass distribution determined by lipoprotein profiling and on HDL-function defined by the cholesterol efflux capacity (CEC) of HDL of patients with homozygous familial hypercholesterolemia (HoFH). Four HoFH patients were treated with increasing dosages of Lomitapide. Lomitapide decreased LDL-C and HDL-C levels with a shift to buoyant HDL. Total CEC of HDL was unaffected. HDL-C levels as determined by conventional methods by the clinical chemistry decreased upon treatment with Lomitapide. However, the additional use of lipoprotein profiling and CEC showed that despite HDL-C lower levels, the relatively more buoyant HDL displayed unaltered function as determined by CEC in comparison with HDL prior to Lomitapide treatment, suggesting no alteration in the anti-atherogenic profile. From this part I conclude that advanced lipoprotein profiling might be a good screening tool for the identification of individuals at increased risk

for T2D before they develop glucose intolerance. The use of advanced lipoprotein profiling in addition to the conventional lipid panel, total cholesterol and triglyceride and LDL- and HDL-C can also help to better understand the effects of new lipid lowering drugs on the detailed lipoprotein profile.

In part II of this thesis I describe the effects of commonly used cardiovascular preventive treatment strategies and genetics in high risk population on plasma levels of lipoprotein(a) [Lp(a)]. In chapter 3.A I investigated the effect of diet aimed at weight loss on Lp(a) levels in T2D patients. While weight loss improves classical CVD risk factors in T2D patients, effects on Lp(a) were unknown and may possibly influence the long term CVD outcome of diet-induced weight loss. Lp(a) levels were determined immunoturbidimetrically in plasma obtained before and after 3-4 months of a calorie-restricted diet in four independent study cohorts. In total 198 obese individuals predominantly T2D patients underwent a diet intervention and 26 obese subjects underwent bariatric surgery. A calorie-restricted diet resulted in weight loss up to 10% and improved conventional CVD risk factors such as LDL cholesterol. Lp(a) levels increased by almost 7 mg/dL. The diet-induced increase in Lp(a) correlated with weight loss. In the bariatric surgery group, no significant change in Lp(a) was found despite considerable weight loss (14.0%). Diet-induced weight loss was accompanied by an increase in circulating Lp(a) levels in obese individuals with and without T2D while classical CVD risk factors improved. This increase in Lp(a) levels may potentially antagonize the beneficial cardio-metabolic effects of a diet-induced weight reduction. In chapter 3.B. I investigated the effect of statin treatment on Lp(a) levels in patients with dyslipidemia taking into account apolipoprotein(a) [apo(a)] isoforms and common LPA SNPs. Lp(a) levels, apo(a) isoform, and the common single nucleotide polymorphisms rs10455872, rs3798220 and T3888P were determined in plasma of patients with dyslipidemia, mostly familial hypercholesterolemia. Two groups were included: the intervention group consisting of dyslipidemic patients starting statin treatment, and the control group consisting of dyslipidemic patients already on statin treatment. In the intervention group plasma Lp(a) levels increased slightly, while they remained unchanged in the control group. However, the increase in plasma Lp(a) levels after initiation of statin treatment occurred selectively in carriers of the low molecular weight apo(a) isoforms (66.4 to 97.4 mg/dL). No interactions with common SNPs in the LPA gene nor with change in LDL-C were detected. Starting statin treatment increases plasma Lp(a) levels exclusively in patients with dyslipidemia that carry the low molecular weight apo(a) isoforms. Since low molecular weight apo(a) isoform is associated with high Lp(a) levels and cardiovascular disease, results of this study suggest that Lp(a) levels increase further upon statin treatment in those subjects with already high Lp(a) levels. In chapter 3.C I investigated the effect of the number of mutations in the genes causing FH on Lp(a) levels. It was already known that FH patients have elevated Lp(a) levels besides the very high LDL-C levels. But the question remains whether this effect on Lp(a) levels is gene-dose-dependent in individuals with either 0, 1 or 2 mutations. Lp(a) levels were measured in subjects with HoFH and their

