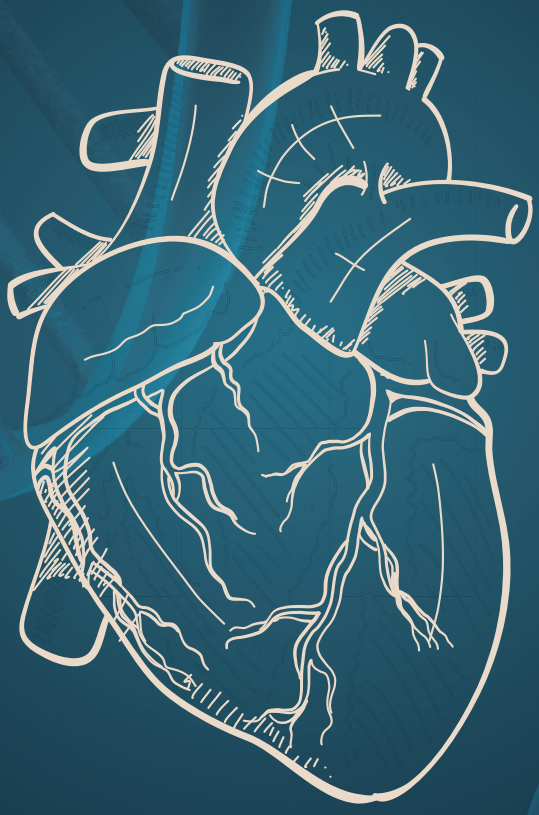
RISK STRATIFICATION IN PATIENTS WITH FAMILIAL HYPERCHOLESTEROLEMIA



SVEN BOS

Risk Stratification in Patients with Familial Hypercholesterolemia

Sven Bos

Risk Stratification in Patients with Familial Hypercholesterolemia
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CHAPTER 1
Introduction

INTRODUCTION

Familial Hypercholesterolemia (FH) (OMIM #143890) is the most common metabolic disorder with a prevalence estimated between in 1:244 and 1:600 (1-3). FH is associated with premature cardiovascular disease (CVD) (4).

FH can be diagnosed by clinical criteria (table 1) and genetically by identification of a pathogenic mutation in the LDLR gene, APOB gene or PCSK9 gene (5-8). Currently over 1200 different mutations are known, most often found in the LDLR gene (www.jojogenetics.nl). Severity can differ depending on the type of mutation. In general apoB mutations are considered to cause a milder phenotype than LDLR or PCSK9 mutations. Within LDLR mutation, null-mutations, mutations which lead to no residual function of the LDL-receptor, are associated with a more severe phenotype with higher high low-density lipoprotein cholesterol (LDL-C) levels compared to LDLR defective mutations with residual LDL-receptor function (9).

The increased LDL-C levels are the driving force of the increased cardiovascular risk in FH patients. To lower CVD risk in FH patients cholesterol lowering agents, mainly statins, are used. The impact of statins on the life expectancy of FH patients can hardly be overestimated. Before the statin era half of men with FH and 12% of women with FH suffered from a myocardial infarction before the age of fifty years (10).

However, despite statin therapy some FH patients still develop CVD (11). The classical risk factors: age, male sex, body mass index (BMI), hypertension, diabetes mellitus, smoking and reduces high-density lipoprotein (HDL) levels all clearly contribute to CVD risk in FH patients (12-14). But even in the absence of these classical risk factors some FH patients will develop cardiovascular events.

Since every patient who is diagnosed with FH immediately starts on statin treatment, more studies were necessary to determine CVD risk in these treated patients. The aim of this thesis was to identify which of these statin treated FH patients were at a higher risk of developing CVD. To investigate this risk I used different approaches as elaborated below.

Table 1 | Dutch Lipid Clinic Network diagnostic criteria for Familial Hypercholesterolemia(15)

Criteria	Points
Family History	
First-degree relative with known premature coronary and vascular disease, OR First-degree relative with known LDL-C level above the 95th percentile*	1
First-degree relative with tendinous xanthomata and/or arcus cornealis, OR Children aged less than 18 years with LDL-C level above the 95th percentile	2
Clinical History	
Patient with premature coronary artery disease*	2
Patient with premature cerebral or peripheral vascular disease[1*]	1
Physical examination	
Tendinous xanthomata	6
Arcus cornealis prior to age 45 years	4
Cholesterol levels mg/dl (mmol/liter)	
LDL-C >= 330 mg/dL (≥8.5)	8
LDL-C 250 – 329 mg/dL (6.5–8.4)	5
LDL-C 190 – 249 mg/dL (5.0–6.4)	3
LDL-C 155 – 189 mg/dL (4.0–4.9)	1
DNA analysis	
Functional mutation in the LDLR, apo B or PCSK9 gene	8
Diagnosis (diagnosis is based on the total number of points obtained)	
Definite Familial Hypercholesterolemia	>8
Probable Familial Hypercholesterolemia	6-8
Possible Familial Hypercholesterolemia	3-5
Unlikely Familial Hypercholesterolemia	<3

[1*] Premature = < 55 years in men; < 60 years in women
LDL-C = low density lipoprotein cholesterol
FH, familial hypercholesterolemia
LDLR = low density lipoprotein receptor
Apo B = apolipoprotein B
PCSK9 = Proprotein convertase subtilisin/kexin type 9

Cardiovascular imaging

One approach is to detect subclinical atherosclerosis in asymptomatic persons with FH. Advanced atherosclerosis on cardiovascular imaging might identify FH patients, who are at exceptional risk of developing cardiovascular events.

Atherosclerotic lesions can be visualized by numerous imaging modalities. Among the commonly used methods are carotid ultrasonography and computed tomography coronary angiography (CTCA).

Carotid ultrasonography

Carotid ultrasonography can be used to measure subclinical atherosclerosis depicted as the presence of carotid plaques or increased carotid intima-media thickness. Both of these outcomes have been associated with CVD risk in the general population (16-18). However, data lacks about the association between carotid ultrasonography outcomes and CVD in FH patients. Moreover, statin-treatment influences the ultrasonography outcomes. Both in FH as in non-FH patients it was shown that statins decrease C-IMT. However, whether carotid ultrasonography outcomes during statin-treatment are still useful for risk prediction has not been established.

Coronary imaging

CTCA is mainly used in symptomatic patients, who present with thoracic chest pain suspected to derive from atherosclerotic disease of the heart. One of the outcomes of the CTCA is the Agatston calcium score, which is calculated based on the intensity, volume and quantity of the calcific (white) signal on the CTCA-scans (19). This score is associated with cardiovascular events, and can improve risk prediction in the general population (20-24). In 2011, we performed a study in 101 asymptomatic FH patients to determine subclinical coronary atherosclerosis showing a wide variety of coronary artery calcification score (CAC-score), and CAC was more abundant in long-term, aggressively statin-treated FH patients than in untreated controls (25). The diversity of CAC scores in FH patients has been party explained by the higher CAC score in those FH patients with LDLR null-mutations compared to LDLR-defective mutations.(26).

Aortic valve calcification

Aortic valve calcification (AoVC) has an estimated prevalence of >50% in the elderly (>75 years) and is associated with 50% higher risk of CVD events (27,28). In homozygous FH, AoVC has a prevalence of 100%, and many of these patients need surgical intervention of functional valvular disease (29,30). Heterozygous FH is associated with less aortic valve dysfunction on echocardiography than homozygous FH (31-34). However, the prevalence and extent of aortic valve calcification (AoVC) is unknown in long-term, statin-treated heterozygous FH patients. Statins seem to have little effect on the progression of AOVC in the general population (35-37). Therefore this group is of particular interest, since statin therapy is the main reason for the prolonged survival in these patients (38). In this thesis, I present the first comparison between the prevalence of AOVC in heterozygous FH and non-FH patients.

Non-traditional risk factors

CVD risk prediction might be improved by measuring non-traditional risk factors. Among these is lipoprotein (a), or Lp(a). Lp(a) was discovered in 1963 by Kare Berg and is a LDL-like protein with an apo(a) moiety. Lp(a) levels are predominantly genetically determined (39), and inversely correlated with the length of the apo(a) moiety. The length of apo(a) is mainly determined by kringle IV type 2 repeats (figure 1). Lp(a) concentration and kringle IV type 2 repeat number are independent risk factors for CVD in the general population, and FH (12,40). In FH, women clearly have a lower CVD burden than men (41-43). but female FH patients, whose Lp(a) levels are elevated, might be susceptible of premature CVD (44). The relationship between Lp(a) and CVD risk may be effected true the binding of oxidized phospholipids which may cause instability of atherosclerotic plaques through increased inflammation (45). Other pathophysiological mechanisms in which Lp(a) could play a role are wound healing and fibrinolysis pathways, however how these pathways play a role in the atherosclerosis pathophysiology is unknown (39,46). Unfortunately, there is a poor Lp(a) lowering responds to statins and other lipid lowering medication. Novel therapeutic agents are currently being developed who are aimed to specifically lower Lp(a) levels but to date no therapy is registered that can exclusively lower Lp(a) levels.

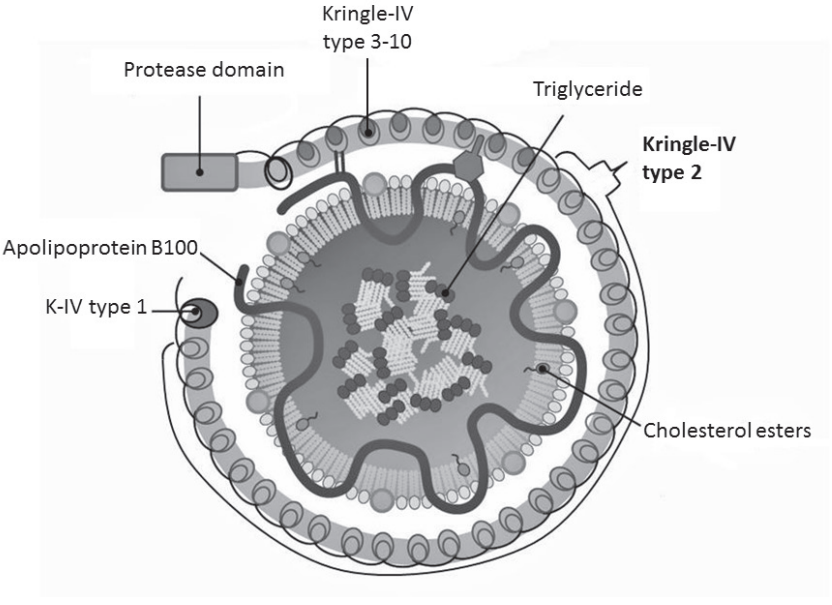

Figure 1 | Schematical structure of Lipoprotein (a).

Another approach of finding novel risk factors is using proteomics techniques. Proteomics aims to find difference in quantity in proteins of different samples, and has been used to identify novel biomarkers in several disease states, including coronary artery disease (47,48). In this thesis I aimed to identify novel markers of cardiovascular disease in long-term statin treated FH patients by applying the proteomic technique to samples of different risk groups of these FH patients. Risk in these patients was identified using coronary angiography with which we investigated a low risk group, an intermediate risk group, and a group with manifested cardiovascular disease.

Cardiovascular imaging and Lp(a)

Lp(a) levels in the general population are associated with AoVC (49), but the relation between AoVC and Lp(a) in FH is unknown as is the relationship between Lp(a) plasma levels and cardiovascular imaging outcomes. In this thesis I investigated whether Lp(a) was associated with the cardiovascular imaging modalities, carotid calcification, coronary calcification and aortic valve calcification.

GENERAL OUTLINE OF THE THESIS

In Chapter 2, I validated our carotid ultrasonography device for use in the studies of Chapter 3 and Chapter 6. In Chapter 3, I investigated whether carotid ultrasonography outcomes were different between statin-treated FH patients and healthy controls, and whether these ultrasonography outcomes correlated with coronary atherosclerosis measured by CTCA. In Chapter 4, I continued to study the CTCA data and investigated whether long-term, statin-treated FH patients had a higher prevalence and extend of AoVC than healthy controls. The association between AoVC and Lp(a) in heterozygous FH patients is shown in Chapter 5. This association is known in the general population but has not been previously investigated in FH patients. Chapter 6 focusses on the association between carotid ultrasonography outcomes and Lp(a) in statin-treated FH patients to investigate whether the residual risk of high Lp(a) levels can be depicted by this non-invasive imaging technique. In Chapter 7, the possible therapeutic possibilities in lowering Lp(a) are described, including novel agents which are currently still in development. The iTRAQ proteomics approach was used in Chapter 8 to explore novel proteins associated with coronary atherosclerosis and CVD endpoint in treated heterozygous FH patients. The summary and discussion of the thesis is presented in English (Chapter 9) and Dutch (Chapter 10).

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1

CHAPTER 2

Validation of a novel fully-automated ultrasound system for the assessment of carotid intima-media thickness and plaques

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A.F.L. Schinkel, G.F. Watts, E.J.G. Sijbrands,
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ABSTRACT

Introduction

Ultrasonography is the most commonly used imaging modality for assessing subclinical atherosclerosis by measuring carotid intima media thickness (C-IMT) and plaques. C-IMT can be reliably measured using automated software, which is present on the portable Panasonic CardioHealth station (CHS). The aim of this study was to determine whether the CHS provides reliable and reproducible data in comparison with another automated software package present on the previously validated Philips iU22 (PiU).

Methods and Results

Carotid ultrasonography was performed by two experienced observers in 85 subjects. C-IMT was measured bilaterally from two different angles, and plaque scans were performed bilaterally.

The intra-class correlation (ICC) of the C-IMT measurements was 0.98 (95% CI: 0.94-0.99) and 0,96 (95% CI: 0.89-0.99) for Observer X and Y, respectively. The ICC of the C-IMT between the two observers was 0.98 (95% CI: 0.95-0.99), and the limitsof agreement (LOA) were 0.007±0.040 mm (p=0.31). The ICC between both systems was 0.89 (95% CI: 0.81-0.93), and the LOA were 0.015±0.052 mm (p=0.03). Inter-observer agreement for the assessment of plaque was high on the CHS (kappa: 0.9±0.1, p=<0.001), and between systems (kappa: 1.0±0.0, p=<0.001).

Conclusion

The CHS has an excellent agreement with the validated PiU. The acquisition time of the CHS is shorter than that of the PiU. We conclude that the CHS is a rapid, reliable and precise method for assessing C-IMT and plaques, making it highly suitable for high-throughput screening and clinical use.

Keywords

- Carotid Intima Media Thickness
- Intra-observer Variability
- Inter-observer Variability

INTRODUCTION

Cardiovascular disease (CVD) is one of the main causes of death worldwide(1). CVD risk can be identified with imaging techniques, like ultrasonography, by detecting subclinical atherosclerosis. Ultrasound is the most commonly used imaging modality to assess carotid intima media thickness (C-IMT) and atherosclerotic plaques (2-5). C-IMT can be measured manually, or with automated software. Automated C-IMT measurements have been shown to produce more reliable, reproducible and faster results than manual measurements(6). The Panasonic CardioHealth Station (CHS) is a portable system capable of measuring the C-IMT automatically (figure 1), but has hitherto not been tested against another validated automated C-IMT measurement system, such as the widely used Philips iU-22 (PiU) ultrasound system(7). We therefore compared the performances of these systems to evaluate whether the CHS produces reliable and reproducible data in C-IMT measurements and in the detection of carotid plaques.

MATERIALS AND METHODS

Study population

Seventy dyslipidaemic patients were recruited between March 2014 and March 2015 from the outpatient clinic for cardiovascular genetics at the Erasmus MC.

Healthy controls were recruited through advertisements. All subjects were over 18 years old, written informed consent was obtained, and the study was approved by the local ethical committee (MEC-2012-309; MEC-2013-556).

All subjects underwent carotid ultrasound imaging twice, on either the CHS (intra-observer variability and inter-observer variability), or on both systems (inter-system variability).

Measurements were performed by two experienced observers (Observer X and Observer Y).

Equipment:

The CHS (Panasonic, Yokohama, Japan) is a portable system capable of automated C-IMT measurements. The CHS is equipped with a broadband 9 MHz linear-array transducer. As a reference, we used the previously validated semi-automated PiU (Philips Medical

Systems, Bothell, USA(7)), equipped with an L9-3 transducer, which used the automated QLAB IMT plugin for C-IMT measurements.

Carotid ultrasound acquisition

All images were acquired based on the 'American Society of Echocardiography consensus statement' protocol (8). In short, subjects were examined lying on an even surface with their head positioned in an angle of approximately 45 degrees facing left when measuring the right side, and vice versa, while performing the ultrasound acquisition.

Carotid ultrasound analysis

The mean C-IMT was measured over a length of 1 cm, at least 0.5 cm proximal of the bifurcation in the common carotid artery. Both sides were measured from two angles: anterior (170°-190°), and lateral (right: 120°-145°; left: 210°-235°).

A plaque scan was performed by placing the transducer transversally in the neck, visualizing the internal, external and common carotid artery. A plaque was marked as present only if the local IMT was more than 50% of the surrounding IMT, or if the C-IMT was above 1.5 mm (9).

Intra-observer and inter-observer variability

For the intra-observer variability the result section of the CHS monitor was covered so that the results were not visible for the observer. After the first procedure the patient was asked to stand up, was then repositioned, and finally re-measured.

The inter-observer variability was assessed by measuring patients twice in succession. First, one of the observers measured the subject whilst the other observer was in the next room. After the first observer finished the procedure the other observer was summoned and subsequently performed the second measurement. From the acquired data we used the individual measurements at the four scan positions, as well as the C-IMT per patient.

Table 2.1 | Inter-observer variability in the Panasonic CHS of all patients.[1*]

	Results per scan position	Results of the mean C-IMT per subject
C-IMT:		
Mean C-IMT (±SD)	0.611 ± 0.141 mm	0.610 ± 0.126 mm
Intra-class coefficient (95%CI)	0.91 (0.88-0.94)	0.98 (0.95-0.99)
Difference between both observers (LOA) (±SD)	0.008±0.081 mm (p=0.25)	0.007±0.040 mm (p=0.31)
Correlation of the C-IMT difference and the mean C-IMT	R= -0.09; (p= 0.26)	R= -0.28; (p= 0.09)
Plaques:		
Plaques found	Obs X: 27 (34%) Obs Y: 30 (38%)	Obs X: 17 (43%) Obs Y: 17 (43%)
Agreement of plaque presence (Intraclass kappa) (±SD)	73 (0.81±0.1) (p<0.001)	38 (0.90±0.1) (p<0.001)

[1*] Patients were 51±15 years old, BMI was 25.8±3.8, and 50% were male.

Inter-system variability

Observer X started scanning the healthy subject with the PiU, and immediately thereafter the subject was repositioned and measured with the CHS. The output of both devices of a healthy subject with a plaque are shown in figure 2.

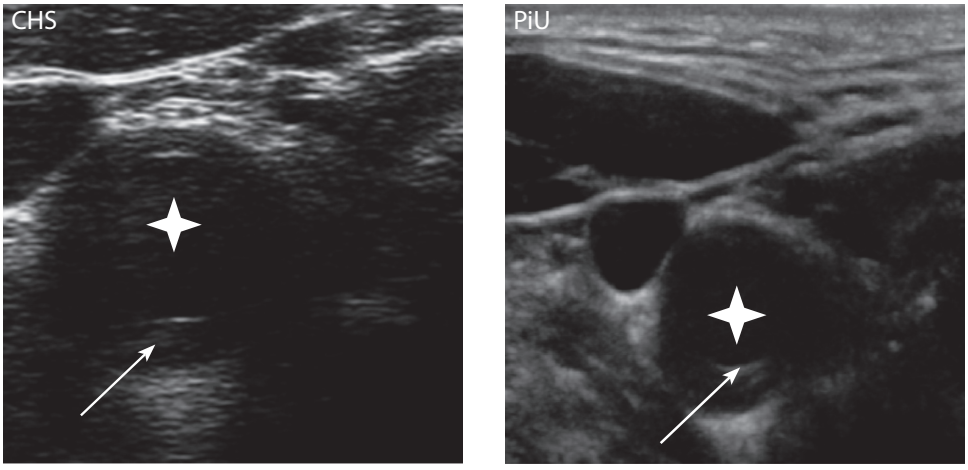


Figure 2.1a | Image outcome of a plaques scan (same location) on the two different systems. The lumen of the carotid artery are marked with stars, and the plaques pointed out with arrows.

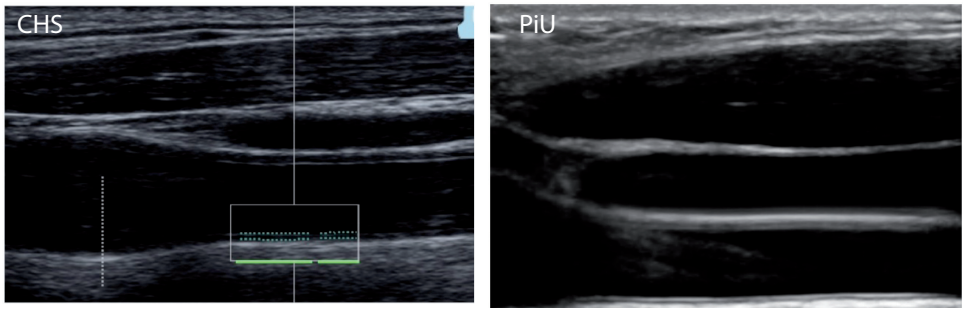


Figure 2.1b | Image output of the C-IMT measurement of the two different systems.
Longitudinal view of the common carotid artery and bifurcation. On the left side of the images the widening of the artery suggest the bifurcation where the "arteria carotis communis" splits into the "arteria carotis inerna", and the "arteria carotis externa". CHS: Panasonic CardioHealthStation; PiU: Philips iU-22 ultrasound system.

Statistics

Binary variables were expressed as number (percentage), and continuous variables as mean ± standard deviation. For C-IMT, the intra-class coefficient (ICC) and Bland Altman analyses were used to determine the mean difference within and between the observers. The ICC was expressed as mean (95% CI). The results of the Bland-Altman analyses were expressed as the limits of agreement (LOA) as mean ± standard deviation. The LOA expresses the difference between the two measurements and a one sample t-test was used to test for statistical significance.

The inter-observer variability was determined on the individual C-IMT measurements, and on the mean C-IMT of a subject.

The association between the differences in C-IMT measurements and the mean C-IMT was determined by linear regression.

Agreement in plaque identification between observers and systems was expressed by the Kappa statistic.

All statistical analyses were performed using SPSS version 21 (SPSS Inc., Chicago, IL, USA).

RESULTS

In total 85 subjects were examined in this study. For determination of intra-observer variability, observer X scanned 15 patients and observer Y 15 other patients; 40 patients

were scanned for the inter-observer variability; and 15 healthy subjects were scanned for the inter-system variability. Baseline values are presented in the footnote of the tables.

Intra-observer variability

The LOA for C-IMT of Observer X were 0.005±0.035 mm (p=0.60), and the ICC was 0.98 (95% CI: 0.94-0.99). Similarly, the LOA for C-IMT of Observer Y were 0.015±0.043 mm (p=0.21), and the ICC was 0.96 (95% CI: 0.89-0.99). Intra-observer variability of plaque presence was not determined, since measurements were made in quick succession and it is unlikely to forget the presence of a plaque in the first measurement.

Inter-observer variability

Results of C-IMT measurements at all four scan positions, the mean C-IMT per patient, and plaque scans are depicted in table 1. The Bland Altman plot is shown in figure 3a. The LOA for C-IMT was not significantly different between the observers. The SD of the LOA was approximately 50% lower for the mean C-IMT of the four scans per subject. Similarly, the ICC improved by taking the mean C-IMT per patient. Plaque presence was similar between observers as indicated by the high intraclass kappa.

In linear regression analysis, the C-IMT difference between observers did not increase with C-IMT value, indicating that the inter-observer variability was independent of C-IMT values.

Table 2.2 | Variability between the Panasonic CHS and the Philips PiU of the four scan positions in the healthy volunteers.[2*]

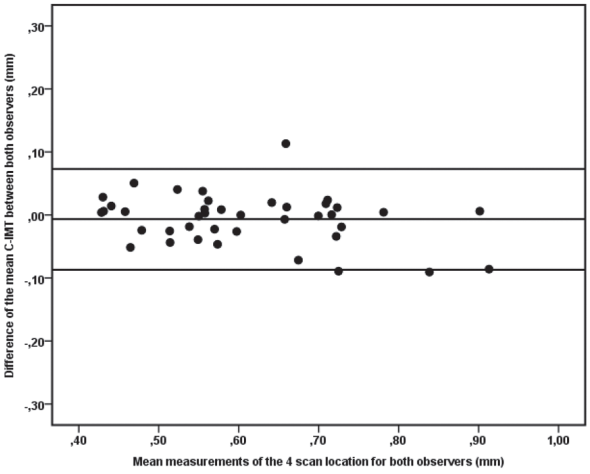
	CHS	PiU	Inter-system
Acquisition time (minutes)	2±1	4±1	(p<0.001)
C-IMT:			
Mean C-IMT (±SD)	0.516±0.077 mm	0.531±0.087 mm	
Difference between both C-IMT measurements (LOA) (±SD)			0.0154 ±0.0522 mm (p=0.03)
Intra-class coefficient (95%CI)			0.89 (0.81-0.93)
Correlation of the C-IMT difference and the mean C-IMT			R= -0.38; (p= 0.16)
Plaques:			
Plaques found	1	1	1

[2*] Subjects were 38±14 years old, BMI was 23.2±2.3, and 47% were male.

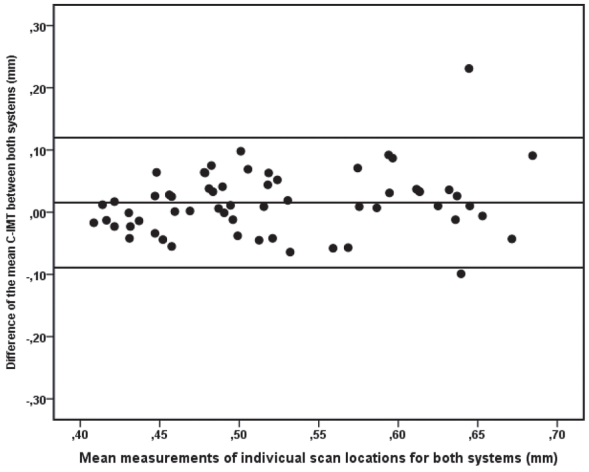
Inter-system variability

Image acquisition time, defined as time between acquiring the first and last image, of the CHS was significantly shorter (2±1 minutes) than the PiU (4±1 minutes; p=<0.001).

Results of the C-IMT measurements, and plaque scans of the healthy volunteers are depicted in table 2, and the Bland Altman plot is shown in figure 3b. For C-IMT the LOA were significantly lower on the CHS than on the PiU. However, the ICC for the C-IMT was similar. One plaque was present in the healthy controls which was seen on both devices.



a). Bland-Altman plot of the inter-observer variability. Outer-lines: +/- 1.96*SD.



b). Bland-Altman plot of the inter-system variability. Outer-lines: +/- 1.96*SD.

Figure 2.2 | Bland Altman Plots

DISCUSSION

This study shows that the portable CHS generated reliable and reproducible data that were in large agreement with the PiU measurements, irrespective of the observer acquiring the images. Interestingly, the ICC for the intra-observer variability was similar to that of the inter-observer variability suggesting a highly reliable measurement technique. High mean C-IMT values were not associated with high measurement variation suggesting that the measurements are reliable at both low and high C-IMT.

These results are in line with an earlier study showing that the fully automated C-IMT-measurements of the CHS were comparable to manual C-IMT measurements(6). The use of manual C-IMT measurements has considerably decreased with the evolvement of automated C-IMT measurement software. However, differences between these automated systems might exist. In our study, the CHS measured the C-IMT significantly smaller than the PiU, although it is questionable whether the mean 0.015 mm difference is clinically relevant. Vanoli et al. also found smaller C-IMT values with the CHS (0.012mm) although in their study this was not statistically significant (6). These results do emphasise caution when comparing C-IMT results obtained with different systems.

Carotid images on the PiU, a semiautomated system, must be stored before the C-IMT can be measured by the software. Storage enables retrospective evaluation, which is not possible on the CHS. The CHS on the other hand is a fully automated portable system that immediately generates the C-IMT value during image acquisition, these images are also stored but in less quality than those of the PiU. Notably, the acquisition time is shorter on the CHS than on the PiU. Moreover, Aldridge et al. showed that training of nonsonographers on 60 subjects with the CHS was sufficient to give results comparable to an expert sonographer(10). Taken together, the CHS has advantages compared to the PiU for performing carotid ultrasound imaging during outpatient visits and as a high-throughput system in large studies.

Whether C-IMT measurements are useful for individual risk assessment remains a matter of debate (11, 12). Recent population studies showed that C-IMT is associated with CVD(8), although C-IMT seems to have no additional value in cardiovascular risk prediction(11, 12). However, the current guidelines of the European Society of Cardiology states that C-IMT-measurements should be considered in asymptomatic adults at moderate risk(13). The discussion about the clinical usefulness of C-IMT is outside the scope of the current study.

The presence of carotid plaques and especially plaque volume are strong predictors of

cardiovascular events (14). However, plaque volume could not be scored on the systems we used since they were not able to obtain 3D ultrasound imaging.

Big inter-observer differences were not related to high C-IMT levels showing that the CHS is reliable at both low and high C-IMT. However, we measured the C-IMT between systems in healthy subjects with relatively low C-IMT values. Therefore, we cannot exclude that higher C-IMT values would expose differences between the systems. Furthermore, healthy volunteers are also less likely to have carotid plaques. Although plaque presence in the inter-observer variability group was not significantly different, there was not a complete agreement between the two experienced observers. This emphasizes caution in interpreting and combining results on plaque presence, especially those results from inexperienced sonographers.

CONCLUSION

The portable CHS provides precise measurements of C-IMT and reliably detects carotid plaques, with a shorter acquisition time than that of the PiU. We conclude that the CHS is a rapid, reliable and precise method for assessing C-IMT and plaques, making it highly suitable for high-throughput screening and clinical use.

Declaration of interest

All authors declare no conflict of interest.

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CHAPTER 3

Carotid artery plaques and intima medial thickness in familial hypercholesteraemic patients on long-term statin therapy: a case control study

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ABSTRACT

Background and aims

Statins reduce subclinical atherosclerosis and premature atherosclerotic cardiovascular disease (ASCVD) in patients with familial hypercholesterolemia (FH). However, some FH patients still develop ASCVD despite statin therapy. We compared subclinical atherosclerosis assessed by carotid plaque presence and intima media thickness (C-IMT), in long-term statin-treated FH patients to healthy controls. Furthermore we analysed whether carotid ultrasonography findings associated with subclinical coronary atherosclerosis.

Methods and results

We assessed the presence of carotid plaques and C-IMT in 221 asymptomatic heterozygous FH patients (48% men; 46±15 years) on long-term (10.0±7.8 years) statin treatment and 103 controls (32% men, 47±16 years). The frequency of carotid plaques and C-IMT did not differ significantly between the FH patients and controls (69 (31%) versus 24 (23%), p=0.1 and 0.58±0.13 versus 0.58±0.12 mm, p=0.9, respectively). In a subgroup of 49 FH patients who underwent cardiac computed tomography, coronary artery calcification correlated with carotid plaque presence (R=0.47; p=0.001), but not with C-IMT (R=0.20; p=0.2).

Conclusions

Carotid plaques and C-IMT did not differ between long-term statin-treated heterozygous FH patients and healthy controls. This shows that long-term statin treatment in these FH patients reduces carotid atherosclerosis to a degree of a healthy population. These findings strongly suggests that sonography of the carotid arteries during follow-up of statin-treated FH patients has limited value.

Keywords

- Carotid plaque presence
- Carotid intima media thickness
- Familial hypercholesterolemia
- Residual risk

INTRODUCTION

Familial hypercholesterolemia (FH) is the most common genetic disorder associated with premature atherosclerotic cardiovascular disease (ASCVD), and is caused by pathogenic mutations in the *LDLR, APOB* or *PCSK-9* gene (1-3). The risk of premature ASCVD is increased due to high low-density lipoprotein cholesterol (LDL-C) levels (4), which can be lowered by statin treatment. Statin therapy can reduce ASCVD risk in heterozygous FH patients to the same risk as in the general population (5). However, there are still FH patients who develop ASCVD despite statin treatment (5). To identify these FH patients, imaging modalities that detect subclinical atherosclerosis may be useful. Carotid ultrasonography can be used the detect plaques and estimate carotid intima media thickness (C-IMT). Increased C-IMT and the presence of carotid artery plaques in particular, are significant predictors of ASCVD in the general population(6-9). Previously it was shown that treatment with a high potency statin during 2 years inhibited progression of C-IMT in FH patients (10,11). Sivapalaratnam, et al. showed that the C-IMT of statin treated FH patients is comparable to that of their healthy spouses (12), suggesting a normalization of risk of ASCVD in the former group. However, C-IMT is not as strongly associated with ASCVD as the presence of carotid plaques (9,13,14), which was not investigated in the aforementioned study. Whether the prevalence of carotid plaques is normalized in FH patients by long-term statin treatment, and whether normalized carotid parameters indeed reflect subclinical coronary atherosclerosis, remains unknown. We therefore compared carotid plaque prevalence and C-IMT between FH patients and healthy controls. Moreover, in a subgroup of FH patients we correlated these parameters with coronary artery calcification.

PATIENTS AND METHODS

Study Population

Between May 2012 and May 2015, asymptomatic heterozygous FH patients were recruited from the outpatient cardiogenetics clinic at the Erasmus Medical Centre in Rotterdam. FH was defined as a score ≥6 on 'The Dutch Lipid Clinic Network criteria' (addendum 1) (15). All patients were on statin treatment. All patients were screened for mutations in the *LDLR*, *APOB* and *PCSK-9* genes. Patients with two mutations, compound heterozygous FH and homozygous FH, were excluded as were patients with symptoms

of ASCVD or a history of ASCVD.

Controls were recruited through public advertisements, and were included between April 2014 and May 2015. Inclusion criteria for the controls were: no major illness, no statin or any other lipid-lowering medication use, and no history of ASCVD.

A total of 221 FH patients were included in our study. Expecting 15%±7% difference in carotid plaque presence (primary endpoint) between FH patients and controls, at least 96 controls were required for a power of 80% and α of 5%. For C-IMT (secondary end point), we considered 0.05 mm to be a clinically significant difference and previously we observed standard deviation of +-0.12 mm, to obtain a power of 80%, α of 5%, at least 69 controls were required.

All subjects were over 18 years old. Written informed consent was obtained from all participants and healthy volunteers. This study was in accordance with the declaration of Helsinki and was approved by the local ethical committee (MEC-2012-309);(MEC-2013-556).

Blood analyses

Fasting blood was collected in EDTA, processed the same day, and plasma samples were stored at -80°C. Lipid levels were measured using standard laboratory techniques.

Carotid ultrasonography

All carotid ultrasound scans and measurements were performed using a Panasonic CardioHealthStation (Yokohama, Japan) that uses a validated automated C-IMT capturing method (16). The scanning protocol is based on the ASE consensus (17), and has been previously published (18). In short, the plaque scans were performed bilaterally in the internal carotid artery, external carotid artery and common carotid artery. Plaques were defined as a local enlargement of the C-IMT of more than 50% of the surrounding C-IMT, or if the C-IMT was above 1.5 mm, and were scored as present or absent (19). C-IMT was measured over 1 cm length, at least 0.5 cm proximal of the bifurcation in the common carotid artery", and measured in the end-diastolic phase which was identified by the vessel motion detector system based on the change in arterial diameter during the cardiac cycle (16). The C-IMT was measured twice on each side", in a 45 degree angle determined by positioning the patients head against a 45 degree wedged pillow, and the mean of these four orientations was used in our study.

CT calcium imaging

A subgroup (n=49, 22%) of the asymptomatic FH patients in this cohort underwent a non-enhanced cardiac computed tomography (CT) scan (Somatom Definition, Siemens Medical Solutions, Forchheim, Germany) in the same period, to quantify the coronary calcium burden, not on indication but for another research study. The calcium score was measured as described previously (20), and expressed as the Agatson score (21). The FH patients who had their calcium score determined were divided in three subgroups. The first group were patients without detectable calcification [n=14). Patients with a positive calcium scan were split in two comparable sized groups (n=17; n=18) based on the calcium score, by using the median calcium score of the FH patients with coronary calcification (Agatston score cut-off of 136).

Statistical analyses

Data with a normal distribution were expressed as mean (±SD), and data with a skewed distribution as median (IQR). Differences between the groups at baseline were compared by a Chi-Square test for binary variables and by ANOVA for continuous variables.

Factors associated with C-IMT and plaques were tested in linear and logistic regression analyses. The regression analyses were repeated separately in the FH patients and the controls to see if there were different predicting variables in the groups.

To test the association of carotid plaques and C-IMT with coronary artery calcification, univariable ordinal regression analyses were performed. Finally, multiple ordinal regression analyses were performed to determine the predictive values of the carotid plaques presence and C-IMT for coronary calcification.

The statistical analyses were performed using SPSS, version 20 (SPSS, Chicago, Illinois).

RESULTS

Clinical characteristics

Data were collected of 221 FH patients, and 103 healthy controls. DNA analysis confirmed FH in 170 patients (77%), with mutations in the *LDLR* and *APOB* gene in 151 and 19 patients, respectively. *PCSK-9* gene mutations were not present in our patients.

Characteristics of FH patients and controls are depicted in table 1. FH patients were of similar age and had similar LDL-C levels as controls. All FH patients used statins on average for 10.0±7.8 years. At inclusion, 74% of patients used rosuvastatin or atorvastatin,

Table 3.1 | Characteristics of the FH patients and non-FH controls.

Characteristics	FH (n=221)	control (n=103)	p
Age (years)	46±15	47±16	0.57
Sex (male)	107 (48%)	33 (32%)	0.004
Body mass index (kg/m^2)	26.3±4.8	25.2±4.6	0.05
Systolic blood pressure (mmHg)	129±14	136±21	0.001
Diastolic blood pressure (mmHg)	78±9	83±12	<0.001
Hypertension[1]	46 (21%)	16 (16%)	0.3
Diabetes mellitus[2]	6 (3%)	1 (1%)	0.3
Smoker (current, former)	72 (33%)	36 (35%)	0.4
LDL-R mutation	151 (68%)	N/A	
APOB mutation	19 (9%)	N/A	
No mutation detected	51 (23%)	N/A	
Total cholesterol levels pre-statin treatment (mmol/L)	8,5±2,1	N/A	
Total Cholesterol (mmol/L)	5.1±1.2[a]	5.6±1.0	0.001
HDL-C (mmol/L)	1.4±0.4[a]	1.6±0.4	0.03
LDL-C (mmol/L)	3.3±1.0[a]	3.3±0.7	0.99
ApoB (mg/L)	1.1±0.3[a]	1.0±0.2	0.16
Triglyceride (mmol/L)[b]	1.1 (0.8)[a]	1.2 (0.8)	0.14
Cholesterol year score[c]	358,1±153,6	288,5±115,6	<0.001
Years on statins			
≤ 1 year	32 (15%)	N/A	
1-2 years	15 (7%)	N/A	
2-5 years	27 (12%)	N/A	
>5 years	147 (67%)	N/A	
High intensity statin dose[d]	164 (74%)	N/A	
Atorvastatin	73 (33%)	N/A	
Fluvastatine	2 (1%)	N/A	
Pravastatine	6 (3%)	N/A	
Rosuvastatine	90 (41%)	N/A	
Simvastatine	50 (23%)	N/A	

[1] Defined as being diagnosed by physician or taking antihypertensive drugs; [2] Defined as being diagnosed by physician or taking anti-diabetic drugs; [a] values of statin treated patients; [b] median (IQR); [c] cholesterol year score was calculated using the formula: Untreated total cholesterol x years without statin treatment + statin-treated total cholesterol x years treated with statins; [d] Defined as atorvastatin ≥40mg, Rosuvastatin ≥20mg, and simvastatin ≥40mg; nd: not determined.

23% used simvastatin and the remainder used fluvastatin or pravastatin. The FH group contained more men than the healthy control group. The main differences between the groups were a higher BMI and lower blood pressure, total cholesterol and HDL cholesterol in the FH group.

Carotid ultrasonography findings

The frequency of plaques in 69 FH patients out of 221 FH patients was not significantly different from 24 out of 103 controls (31% versus 23%; p= 0.09). The mean C-IMT was similar in the FH patients to the healthy controls (0.58±0.13mm and 0.58±0.12mm, respectively; p=0.90). Adjustment for age, male sex, body mass index, systolic and diastolic blood pressure, total cholesterol, and HDL-cholesterol did not materially change these results (data not shown).

In the FH group, plaque presence was associated with age, male sex, body mass index, systolic blood pressure, type 2 diabetes mellitus, hypertension, smoking, total cholesterol, LDL-cholesterol, triglyceride levels, years on statins, and CYS. In the multivariate logistic regression model, we used all the significant co-variables except years on statins and CYS. These two variables were excluded because of associations with other variables, which were used to calculate them. Notably, substituting these variables for age and highest cholesterol levels did not change the results. After adding the remaining significant variables from the univariate analyses, only age remained significantly associated with the presence of plaque (table 2a). Similarly, in the FH group, the mean C-IMT was positively associated with age, male sex, body mass index, systolic blood pressure, diastolic blood pressure, hypertension, smoking, total cholesterol, LDL-cholesterol, triglyceride levels, years on statins, and CYS. In the multiple linear regression model the remaining associated variables were age and hypertension (table 2b). In the control group, results were similar to FH for plaques.

Coronary artery calcification findings

In the subgroup of 49 FH patients, the plaque presence significantly correlated with the coronary calcium-score (Spearman correlation coefficient R=0.47; p=0.001), but C-IMT did not (R=0.20; p= 0.2).

Of the FH patients, who underwent cardiac CT, 14 patients showed no coronary calcifications (Agatston-score: 0); 17 had mild calcification (Agatston -score: 1-136); and 18 had more severe calcification (Agatston -score>136). In univariable ordinal regression analyses, only the presence of carotid plaques (proportional odds: 7.96, 95%

CI 2.41-26.32; p=0.001) and age (proportional odds: 1.06, 95% CI 1.01-1.12; p 0.03) were significant predictors of coronary calcium category. C-IMT was not predictive of a higher calcium-score (proportional odds: 9.23, 95% CI 0.06-1511; p=0.4). In multiple ordinal

Table 3.2 | Multiple regression analyses

A: Multiple Logistic regression of predictors of the presence of carotid plaques in the FH patients, and controls.

	FH		R^2 = 40%	Controls		R^2 = 34%
	OR	95% CI OR	p	OR	95% CI OR	p
Age (Years)	1.11	1.07-1.15	**<0.001**	1.10	1.03-1.17	**0.01**
Sex (Male)	1.79	0.82-3.93	0.1	1.56	0.37-6.66	0.5
Body Mass Index (kg/m^2)	0.99	0.90-1.08	0.8	1.13	0.99-1.29	0.1
Systolic Blood Pressure (mmHg)	1.02	0.99-1.05	0.3	0.99	0.96-1.02	1.0
Hypertension	1.14	0.44-2.93	0.8	2.75	0.66-11.50	0.2
Smoker (Current, Former)	1.44	0.69-2.98	0.3	0.74	0.21-2.68	0.7
Total Cholesterol (mmol/L)	0.53	0.18-1.53	0.2	3.10	0.49-19.61	0.2
LDL-C (mmol/L)	2.71	0.85-8.62	0.1	0.20	0.02-2.40	0.2
Triglycerides (mmol/L)	1.32	0.85-2.10	0.2	0.75	0.25-2.21	0.6

Bold entries in the table highlight a significant p level < 0.05

B: Multiple linear regression analyses of predictors of C-IMT in the FH patients, and controls.

	FH		R^2 = 36%	Controls		R^2 = 50%
	OR	95% CI OR	p	OR	95% CI OR	p
Age (Years)	1.00	1.00-1.00	**<0.001**	1.01	1.01-1.01	**<0.001**
Sex (Male)	0.98	0.95-1.01	0.1	1.00	0.95-1.04	0.6
Body Mass Index (kg/m^2)	1.00	1.00-1.00	0.3	1.00	1.00-1.01	0.2
Systolic Blood Pressure (mmHg)	1.00	1.00-1.00	0.2	1.00	1.00-1.00	0.4
Hypertension	1.05	1.01-1.10	**0.02**	0.99	0.93-1.05	0.9
Smoker (Current, Former)	1.00	0.97-1.03	0.1	1.01	0.96-1.05	0.7
Total Cholesterol (mmol/L)	1.02	0.95-1.10	0.9	0.94	0.85-1.04	0.1
LDL-C (mmol/L)	0.99	0.92-1.07	0.5	1.01	0.89-1.14	0.1
Triglycerides (mmol/L)	0.99	0.96-1.02	0.9	1.00	0.94-1.05	0.3

Bold entries in the table highlight a significant p level < 0.05

regression analyses, carotid plaque presence as a dichotomous variable was a strong predictor of calcium severity independently of age (proportional odds: 11.94 (95% CI 3.26-43.69); p < 0.001) (table 3).