heterozygous (heFH) and unaffected relatives. Median Lp(a) levels in unaffected relatives, HeFH, and HoFH patients were 19.9, 24.4, and 47.3 mg/dL, respectively. There was a trend towards increased plasma Lp(a) levels in homozygous FH patients compared to both, heterozygous FH as well as unaffected relatives was observed. Whether the increased Lp(a) levels in HoFH patients add to the increased CVD risk and whether this risk can be reduced by therapies that lower both LDL-C and Lp(a) levels, remains to be elucidated. Taken the results of chapter 3.A-C together, I can conclude that there is a need for expanding the standard lipid panel and thereby including Lp(a) levels, to identify those individuals with increased Lp(a) levels before initiating treatment strategies with a risk of further increase of Lp(a) levels.

In part III of this thesis I discuss treatment options for lowering Lp(a) and treatment of severe dyslipidemia. In chapter 4.A, I provided an overview of treatment options for Lp(a) lowering. In this review I show that Lp(a) is lowered by estrogens, niacin, and lipoprotein apheresis. CETP inhibitors and PCSK9 antibodies, currently studied in phase 3 trials, also lower Lp(a) concentrations by 30-50%. Besides lowering Lp(a) all of these compounds have modifying effects on multiple lipoprotein classes. An antisense oligonucleotide directed to apo(a) has recently been developed to specifically lower circulating Lp(a) levels. This compound inhibited Lp(a) mRNA up to 90%, and plasma Lp(a) levels up to 82% in human volunteers independent of Lp(a) levels at baseline. In conclusion there are multiple agents, including the next generation RNA based antisense therapeutics, that have Lp(a) lowering properties. It remains to be established whether specifically lowering Lp(a) therapies reduce CVD events. In chapter 4.B, I present the lifetime journeys of lipid lowering therapy in siblings with compound heFH. They were treated from the age of 5 and 3 years, respectively with a wide array of lipid lowering-drugs. Up to now they have tried almost all available and upcoming lipid-lowering agents, except LDL apheresis. This report shows the eternal crusade of finding optimal treatment for very severely affected HoFH, LDLR negative patients, and illustrates the progress in the development of lipid-lowering medication in the last decades, but also indicates that additional therapy is still very much needed. Notably, close monitoring of side effects, adverse events and the effect of combinations of drugs in childhood as well as early access to novel drugs in adolescence and adulthood was feasible and enabled to improve the treatment of these patients as they remain free of CVD until now. Many promising lipid-lowering drugs are being studied currently, some are available for the clinical use such as PCSK9 inhibitors. I believe that there is a new future coming for those patients with severe dyslipidemia such as HoFH and difficult to treat dyslipidemia such as high Lp(a) levels.