Table 3.3 | Predictors of the extent of coronary calcium

Multiple ordinal regression in statin-treated FH patients (n=49)			
Predictor variables	**Proportional Odds**[1]	**95 % CI**	**p-Value**
Plaque presence	11.94	3.26-43.69	0.0002
Age (Years)	1.09	1.03-1.16	0.005

[1] The Proportional Odds was calculated with an ordinal logistic regression analysis, using three ranked categories of Agatston-score (AU = Agatston units): group 1 (AU=0, n=14), group 2 (0 <AU ≤ 136; n = 17), group 3 (AU > 136; n = 18). This model predicts how much an increase in the explanatory variable leads to an increase of probability of being in the higher calcification group.
R2 = Nagelkerke R square, CI = confidence interval
R2 = 42%

DISCUSSION

This study demonstrates that the prevalence of carotid plaques and C-IMT did not differ between long-term statin-treated FH patients and healthy controls. Moreover, carotid plaques but not C-IMT correlated with coronary calcium in a subgroup of FH patients who underwent cardiac CT.

In our cohort of FH patients with mean age of 46 years, we found that 31% showed carotid artery plaques , which was not significantly different from our controls. We observed a not significant 8% difference in plaque prevalence. It is unlikely that such a small difference contributes to a clinical relevant difference in risk of cardiovascular events in individual patients. However, at a population scale this still may point at incomplete normalisation of the risk. Some of our control subjects did exhibit ASCVD risk factors such as hypertension and smoking that could have led to an increase in carotid plaques or C-IMT. In contrast the prevalence of carotid plaques in our healthy volunteers (23%) in our study seems to be in line with previous studies in healthy volunteers. In a Swedish cohort with a mean age of 57 years, plaques were present in 43% of the participants (22), and in an American multi-ethnic population with mean age 61 years, prevalence was 42% (9). The prevalence of plaques is higher in untreated newly diagnosed FH patients(17). In the pre-statin era, carotid plaques were present in 70% of FH patients all younger than 30 years, compared to only 12% in age-matched controls (23). This also suggests that initial differences in the

presence of carotid plaque between the young untreated FH patients and controls have diminished later in life, owing to statin treatment of the FH patients. It is possible that the small, not significant difference found in this study is mainly caused by those FH patients who did not start early with aggressive statin treatment. Therefore, we cannot be sure that our results are applicable to all treated FH patients. It is possible that the control subjects with carotid plaque also have increased coronary artery calcification, but these data were not available. Further research is needed to determine whether carotid plaque presence predicts future cardiovascular events in statin-treated FH-patients.

We observed no statistical differences in C-IMT between long-treated FH patients and healthy controls. These results are in line with a previous report using 40 FH patients treated for more than 5 years with statins (9). With 0.58 mm, the mean C-IMT values we measured are similar to several other studies on healthy subjects with approximately the same age, indicating that our control group does not have increased C-IMT values (9,22,24,25). Moreover the mean C-IMT value of our long-term statin treated FH patients are much lower than in the ASAP study population (10), and even lower than in the ENHANCE study population (26), or than in FH patients that have been treated for at least 5 years with statins (12). Characteristics of the ENHANCE population were similar to our cohort of FH patients, except that the treated LDL-C cholesterol in our patients (3.3±1.0 mmol/L) is lower than in statin + ezetimibe treated arm of the ENHANCE (3.7±1.4 mmol/L; $p<0.01$). C-IMT values of the ENHANCE study (0.67±0.16 mm) were higher than in our study (0.58±0.13 mm; $p<0.01$) which might be explained by the combination of higher on-treatment LDL-C values, different measuring systems, or possibly, a longer period of statin treatment.

We previously reported that subclinical coronary artery disease, as determined by coronary CT angiography, was increased in asymptomatic FH patients compared with statin-treated (3±4 years), non-anginal chest pain patients (27). In another study in the same patients, no differences in C-IMT were found (28), which is in line with our findings. In the current study we could not compare coronary CT angiography results between the FH patients and controls as calcium scoring was not performed because of the undesirable radiation exposure in the healthy controls.

In the present study, a subgroup of these FH patients (n=30) was included and 19 FH patients were added, who only received non-enhanced cardiac CT scans. In these patients coronary artery calcification was strongly correlated with carotid plaque presence, but not with C-IMT. The lack of association of C-IMT and coronary artery calcification was also previously shown in non-statin treated FH patients (29,30).

The association of carotid plaque presence and C-IMT on ASCVD outcome has not been studied in statin-treated FH patients. Despite this lack of evidence many cohort and intervention studies have used C-IMT as a proxy of ASCVD outcome in statin-treated FH patients (18,26,31,32). Since in our study in statin-treated FH patients C-IMT did not associate with coronary calcification, C-IMT might not be useful as a marker for ASCVD risk in these patients.

The case-control design of our study limits the possibility to observe changes in plaque presence and C-IMT over time. Since no prospective ultrasound data is available for our patients, we cannot assess whether ultrasonographic changes over time are associated with cardiovascular risk. Clinical characteristics differed slightly between patients and controls. The controls were of similar age, but more often women. Therefore, it is unlikely that better gender-matched controls will unmask a hidden burden in our treated FH cohort. Since the results were similar in both groups and the C-IMT values were similar to other healthy populations (9,22,24,25), and adjustment for these differences had no influence, it is unlikely that these differences have affected the outcome of our study. We did not include data of compound heterozygous FH patients and homozygous FH patients, because their baseline and treated LDL-C as well as their ASCVD risk is not comparable to heterozygous FH patients.

Carotid plaques were only scored as present or absent whilst plaque volume measurements may be a better way to score cardiovascular risk (14,33). However, this can only be done with 3D ultrasonography which was not available on our ultrasound system. Therefore, we cannot exclude that other ultrasonographic techniques or locations may yield different results.

CONCLUSION

Carotid plaques and C-IMT did not differ between long-term statin-treated heterozygous-FH patients and healthy controls. This shows that long-term statin treatment in these FH patients reduces carotid atherosclerosis to a degree of a healthy population. These findings strongly suggests that sonography of the carotid arteries during follow-up of statin-treated FH patients has limited value.

Conflicts of interest

There was no relationship with industry that could cause a conflict of interest.

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CHAPTER 4

Increased aortic valve calcification in familial hypercholesterolemia: Prevalence, extent and associated risk factors in a case-control study

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ABSTRACT

Background

Familial hypercholesterolemia is typically caused by LDL receptor (LDLR) mutations that result in elevated levels of LDL cholesterol (LDL-C). In homozygous FH, the prevalence of aortic valve calcification (AoVC) reaches 100% and is often symptomatic.

Objectives

The objective of this study was to investigate the prevalence, extent and risk-modifiers of AoVC in heterozygous FH (he-FH) that are presently unknown.

Methods and results

145 asymptomatic patients with he-FH (93 men; mean age 52, ±8 years) and 131 non-familial hypercholesterolemia controls (78 men; mean age 56, ±9 years) underwent CT computed tomography calcium scoring. AoVC was defined as the presence of calcium at the aortic valve leaflets. The extent of AoVC was expressed in Agatston units, as the AoVC-score. We compared the prevalence and extent of AoVC between cases and controls. In addition, we investigated risk modifiers of AoVC, including the presence of LDLR mutations without residual function (LDLR-negative mutations), maximum untreated LDL-cholesterol (maxLDL), LDL-C, blood pressure, and coronary artery calcification (CAC).

Prevalence (%) and AoVC-score (median, IQR) were higher in he-FH patients than in controls: 41%, 51(9-117); and 21%, 21(3-49) ($p<0.001$ and $p=0.007$). Age, untreated maxLDL, CAC and diastolic blood pressure were independently associated with AoVC. LDLR-negative mutational he-FH was the strongest predictor of the AoVC-score (OR: 4.81; 95% CI: 2.22-10.40; p = <0.001).

Conclusion

Compared to controls, he-FH is associated with a high prevalence and a large extent of subclinical AoVC, especially in patients with LDLR-negative mutations, highlighting the critical role of LDL-C metabolism in AoVC etiology.

Keywords

- Aortic valve calcification;
- Calcific aortic stenosis;
- Coronary artery calcification;
- Familial hypercholesterolemia;
- Low-density lipoprotein receptor;
- LDLR-negative mutation.

INTRODUCTION

Aortic valve calcification (AoVC) has an estimated prevalence of more than 50% in the elderly (> 75 years), and is associated with an elevated risk of coronary (72%) and cardiovascular events (50%) (1,2). In addition, the degree of AoVC correlates with stenosis severity, disease progression and the development of coronary and cardiovascular events (3-5).

In the general population AoVC is associated with age, male gender, smoking, hypertension, diabetes, obesity and hypercholesterolemia (6,7). Patients with familial hypercholesterolemia (FH) have extremely high levels of low-density lipoprotein cholesterol (LDL-C) and may be at high risk of developing AoVC. FH is an autosomal inherited disorder caused by mutations in the LDL receptor (LDLR) gene, the apolipoprotein B (APOB) gene, or the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene (8). LDLR mutations can be classified as mutations with residual LDLR function (LDLR-defective mutations) or without LDLR function (LDLR-negative mutations) (9).

In patients who are homozygous for FH, the prevalence of AoVC reaches 100% and surgical intervention of functional valvular disease is often needed (10,11). Compared to homozygous FH, heterozygous FH (he-FH) is associated with less aortic valve dysfunction on echocardiography (12-15). However, the prevalence of AoVC in he-FH is unknown.

The purpose of this single-centre study was to determine the prevalence and extent of AoVC in asymptomatic statin-treated patients, heterozygous for FH. In addition, we evaluated which variables were associated with the presence and extent of AoVC. In the molecular context of the patients, we compared AoVC between he-FH patients with and without LDLR-negative mutations.

METHODS

Study population

Between February 2008 and June 2011 we included 145 consecutive patients with he-FH. Additionally, between November 2006 and January 2011 we included 131 consecutive patients with non-anginal chest pain (NACP) as a control group. Patients with NACP were used as a substitute for asymptomatic patients without he-FH because the radiation exposure limits the choice of controls to patients with an indication for cardiac computed tomography (CT).

NACP patients were referred by their general practitioner for the evaluation of chest pain and underwent stress testing and cardiac CT. They did not have a history of coronary artery disease (CAD). NACP was defined as chest pain or discomfort that was not 1) provoked by exertion or emotional stress, or 2) relieved by rest or nitroglycerin(16).

He-FH patients were recruited from our tertiary outpatient lipid clinic. He-FH was determined either by the presence of a confirmed LDLR or APO-B gene mutation (the patients did not have PCSK-9 mutations), or clinically as having a untreated LDL-C above the 95th percentile for gender and age in combination with at least one of the following: the presence of typical tendon xanthomas in the patient or a first degree relative; an LDL-cholesterol level above the 95th percentile for gender and age in a first degree relative; proven CAD in a first degree relative under the age of 60 (17).

DNA samples were taken of all patients with a clinical suspicion of he-FH and were sent to a central laboratory for mutational screening (18). A complete overview of the mutations found and clinical characteristics of both LDLR-negative and LDLR-defective he-FH has been previously published (19). Plasma lipid levels were measured on fasting blood samples at time of inclusion. Cholesterol levels before statin treatment were obtained from patient medical records, and used as the variable maximum untreated total cholesterol, and untreated maximum LDL cholesterol (maxLDL).

Exclusion criteria were: symptoms of CAD, history of CAD, rheumatic fever or known aortic valve pathology, although cardiac ultrasounds were not routinely performed prior to inclusion. Patients with a secondary cause of hypercholesterolemia such as renal, liver or thyroid disease were also excluded from the study. Further exclusion criteria were renal insufficiency (serum creatinine > 120 unmold/L), known contrast allergy and irregular heart rhythm (atrial fibrillation). In asymptomatic he-FH patients, the inclusion age was 40-70 years for men. Women were included after childbearing age (45-70 years) because of potential radiation-induced harm to the fetus or ovaries.

This study complies with the Declaration of Helsinki, the institution's human research committee approved the study protocol and all patients gave written informed consent.

CT calcium score

To quantify the AoVC, as well as the coronary calcium score, a cardiac CT scan without contrast medium was performed, which enabled calcium scoring at high accuracy and reproducibility (20,21). All CT scans were performed on a dual source CT scanner (first 232 scans: Somatom Definition, last 44 scans: Somatom Definition FLASH, Siemens Medical Solutions, Forchheim, Germany), with application of a prospectively ECG-triggered scan protocol with a tube current of 76 mAs at 70% of the RR-interval. Images were reconstructed with a slice thickness of 3 mm and an increment of 1.5 mm using a medium convolution kernel (B35f).

Lesions were classified as AoVC if located within the aortic valve leaflets, exclusive of the aortic annulus or coronary arteries, and contained 3 or more contiguous pixels with an attenuation value of more than 130 Houndsfield units (2,21). The AoVC-score was defined as the quantity of AoVC expressed in Agatston units, by the same lesion definition as for coronary artery calcium quantification (CAC), using dedicated software (MMWP, Siemens Medical Solutions, Forchheim, Germany) (22). The CT reading was performed blinded with regard to patient characteristics. The absence of AoVC was assigned a score of 0. Additional information about the scan protocol, including the quantification of coronary artery calcium (CAC) has been previously published (23).

Contrast-enhanced scans were consulted if the exact location of calcified lesions, in the valve or aortic root, were unclear.

Statistical analysis

Categorical variables were expressed as numbers (percent). Normally distributed continuous variables were shown as mean (± SD) and skewed variables as median (IQR). To determine the differences between he-FH patients and NACP patients we used a Pearson Chi-Square test to compare binary variables. Continuous variables with a normal distribution were tested with a T-test, and skewed variables were tested with a Mann-Whitney-U test. Statistical significance was considered at a two-sided P-value of < 0.05.

We compared the prevalence of AoVC and the AoVC-scores between he-FH and NACP patients, in relationship to age. Age categories were chosen on the basis of equal patient numbers in all groups (N = 92, N = 92 and N = 92).

To evaluate which variables were associated with AoVC we used a univariable ordinal logistic regression model. Subsequently, we divided the AoVC-score (Agatston units) into three groups based on equal distribution of patients in whom AoVC was present: 1) AoVC-score = 0 (N = 190), 2) AoVC-score of > 0 – 37 (N = 43), and 3) AoVC-score > 37 (N = 43). Variables associated with AoVC where analyzed in the entire cohort with a multivariable ordinal logistic regression model to identify a set of predictors of AoVC. We have chosen an ordinal regression model over a linear regression model to investigate a dose response relationship between the highly skewed AoVC variable and other variables. Correcting skewness by logistically transforming would have been possible as well, but we did not like to change the data into an artificial score in 190 persons whose Agatston score was 0.

In addition, the association between the presence of CAC and AoVC was examined for he-FH patients and controls. The differences in distributions of AoVC in the presence and absence of CAC were analyzed with a Pearson Chi-Square test, and differences between the AoVC-score in the presence and absence of CAC with the Mann-Whitney-U test.

Finally, we compared the influence of LDLR-negative, and LDLR-defective mutational he-FH on AoVC. Data were analyzed using SPSS (version 22, SPSS, Chicago, USA).

RESULTS

Baseline characteristics of he-FH patients and controls

Age, systolic blood pressure, and the prevalence of hypertension and diabetes were higher in control patients, than in the he-FH group (table 1). He-FH patients had higher untreated maxLDL levels, more frequently used statins, and more often reported a positive family history for CAD compared to the controls. Gender, body mass index and treated cholesterol levels were similar in both groups.

AoVC in he-FH patients and controls

AoVC was compared between he-FH patients and controls (table 2). AoVC was more prevalent in he-FH patients (41%, N = 59) than in controls (21%, N = 27, P < 0.001), irrespective of the age category. Limiting the analysis to patients with AoVC present, the AoVC-score (median, IQR) was higher in he-FH patients than in controls: 51 (9 - 117) and 21 (3 - 49), respectively (P = 0.007).

Table 4.1 | Clinical characteristics

	He-FH (N = 145)	Control (N = 131)	p-value
General			
• Age (years)	52 ± 8	56 ± 9	< 0.001
• Gender (male)	93 (64)	78 (60)	0.432
• Body mass index (kg / m^2)	27 ± 4	27 ± 5	0.309
• Smoking (current / former)	41 (28)	45 (35)	0.277
• Hypertension[1*]	38 (26)	59 (45)	0.001
• Systolic blood pressure (mmHg)	129 ± 7	141 ± 20	< 0.001
• Diastolic blood pressure (mmHg)	80 ± 10	81 ± 12	0.282
• Diabetes Mellitus	6 (4)	17 (13)	0.008
• Positive family history of premature coronary artery disease [2*]	102 (70)	65 (50)	< 0.001
Lipids			
• Maximum untreated total cholesterol (mmol / L)	9.8 ± 2.3	5.5 ± 1.2	< 0.001
• Maximum untreated LDL cholesterol (mmol / L)	7.1 ± 2.2	3.7 ± 1.1	< 0.001
• Statin use	142 (98)	44 (34)	< 0.001
• Total cholesterol (mmol / L)	5.5 ± 1.4	5.3 ± 1.2	0.264
• HDL (mmol / L)	1.4 ± 0.4	1.4 ± 0.4	0.388
• LDL (mmol / L)	3.5 ± 1.3	3.5 ± 1.1	0.980
• Triglyceride (mmol / L)	1.09 (0.80 - 1.65)	1.32 (0.87 - 1.79)	0.107

Values are mean SD, n (%), or median (interquartile range). 1*Blood pressure >140/90 mm Hg and/or antihypertensive treatment. 2*Proven coronary artery disease in first-degree relative aged <60 years.
Control = patients with nonanginal chest pain; HDL = high density lipoprotein; he-FH = heterozygous familial hypercholesterolemia; LDL = low-density lipoprotein; LDL-C = low-density lipoprotein cholesterol.

Table 4.2 | Calcification of the aortic valve leaflets

	He-FH (N = 145)	Control (N = 131)	p-value
AoVC present			
AoVC present, per age category:	59 (41%)	27 (21%)	< 0.001
• 40 - 50 years	16 (26%)	0 (0%)	0.002
• 51 - 58 years	21 (48%)	8 (17%)	0.001
• 59 - 70 years	22 (56%)	19 (36%)	0.005
AoVC-score	51 (9 - 117)	21 (3 - 49)	0.007
AoVC-score per category:			< 0.001
• Agatston = 0	86 (59)	104 (79)	-
• Agatston > 0 – 37	24 (17)	19 (15)	-
• Agatston > 37	35 (24)	8 (6)	-

Continuous data are expressed as median (IQR), dichotomous data as N (%), age categories chosen on the basis of equal patient numbers in all groups (N = 92).
He-FH = heterozygous familial hypercholesterolemia, control = patients with non-anginal chest pain. AoVC = aortic valve calcification, AoVC-score categories chosen on the basis of equal distribution of patients with AoVC (N = 43).

Risk factors for AoVC

Risk factors for AoVC are shown in table 3. The AoVC burden by Agatston score was associated with age, untreated maxLDL, LDLR-negative mutational he-FH, CAC and diastolic blood pressure. Gender, smoking, hypertension, diabetes mellitus and obesity were not associated with the extent of AoVC.

In the multivariable ordinal regression model, all variables explained 27% of the variance of AoVC and all remained significantly associated with AoVC. Among the variables, LDLR-negative mutation carrier status was a strong predictor of the extent of AoVC (OR: 4.81; 95% CI: 2.22-10.40; p= <0.001). Analyses restricted to the he-FH patients, LDLR-defective, and LDLR-negative had similar results (data not shown).

Association between coronary and aortic valve calcification

The presence of CAC was associated with a higher prevalence of AoVC, both in he-FH and control patients (table 4). Of the patients without CAC, no more than 4% showed AoVC. However, in the absence of AoVC, still more than 39% of patients exhibited CAC.

Aortic valve calcification and LDL receptor mutational status

Out of 145 he-FH patients, fifty-nine patients (41%) had an LDLR-negative mutation. Compared to he-FH patients with LDLR-defective mutations, LDLR-negative mutational he-FH was associated with: higher total cholesterol (5.8 ± 1.6 and 5.3 ± 1.3 mmol/L, p=0.026), LDL-C (3.9 ± 1.4 and 3.2 ±1.1 mmol/L, p=0.002) and untreated maxLDL (8.0 ± 2.5 and 6.6 ± 1.7 mmol/L, p= <0.001). In addition, he-FH patients with LDLR-negative mutations were younger (51 ± 7 and 53 ± 8 years, p=0.040), started using statins at younger age (40 ± 9.8 and 46 ± 9.4 years, p=<0.001), and used statins for a longer period of time (10. ± 7 and 7 ± 7 years, p=0.010). All other variables from table 1 were not statistically different between groups.

He-FH patients with LDLR-negative mutations had higher prevalence of AoVC (31 (53%)) as compared to LDLR-defective mutations (28 (33%); p< 0.001) and controls (27 (21%); p = 0.016). The difference in AoVC prevalence between LDLR-defective mutational he-FH and the controls was also significant (p = 0.048). Additionally, AoVC-scores increase faster with age in LDLR-negative he-FH than in LDLR-defective he-FH (data not shown).

Table 4.3 | Predictive value of risk factors for AoVC-score.

N = 276	Univariable ordinal regression in the entire cohort			Multivariable ordinal regression in the entie cohort†		
	Proportional Odds §	95 % CI	p-Value	Proportional Odds §	95 % CI	p-Value
LDLR-negative mutation	3.87	2.19-6.84	< 0.001	4.81	2.22-10.40	< 0.001
Age (Years)	1.07	1.04-1.11	< 0.001	1.12	1.08-1.16	< 0.001
Sex (Male)	1.40	0.83-2.37	0.212			
BMI	1.05	0.99-1.11	0.106			
Smoking (Current/ Former)	1.24	0.73-2.11	0.433			
Hypertension ‡	0.84	0.58-3.32	0.518			
Diabetes Mellitus	0.72	0.30-1.70	0.452			
Maximum Untreated Cholesterol	1.33	1.21-1.46	< 0.001			
Maximum Untreated LDL Cholesterol	1.26	1.13-1.39	< 0.001	1.18	1.03-1.34	0.01
Treated Total Cholesterol	1.09	0.90-1.31	0.373			
HDL	1.02	0.54-1.95	0.943			
LDL	1.10	0.90-1.36	0.349			
TG	0.98	0.81-1.19	0.842			
Systolic Blood Pressure	1.01	0.99-1.03	0.065			
Diastolic Blood Pressure	1.03	1.00-1.05	0.031	1.04	1.01-1.07	0.01
CAC (per 100 Agatston Units Increase)	1.26	1.16-1.36	< 0.001			
CAC (Present)	6.97	3.18-15.29	< 0.001			

Agatston units: group 1 (AoVC score ¼ 0), group 2 (AoVC score >0 to 37), and group 3 (AoVC score >37). †= No significant interaction between the presence of AoVC and other risk factors. § = Because 3 ranked categories (3 AoVC groups) are being analyzed, the proportional odds variable is a relative risk that is calculated with an ordinal logistic regression analysis. This model predicts how much an increase in the explanatory variable leads to an increase of probability in the higher AoVC group.
‡ = Blood pressure >140/90 mm Hg and/or antihypertensive treatment. BMI = body mass index; CAC = coronary artery calcification (Agatston units); CI = confidence interval; LDLR = low-density lipoprotein receptor; other abbreviations as in Tables 1 and 2.

Table 4.4 | Association between the presence of CAC and AoVC for FH patients and controls.

	CAC present	CAC absent	p-value
He-FH (N = 145)			
• AoVC present	56 (39%)	3 (2%)	< 0.001
• AoVC absent	61 (42%)	25 (17%)	
• AoVC-score	55 (13 - 125)	3 (0 - 3)	0.019
Control (N = 131)			
• AoVC present	22 (17%)	5 (4%)	0.002
• AoVC absent	51 (39%)	53 (40%)	
• AoVC-score *	15 (2 - 44)	24 (10 - 98)	0.257

∗ Median AoVC-score of patients with AoVC present in Agatston units.
Continuous data are expressed as median (interquartile range), AoVC = aortic valve calcification, CAC = coronary artery calcification (Agatston units), FH = familial hypercholesterolemia. He- FH = heterozygous familial hypercholesterolemia, control = patient with non-anginal chest pain.

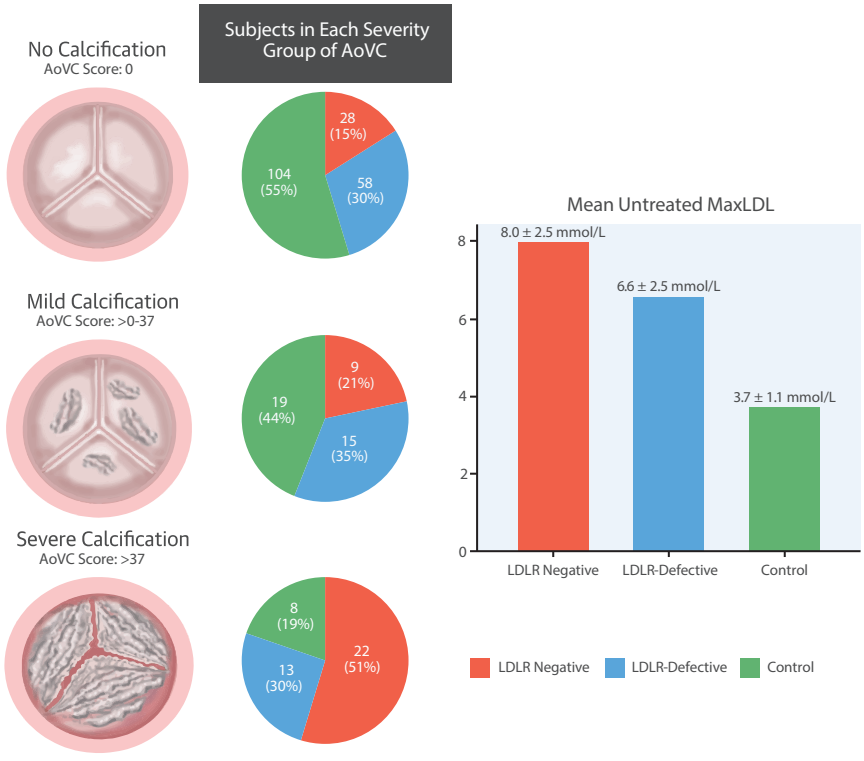
DISCUSSION

The main findings of this study can be summarized as: 1) the prevalence and extent of AoVC was higher in he-FH patients than in the non-familial hypercholesterolemia; 2) age, untreated maxLDL, LDLR-negative mutational he-FH and diastolic blood pressure were positively associated with AoVC; 3) the level of treated LDL-C was not predictive of the prevalence and extent of AoVC; and 4) the absence of CAC was associated with a low prevalence of AoVC.

In a recent study by Smith et al., using Mendelian randomization, it has been shown that a genetic predisposition to elevated LDL-C was associated with the presence of AoVC and the incidence of functional aortic stenosis in large community based cohorts (24). The authors concluded that their results provided evidence supportive of a causal association between LDL-C and aortic valve disease. We also performed a Mendelian randomization approach, which is a combination of 1): An association of the genetic background (the LDL-receptor mutation), with the intermediate trait (untreated maxLDL) and the outcome parameter (AoVC); and 2): An association between the intermediate trait (untreated maxLDL) and outcome parameter (AoVC), corrected for confounding by multiple regression analysis. This Mendelian randomization approach mimics a RCT on a genetic level, and suggests a causal role of LDL-C in beginning aortic-valve pathology.

In our study, he-FH patients were exposed to extremely high levels of LDL prior to statin treatment, especially those with LDLR-negative mutational he-FH. This could have

CENTRAL ILLUSTRATION Severity of AoVC in Patients With he-FH and Control Subjects, and Their Untreated LDL-C Levels



ten Kate, G.-J.R. et al. J Am Coll Cardiol. 2015; 66(24):2687–95.

In this study, 145 asymptomatic patients with heterozygous familial hypercholesterolemia (he-FH) and 131 nonfamilial hypercholesterolemia control subjects underwent computed tomography calcium scoring of the aortic valve. Severity of aortic valve calcification (AoVC) per group is shown in the pie charts, and the bar graph illustrates the untreated low-density lipoprotein (LDL-C) levels for the groups. LDLR ¼ low-density lipoprotein receptor; MaxLDL ¼ untreated maximum low-density lipoprotein cholesterol.

caused the higher prevalence and quantity of AoVC that we found in he-FH patients, and particularly those with LDLR-negative mutations. Since the moment of being diagnosed with hypercholesterolemia patients have been treated with statins, which dramatically lowered LDL-C levels, and thereby reduced the predictive value of LDL-C towards AoVC. This could explain why untreated maxLDL and LDLR-negative mutational he-FH were, and the level of statin treated LDL-C was not predictive of AoVC in our study.

Diastolic blood pressure was mildly but significantly associated with AoVC in our overall normotensive subjects. One might speculate that the increased diastolic blood pressure promotes stress on the aortic side of the valve leaflets, which is where aortic valve lesions are most commonly found (25). This increased stress on the aortic valve

can lead to tissue remodelling and promote inflammation, leading to calcification, stenosis, and ultimately valve failure (26).

Messika-Zeitoun et al. investigated determinants and progression of AoVC in a population based follow-up study, using electron-beam-CT. De novo AoVC was found to be associated with elevated LDL-C levels, whereas established AoVC progressed independently of atherosclerotic risk factors and faster with increasing initial extent of AoVC (27). This led to the hypothesis that elevated levels of LDL-C have their atherogenic effect during the early phase of AoVC, before the start of statin treatment.

As shown in the results section, patients with LDLR-negative mutational he-FH started using statins at younger age and used statins for a longer period of time. However, despite their more intense statin treatment, these patients showed a higher prevalence of AoVC, which more rapidly increased with age. To investigate the effect of statin treatment on AoVC in our cohort of he-FH patients of whom we knew were exposed to high levels of LDL-C early in life, we included "duration of statin use" in a multivariable ordinal regression model. Duration of statin use was however not associated with AoVC after correction for age, untreated maxLDL, LDLR-negative mutational he-FH and diastolic blood pressure. All other variables remained statistically significant (data not shown).

In addition, three major prospective randomized trials could not demonstrate any impact of lipid lowering therapy on the rate of progression of AoVC (28-30). However, macrophage and osteoclast infiltration of the AoVC were reduced by atorvastatin in cholesterol fed mice (31). Apparently, other pathogenic risk mechanisms prevail once AoVC has been established. It is known that during the later stages of calcific aortic stenosis, a process of osteoblastic activity prevails over the initial atherosclerotic process, resulting in progressive calcification of the valve that seems unrelated to LDL-C levels or statin treatment and fits the observed independence of AoVC from lipid profile or statin treatment. The extensive AoVC in our young study population suggest that statins have their main effect in preventing aortic valve pathology prior to the development of aortic valve stenosis. The three prospective randomized trials were restricted to patients with beginning aortic valve pathology in whom statins could not exert a preventive effect anymore. Even though the exact role of serum lipids in the pathogenesis of aortic valve disease is unknown, it is evident that lipid depositions are found within and in proximity to aortic valve lesion which is not the case in healthy valve leaflets (25). This suggests a critical role for lipids in the early onset of aortic valve pathology.

The concept of two different phases in the development of AoVC progression is not

only essential in comprehending the effect of statin treatment, but could also explain the discordant association between AoVC and CAC. As shown in table 4, the absence of CAC was associated with very low prevalence of AoVC. However, the absence of AoVC was not predictive of the absence of CAC. Perhaps, during the early phase of AoVC, risk factors for CAC are preconditions for the development of AoVC. However, if AoVC develops after the initial atherosclerotic phase, its progression seems to be regulated by risk factors that differ from those causing CAC (27,33).

We found that the prevalence and extent of subclinical AoVC is clearly increased in he-FH patients, especially in patients carrying LDLR-negative mutations. It should be emphasized that AoVC is generally without symptoms, and only a fraction of patients with AoVC ultimately develop clinical aortic stenosis. The reported prevalence of hemodynamically significant aortic valve stenosis on echocardiography is low in he-FH (15). Since statin therapy became available, the risk of cardiovascular disease mortality has been substantially reduced in he-FH patients (35).

Detection and treatment of he-FH patients at young age may not only slow progression of CAD but also could be effective to prevent or slow the development of AoVC during the early phase of disease. This underlines the clinical importance of studies on the effectiveness of statin use for the primary prevention of AoVC, especially in patients with LDLR-negative mutational he-FH.

Study limitations

This study is a cross-sectional observation of AoVC in patients aged between 40 and 70 years, without clinical outcome data, and without functional assessment of stenosis with echocardiography. The cross-sectional design of our study did not allow for proper evaluation of the effects of statins on AoVC, which would require a prospective study and sequential imaging. Additionally, we do not have sufficient follow-up in our cohort to assess the clinical consequences of the observed AoVC, which a limitation of the observational design of the present study.

Only asymptomatic patients were selected and it remains to be seen if patients with AoVC will eventually develop clinical aortic valve disease, since the majority of AoVC will not lead to aortic stenosis (36).

The current selection of he-FH patients, who were referred to our university lipid clinic, may have more severe AoVC as compared to he-FH patients in the general population. This single center study from a tertiary hospital could have resulted in overestimation of the total prevalence and extent of AoVC. However, this potential selection bias should

equally hold for LDLR-negative and LDLR-defective mutations.

We have analyzed patients with a major locus effect in the cholesterol metabolism, but we cannot exclude contributions from variants of other genes, like *NOTCH1* (37,38). Especially calcified bicuspid aortic valves have very high heritability. Bicuspid aortic valves have an estimated prevalence of 1-2%. The linkage peaks of aortic stenosis and the *NOTCH1* gene have not been found on chromosome 19 on which the LDL receptor is located (19p13.2). Without co-segregation with the mutations in the LDL receptor, it is unlikely that our cohort was enriched with variants of these other genes.

Recent studies on coronary atherosclerosis showed that the calcified plaque component increased after long-term statin therapy (39,40). In our study, patients with he-FH and particularly patients with LDLR-negative mutations, received higher dosages of statins for longer periods of time and statin use could therefore be a more complex confounder in our analyses. However, in our multivariable ordinal regression model, the time of statin treatment was not significantly associated with AoVC in he-FH patients.

As compared to the general population, we did not find male gender to be a risk modifier of AoVC. This is likely caused by the relatively small size of our study as compared to the large population based Heinz Nixdorf Recall Study or the Multi-Ethnic Study of Atherosclerosis (2,5,27).

CONCLUSION

We found that: he-FH is associated with a high prevalence and a large extent of subclinical AoVC; age, diastolic blood pressure, untreated maxLDL, and LDLR-negative mutations were associated with the extent of AoVC; the difference between LDLR-negative and defective mutations provides important evidence for the critical role of LDL-C metabolism for the pathogenesis of AoVC and; the absence of CAC was associated with low prevalence of AoVC suggesting shared pathophysiological determinants. Worldwide, the majority of he-FH patients, who have been treated with statins for a substantial period of their life, are still too young to express valve diseases. However, due to the prolonging survival in these patients, because of statin treatment, our results suggest that aortic valve pathology will be a common problem in aging he-FH patients, especially in those with LDLR-negative mutations.

PERSPECTIVES

Clinical Competency:

This is the first study that shows a high prevalence and extent of aortic valve calcification in he-FH patients. With the prolonging survival in these patients due to statin treatment, and its lacking effect in halting the progression of AoVC, our study results suggest that aortic valve pathology will be a common problem in the aging he-FH patient.

Translational Outlook:

The mechanism by which LDL-C initiates AoVC requires furthers investigation.

Translational Outlook 2:

Prospective imaging studies of aging he-FH patients are needed to elucidate the clinical outcome of aortic valve pathologies in these patients.

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CHAPTER 4B

Calcific Aortic Valve Disease in Familial Hypercholesterolemia: The LDL-Density-Gene Effect*

Nalini M. Rajamannan

Refers to

Sven Bos, Gert-Jan R. ten Kate, Admir Dedic, Lisan A. Neefjes, Akira Kurata, Janneke G. Langendonk, Anho Liem, Adriaan Moelker, Gabriel P. Krestin, Pim J. de Feyter, Jeanine E. Roeters van Lennep, Koen Nieman, Eric J. Sijbrands

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KEY WORDS

- aortic valve
- calcification
- familial hypercholesterolemia
- LDL density theory
- low-density lipoprotein

Calcific aortic valve disease (CAVD) is the most common indication worldwide for valve intervention. For years, the mechanism for this calcification was thought to be due to a passive degenerative process. However, in the 21st century, the National Heart, Lung, and Blood Institute of the National Institutes of Health recognized that CAVD is an active biologic osteogenic process (1). Initiation of osteogenesis in the aortic valve depends on risk factors similar to those known to promote coronary artery disease, which cause myofibroblasts to differentiate via an osteogenic gene activation that results in valve calcification (1,2).

In this issue of the *Journal*, a study from the Netherlands by ten Kate et al. (3) tested the prevalence, extent, and risk modifiers of CAVD in patients with heterozygous familial hypercholesterolemia (he-FH). Clinically, the he-FH phenotype is encountered more often than the homozygous phenotype due to rapid progression of coronary artery disease in the homozygous patient population. The investigators therefore sought to determine the prevalence of CAVD in patients with he-FH by measuring the amount of calcification burden via computed tomography measurements of the coronary artery and aortic valve, low-density lipoprotein receptor (LDLR) function, and lipid levels and assessing their association with CAVD.

LDL Receptor Density

The investigators discovered that the prevalence of aortic valve calcification (AoVC) and the AoVC score (median [interquartile range]) were both higher in patients with he-FH than in control subjects: 41% versus 21%, respectively ($p < 0.001$) and 51 (9 to 117) versus 21 (3 to 49) ($p = 0.007$) (3). LDLR-negative mutational he-FH was the strongest predictor of the AoVC score (odds ratio: 4.81; 95% confidence interval: 2.22 to 10.40; $p < 0.001$). He-FH was associated with a high prevalence and a large extent of subclinical AoVC, especially in patients with LDLR-negative mutations, compared with the control subjects. Moreover, the AoVC scores increased faster with age in the LDLR-negative he-FH patients than in the LDLR-defective he-FH patients.

Calcification Density

The LDLR-negative mutation carrier status was a strong predictor of the extent of AoVC (3). The association between coronary artery calcification and AoVC was associated with a higher prevalence of AoVC, both in patients with he-FH and in control subjects. The authors hypothesized that the high level of coronary artery calcification may be due to confounding variables such as differences in statin therapy in the he-FH population

versus the control population. The concept of 2 different phases of AoVC progression is not only essential but could explain the discordant findings. The National Heart, Lung, and Blood Institute Aortic Stenosis Working Group for CAVD (1) also emphasized this concept in early valve sclerosis versus late valve stenosis.

Lipid Density

Compared with he-FH patients with LDLR-defective mutations, patients with LDLR-negative mutational he-FH had higher levels of total cholesterol and maximum untreated low-density lipoprotein cholesterol (3). In addition, he-FH patients with LDLR-negative mutations began statin treatment at a younger age and used statins for a longer period of time. Figure 1 illustrates the results of the study in patients with he-FH, including the effect of functional low-density lipoprotein (LDL) receptors and proportional increases in LDL with the degree of AoVC.

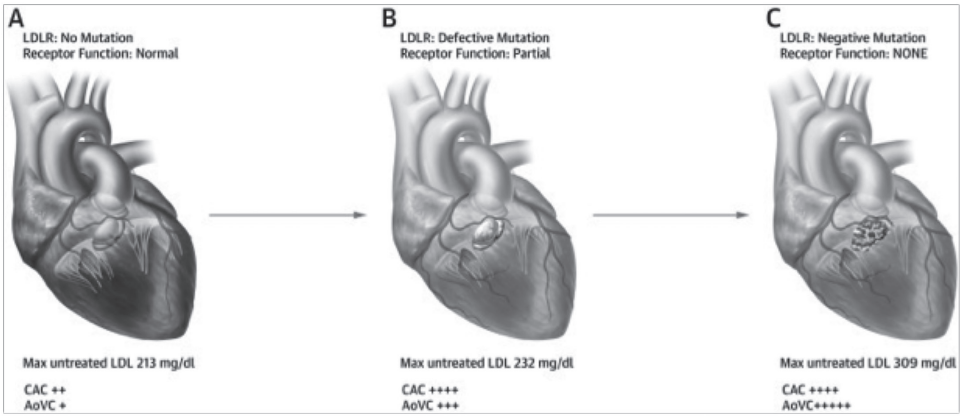

Figure 1 | The LDL-Density-Gene Effect

(A) The control heart with the normal low-density lipoprotein receptor (LDLR).
(B) Heterozygous familial hypercholesterolemia–defective receptor mutation and mild calcific aortic valve disease.
(C) Heterozygous familial hypercholesterolemia–negative receptor mutation and severe calcific aortic valve disease. + = a semi-quantitative measurement of approximately 10% effect; AoVC = aortic valve calcification; density = concentration; CAC = coronary artery calcification; LDL = low-density lipoprotein.

To the best of our knowledge, this study by ten Kate et al. (3) is the first to correlate in patients the role of LDL and the effect of the LDL receptor genetic contribution in terms of phenotypic expression of calcification in the valve and in the coronary arteries. The LDL-density theories (4–6) provide a hemodynamic explanation for why abnormal calcification develops secondary to high LDL density concentration up-regulating

osteogenesis. The effect of fluid flow in the heart is responsible for the variable phenotype expression, depending on the radius of the specific anatomic location in the heart (i.e., artery vs. valve).

Fluid hemodynamics in the heart depends on multiple factors, as derived by the Bernoulli equation for fluid flow (7). Bernoulli described flow through a column as being directly proportional to the change in pressure across the column and indirectly proportional to the resistance. The formula for flow through the heart is similar to Ohm's law for electricity, as shown in Equation 1.

Equation 1$Q=\Delta PR$

The entire formula for resistance for steady-state flow through a circular tube is shown in Equation 2, where η = viscosity and r = radius of the tube.

Equation 2$R=8\eta L\pi r4$

Equations 1 and 2 can be combined to provide the flow rate through a circular tube in terms of a pressure drop, which is described as Poiseuille's law:

Equation 3$Q=\pi r48\eta L\Delta P$

The differences in the rate of fluid flow depend on the radius of the anatomic structure, which is inversely proportional to the resistance. In addition, it is important to note the inverse r4dependence of the resistance to fluid flow. If the radius of the tube is halved, the pressure drop for a given flow rate and viscosity is increased by a factor of 16, because the flow rate is then proportional to the fourth power of the radius. The LDL-Density-Radius Theory (4) and the LDL-Density-Pressure Theory (5) provide the molecular hypothesis of the role of lipids in the differentiation of valve myofibroblasts into osteoblast-like cells responsible for the calcifying phenotype. Expression of the calcification in the coronary artery (8) occurs at a faster rate than the aortic valve secondary to the effect of the radius in these 2 anatomic locations in the heart.

The present study (3) measured the level of calcification, and the results correlate the LDL concentration, LDL receptor gene expression, and finally a Mendelian randomization analysis to suggest a causal role of LDL-C in beginning aortic valve pathology. The first case report to demonstrate by histology the presence of atherosclerosis in the aortic

valve is in a post-mortem analysis of a patient's aortic valve who had the diagnosis of familial hypercholesterolemia (9).

If atherosclerosis is an initiating event in this patient population, would lipid-lowering strategies be effective for the slowing of disease progression? In the present study (3), patients with he-FH were exposed to extremely high levels of LDL before statin treatment, especially those with mutational he-FH. Since these patients were first diagnosed with hypercholesterolemia, they have been treated with statins; this approach dramatically lowered LDL-C levels and thereby reduced the predictive value of LDL-C toward AoVC. The authors (3) proposed that the benefits of the statins are for patients who received the statins early in the atherosclerotic process (9), before the development of calcification and eventually severe stenosis. Furthermore, they hypothesized that the results of randomized controlled trials (6) that tested the effect of statins in CAVD may be due to the initiation of treatment in patients with advanced calcific disease.

In conclusion, the present study (3) is the first to combine biochemical analysis with genetic LDL receptor function and the calcifying phenotype in the heart. The study further confirms the hypothesis regarding the possible modification and slowing of CAVD progression with the use of long-term lipid lowering if the therapy is initiated in the early stages of pre-clinical CAVD, the atherosclerotic phase (9).

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Dr. Rajamannan is the inventor on a patent for methods to slow progression of aortic valve disease; the patent is owned by the Mayo Clinic, with no royalty payments to the inventor.

CHAPTER 5

Lipoprotein(a) levels are associated with aortic valve calcification in asymptomatic patients with familial hypercholesterolemia

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ABSTRACT

Objectives

Lipoprotein(a) [Lp(a)] is an independent risk factor for aortic valve stenosis and aortic valve calcification (AVC) in the general population. In this study, we determined the association between AVC and both plasma Lp(a) levels and apolipoprotein(a) [apo(a)] kringle IV repeat polymorphisms in asymptomatic statin-treated patients with heterozygous familial hypercholesterolaemia (FH).