NEDERLANDSE SAMENVATTING

In deel I van dit proefschrift onderzoek ik de toegevoegde waarde van het gebruik van uitgebreide analyse van het lipoproteïnen profiel voor de diagnose dyslipidemie. Daarnaast heb ik onderzocht wat het effect is van toename van glucose intolerantie en gebruik van medicatie op het lipoproteïnen profiel. In hoofdstuk 2.A geef ik antwoord op de vraag of diabetische dyslipidemie al aanwezig is bij normoglycemische personen uit families met type 2 diabetes (T2D) patiënten die daarom een verhoogd risico hebben op de ontwikkeling van deze aandoening. Het is bekend dat kenmerken van diabetische dyslipidemie al enkele jaren voor de ontwikkeling van T2D aanwezig zijn, maar het is niet bekend of dit al het geval is voordat er sprake is van glucose intolerantie. Ik heb ook onderzocht of de aanwezige kenmerken van diabetische dyslipidemie geassocieerd zijn met de insuline gevoeligheid (insulin sensitivity index= ISI) en met de beta cel functie (Disposition index=DI) en de "fractional synthesis rate" van insuline (FSR). De glucose tolerantie status was bepaald met een uitgebreide orale glucose tolerantie test en gedefinieerd als normoglycemie, prediabetes en T2D. In vergelijking met de gezonde controles hadden de normoglycemische familieleden lagere plasma waarde van HDL_3-C en HDL_2-C. In de Hindoestaanse maar niet in de Nederlandse families correleerde totaal HDL-C met ISI en DI. Echter, HDL_3-C en HDL_2-C correleerden alleen met DI. HDL_2-C en TG correleerden met FSR. In de Hindoestaanse families waren HDL_3-C en HDL_2-C sterker geassocieerd met beta cel functie en FSR dan met insuline gevoeligheid. In dit hoofdstuk laat ik zien dat lage circulerende HDL-C waarden een kenmerk van diabetische dyslipidemie zijn, al aanwezig bij normoglycemische eerstegraads familieleden van T2D patiënten. Deze kenmerken van diabetische dyslipidemia nemen toe samen met glucose intolerantie en gaan vooraf aan T2D, maar ook aan prediabetes, in families met een hoog risico op T2D. Deze resultaten wijzen op de noodzaak voor de screening van mensen uit hoog risico families die een hoog risico hebben op het ontwikkelen van T2D. Ik concludeer hieruit dat de veranderingen in HDL subgroepen mogelijk kan bijdragen aan het verslechteren van de beta cel functie in deze families. Het gebruik van de uitgebreide lipoproteïnen bepaling kan bijdragen aan het identificeren van die personen die later glucose intolerantie zullen ontwikkelen. In hoofdstuk 2.B, onderzoek ik het effect van het nieuwe cholesterol verlagende middel "Lomitapide" op de hoeveelheid, samenstelling en functie van HDL in vier patiënten met homozygote familiaire hypercholesterolemie (HoFH). HDL subklassen werden bepaald met behulp van de uitgebreide lipoproteïnen bepaling, en HDL functie werd gemeten als cholesterol efflux capaciteit (CEC). Lomitapide verlaagde LDL-C en HDL-C waardes met een verschuiving van HDL naar een lagere dichtheid. Totaal CEC van HDL was onveranderd ondanks lagere concentraties van HDL-C. Dit suggereert dat het totale anti-atherogene effect van HDL onveranderd bleef. Hieruit concludeer ik dat het gebruik van het uitgebreide lipoproteïnen profiel bijdraagt aan het ophelderen van de effecten van nieuw ontwikkelde cholesterol verlagende medicatie.