Methods

A total of 129 asymptomatic heterozygous FH patients (age 40–69 years) were included in this study. AVC was detected using computed tomography scanning. Lp(a) concentration and apo(a) kringle IV repeat number were measured using immunoturbidimetry and immunoblotting, respectively. Univariate and multivariate logistic regression were used to assess the association between Lp(a) concentration and the presence of AVC.

Results

Aortic valve calcification was present in 38.2% of patients, including three with extensive AVC (>400 Agatston units). Lp(a) concentration was significantly correlated with gender, number of apo(a) kringle IV repeats and the presence and severity of AVC, but not with coronary artery calcification (CAC). AVC was significantly associated with plasma Lp(a) level, age, body mass index, blood pressure, duration of statin use, cholesterolyear score and CAC score. After adjustment for all significant covariables, plasma Lp(a) concentration remained a significant predictor of AVC, with an odds ratio per 10-mg dL1 increase in Lp(a) concentration of 1.11 (95% confidence interval 1.01– 1.20, P = 0.03).

Conclusion

In asymptomatic statin-treated FH patients, plasma Lp(a) concentration is an independent risk indicator for AVC.

Keywords

- aortic valve calcification, asymptomatic
- coronary artery calcification
- familial hypercholesterolaemia
- lipoprotein(a)

INTRODUCTION

Aortic valve calcification (AVC), characterized by calcium deposition and thickening of the aortic valve, is a significant risk factor for aortic valve stenosis and cardiovascular disease (CVD). AVC prevalence in the elderly population (> 75 years old) is approximately 50% and 25% of them develop aortic valve stenosis [1]. AVC prevalence and severity are associated with coronary artery disease (CAD) [2], all-cause mortality [3] and aortic stenosis [4]. AVC shares several risk factors with atherosclerosis including age, male gender, dyslipidemia, smoking, hypertension, diabetes and obesity [5]. In heterozygous familial hypercholesterolemia (FH) patients, the onset and progression of valvular calcification are not completely explained by the above-mentioned risk factors, particularly not in statin-treated FH patients, whose low density lipoprotein (LDL)-cholesterol levels are markedly reduced, compared to the untreated condition. Additional risk factors for valvular calcification in these patients remain to be identified.

Lipoprotein(a) [Lp(a)] is an LDL-like particle with an extra protein named apolipoprotein(a) [apo)(a)], covalently bound to apolipoprotein B-100 (apoB). Lp(a) concentrations are largely genetically determined by variations in the kringle IV (KIV) repeat number in the LPA gene encoding for apo(a) [6]. The apo(a) size is inversely associated with plasma Lp(a) levels: KIV repeat numbers below 23 are associated with, on average, elevated plasma Lp(a) levels [6]. Elevated plasma Lp(a) levels are considered a major risk factor for CVD in the general population [7, 8] as well as in FH patients [9-11]. It has been suggested that Lp(a) mediates CVD via its effects on atherosclerotic stenosis, fibrinolysis and wound healing [12]. A recent genome wide association study reported an association of a SNP in the LPA gene and plasma Lp(a) concentrations with AVC and aortic stenosis across multiple ethic groups [13]. Moreover, plasma Lp(a) levels have been associated with calcific aortic valve disease and accumulation of apo(a) has been observed in early lesions of aortic valve stenosis [14-16].

The aim of this study was to investigate whether Lp(a) concentrations and the KIV repeat copy number variation are associated with AVC in a cohort of asymptomatic statin-treated heterozygous FH patients.

MATERIALS AND METHODS

Study population

Between February 2008 and June 2011, 145 patients with a diagnosis of FH were recruited from the outpatient clinic, Erasmus Medical Center, Rotterdam, The Netherlands. As previously described [17], the inclusion criteria for FH were: either patients with a documented LDL receptor mutation or patients with LDL-cholesterol > 95th percentile for gender and age in combination with either 1) the presence of typical tendon xanthomas in the patient or in a 1st degree relative or 2) LDL-cholesterol > 95th percentile for gender and age in a 1st degree relative or 3) proven coronary artery disease in a 1st degree relative under age of 60. Patients with previous symptomatic CVD or with symptoms suggestive of ischemic heart disease at the time of inclusion were excluded from the study. All participants provided written informed consent prior to inclusion (Reference number MEC 2007-183). Of 129 FH patients, plasma samples were available for Lp(a) measurement; these patients were included in this study.

Computed tomographic scanning and calcification score measurement

All computed tomographic (CT) scannings were performed using a dual-source CT scanner (Somatom Definition, Siemens Medical Solutions, Forchheim, Germany). AVC and coronary artery calcification (CAC) were determined as previously described [17]. The calcium scores were calculated and expressed as Agatston Units (AU) as previously described [18]. An AU score of more than 400 was regarded as an extensive calcification [19].

Lp(a) measurement

Venous blood samples were collected after an overnight fast. After centrifugation, plasma samples and buffy coats were collected and stored at -80oC until analysis. Lipid parameters were measured by standard laboratory techniques. Plasma Lp(a) concentrations were measured using a particle enhanced immunoturbidimetric assay, independently of apo(a) KIV repeats (Diagnostic System #171399910930) [20]. In the samples with low Lp(a) concentration, Lp(a) levels were determined using an enzyme-linked immunosorbent assay (ELISA) [21] that has a markedly lower detection limit.

Detection of apolipoprotein(a) KIV repeats

The number of apo(a) KIV repeats was determined by immunoblotting, using a volume

of plasma containing 30 ng apo(a) protein. Plasma samples were mixed with SDS gel-loading buffer and heated at 98oC for 5 minutes. Gel electrophoresis was performed under a reducing condition with SDS and a 1.75% agarose gel. Proteins were then transferred to a PVDF membrane (Millipore) using a semi-dry blotting system (Millipore Graphite Electroblotter II) at 13 V for 1 hour. The membrane was subsequently incubated with blocking buffer containing 1% Bovine Serum Albumin (BSA) for 1 hour at 38oC to reduce non-specific binding. Primary antibody incubation was performed using monoclonal antibody 1A2 against apo(a) KIV [22], followed by incubation with goat anti-mouse IgG antibody coupled to Horseradish peroxidase (HRP) (Pierce, 1:3000). Enhanced chemiluminescence (ECL) and visualization on film were used for detection. A mixture of human plasma samples with 5 isoforms of known number of apo(a) KIV repeats was used as reference material.

Statistical analysis

Data are presented as mean + standard deviation or median (Interquartile range, IQR) for continuous variables and as number (%) for categorical variables. Mann-Whitney U and Fisher's exact tests were performed to analyze the differences between 2 continuous and categorical parameters, respectively. The presence of AVC or CAC was defined as AVC or CAC score of more than 0 AU. The severity of AVC or CAC was expressed by the AU score as continuous variables. Apo(a) phenotypes were categorized into 2 groups: low molecular weight (LMW) apo(a) (KIV < 22 repeats) and high molecular weight (HMW) apo(a) (KIV > 22 repeats). When 2 apo(a) isoforms were detected in the immunoblot, the smaller isoform was used for categorization as discussed recently [6]. Hypertension was defined as a systolic blood pressure (SBP) above 140 mm Hg or a diastolic blood pressure (DBP) above 90 mm Hg or those who were receiving antihypertensive therapy at the time of inclusion. Cholesterol-year score, a measurement of life-long cholesterol burden, was calculated using the formula (untreated total cholesterol x years without statins) + (statin-treated total cholesterol x years with statins) [23]. Correlations between Lp(a) concentrations and other variables were determined using Spearman correlation test. In order to account for different levels of CAC score, regression models were fitted on Lp(a) concentration, the presence and the severity of AVC separately, using CAC score as an independent variable. Subsequently, the residuals were used to calculate Spearman correlation coefficient (r). Significant predictors for the presence of AVC (AVC score > 0) were identified using univariate logistic regression analysis. The variables entered to the model were classical risk factors for CVD or parameters previously suggested [24].

The independent association of Lp(a) concentrations with AVC was further investigated using multivariate logistic regression analysis with increasing numbers of significant AVC predictors from the univariate analysis. All data were analyzed using IBM SPSS v 21.0 software. Results were considered statistically significant at $p < 0.05$.

RESULTS

Characteristics of study population

The general characteristics of the study population are presented in table 1. The population consisted of 81 men and 48 women. The median age was 51 years (IQR = 46-59) (range from 40-69 years). The majority of patients (97.7%) was treated with statins. The median duration of statin-use was 7 years (IQR = 2-14) (range from 0-30 year). Data on calcification scores were available for all participants. A total of 50 patients (39.8%) had developed AVC, of which 3 (2.3%) patients had extensive calcification of more than 400 AU. Prevalence of CAC was 79.1% with 28 patients having developed extensive CAC. No bicuspid aortic valves were identified.

Plasma Lp(a) concentration and apo(a) KIV isoforms

The median plasma Lp(a) concentration was 26.7 mg/dL, ranging from 0.5 to 419.8 mg/dL (IQR = 8.1-63.9). Plasma Lp(a) levels were significantly higher in patients with AVC than in those without (43.4(10.6-105.0) vs. 24.5(5.5-49.1) mg/dL, $p = 0.02$). Thirty six (27.9%) patients had one or two isoforms of LMW apo(a) and 90 (69.8%) patients had only HMW apo(a) isoforms. We were unable to measure the apo(a) KIV repeat number of the other 3 patients. Therefore, the frequency of the LMW apo(a) phenotypes in the population was 0.29. The frequency of LMW apo(a) phenotypes was not significantly different between patients with and without AVC (31% vs. 27%, $p = 0.68$). As expected, patients with LMW apo(a) phenotypes had higher plasma Lp(a) concentrations than those with HMW apo(a) (91.5(26.6-129.8) vs. 18.0(4.2-41.2) mg/dL, $p < 0.001$). In addition, the number of apo(a) KIV repeats was inversely correlated with Lp(a) concentrations ($r = -0.57$, $p < 0.001$). Lp(a) concentrations positively correlated with both the presence ($r = 0.21$, $p = 0.02$) and the severity ($r = 0.19$, $p = 0.04$) of AVC.

Table 5.1 | General characteristics of the study population

n	129
Male (n, %)	81 (62.8%)
Age (y)	51 (46-59)
Systolic blood pressure (mmHg)	129±12
Diastolic blood pressure (mmHg)	80±8
BMI (kg/m2)	26.3±3.7
Total cholesterol (mmol/L)	5.47±1.41
LDL-cholesterol (mmol/L)	3.49±1.24
HDL-cholesterol (mmol/L)	1.38±0.37
Triglyceride (mmol/L)	1.05 (0.78-1.55)
Fasting blood sugar (mmol/L)	5.31±0.69
Lipoprotein(a) (mg/dL)	26.7 (8.1-63.9)
Statin medication (n, %)	126 (97.7%)
Duration of statin use (y)	7 (2-14)
Previous and current smoker (n, %)	33 (25.6%)
Hypertension (n, %)*	34 (26.4%)
Bicuspid aortic valve (n, %)	0 (0%)
Aortic valve calcium (n, %)	
0 AU	79 (61.2%)
> 0 – 100 AU	38 (29.5%)
> 100 – 400 AU	9 (7.0%)
> 400 AU	3 (2.3%)
Coronary artery calcium (n, %)	
0 AU	27 (20.9%)
> 0 – 100 AU	45 (34.9%)
> 100 – 400 AU	29 (22.5%)
> 400 AU	28 (21.7%)

* Hypertension: SBP >140mmHg and/or DBP > 90 mmHg or the use of antihypertensive medication.

Association between Lp(a) and CVD-related parameters

Lp(a) concentrations were positively correlated with gender (r = 0.20, p = 0.02), as female FH patients had higher Lp(a) concentrations than male patients (35.5(11.7-74.8) vs. 19.7(4.3-50.5) mg/dL, p = 0.02). In contrast, no significant associations were found between Lp(a) concentrations and the presence (r = 0.05, p = 0.56) or severity (r = 0.09, p = 0.32) of CAC. After adjustment for CAC, Lp(a) concentrations remained positively correlated with the presence of AVC (r = 0.19, p = 0.03) but not with the severity of AVC (r

= -0.02, p = 0.82). No significant correlations were found between Lp(a) concentrations and age, BMI, blood pressure, smoking or other lipid parameters.

Association of AVC with Lp(a) and other CVD parameters

Next we analyzed the significant predictors for the presence of AVC using logistic regression analysis (table 2). In a univariate model, age, BMI, blood pressure, duration of statin use, CYS, CAC and Lp(a) concentrations were found to be significant predictors for AVC.

Table 5.2 | Univariate logistic regression analysis of the presence of aortic valve calcification (AVC) in patients with familial hypercholesterolemia (FH).

	Odds ratio	95%CI	p
Age (year)	1.10	1.04-1.16	<0.001
Gender (male)	1.67	0.79-3.55	0.18
BMI (kg/m^2)	1.11	1.01-1.23	0.04
Systolic blood pressure (mmHg)	1.06	1.02-1.10	0.001
Diastolic blood pressure (mmHg)	1.05	1.01-1.11	0.03
Total cholesterol (mmol/l)	1.15	0.89-1.49	0.28
LDL-cholesterol (mmol/l)	1.34	0.98-1.85	0.07
HDL-cholesterol (mmol/l)	0.90	0.35-2.32	0.82
Triglyceride (mmol/l)	1.04	0.69-1.56	0.86
Fasting blood glucose (mmol/l)	1.26	0.76-2.10	0.37
Lipoprotein(a) (per 10 mg/dL)	1.12	1.04-1.20	0.004
LMW apo(a) phenotype	1.23	0.56-2.72	0.60
Duration of statin use (year)	1.08	1.02-1.13	0.004
Previous and current smoker	0.87	0.38-1.97	0.73
Hypertension	1.35	0.61-3.00	0.46
CYS	1.006	1.003-1.01	<0.001
CAC score (AU)	1.004	1.004-1.005	<0.001

* Hypertension: SBP >140mmHg and/or DBP > 90 mmHg or the use of antihypertensive medication; CYS: cholesterol-year score, was calculated using the formula: Untreated total cholesterol x years without statin treatment + statintreated total cholesterol x years treated with statins; CAC: coronary artery calcification For each continuous parameter, odds ratio was calculated per 1 unit increase, except for Lipoprotein(a).

The independent association of Lp(a) concentration with AVC was analyzed using multiple logistic regression analysis with increasing number of parameters for adjustment (table 3). As age, CYS and duration of statin-use were highly mutually correlated, CYS was used

as a representative of the 3 parameters. The association between Lp(a) concentration and AVC was not influenced by CYS and/or CAC adjustment. Moreover, after adjustment for all significant determinants of AVC from the univariate model (age, BMI, SBP, DBP, duration of statin use, CYS and CAC score), Lp(a) concentrations remained a significant predictor for AVC. A 10 mg/dL-increase in Lp(a) concentration was associated with an 11% increased risk of developing AVC (95%CI = 1.01-1.20, p = 0.03).

Table 5.3 | Adjusted association of Lp(a) concentration with aortic valve calcification (AVC) in patients with familial hypercholesterolemia (FH).

Adjusted for	Odds ratio[5*]	95%CI	p
CYS	1.10	1.02-1.20	0.02
CAC score	1.14	1.05-1.23	0.002
CYS and CAC score	1.13	1.04-1.22	0.005
Age, BMI, SBP, DBP, Duration of statin use, CYS and CAC score	1.11	1.01-1.20	0.03

Odds ratios were calculated per 10 mg/dL increase of Lp(a) concentration, CYS: cholesterol-year score, CAC: coronary artery calcification, SBP: systolic blood pressure, DBP: diastolic blood pressure

In clinic, Lp(a) cutoff values of 30 or 50 mg/dL are used. Plasma Lp(a) concentrations over 30 mg/dL were not significantly associated with an increased risk of AVC (OR(95%CI) = 1.80(0.88-3.70), p = 0.11). Plasma Lp(a) concentrations above 50 mg/dL were associated with a 2.57-fold increased risk of AVC (95%CI = 1.20-5.52, p = 0.02). However, after adjustment for parameters selected from the univariate analysis, the association no longer reached statistical significance (OR(95%CI) = 2.03 (0.80-5.18), p = 0.14).

DISCUSSION

Little is known about the development of valvular calcification in patients with heterozygous FH. The exposure to classical risk factors is not a sufficient explanation for the development of AVC in these statin-treated patients. In the present study, we report, for the first time, a significant association of plasma Lp(a) concentrations with AVC in asymptomatic statin-treated heterozygous FH patients. After adjustment for age and other CVD-related parameters, Lp(a) concentration was still associated with AVC, suggesting that Lp(a) might be used as an independent risk marker for AVC in these

patients. Our results are in line with previous findings showing that Lp(a) concentrations might have a casual role in AVC development [13], which may result in symptomatic end-stage aortic valve disease [12, 15, 25, 26].

Statin treatment has substantially improved the prognosis of patients with heterozygous FH [27]. Nonetheless, we observed impressive CAD in asymptomatic, long-term statin-treated FH patients [28]. It might be that additional risk factors that are independent of LDL levels determine the residual CVD risk in treated FH. In the Jupiter trial, statin negligibly influenced the average Lp(a) concentrations, whereas Lp(a) levels determined the residual risk [29]. In our treated FH patients, we found that Lp(a) levels were associated with AVC but not with CAC. It is, therefore, tempting to speculate that Lp(a) contributed to the residual CVD risk of treated FH through AVC and subsequent aortic valve stenosis.

It is well-established that plasma Lp(a) concentrations are largely genetically determined by the variation in the number of KIV repeats that are based on genetic variants at the LPA locus. However, we cannot exclude that Lp(a) is solely an associated factor for AVC in the present study. In the future, a large genetic study, i.e. a Mendelian randomization approach, is required to investigate the causality. Interestingly, LMW apo(a) phenotypes were not significantly associated with AVC, despite the strong correlation between apo(a) phenotypes and Lp(a) concentration. Moreover, the frequency of LMW apo(a) is similar in patients with and without AVC. We cannot exclude that this is a chance finding, because 14 of the 29 patients who expressed an LMW isoform also expressed an HMW isoform. The large group of mixed isoforms reduces the power for detecting an association between the specific LMW isoform and AVC. To study this association in FH a larger cohort is required. Alternatively, the LMW apo(a) phenotype was previously found to be a strong predictor for CVD outcomes [8, 30, 31], apo(a) KIV repeat number was not significantly associated with aortic valve stenosis in the general population [12].

As CAC and AVC have several pathological similarities and share risk factors [32-34], and CAC had a strong predictive value towards AVC [35, 36], we tested whether Lp(a) concentration was generally associated with calcification or specifically associated with calcification at the aortic valve. Interestingly, Lp(a) was associated only with AVC but not CAC in our patients. Moreover, the association between Lp(a) concentration and AVC was not influenced by the adjustment for CAC. These results suggest that high Lp(a) levels might point at FH patients specifically at risk for AVC. How Lp(a) is involved in the mechanism of AVC development remains to be established.

Limitation

The capability of the CT scan to detect small calcified plaques is limited [37], therefore the presence of AVC might be underestimated. Moreover, we included only middle-aged, asymptomatic statin-treated FH patients. The results may be different in symptomatic, elderly or untreated patients. Replication in a larger group of FH patients is required to confirm and extend our findings.

Clinical implication

AVC and aortic valve stenosis are a major risk factor for CVD. However, no efficient treatment other than aortic valve replacement is available. With this study, we provide evidence that plasma Lp(a) concentrations may be a clinically useful risk factor of AVC and subsequent residual CVD risk assessment in heterozygous FH patients. Although statin treatment has been found to reduce AVC in vitro and in vivo [38, 39], it does not affect the clinical outcome of AVC [40, 41], and we speculated that this might be explained by the fact that statins do not reduce Lp(a) concentrations [29, 42]. In contrast, the progression of CAC is attenuated by statins, which is mainly the result of the cholesterol-lowering effect of statins [43, 44]. As the association between Lp(a) and AVC found in this study was independent of cholesterol-year score (CYS), therapeutic strategies to lower plasma Lp(a) concentration may be a potential treatment for AVC in FH patients. Notably, the commonly used Lp(a) cut-off points of 30 and 50 mg/dL (80th percentile) were not independently associated with AVC. These cut-off values were suggested based on the association between Lp(a) and myocardial infarction [45, 46]. However, data to support a clear Lp(a) cut-off point associated with increased AVC are not available and need further investigation. In addition, FH patients have higher plasma Lp(a) levels than the general population [47, 48]. Therefore, thresholds have to be validated in this particular high risk group to be discriminative. In our population, an Lp(a) higher than the 80th percentile (~80 mg/dL) was independently associated with an increased risk of AVC presence (data not shown). On the other hand, based on our results, lowering Lp(a) concentration is expected to have a limited effect on the development of CAC in asymptomatic FH patients.

In conclusion, we report a significant association between plasma Lp(a) concentration and AVC in asymptomatic statin-treated FH patients, independently of age and other CVD-related risk factors. Although AVC and CAC were strongly mutually associated, Lp(a) concentration was not correlated with CAC, suggesting a specific role of Lp(a) in AVC in these patients.

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5

CHAPTER 6

Lipoprotein (a) levels are not associated with carotid plaques and carotid intima media thickness in statin-treated patients with familial hypercholesterolemia

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ABSTRACT

Background

Lipoprotein (a), also called Lp(a), is a cardiovascular disease (CVD) risk factor. Statins do not lower Lp(a), this may at least partly explain residual CVD risk in statin-treated patients with familial hypercholesterolemia (FH). We investigated the association of Lp(a) levels with atherosclerosis in these patients.

Methods and results

We performed ultrasonography in 191 statin-treated FH patients (50% men; 48±15 years) to detect carotid plaques and determine carotid intima-media thickness (C-IMT). Patients with high versus low Lp(a) levels (≤ 0.3 g/L) had similar plaque prevalence (36 and 31%, p=0.4) and C-IMT (0.59±0.12 and 0.59±0.13 mm, p=0.8). Patients with and without plaques had similar Lp(a) levels (median 0.35 (IQR: 0.57) and 0.24 (0.64) g/L, respectively, p = 0.4).

Conclusions

The Lp(a) levels were not associated with atherosclerosis in the carotid arteries of statin-treated FH patients. This suggests that adequate statin treatment delays carotid atherosclerosis in FH independently of Lp(a) levels.

Keywords:

- Carotid plaque presence
- Carotid intima media thickness
- Lipoprotein (a)
- Familial hypercholesterolemia
- Residual risk

INTRODUCTION

Familial hypercholesterolemia (FH) is the most common genetic disorder associated with premature cardiovascular disease (CVD). FH is caused by mutations in the LDLR, APOB or PCSK-9 gene [2-4]. FH patients have raised low-density lipoprotein (LDL)-cholesterol levels, which strongly increases the risk of premature CVD [5]. To reduce CVD risk, preventive statin therapy is indicated, but despite statin treatment some FH patients still develop CVD [6-8]. This residual risk might be partly explained by alternative risk factors like lipoprotein (a), also called Lp(a).

Lp(a) is a LDL-like protein with an apo(a) moiety, and Lp(a) levels are predominantly genetically determined [9]. High Lp(a) levels have been shown to explain residual CVD risk in FH patients as well as in the general population[10, 11], and are unaffected by statin therapy[12].

Atherosclerosis can be visualized by carotid ultrasonography as the presence of plaques and the intima media thickness (C-IMT), which are both associated with CVD[13-16]. Previous studies have shown an association between Lp(a) levels and C-IMT in subjects in the general population and in those with severe hypercholesterolemia [17, 18]. However, it is unknown whether Lp(a) levels are associated with carotid plaque presence and C-IMT in statin-treated patients.

The aim of this study is to assess whether Lp(a) levels are related to atherosclerosis depicted by carotid ultrasonography in statin-treated FH patients.

METHODS

Study Population

Between May 2012 and October 2014, FH patients were included from the outpatient cardio-genetics clinic at the Erasmus Medical Center in Rotterdam. FH was defined as a score ≥6 on 'The Dutch Lipid Clinic Network criteria' [19]. All patients were screened for mutations in the *LDLR*, *APOB* and *PCSK-9* genes. Patients with homozygous and compound heterozygous FH were excluded. Written informed consent for blood storage and the use of clinical data was obtained and approved by the local ethical committee (MEC-2012-309).

Blood analyses

Fasting blood was collected, processed the same day and EDTA plasma samples were stored at -80°C. Within 30 months Lp(a) levels were determined in once thawed samples using an apo(a)-size independent immunoturbidimetric assay (Lp(a) 21 FS, DiaSys, Holzheim, Germany). Other lipid levels were measured according to the standard laboratory techniques.

Carotid ultrasonography

All carotid ultrasound measurements were performed on the Panasonic CardioHealthStation (Yokohama, Japan), with a validated automated C-IMT capturing method [20] (figure 1). Subjects were examined in supine position with their head positioned in an angle of approximately 45 degrees. A plaque scan was performed by placing the transducer transversally, visualizing the common, internal, and external carotid artery. Plaques were defined as a local enlargement of the C-IMT of more than 50% of the surrounding C-IMT, or if the C-IMT was above 1.5 mm, and were scored as present or absent. The mean C-IMT was measured over 1 cm length, at least 0.5 cm proximal of the bifurcation in the common carotid artery. Both sides were measured in two angles: anterior (170°-190°), and lateral (right: 120°-145°; left: 210°-235°), as indicated in real time by the device (Figure 1). In the present study, the C-IMT indicates the mean of the left and right mean C-IMT.

Statistical analysis:

Data with a normal distribution were expressed as mean (±SD), and data with a skewed distribution as median (IQR). Groups were compared a Chi-Square test or ANOVA. Skewed data was logistically transformed.

High baseline Lp(a) was defined as > 0.3 g/L and low as ≤ 0.3 g/L. This level has been reported to be the approximate median Lp(a) level in the general population [21, 22].

The associations between Lp(a) and the presence of carotid plaques and C-IMT were examined by logistic and linear regression methods, respectively. Additionally, these analyses were repeated in a subgroup of those with an LCL-C above 4mmol/L. Finally, associations between other variables and the presence of carotid plaques and C-IMT were assessed.

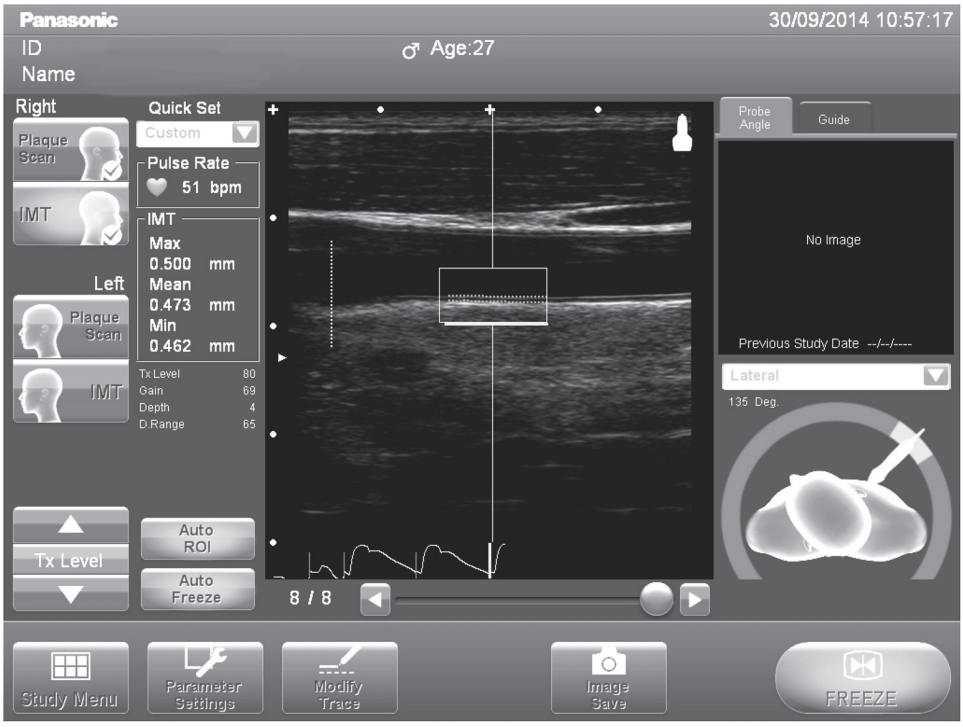

Figure 6.1 | Example of a C-IMT measurement of the Panasonic CardioHealthStation.

Output of the Panasonic CardioHealthStation showing one C-IMT measurement from the right common carotid artery from a lateral (135°) angle, over 1 cm. In the centre left side of the image results were displayed. The mean value displayed was combined with the other three measurements, and the mean of these four variables where used for analyses.

RESULTS

Data were collected from 191 FH patients. FH was genetically confirmed in 149 patients (78%), with mutations in the LDLR and APOB gene in 130 and 19 patients, respectively. *PCSK-9* gene mutations were not found. The patients were 48±15 years old, and 50% were men (Table 1). Plaques in the carotid arteries were present in 64 (33.5%) patients, and the C-IMT was 0.59±0.13 mm. The coronary calcium score, measured in a subgroup of 33 asymptomatic patients was 49 (344) Agatston units, and was not associated with carotid plaques (p=0.8), and C-IMT (p=0.737).

The Lp(a) levels ranged from 0.002 to 3.732 g/L with a median of 0.258 (0.620)g/L. We compared the patients with high and low Lp(a) levels (>0.3 g/L versus ≤ 0.3 g/L)

Table 6.1 | Baseline Characteristics

	Lp(a) >0.3g/L (n=91)	Lp(a) ≤0.3g/L (n=100)	p
Age (years)	50±16	46±15	0.072
Sex (male)	45 (49%)	51 (51%)	0.472
Body mass index (kg/m^2)	26.1±4.0	26.6±5.1	0.457
Systolic blood pressure (mmHg)	128±13	129±13	0.452
Diastolic blood pressure (mmHg)	78±8	78±9	0.842
Hypertension[1]	26 (28%)	28 (25%)	0.529
Diabetes mellitus[2]	4 (4%)	4 (4%)	0.586
Smoker (current and former)	35 (38%)	41 (41%)	0.469
Stigmata[3]	20 (22%)	21 (21%)	0.554
Alcohol abuse[4]	5 (5%)	9 (9%)	0.275
Inactivity[5]	29 (32%)	23 (23%)	0.087
Maximal intensity statin[6]	68 (75%)	69 (69%)	0.423
Years of statin treatment	10±8	10±7	0.815
Ezetimibe	36 (40%)	37 (37%)	0.415
LDL-R mutation	57 (63%)	73 (73%)	0.084
APOB mutation	11 (12%)	8 (8%)	0.242
No genetic confirmation	23 (25%)	19 (19%)	0.192
Positive family history[7]	61 (67%)	67 (67%)	0.446
Premature CVD[8]	18 (20%)	12 (12%)	0.106
Any CVD	18 (20%)	14 (14%)	0.191
Coronary calcium score (Agatston Units)[9]	50 (228) (n=20)	40 (436) (n=13)	0.882
Total Cholesterol (mmol/L)	5.00±0.94	5.09±1.15	0.567
HDL (mmol/L)	1.47±0.43	1.41±0.47	0.398
LDL (mmol/L)	3.20±0.85	3.23±1.02	0.794
Triglyceride (mmol/L)[9]	0.99 (0.64)	1.09 (0.94)	0.056

1 Defined as being diagnosed by physician or taking antihypertensive drugs;
2 Defined as being diagnosed by physician or taking anti-diabetic drugs;
3 Presence of Xanthoma/Xanthalasmata/arcus lipoides;
4 >2U alcohol/day ;
5 <30min of physical activity/day;
6 The use of Atorvastatin ≥ 40mg or Rosuvastatin ≥ 20mg or Sinvastatin 80mg;
7 Any CVD in a first or second degree relative;
8 CVD <55 years (men) or < 60 (woman);
9 Mean (IQR).

and we did not find significant differences in the baseline characteristics between these groups (Table 1). Plaque prevalence was similar in both groups: 33 (36%) vs 31 (31%) (p=0.4). There were no differences in C-IMT between the high Lp(a) group and the low Lp(a) group; 0.59±0.13 mm and 0.59±0.13mm (p=0.8). Qualitatively similar results were obtained when restricting the analyses to the mutation confirmed FH patients, or when other cut-off values of Lp(a) (0.5g/L and 1.0g/L, data not shown) were used. In the regression analyses, there were no statistically significant associations between Lp(a) levels and plaque presence (p=0.5) and C-IMT (p=0.4). These results did not change in the sub-analysis in those with a treated LDL-C above 4mmol/L. In addition, no other variable except age was associated with carotid plaques (p= <0.001), and C-IMT (p= <0.001). Finally, Lp(a) levels did not differ between patients with and without plaques present in the carotids: (0.349 (0.571) and 0.243 (0.641)) g/L, p = 0.4).

DISCUSSION

In our study, Lp(a) levels are not associated with presence of carotid plaque and C-IMT in statin-treated FH patients. This is unexpected since Lp(a) is a residual risk factor in treated FH patients[10, 11], and Lp(a) levels are associated with C-IMT [17, 18]. As our FH patients received aggressive long-term statin therapy, they may have effectively delayed carotid atherosclerosis and vessel wall thickening, such that the association with Lp(a) levels is no longer evident. In a previous study in a subgroup of our FH patients, Lp(a) is also not associated with subclinical coronary atherosclerosis, depicted by coronary calcium scoring [23].

An explanation for the lack of association between Lp(a) and carotid plaques is the relative young age of our FH patients. The prevalence of carotid plaques in our patients with mean age 48 years is 33.5%. Recent studies has shown a 43% prevalence in a Swedish population with a mean age 57 years [1], and a 42% prevalence in 61±10 year old healthy American multi-ethnic population [16]. In adverse plaques were increased in a group of young, newly diagnosed and mostly untreated, FH patients [24]. This suggest that our study group has a low prevalence of carotid plaques which might be due to the aggressive statin treatment.

The very low C-IMT did not associate with Lp(a). In healthy young men with mean age 29 years, a C-IMT of 0.54 mm is observed [25]. Additionally, the healthy controls of the ARIC study with a mean age of 56 years have a C-IMT of 0.60 mm [17]. Therefore, the

C-IMT in our study of aggressively treated FH patients seems in the range of a healthy population. This is important since in young, newly diagnosed and mostly untreated, FH patients the C-IMT values were increased [24]. At our outpatient clinic, we perform an active screening among relatives of FH patients**.** Early identification, a subsequent early start of aggressive statin treatment and patient education improving lifestyle may have resulted in a C-IMT within the normal range. Additionally coronary artery calcification does not associate with carotid plaques and C-IMT, even though we do see severe coronary lesions in some patients, which questions the clinical applicability of carotid sonography in statin-treated FH patients.

Lp(a) levels are not influenced by statins [12]. In line, we find large variations of the Lp(a) levels. Hence, statin treatment may compensate for the atherogenic effects of Lp(a) by either unknown mechanisms influencing of Lp(a) function or fully independent of Lp(a).

Although Lp(a) is not associated with subclinical atherosclerosis determined by C-IMT, Lp(a) levels might still contribute to residual CVD risk in treated FH patients. The relationship between Lp(a) and CVD risk may be effected via pathophysiological mechanisms other than atherosclerosis. Proposed mechanisms are wound healing and fibrinolysis, in which Lp(a) also plays a role [9, 22].

Limitations

Our study has some limitations. First, this study is an association study and therefore, we cannot prove or dismiss that Lp(a) has a causal effect in the residual CVD risk with this data. In addition, prospective ultrasound data was not available in these patients, and we cannot exclude that changes in plaque presence of C-IMT are associated with Lp(a). Another limitation is that carotid plaques are only scored categorical (present/absent), whereas plaque volume would be a more precise measurement. However, the device we used is not able to obtain 3D ultrasound plaque volume. Therefore, we cannot exclude different results when this 3D technique would have been used. In addition, we only used carotid ultrasound and did not look at other arteries liable to atherosclerosis. Therefore we cannot exclude different results if other arterial bed were examined.

Finally the Lp(a) measurement used is, like most commercially available measurements, not a fully KIV-2 independent measurement, which leads to an overestimation of the low Lp(a) levels and a underestimation of high Lp(a) levels. However, since our primary analyses uses a cut-off value near the median Lp(a) level, it is unlikely that our measurement method reclassified patients into the other group.

CONCLUSION

In statin-treated FH patients, we cannot prove that Lp(a) is a residual risk factor of CVD, since Lp(a) levels are not associated with the presence of carotid plaque presence and the C-IMT. This is most likely explained by the long-term adequate statin treatment resulting in a low C-IMT, and the relative low prevalence of plaques in the carotid arteries.

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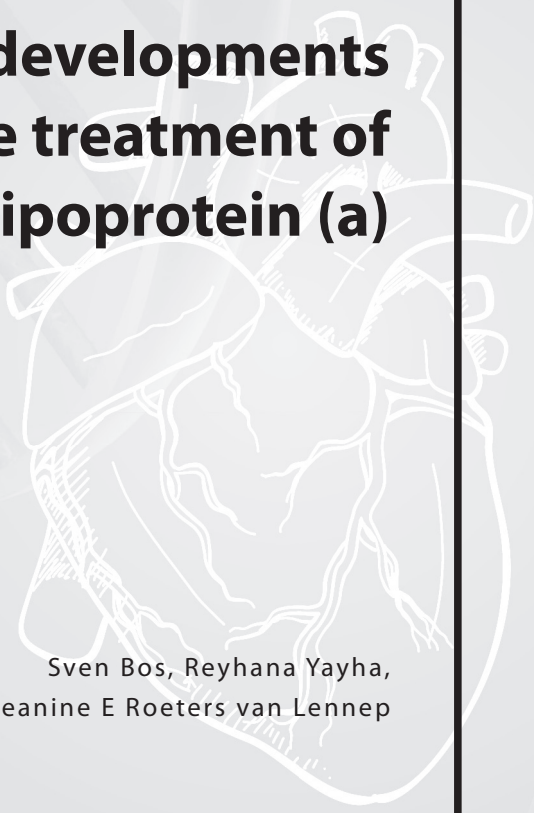
CHAPTER 7

Latest developments in the treatment of lipoprotein (a)



Sven Bos, Reyhana Yayha,
Jeanine E Roeters van Lennep

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.

Keywords (3-5)

- Lipoprotein (a)
- Treatment
- Cardiovascular disease
- Risk factor

INTRODUCTION

Lipoprotein (a) (Lp(a)) is an low density lipoprotein (LDL) like particle with an apolipoprotein (apo(a)) moiety attached to it (figure 1[1])[2]. 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 [3]. The size of the apo(a) is inversely related with plasma Lp(a) levels [3]. 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 [4-7]. 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.

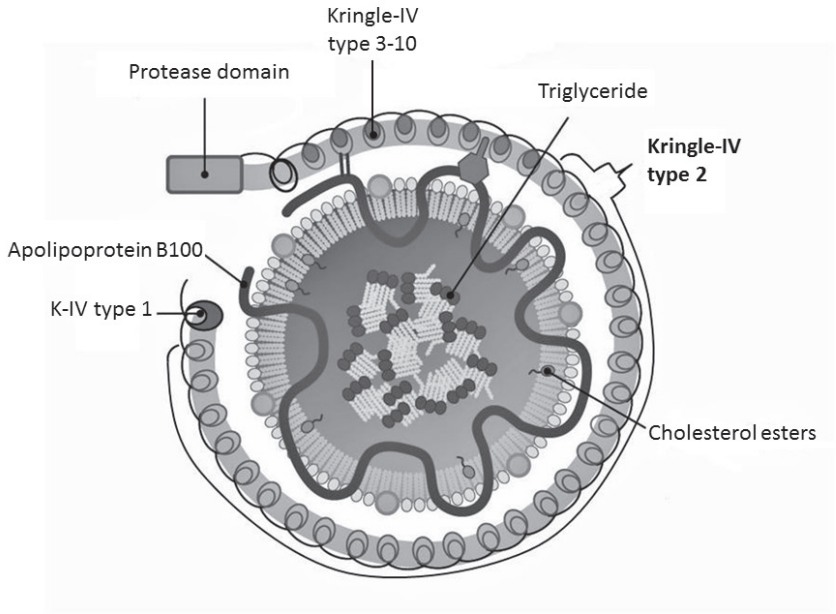


Figure 7.1 | Lipoprotein(a) particle.
Adapted from J E Roeters van Lennep and M T Mulder[1].

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[8, 9].

Studies on the influence of diet on Lp(a) have produced conflicting result for a long time, and it remains to be established if diet indeed modifies Lp(a) or not [9, 10]. 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 [11]. 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. [12], 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 [13]. 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 [13]. Although, the mechanism is uncertain Hoover-Plow and Menggui Huang proposed influence of estrogen on the LPA promoter[14]. 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 [15]. 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 [15]. 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 [15]. This effect seems to be synergistic to either statins or ezetimibe because administration of eprotirome as monotherapy does not influence Lp(a) levels [16]. 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 [17]. 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 [18, 19]. 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 [20, 21]. In the first study patients who were receiving a standard statin dose were switched to the maximum dosage of rosuvastatin, i.e. 40mg [20]. In this study, optimizing statin dose led to a decrease of LDL-C (23%), but did not show an effect on Lp(a)[20]. In the second study the effect of morning and evening dosages of simvastatin were compared, in previously untreated patients [21]. 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[21]. 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) [22]. Another indication for lipoprotein apheresis is Lp(a)-hyperlipoproteinemia (Lp(a) > 0,6g/L) with progressive CVD [23]. In these patients, who are adequately treated with statins, lipoprotein apheresis reduces Lp(a) by 70% directly post-treatment [22, 23], this led to a decrease of major adverse coronary events by 78% [22]. 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 [24]. 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 [25]. 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 [25]. 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 [25, 26]. 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% [27]. 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)[2]. However given the outcome of the recent RCT's it is questionable if this recommendation is correct [25, 28]. 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 [29].

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%) [30, 31]. 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 [30, 31]. 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-C 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 [32]. 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%) [32]. 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) [33]. 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[34]. Furthermore, an 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% [33]., 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[35]. 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 [36]. To inhibit PCSK-9 activity, monoclonal antibodies have been developed that specifically target the PCSK-9 protein [36-38]. 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% [36-38]. 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%)[39]. 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 [37]. 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% [40] [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.

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.

Table 7.1 | Overview of different drugs and their effects op Lp(a) levels

Compound	Effect Lp(a)*	Effect other lipoproteints	Dicussed 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 7.1 | Continued

Compound	Effect Lp(a)*	Effect other lipoproteints	Dicussed study on effect Lp(a)	Assay used in discussed study	Effect cardiovascular events	Conclusion
Lipoprotein Apheresis	Decrease 70% (directly post treatment) Decrease 26% (one week average)	Decrease LDL-C 67%	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

Table 7.1 | Continued

Compound	Effect Lp(a)*	Effect other lipoproteints	Dicussed study on effect Lp(a)	Assay used in discussed study	Effect cardiovascular events	Conclusion
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	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

Table 7.1 | Continued

Compound	Effect Lp(a)*	Effect other lipoproteints	Dicussed study on effect Lp(a)	Assay used in discussed study	Effect cardiovascular events	Conclusion
Anti-sense Apo(a)	Decrease 82%	Unknown	Presented by Tsimikas at the American heart Association 2014. [http://ir.isispharm.com/phoenix. tml?c=222170&p=irol-newsArticle&ID=1877550&highlight]	Unknown	Unknown	Decreases Lp(a) The only Lp(a) specific treatment

Lp(a), Lipoprotein (a); TG, Triglyceride; LDL-C, Low density lipoprotein-cholesterol; HDL, High density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; CETP, Cholesterol ester transfer protein; PCSK-9, proprotein convertase subtilisin/kexin type 9; FH, familial hypercholesterolaemia
*Important note: Percentage decrease in Lp(a) from baseline cannot be compared between studies when other measurement techniques are used.

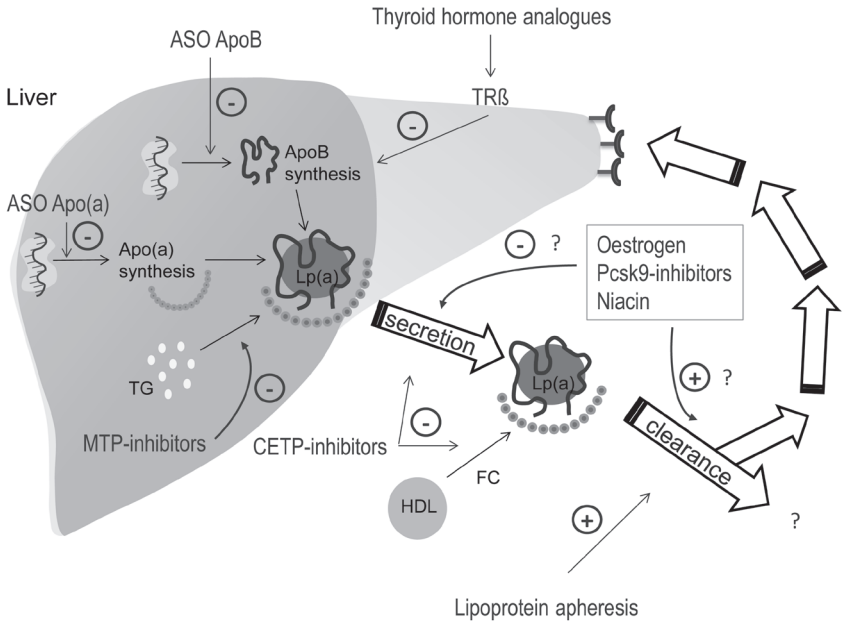


Figure 7.2 | Known and proposed mechanisms of compounds that lower Lp(a).