In het tweede deel van dit proefschrift, onderzoek ik de effecten op de plasma concentraties van lipoproteïne (a) [Lp(a)] van de meest gebruikte behandelingsstrategieën in de cardiovasculaire preventie en het effect van genetica in hoog risico populaties. In hoofdstuk 3.A, onderzoek ik het effect van een gewicht verlagend dieet op de plasma Lp(a) concentraties in T2D patiënten. Terwijl gewichtsverlies een verbetering laat zien van de conventionele risicofactoren voor hart- en vaatziekten (HVZ) in T2D patiënten, was het effect op Lp(a) niet bekend. Plasma Lp(a) concentraties werden bepaald voor en 3-4 maanden na het volgen van een laag calorisch dieet, in vier onafhankelijke studie cohorten. In totaal hebben 198 mensen met obesitas en T2D een dieet interventie ondergaan en ondergingen 26 personen met obesitas bariatrische chirurgie. Een laag calorisch dieet resulteerde in een gewichtsverlies tot 10% en verbeterde de bekende risicofactoren voor HVZ zoals LDL-C. Lp(a) concentraties stegen echter met bijna 7 mg/dL. Deze stijging in Lp(a) correleerde met het gewichtsverlies. In de groep die bariatrische chirurgie onderging was echter geen significante verandering in plasma Lp(a) concentratie ondanks het aanzienlijke gewichtsverlies van 14%. Dieet-geïnduceerd gewichtsverlies gepaard met een verhoging van Lp(a) concentraties werd gevonden in obese patiënten met en zonder T2D. Deze stijging in Lp(a) concentratie kan mogelijk het gunstige cardio-metabolische effect van dieet geïnduceerd gewichtsverlies verminderen. In hoofdstuk 3.B, onderzoek ik het effect van behandeling met statines op de plasma Lp(a) concentratie in patiënten met dyslipidemie, hoofdzakelijk familaire hypercholesterolemie, in relatie tot de apolipoproteïne (a) isoform en LPA single nucleotide polymorphisms (SNPs) rs10455872, rs3798220 and T3888P. Twee groepen werden geïncludeerd: de interventie groep bestaande uit patiënten met dyslipidemie die voor het eerst startten met statine behandeling en de controle groep bestaande uit patiënten met dyslipidemie die al langer behandeld werden met statine. Start van de behandeling met statine resulteerde in een stijging van Lp(a) concentratie, maar alleen in de groep met de apo(a) isoform' met laag moleculair gewicht (LMW) (66.4 tot 97.4 mg/dL). De verandering in Lp(a) was niet geassocieerd met de LPA SNPs of met de verandering in LDL-C. Omdat de LMW isoform geassocieerd is met hoge Lp(a) waardes en HVZ, suggereren deze resultaten dat de Lp(a) concentratie nog verder stijgt na start van statine behandeling in mensen die al een hoge Lp(a) concentratie hebben. In hoofdstuk 3.B onderzoek ik het effect op de plasma Lp(a) concentratie van een aantal mutaties in de genen die FH veroorzaken. Bekend is dat mensen met FH verhoogde Lp(a) waarden in het bloed hebben naast verhoogde LDL-C waarden. De vraag is of de mate waarin het Lp(a) verhoogd is, afhankelijk is van het aantal gen mutaties (2, 1 of 0). Plasma Lp(a) concentraties waren 47.3 en 24.4 mg/dL in HoFH en HeFH patienten, en 19.9 mg/dL in de gezonde familieleden. Toekomstig onderzoek moet uitwijzen of deze hogere Lp(a) waarden in HoFH patiënten bijdragen aan een verhoogd risico op HVZ en of dit risico vermindert door therapie die zowel Lp(a) als LDL-C verlaagt. Concluderend uit de resultaten van hoofdstukken 3.A-C: Het is noodzakelijk om de standaard lipiden bepaling uit te breiden met de Lp(a) bepaling om die mensen te identificeren met een verhoogde Lp(a) waarde voorafgaand aan een behandeling die mogelijk Lp(a) nog verder doet stijgen.