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[41].

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.

Key Points: (3-5 bullets in 1 sentence)

- Lp(a) is a risk factor of 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 available 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.

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Conflict of interest

There are no conflicts of interest

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7

CHAPTER 8

Novel protein biomarker predictors of coronary artery disease in statin-treated patients with familial hypercholesterolemia

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ABSTRACT

Background

Familial hypercholesterolaemia (FH) is the most common and serious monogenic disorder of lipid metabolism. The incidence of CAD varies among both treated and untreated FH patients.

Objective: The aim of the study was to utilise proteomics to identify novel protein biomarkers that differentiate genetically confirmed FH patients at high CAD risk from low CAD risk.

Methods

Sixty genetically confirmed FH patients were recruited and stratified into; (i) asymptomatic FH with low atherosclerotic burden (FH, n=20); (ii) asymptomatic FH with high atherosclerotic burden (FH + Ca, n=20); and (iii) FH with previously confirmed symptomatic CAD (FH + CAD, n=20).

Results

Six new potential proteins were identified; leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG). There were significant associations between gender and C4B (Z=2.31, p=0.021), C1QB (Z=2.49, p=0.013), CD14 (Z=2.17, p=0.03) and HRG (Z=2.14, p=0.033). There were significant associations between smoking and LRG1 (χ^2_2=6.59, p=0.037), CB4 (χ^2_2=7.85, p=0.02) and HRG (χ^2_2=6.11, p=0.047). All the peptides were significantly associated with progression of CAD, independently of age and smoking. However, the absence of the proteins was the strongest marker. The most accurate predictor of CAD was HRG (AUROC=0.922), while LRG1, C4B and C1QB were excellent predictors of CAD (AUROC>0.9). For prediction of either coronary atherosclerosis or CAD; LRG1, C4B, C1QB and HRG were relatively good predictors.

Conclusions

The present study has identified six novel protein biomarkers that are associated with atherosclerotic disease progression and subsequent coronary events in patients with FH.

Keywords:
- familial hypercholesterolaemia,
- proteomics,
- biomarkers,
- atherosclerosis,
- coronary artery disease

INTRODUCTION

Familial hypercholesterolemia (FH) is the most common and serious monogenic disorder of lipid metabolism[1, 2] with a worldwide prevalence of at least 1 in 300.[3] It is caused by mutations in the LDL receptor (*LDLR*) gene, the apolipoprotein B (*APOB*) gene, or the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene.[4-6] These mutations result in significantly elevated low-density lipoprotein (LDL) cholesterol levels that cause premature atherosclerotic coronary artery disease (CAD).[7]

However, FH remains a frequently under diagnosed cause of CAD, and of those diagnosed, many are inadequately treated.[8] In addition, the incidence of CAD and life expectancy varies among patients with both treated and untreated FH.[9-11] Untreated, 50% of male FH patients and 20% of female FH patients develop fatal coronary heart disease by 60 years of age. While treatment with statins more than halves the risk of coronary events in adults with FH,[12] treated asymptomatic FH patients display significant variability in the extent of subclinical coronary atherosclerosis despite the use of aggressive statin therapy.[9] Current known plasma biomarkers, in addition to classical risk factors, do not explain the residual CAD risk in people with FH. Indeed, the large variation in CAD incidence within the FH population suggests there are other factors, in addition to elevated cholesterol, that may play a role in development of atherosclerosis in FH. There is an urgent need for improved cardiovascular screening in asymptomatic individuals, however the development of novel markers to identify cardiovascular risk must add to the prognostic value provided by standard risk markers.[13, 14]

In the past decade, quantitative proteomic techniques including, isobaric tag for relative and absolute quantification (iTRAQ), have been used to identify novel biomarkers in several disease states, including CAD.[15, 16] Using isotope labelled molecules, iTRAQ allows for the quantification of multiple proteins from various sources, in a single experiment.[17] Previous iTRAQ studies have shown differences in expected CAD associated proteins,

including those involved in inflammation, coagulation and lipid metabolism,[15, 16] while other studies have identified novel predictors.[15] To date, no such study has investigated the use of iTRAQ proteomics in predicting CAD risk in a FH population.

Therefore, the aim of the present study was to utilise proteomics to identify candidate protein biomarkers that may differentiate genetically confirmed FH patients at high CAD risk from those with low CAD risk.

MATERIALS & METHODS

Study population

Sixty FH patients (40-70 yrs) from the Vascular Genetics Outpatient Clinic at the Erasmus MC were recruited. All participants had a genetically confirmed mutation in the *LDLR*-gene. The 60 patients were selected and stratified into 3 subgroups; (i) asymptomatic FH with a low atherosclerotic burden as defined a coronary diseased segment score of 0 (FH, n=20); (ii) asymptomatic FH with a high atherosclerotic burden as defined by a coronary diseased segment score >7 (FH + Ca, n=20); and (iii) FH with previously confirmed symptomatic CAD (myocardial infarction, percutaneous coronary intervention or coronary bypass surgery) (FH + CAD, n=20). Exclusion criteria included; a secondary cause of hypercholesterolaemia, and renal, liver and thyroid disease. Within the asymptomatic groups, additional exclusion criteria included; symptoms of CAD, history of CAD, renal insufficiency (serum creatinine >120 mmol/L), known contrast allergy and atrial fibrillation. The study was conducted in line with the Declaration of Helsinki. All patients gave a written informed consent and the study protocol was approved by the Erasmus MC Ethical Review Board.

Coronary CT Angiography (CCTA)

CCTA scan protocols and outcomes have previously been described.[18] Briefly, all asymptomatic FH patients underwent CCTA to determine their atherosclerotic burden. Scans were performed on a dual source CT scanner (Somatom Definition, Siemens Medical Solutions) and analysed separately by two experienced readers blinded to the patient's status. Coronary calcium was measured in Agatston units using dedicated software.[19] In addition, using a modified 17 coronary segment model[20] the percentage of maximum luminal diameter narrowing was visually estimated and graded as either; 0%, 1-20%, 21-50%, 51-70% or >70%. Based on the narrowing per segment, 3 scores were

then used; (i) the diseased segment score (DSS), granting 1 point for each narrowing >20%; (ii) the CAD severity score, granting 1, 2 or 3 points per segment narrowing of 21-50%, 51-70% and 70%, respectively; and (iii) the CAC extent score, granting 1, 2, 3 or 4 points per segment narrowing of 1-20%, 21-50%, 51-70% and >70%, respectively.

iTRAQ Proteomics

Isobaric tag for relative and absolute quantification (iTRAQ) was performed by Proteomics International (PI) on fasting EDTA plasma that had previously been stored at -80°C. The process involved an initial discovery phase followed by a validation phase. In the discovery phase the samples were depleted of the top 14 high abundance proteins, diafiltrated, reduced, alkylated and trypsin digested. The samples from each group were then labelled with iTRAQ reagents and combined to make a pooled sample (100 µL) for each individual group (FH, FH + Ca and FH + CAD) and an overall pooled sample (all 60 samples). Samples were then desalted on a Strata-X 33 µm polymeric reversed phase column (Phenomenex) and dissolved in buffer (10 mM KH_2PO_4, pH3 in 10% acetonitrile) before separation by strong cation exchange liquid chromatography (SCX, Agilent 1100 HPLC System) using a PolySulfoethyl column (4.6 x 100 mm, 5 µm, 300A). Peptides were eluted with a linear gradient of 0 – 400 mM KCl. Eight fractions containing the peptides were collected and desalted on Strata-X columns. The fractions were then analysed using electrospray ionisation mass spectrometry (Agilent 1260 Infinity HPLC system) coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer, before being loaded onto a ProtID-Chip-150 C18 column (Agilent) and separated with a linear gradient (water/acetonitrile/0.1% formic acid v/v).

In the validation phase, samples (20 µL) were again depleted of the top 14 high abundance proteins, diafiltrated, reduced, alkylated and trypsin digested. Samples were then desalted on a Strata-X 33 µm polymeric reversed phase column (Phenomenex) and analysed by electrospray ionisation mass spectrometry (LC/MS) using a Dionex UltiMate 3000 nanoflow HPLC system coupled to a 4000 Q-TRAP mass spectrometer (AB Sciex). Duplicate runs were performed for all samples. A 1 µL volume containing 1:1 (v/v) ratio of tryptic unlabelled and ^{18}O-labelled reference standard plasma peptides was then loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm column and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 90 mins. A reference plasma sample was used as a control to determine the representative peptides of the new proteins and as an ^{18}O-labelled reference standard for relative peptide quantification. MRM transitions for unlabelled and ^{18}O-labelled peptides were created and searched for

in the mass spectrometer. Peptide peak area ratio analysis was performed using Skyline software.

Statistical analysis

Categorical variables were described using percentages. The distributional characteristics of continuous variables were examined using tests for skewness and kurtosis.[21] Many were not normally distributed so all were described using the median and inter-quartile range (IQR). Univariable analysis of the association between the peptides and disease group was based upon the non-parametric Kruskal-Wallis test, and the Mann-Whitney test was used to examine dichotomous covariates. Correlation between continuous variables was assessed using the non-parametric Spearman rank-order correlation coefficient. Inflation of the critical significance level by multiple comparisons was addressed using the sequential rejection modification of the Bonferroni method developed independently by Holm and Simes.[22, 23] Subsequent inferential analysis was based upon the underlying model of the disease process as a sequence of increasingly severe stages, which are irreversible, viz. normal, calcification, CAD. The appropriate statistical model is the continuation ratio regression model[24] (OCR) which is a variation of the Cox proportional hazards model for discrete ordinal outcome data.[25] The results are provided as hazard ratios (HR) with 95% confidence intervals (95% CI). Covariates that had the potential to confound the analysis were also examined using the OCR model. In order to preserve statistical power and in keeping with the development of methods for analysis of observational data, a proximity score was estimated for each peptide following the recommendations of Little and Rubin[26] and Stuart.[27] Estimation of covariate adjusted HRs used the proximity score as a single covariate. Analysis using the receiver operating characteristic (ROC) curve was used to estimate the predictive accuracy of proteins that showed an association with disease progression. The methods developed by Pepe[28] were used to estimate the area under the ROC curve (AUROC) to compare the peptides with each other, and to examine alternative methods for combining the peptide results. Robust bootstrapped estimation of the standard errors was used to avoid overfitting. The same methods were used to estimate covariate adjusted AUROCs. It became apparent during the analysis that the association between the potential biomarkers and disease progression was negative and so an inverse transformation was used to prepare the ROC curves, resulting in axes which are reversed from the usual form. Because of the semi-continuous nature of the non-negative 'clumping at zero' measures of the proteins, analysis of the association between measures of CAD

severity and other disease characteristics with the proteins was conducted using a tobit regression model with truncation at zero.[29] Statistical significance was determined by a p-value less than 0.05 for the multivariate models. All analysis was conducted using the Stata package (Version 13.1, StataCorp, College Station, Tx, USA).

RESULTS

Patient characteristics

The patient characteristics are presented in Table 1. There was a significant difference in mean age between the groups in addition to a higher number of male patients. Approximately three quarters of the participants had a family history of CAD, while one third had known hypertension. There were no significant differences in fasting glucose levels between the groups (data not shown) and there were only 2 subjects with diabetes (both in the FH + Ca group). Thirty percent of the cohort were current or ex smokers and there were significantly more in the FH + CAD group (70% (46-86) vs. 30% (13-54), p=0.004). As expected, the FH patients with low atherosclerotic burden (FH) had a significantly lower mean calcium score compared with the FH patients with high atherosclerotic burden (FH + Ca) (1.96 (1.18, 3.26) v 590.4 (408.8, 582,8), p=0.0001).

Table 8.1 | Patient characteristics

	FH only	FH + Ca	FH + CAD	Total	p
N	20	19	20		
Age (yrs)	46.1 (43.6, 48.6)	55.2 (51.4, 58.9)	57.3 (52.3, 62.3)		0.0005
Gender	n (%)	n (%)	n (%)		
• Male	11 (32.4)	14 (41.2)	9 (26.5)	34 (100)	0.186
• Female	9 (36.0)	5 (20.0)	11 (44.0)	25 (100)	
Hypertension					
• No	19 (40.4)	12 (25.5)	16 (34.0)	47 (100)	0.047
• Yes	1 (8.3)	7 (58.3)	4 (33.3)	12 (100)	
Family History					
• No	5 (33.3)	5 (33.3)	5 (33.3)	15 (100)	0.994
• Yes	15 (34.1)	14 (31.8)	15 (34.1)	44 (100)	
Smoker					
• Never	11 (33.3)	16 (48.5)	6 (18.2)	33 (100)	0.001
• Former	6 (50.0)	3 (25.0)	3 (25.0)	12 (100)	
• Current	3 (21.4)	0 (0)	11 (78.6)	14 (100)	

Table 8.1 | Continued

	FH only	FH + Ca	FH + CAD	Total	p
BMI	24.9(23.7, 26.1)	27.2(25.1, 29.2)	26.3(24.9, 27.8)		0.17
Lipids					
• TC	5.39(4.92, 5.86)	5.38(4.87, 5.90)	4.91(4.46, 5.35)		0.39
• HDL	1.45(1.31, 1.60)	1.22(1.07, 1.36)	1.23(1.09, 1.38)		0.043
• TG	1.02(0.84, 1.22)	1.20(0.92, 1.57)	0.96(0.80, 1.16)		0.51
• LDL	3.36(2.89, 3.82)	3.62(3.16, 4.08)	3.25(2.89, 3.61)		0.57
Years on statins	8.9 (5.80, 12.0)	10.8 (7.29, 14.4)	10.5 (7.79, 13.1)		0.48
Medication					
• Oral antidiabetics	20 (100%)	19 (100%)	19 (95%)	58 (98%)	0.37
• Lipid lowering	20 (100%)	18 (100%)	20 (100%)	58 (100%)	
• RR lowering	3 (15%)	4 (22%)	15 (75%)	36 (62%)	<0.001
• Blood thinners	0 (0%)	3 (17%)	20 (100%)	23 (40%)	<0.001

BMI, body mass index; CAD, coronary artery disease; FH, familial hypercholesterolemia; HDL, high-density lipoprotein; LDL, low-density lipoproteins; RR, relative risk; RR lowering, blood pressure–lowering drugs; TC, total cholesterol; TG, trigylcerides.
Results presented as frequency (%) for categorical and mean (95% confidence interval) for continuous variables, respectively. Differences between the groups were assessed using analysis of variance with Holm-Simes post-hoc comparisons.

Identification of potential protein biomarkers

In the initial discovery phase, 164 proteins were detected from a total of 47,708 spectra. In the subsequent validation phase, all differentially expressed proteins were shortlisted and a list of common differentially expressed proteins was extrapolated. Of the 17 proteins identified, 9 were already available as existing PI assays. The remaining 8 new proteins were assessed in silico and representative peptides and transitions were determined for 4 of them, while representative peptides could not be determined for the remaining 4 proteins. The final list of 13 proteins was represented by 20 peptides and 106 transitions and from this a final 6 proteins were selected as potential biomarker candidates. These proteins were; leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG).

Distribution and associations of protein biomarkers

All of the 6 proteins displayed a bi-modal distribution and Figure 1 shows the median, inter-quartile range and range for each one. One sample in the FH + Ca group could not be analysed. There was significant rank-order correlation between the protein biomarkers,

with the exception of CD14. Not all proteins could be detected in a substantial proportion of participants; in particular, monocyte differentiation antigen (CD14) was not detected in more than 75% of the participants, including none of the FH + CAD patients.

Within the whole cohort, there were significant associations between gender and C4B (Mann-Whitney Z=2.31, p=0.021), C1QB (Z=2.49, p=0.013), CD14 (Z=2.17, p=0.03) and HRG (Z=2.14, p=0.033). There were also significant associations between smoking and LRG1 (Kruskal-Wallis χ^2_2=6.59, p=0.037), CB4 (χ^2_2=7.85, p=0.02) and HRG (χ^2_2=6.11, p=0.047). There were no significant associations between any of the protein biomarkers and age or lipid levels.

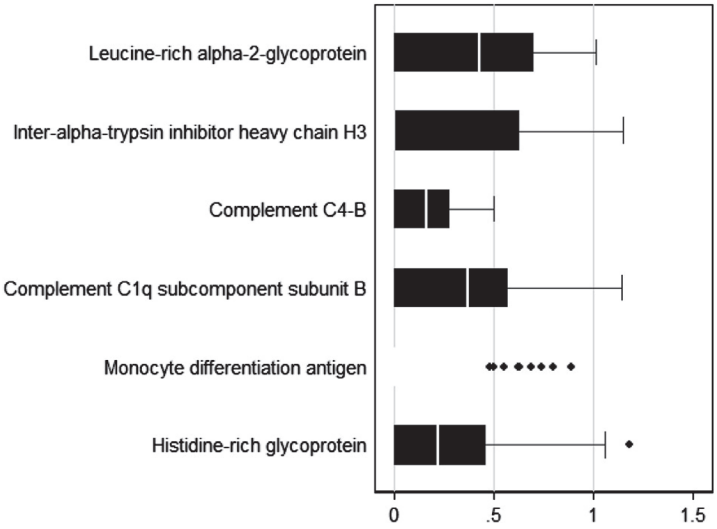

Figure 8.1 | Relative abundance of plasma protein biomarkers in all patients with familial hypercholesterolemia.

Analysis of protein biomarkers as indicators of CAD

Table 2 shows the results of the OC regression analysis for the level of each protein and also for the total number of proteins that could be detected. Crude HRs were generally lower than the adjusted HR, which reflects the increase in risk of coronary atherosclerosis and CAD with increasing age and with smoking. Two p values are shown in the table, the first tests the null hypothesis that the HR across the groups is equal (HR=1) and the second tests the null hypothesis that the change in the HR is the same for each transition from FH to FH + Ca to FH + CAD. Overall, it is clear that all of the peptides were significantly associated with progression of CAD, independently of age and smoking exposure, using a critical p value adjusted for multiple comparisons.

Table 8.2a | Univariate CAD HR models for group by each peptide

Peptide	Hazard ratio	95% CI for HR		p^1 HR=1	p^2 HR=For each group
LRG1	0.054	0.017	0.170	<0.001	0.302
ITIH3	0.213	0.094	0.481	<0.001	0.054
C4B	0.005	0.000	0.050	<0.001	0.020
C1QB	0.069	0.021	0.230	<0.001	0.334
CD14	0.149	0.049	0.458	0.001	0.145
HRG	0.132	0.048	0.365	<0.001	0.001
Peptides detected	0.054	0.017	0.170	<0.001	0.302

Table 8.2b | Multivariable CAD HR models for each peptide adjusted for age and smoking status

LRG1	0.102	0.028	0.377	0.001	0.003
ITIH3	0.292	0.123	0.692	0.005	0.111
C4B	0.027	0.002	0.416	0.010	<0.001
C1QB	0.142	0.034	0.601	0.008	0.027
CD14	0.222	0.058	0.850	0.028	0.199
HRG	0.262	0.086	0.794	0.018	<0.001
Peptides detected	0.102	0.028	0.377	0.001	0.003

Results are presented as hazard ratios (HR) with 95% Confidence Intervals (95% CI) using continuation ratio regression model before and after adjustment for age and smoking. All of p^1 were statistically significant after correction for multiple comparisons.

LRG1 – leucine-rich alpha-2-glycoprotein, ITIH3 – inter-alpha-trypsin inhibitor heavy chain H3, C4B – Complement C4-B, C1QB – Complement C1q subcomponent subunit B, CD14 – Monocyte differentiation antigen, HRG – histidine-rich glycoprotein

However, the analysis also showed that it was the absence of the proteins that was the strongest indicator and this is demonstrated in Table 3 where it can be seen that for all proteins, other than CD14, there are few patients in the FH only group who don't show the protein and there are few in the FH + CAD group who do show the protein.

Results of the association between the amount of protein and other CAD severity markers are shown in Table 4. It is clear that the proteins are not associated with clinical indicators of CAD severity. A tobit truncated regression analysis demonstrated a significant negative association with the Framingham points score for C4B (coefficient=-11.4, p=0.031), C1QB (coefficient=-6.99, p=0.011) and HRG (coefficient=-4.83, p=0.033) in the FH and FH + Ca patients (the only patients for which this was appropriate).

Table 8.3 | Associations between protein biomarkers and CAD severity in FH patients.

		LRG1	ITIH3	C4B	C1QB	CD14	HRG
Disease severity score	rho	-0.078	0.140	-0.028	-0.117	-0.180	-0.060
	p	0.636	0.395	0.868	0.479	0.274	0.717
CAD severity score	rho	-0.094	0.122	-0.025	-0.137	-0.181	-0.084
	p	0.569	0.460	0.879	0.405	0.269	0.610
SIS score	rho	-0.107	0.125	-0.066	-0.172	-0.189	-0.100
	p	0.519	0.447	0.692	0.296	0.248	0.544
CAD extent 05	rho	-0.146	0.037	0.014	-0.146	-0.125	-0.026
	p	0.377	0.821	0.934	0.377	0.447	0.874
CAD extent 04	rho	-0.144	0.038	0.015	-0.143	-0.125	-0.026
	p	0.381	0.819	0.928	0.384	0.447	0.877

Univariate analysis of the association between peptides and CAD severity., LRG1 :Leucine-rich alpha-2-glycoprotein, ITIH3 :Inter-alpha-trypsin inhibitor heavy chain H3, C4B :Complement C4-B, C1QB :Complement C1q subcomponent subunit B, CD14 :Monocyte differentiation antigen, HRG :Histidine-rich glycoprotein, Rho: Spearmans's rho rank-order correlation coefficient, p: Significance for the H0 test that rho=0.

A logistic regression analysis comparing these three proteins and the Framingham score indicates that for C4B and C1QB, the Framingham score is superior as an indicator of arterial calcification. With HRG we found a significant interaction with the Framingham score so that together the two measures provide enhanced discrimination between asymptomatic FH patients and those with calcification (HRG: p=0.011, Framingham: p=0.007 and interaction: p=0.014, AUC=0.858).