In het derde deel van dit proefschrift bediscussieer ik de behandelopties voor het verlagen van Lp(a) en de behandeling van ernstige dyslipidemie. In hoofdstuk 4.A, geef ik een overzicht van de behandelopties voor verlaging van Lp(a). In dit review laat ik zien dat Lp(a) wordt verlaagd door oestrogenen, niacine en lipoproteïnen aferese. CETP remmers en PCKS9 antilichamen verlagen ook de Lp(a) waarden met 30-50%. Al deze behandelingen hebben tevens effect op de verschillende lipoproteïnen subgroepen. Een antisense oligonucleotide gericht tegen apo(a) is recent ontwikkeld om specifiek het circulerende Lp(a) te verlagen. Dit laatste remt de Lp(a) mRNA met 90% en zorgt voor daling met 82% van Lp(a) waarde in gezonde vrijwilligers onafhankelijk van de baseline Lp(a) waarde. Het moet nog worden vastgesteld of specifieke verlaging van Lp(a) uiteindelijk zal leiden tot verlaging van HVZ. In hoofdstuk 4.B, presenteer ik het verloop van verschillende cholesterol verlagende therapieën in een broer en zus met compound HeFH. Vanaf de leeftijd van 5 en 3 jaar werden ze behandeld, met een breed scala aan cholesterol verlagende medicatie. Tot nu toe hebben ze bijna alle beschikbare en opkomende cholesterol verlagende medicijnen geprobeerd, met uitzondering van LDL aferese. Dit hoofdstuk beschrijft de eeuwige zoektocht naar de optimale behandeling van LDLR negatieve patiënten. Dit hoofdstuk illustreert de progressie in de ontwikkeling van cholesterol verlagende medicatie in de afgelopen decennia, maar geeft ook aan dat additionele therapie nog steeds noodzakelijk is. Hierbij is het uitermate belangrijk dat er goede monitoring van bijwerkingen en nadelige effecten plaatsvindt. Daarnaast is het belangrijk dat het effect van combinatie van medicatie vanaf de kindertijd bijgehouden wordt en dat er vroege toegang is tot nieuwe medicatie tijdens adolescentie en op volwassen leeftijd. Als gevolg hiervan kan de therapie bij deze hypercholesterolemische patiënten die nog steeds geen HVZ hebben ontwikkeld steeds worden verbeterd. Er zijn verschillende veelbelovende cholesterol verlagende medicijnen die momenteel bestudeerd worden, waarvan sommigen al beschikbaar zijn voor gebruik in de kliniek zoals PCSK9 remmers. Ik geloof dat er een nieuwe toekomst is voor de behandeling van patiënten met ernstige en moeilijk te behandelen dyslipidemie zoals HoFH en hoge Lp(a) waarden.

LIST OF PEER REVIEWED PUBLICATIONS

Yahya R, Berk KA, Verhoeven AJM, Touw J, Leijten FP, van Rossum EF, Wester VL, Lips MA, Pijl H, Timman R, Erhart G, Kronenberg F, Roeters van Lennep JE, Sijbrands EJG, Mulder MT. Effect of diet-induced weight loss on lipoprotein(a) levels in obese individuals with and without type 2 diabetes. Diabetologia. 2017 Jun;60(6):989-997. doi: 10.1007/s00125-017-4246-y. Epub 2017 Apr 6.

Yahya R, Mulder MT, Sijbrands EJ, Williams M, Roeters van Lennep JE. Low-density lipoprotein receptor-negative compound heterozygous familial hypercholesterolemia: Two lifetime journeys of lipid-lowering therapy. J Clin Lipidol. 2017 Jan - Feb;11(1):301-305. doi: 10.1016/j.jacl.2017.01.004. Epub 2017 Jan 12.

Yahya R, Favari E, Calabresi L, Verhoeven AJM, Zimetti F, Adorni MP, Gomaraschi M, Averna M, Cefalù AB, Bernini F, Sijbrands EJG, Mulder MT, Roeters van Lennep JE. Lomitapide affects HDL composition and function. Atherosclerosis. 2016 Aug;251:15-18. doi: 10.1016/j.atherosclerosis.2016.05.005. Epub 2016 May 11.

Sjouke B, **Yahya R**, Tanck MWT, Defesche JC, de Graaf J, Wiegman A, Kastelein JJP, Mulder MT, Hovingh GK, Roeters van Lennep JE. Plasma lipoprotein(a) levels in patients with homozygous autosomal dominant hypercholesterolemia. J Clin Lipidol. 2017 Mar - Apr;11(2):507-514. doi: 10.1016/j.jacl.2017.02.010. Epub 2017 Feb 27.

Bos S, **Yayha R**, van Lennep JE. Latest developments in the treatment of lipoprotein (a). Curr Opin Lipidol. 2014 Dec;25(6):452-60. doi: 10.1097/MOL.0000000000000126.

Versmissen J, Vongpromek R, **Yahya R**, van der Net JB, van Vark-van der Zee L, Blommesteijn-Touw J, Wattimena D, Rietveld T, Pullinger CR, Christoffersen C, Dahlbäck B, Kane JP, Mulder M, Sijbrands EJ. Familial hypercholesterolaemia: cholesterol efflux and coronary disease. Eur J Clin Invest. 2016 Jul;46(7):643-50. doi: 10.1111/eci.12643. Epub 2016 Jun 20.

PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name:	Reyhana Yahya
Erasmus MC Department:	Internal Medicine
Research School:	COEUR
PhD period:	2012 – 2018
Promotor(s):	Prof. E.J.G. Sijbrands
	Dr. J. E. Roeters van Lennep
Supervisor(s):	Dr. A.J.M. Verhoeven
	Dr. M.T. Mulder

	Year	Workload (hours/ ECTS)
1. PhD training		
General academic skills		
- Course Radioactivity	2013	1,5
- BROK course	2015	1.5
- Biomedical English Writing and Communication	2015	3.0
In-depth courses (e.g. Research school, Medical Training)		
- Coeur Research seminar	2012	0,4
- Coeur Lecture 'Smooth muscle cells'	2012	0,1
- Research seminar 'Glucose metabolism & vascular disease'	2012	0,4
- Coeur lecture 'Wine polyphenols, are they the guardian angles your endothelium?'	2012	0.1
- Research seminar 'pulmonary & right ventricle interaction'		
- Coeur Lecture by Jay Heinecke	2013	0.4
- Coeur Research Seminar	2013	0.1
- Research seminar 'Non-invasive imaging of MI	2013	0.4
'	2012	0.4
- Coeur debate: 'cardiovascular controversies'	2014	0.4
- Research Seminar 'current cardiac and vascular aging'	2014	0.4
- Seminar personalised Medicine	2015	0.2
- Coeur Lecture 'Lifelines'	2015	0.1

Presentations		
- JMS oral presentation (4x)	2012, 2013, 2014 2013, 2014, 2015	1.2
- Poster presentation science days (3x)	2013 2013, 2014 2013, 2014	0.9 0.3
- Poster presentation Cost Action	2013	0.6
- Poster presentation CVC (2x)	2013	0.6
- Poster presentation EAS (2x)	2014	0.3
- Oral presentation (seminar in Parma)	2014	0.3
- Poster presentation ELC		0.3
- Poster presentation IAS		0.3
- Oral presentation NLC		
International conferences		
- EAS Lyon (4 days)	2013	1.2
- Cost Action Lille (2 days)	2013	0.6
- ELC Tutzing (4 days)	2013	1.2
- ISA Rome (3 days)	2014	0.9
- EAS Madrid (4 days)	2014	1.2
- ISA Amsterdam (3 days)	2015	0.9
Seminars and workshops		
- Science days Internal medicine Antwerp (2 days)	2012 2013 2014 2015	2.4
- HDL Training Groningen School (3 days)	2012	0.9
- Coeur course 'Vascular clinical epidemiology' (2 days)	2012	1.5
- NLC Leiden (1 day)	2012	0.3
- Coeur course 'Atherosclerose & aneurismal disease' (2 days)	2012	1.5
- CVC Noordwijkerhout (2 days)		
- Nihes Summer Course, ESP01 'principles of research in medicine and Epidemiology' (5 days)	2013 2013	0.6 0.7
- Nihes Summer Course, ESP03 'Introduction to Data-analysis' (5 days)	2013	1.0
- NLC Leiden (1 day)	2013	0.3
- CVC Ermelo (2 days)	2014	0.6
- Coeur PhD day (Workshops: Getting your PhD at EMC, Defend your thesis)	2014 2014	0.3 0.3
- NLC Leiden (1 day)		
- Course medical Library (Endnote, Pubmed and other EBM databases) (4 days)	2014, 2015	1.0
Other		
Short term scientific mission (STSM) cholesterol efflux experiments in Parma (3 weeks)	2013	4.5

2. Teaching activities		
	Year	**Workload (Hours/ ECTS)**
Lecturing		
Teaching Lipoprotein profile by density gradient ultracentrifugation to master students and MLO students	2012-2014	0.2
Supervising		
- Joanne and Karlijn (JMS students): 4 weeks supervision mini JMS project	2012	0.1
- Rajiv Biharie (master student, project: Lipoprotein profile in first degree relatives of T2D patients, Caucasians versus South Asians): 20 weeks supervision master theses.	2012	1.0
- Jildau, Hieleke and Helianne (TU students: calculation lipoprotein subclasses from detailed lipoprotein profile using matlab): supervision 3 weeks	2013	0.1
- Demi, Marissa and Remco (TU students: lipoprotein profile by density gradient ultracentrifugation of Caucasian healthy adults compared to first degree relatives of T2D patients): supervision 3 weeks	2014	0.1
- Niels van der Schaft (medical student): 35 weeks supervising data selection and patient inclusion.	2014	1.3
Total:		*38.6*

ABOUT THE AUTHOR

Reyhana Yahya is geboren en opgegroeid in Bagdad, de hoofdstad van Irak. Op 14 jarige leeftijd is zij samen met haar ouders en twee zusjes naar Nederland geïmmigreerd. Na het leren van de Nederlandse taal, is ze in 2003 gestart in Atheneum klas 3 op het Walburg College te Zwijndrecht. In 2006 heeft ze haar VWO diploma behaald. Vanaf 2006 volgde ze de geneeskunde opleiding aan het Erasmus MC in Rotterdam, hetgeen in 2012 werd afgerond. In 2007 heeft ze een buitenlandstage gelopen op de afdeling Obstetrie en Gynaecologie, Brighton Sussex University hospital, Brighton, Engeland (Dr. Tony Kelly.). Deze werd afgerond met een literatuuronderzoek over verschillen van bevallingsmethodes en moedersterfte tussen Engeland en Nederland. Ook werkte ze als vrijwilliger voor STOLA "stichting stages voor medische studenten in "ontwikkelingslanden" organiseerde en begeleidde. Ze heeft diiverse extracurriculaire cursussen gevolgd waaronder de tropencursus en het ECG cursus. Daarnaast had ze verschillende bijbaantjes, zoals werken in een studententeam op de kinderafdeling in het SFG, het geven van bijles in bèta vakken aan middelbare scholieren, en assistent 'genetic field worker' voor familiare hypercholesterolemie op de Afdeling Inwendige Geneeskunde, sectie Vasculaire Ziekten. Haar masteronderzoek werd in 2011 verricht bij dezelfde sectie onder leiding van Dr. Roeters van Lennep met als onderwerp het effect van statine op Lp(a). Dit onderzoek werd voortgezet tijdens haar promotie (Hoofdstuk 3.B). Aansluitend begon zij hier aan haar promotieonderzoek ,van 2012 tot en met 2015 verrichte Reyhana het onderzoek dat in dit proefschrift wordt beschreven. In 2013 heeft ze een deel van haar onderzoek aan de universiteit van Parma, Italië (prof. dr. Bernini), met als onderwerp cholesterol efflux capaciteit van HDL in patiënten met lomitapide behandeling (Hoofdstuk 2.B) en verder bezocht ze verschillende nationale en internationale congressen. Na beëindiging van haar onderzoeksperiode heeft ze een jaar als basisarts gewerkt op de psychiatrische afdelingen 'high intensive care' en 'open opname afdeling' van het Bavo Europoort in Capelle aan den IJssel. Sinds 2017 volgt ze de huisartsopleiding aan het Radboud UMC in Nijmegen en op dit moment is zij bezig met het afronden van het tweede jaar van de huisartsopleiding in Den Bosch.

Reyhana is samen met haar partner Hans trotse ouder van dochter Mila die in 2016 werd geboren.