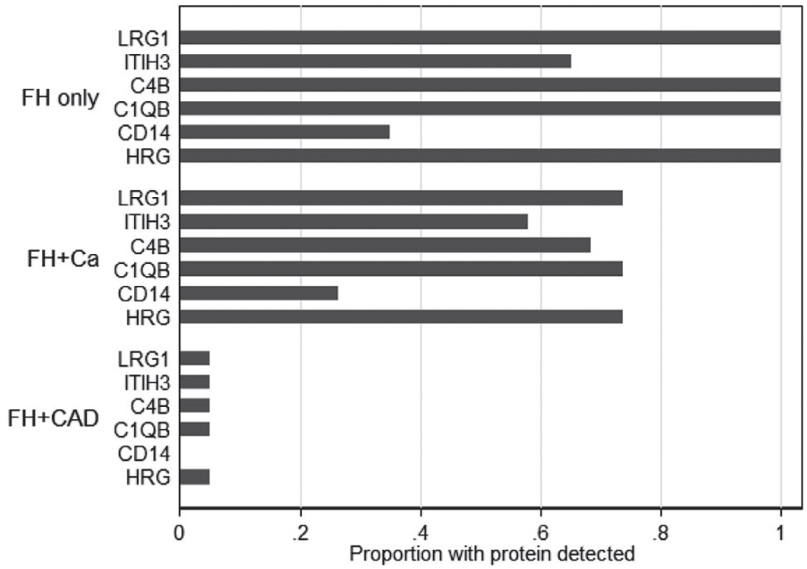

Figure 8.2 | Proportion of patients within each disease severity group with any of the indicator proteins detected.

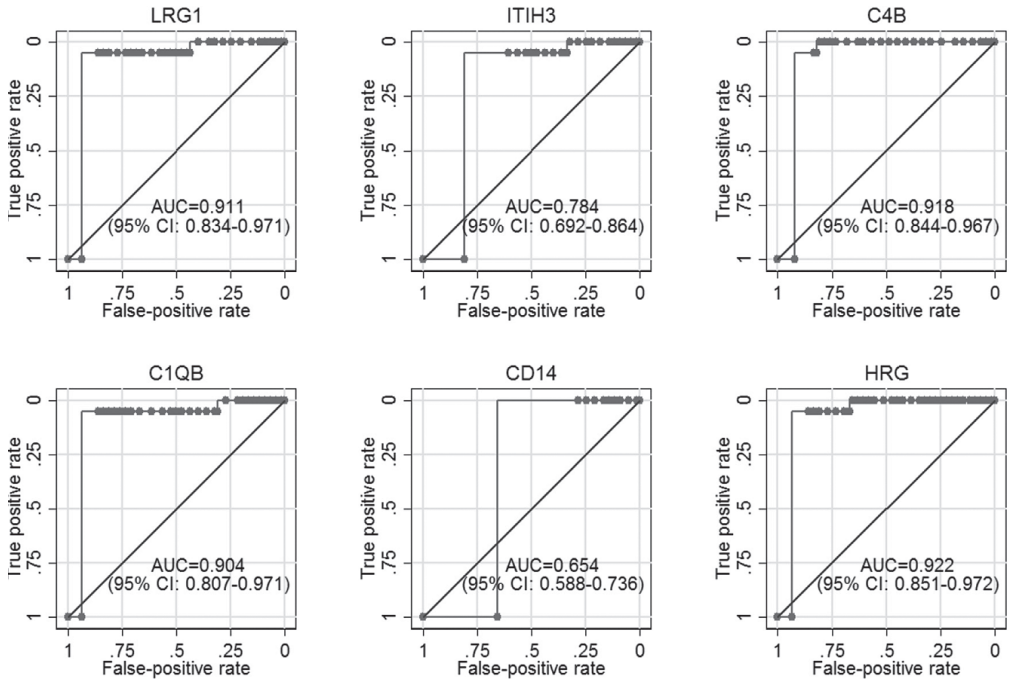

Figure 8.3 Area under the ROC (AUROC), estimating the predictive accuracy of each protein with CAD progression.

AUC: Area under the curve; C1QB: Complement C1q subcomponent subunit B; C4B: Complement C4-B; CAD: Coronary artery disease; CD14: Monocyte differentiation antigen; CI: Confidence interval; HRG: Histidine-rich glycoprotein; ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3; LRG1: Leucine-rich alpha-2-glycoprotein.

As might be expected, there are significant associations between treatment and disease progression for both RR-lowering drugs ($p<0.001$) and blood thinners ($p<0.001$) (Table 6). Both treatments are also associated with each other and they show a significant association with the proteins of interest in this study. In addition, the RR-lowering drugs but not the blood thinners show significant interactions with LRG1, ITIH3, C4B and C1QB but not with CD14 or HRG (Table 5).

An analysis of the AUROC for FH + CAD for each protein, comparing the protein as measured with the protein 'detectable' vs. 'not detectable' showed there was little difference between the two (Table 5). This analysis includes the AUROC for the total number of peptides detected and whether any of the peptides were detected. The most accurate indicator of CAD is HRG (as measured) with an AUROC of 0.922 (0.862, 0.983), but there were a number of other proteins that were very similar (see Figure 2).

Table 8.4 | Tests comparing ROC AUC

Predictor of CVD	AUC	95% CI for AUC		s.e.
		LCL	UCL	
LRG1 (as measured)	0.911	0.838	0.984	0.037
LRG1 (zero vs. more than zero)	0.911	0.839	0.983	0.037
ITIH3 (as measured)	0.784	0.693	0.875	0.046
ITIH3 (zero vs. more than zero)	0.783	0.691	0.874	0.047
C4B (as measured)	0.918	0.857	0.979	0.031
C4B (zero vs. more than zero)	0.898	0.823	0.974	0.039
C1QB (as measured)	0.905	0.823	0.986	0.041
C1QB (zero vs. more than zero)	0.911	0.839	0.983	0.037
CD14 (as measured)	0.654	0.580	0.727	0.037
CD14 (zero vs. more than zero)	0.654	0.580	0.727	0.037
HRG (as measured)	0.922	0.862	0.983	0.031
HRG (zero vs. more than zero)	0.911	0.839	0.983	0.037
Total peptides detected	0.912	0.840	0.983	0.037
Any peptides detected	0.911	0.839	0.983	0.037

Predictive accuracy of proteins with disease severity using area under the ROC (receiver operating curve), to compare peptides with each other.
AUC: Area under curve, LCL: Lower confidence level, UCL: Upper confidence level, s.e.: Standard error, LRG1: Leucine-rich alpha-2-glycoprotein, ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3, C4B: Complement C4-B C1QB: Complement C1q subcomponent subunit B, CD14: Monocyte differentiation antigen, HRG: Histidine-rich glycoprotein

Overall ROC analysis revealed LRG1, C4B and C1QB were excellent indicators of CAD in patients with FH (AUROC > 0.9), with ITIH3 only a relatively good indicator. In addition, the total number of proteins detected and whether any of them were detected was also excellent. For indication of either coronary atherosclerosis or CAD; LRG1, C4B, C1QB and HRG were relatively good (Figure 3). Although C1QB is a possible indicator, none of the protein biomarkers were considered to be good for indicating coronary atherosclerosis alone. When the AUC is adjusted for the use of RR-lowering drugs, bootstrapped estimates of the AUC showed that CD14 and ITIF3 are not accurate indicators of CAD when adjusted for the presence of RR-lowering drugs, but HRG (AUC=0.920, 0.846-0.974) and C4B (0.916, 0.837-0.961) are excellent indicators of CAD and are little influenced by RR-lowering drugs.

Table 8.5 | Associations between protein biomarkers, treatment and disease progression

	HR	$LCL_{95\%}$	$UCL_{95\%}$	P^2	AUC for CAD (CI)
RR lowering drugs alone	5.04	2.18	11.7	<0.001	0.783 (0.668-0.897)
Blood thinner drugs alone				<0.001	
LRG1	0.188	0.053	0.668	0.01	0.907(0.818-0.960)
RR lowering drugs	18.9	2.12	168	0.008	0.783 (0.682-0.899)
Protein/drug interaction	0.005	0.0001	0.221	0.006	
ITIH3	0.555	0.207	1.48	0.241	0.778 (0.688-0.868)
RR lowering drugs	11.1	2.86	43.0	<0.001	0.783 (0.682-0.899)
Protein/drug interaction	0.044	0.005	0.405	0.006	
C4B	0.027	0.002	0.464	0.013	0.916 (0.837-0.961)
RR lowering drugs	9.34	2.04	43.0	0.004	0.783 (0.645-0.884)
Protein/drug interaction	0.000	0.0001	0.001	0.001	
C1QB	0.135	0.03	0.608	0.009	0.902 (0.815-0.974)
RR lowering drugs	13.1	2.18	79.1	0.005	0.783 (0.657-0.884)
Protein/drug interaction	0.000	0.0001	0.048	0.001	
CD14	0.258	0.072	0.923	0.037	0.658 (0.605-0.763)
RR lowering drugs	4.73	1.87	12.0	0.001	0.783 (0.682-0.896)
Protein/drug interaction	0.522	0.028	9.70	0.663	
HRG	0.235	0.063	0.879	0.031	0.920 (0.846-0.974)
RR lowering drugs	5.51	1.78	17.1	0.003	0.783 (0.668-0.884)
Protein/drug interaction	0.316	0.033	3.001	0.316	

P: p value for likelihood ratio test that HR=0, LRG1: Leucine-rich alpha-2-glycoprotein, ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3, C4B: Complement C4-B, C1QB: Complement C1q subcomponent subunit B, CD14: Monocyte differentiation antigen, HRG: Histidine-rich glycoprotein

DISCUSSION

Using highly sensitive proteomic techniques, the present study has revealed six plasma proteins that were significantly associated with coronary artery disease progression in statin-treated FH patients. This is the first study to describe such an association and the findings may represent a novel tool for predicting the development of CAD or the residual CAD risk, independent of classical risk factors and clinical indicators, in this high-risk population.

Atherosclerotic cardiovascular disease (CVD) is a leading cause of morbidity and mortality globally.[30] A recent study has shown that proteomic profiling identified both single and multiple marker protein panels that were associated with new-

onset atherosclerotic CVD in participants from the Framingham Heart Study. These included many novel protein biomarkers, which when viewed as a panel of aggregate proteins, improved myocardial infarction and atherosclerotic risk prediction above and beyond established risk factors.[30] In the present study, we have highlighted six protein biomarkers as potential predictors of CAD risk in our statin-treated FH population. Since FH is already associated with an extremely elevated risk of developing CAD,[7] albeit one with significant inter-individual variation,[9-11] this represents an exciting new finding. This finding could potentially provide a tool for identifying those at high risk of developing CAD, which would then allow for personalised treatment to prevent early CAD events. This is particularly important given that intervention strategies at the preclinical stage are more likely to confer benefit. Interestingly, while all six peptides were associated with disease progression (from no atherosclerotic burden, to presence of severe coronary atherosclerosis, up to symptomatic coronary event), it was the absence of these proteins that suggested the highest risk, independent of age or smoking.

Of the six proteins found, the strongest association with CAD appeared to be HRG, followed by LRG1, C4B and C1BQ. ITIH3 appeared to have little association with disease progression. Our analysis suggests that the association with LRG1, C4B and C1BQ may be an artefact because of the application of risk reduction therapy but this does not appear to be the case for HRG. HRG, or histidine-rich glycoprotein, is a serum protein belonging to the cystatin superfamily, which plays a regulatory role in hemostasis and innate immunity.[31] A previous study in mice has shown that the *Hrg*$^{-/-}$ mice had higher anti-thrombin activity, shorter pro-thrombin time and reduced bleeding time, compared to their heterozygous and wild type counterparts. These findings suggest that HRG plays a role as both an anticoagulant and anti-fibrinolytic modifier, and may also regulate platelet function. As a result, the authors suggest that an absence of HRG could trigger monocyte proliferation to compensate for a decrease in phagocyte activation. Coupled with the suggestion that HRG binds several components of the coagulation and fibrinolysis cascades,[31] this implies that its absence could also play a role in the development of atherosclerosis in humans. In agreement with this is our finding of significantly reduced levels of HRG in the FH + CAD group compared to the asymptomatic FH group. Indeed, only one patient in the FH + CAD group had detectable levels of HRG, while all but one had detectable levels in the FH group. Within the FH + Ca group, 40% had detectable levels of HRG, suggesting that either a reduction in circulating HRG occurs with disease progression or there's a possible 'switching off" of HRG production leading to the development of atherosclerosis.

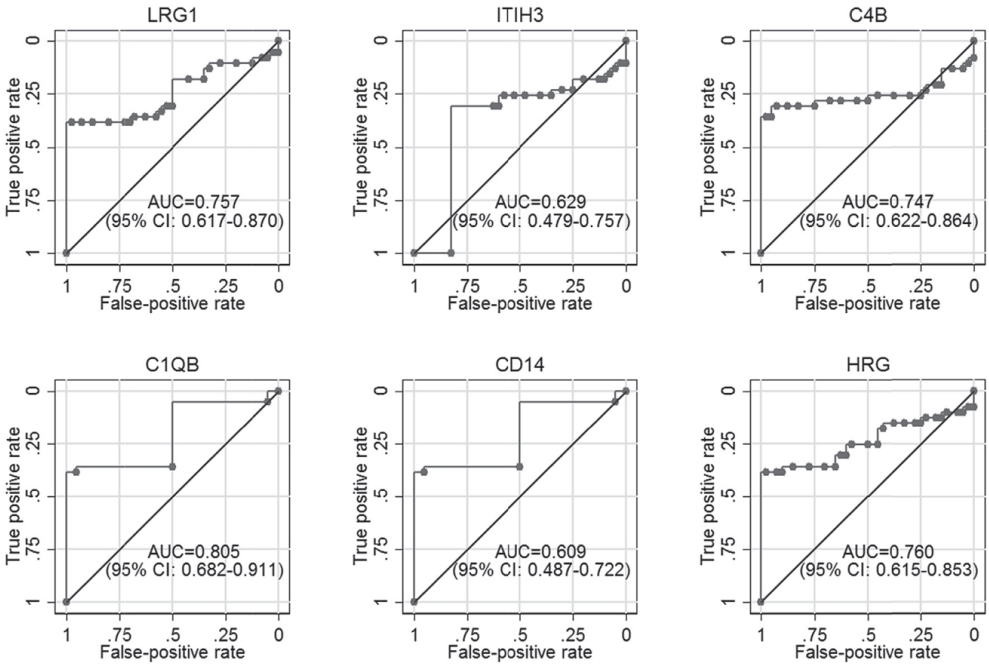

Figure 8.4 | Area under the receiver operating characteristic curve, estimating the predictive accuracy of each protein with coronary artery calcium or coronary artery disease.
AUC: Area under the curve; C1QB: Complement C1q subcomponent subunit B; C4B: Complement C4-B; CAD: Coronary artery disease; CD14: Monocyte differentiation antigen; CI: Confidence interval; HRG: Histidine-rich glycoprotein; ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3; LRG1: Leucine-rich alpha-2-glycoprotein.

LRG1, or leucine-rich α-2 glycoprotein is a highly conserved member of the leucine-rich repeat family of proteins, many of which have been found to play a role in protein-protein interaction, signalling and cell adhesion.[32] LRG1 has been shown to increase proliferation in cultured endothelial cells and is up-regulated and pro-angiogenic in mouse models of retinal disease.[33] A recent human study has found elevation of LRG1 is associated with arterial stiffness, endothelial dysfunction and peripheral vascular disease in patients with type 2 diabetes.[34] In the present study however, we saw almost a complete absence of LRG1 in the FH + CAD group when compared to the asymptomatic groups. Again, only one patient in the FH + CAD group had detectable levels of LRG1. The reason for this discrepancy between the previous animal and human studies and our study is unclear. It is possible that an initial elevation of LRG1 promotes arterial stiffening and endothelial dysfunction, both early hallmarks of atherosclerosis, but then decreases as the disease establishes and progresses. The findings from the

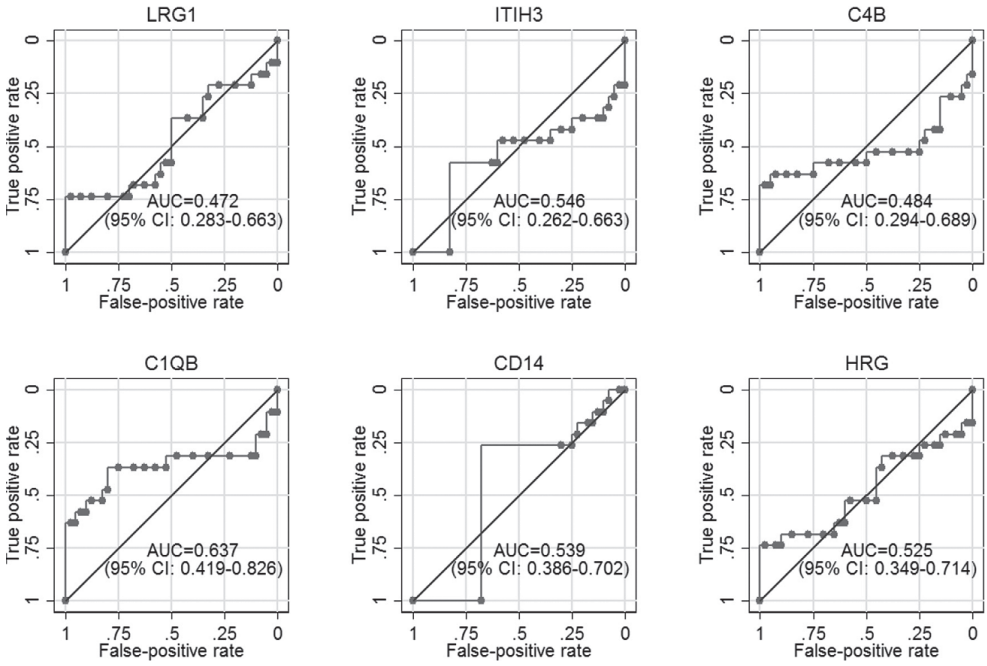

Figure 8.5 | Area under the receiver operating characteristic curve, estimating the predictive accuracy of each protein with coronary artery calcium.
AUC: Area under the curve; C1QB: Complement C1q subcomponent subunit B; C4B: Complement C4-B; CAD: Coronary artery disease; CD14: Monocyte differentiation antigen; CI: Confidence interval; HRG: Histidine-rich glycoprotein; ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3; LRG1: Leucine-rich alpha-2-glycoprotein.

Framingham Heart Study support this, as LRG1 was only found to be predictive of new-onset atherosclerosis, and not myocardial infarction.[30]

Inflammation plays a key role in both the development and progression of atherosclerosis, with initial modification of LDL resulting in its preferential uptake in the intimal layer of the arterial wall, resulting in an immediate innate immune response, which ultimately leads to the development of fatty lesions and atherosclerotic plaques.[35] Children with FH have been shown to have an inflammatory imbalance, which may contribute to the accelerated atherosclerosis development.[36] Furthermore, oxidative modification of LDL has been shown to be related to inflammatory gene expression and subsequent atherosclerosis development in both children and young adults with FH.[37] Complement C4-B (C4B) and Complement C1q subcomponent subunit B (C1BQ) are part of the complement system, which plays a role in our innate defence. As oxidatively modified LDL promotes inflammation, the innate immune system is the

initial process for neutralising and eliminating these toxic particles.[35] The complement system is also thought to contribute to endothelial dysfunction, and is activated in early fatty streaks and late stages of atherosclerosis.[38] In the present study, we have shown that both C4B and C1BQ are excellent predictors of CAD. However, once again it was the absence of both of these proteins in the FH + CAD group compared to the FH group that was predictive. While inflammation plays a key role in atherosclerosis, it is also possible that chronic exposure to stressors and inflammatory markers in the arterial wall may lead to a loss of immune homeostasis. As such, both C4B and C1BQ may play a role in the early, subclinical stages of CAD (the asymptomatic groups) when atherosclerosis is first developing, but are absent in the FH + CAD group where disease is established. Supporting this is the suggestion that the complement system has a dual role in atherosclerosis, including the removal of debris as well as amplification of the inflammatory response. Furthermore, some components of the complement system, including C1Q appear to have a protective effect.[38] Clearly the role of the complement system in the development of atherosclerosis is complex and the role of these proteins in disease progression warrants further investigation.

CD14, or monocyte differentiation antigen, is a protein expressed in monocytes and macrophages and also involved in inflammation.[39] A recent study has shown that CD14 may be a potential marker of CAD where urinary CD14 levels were significantly higher in patients with angiographic CAD compared with controls.[39] Interestingly, we saw no detectable amounts of circulating CD14 in >75% of our participants, including none of the patients in the FH + CAD group. This is in contrast to the previous study, however it should be noted that we did not perform CD14 analysis on urine within our study population. In the previous study, the authors noted no significant differences in plasma CD14 between their two groups. Furthermore, while their CAD group had angiographically proven CAD, they had not had a previous coronary event.[39]

The lack of association between any of the protein biomarkers and classical risk factors of CAD is interesting. Furthermore, while most of the proteins were excellent to good predictors of CAD, they were less robust as predictors of coronary atherosclerosis. The reason for this is unclear. However, it may be in part due to the fact that one or more proteins play different roles at different stages of the development of atherosclerosis. Our findings suggest that the proteins identified as potential biomarkers may be more relevant at the preclinical stage of disease development, which is where they would have the greatest clinical utility. Future studies examining healthy control populations as well as protein levels and disease progression in FH patients are needed to tease out

these relationships. Furthermore, it is worth considering that while FH is characterised by advanced atherosclerosis and CAD, it is a genetically distinct disease. It is therefore possible that there are additional underlying factors, unique to FH and its associated mutations, which manifest differently to a patient with CAD not associated with FH.

While it is too early in this investigation to conclude that a predictive relationship exists between the loss of these peptides and disease progression, the results suggest there are grounds to support such a hypothesis. Firstly, a statistical association exists between a number of related proteins and disease. Secondly, there is a clear lack of significant association between the severity of CAD and the potential biomarkers (Table 4). This supports a hypothesis of prediction of the change in state rather than progression of severity of the condition *per se*. Thirdly, the significant association of age and smoking also support the hypothesis that these potential markers may be able to predict the change in disease status since age is a surrogate marker of elapsed time for each patient and smoking is a well established predictor of CAD. It is clear that a prospective study of asymptomatic FH patients to investigate CAD progression is warranted.

There are several limitations associated with the present study that must be acknowledged. These include; the cross-sectional study design and inclusion of a highly selected population with a relatively small sample size. Furthermore, we did not test against other biomarkers, including genetic ones. This is particularly important given the need for novel strategies to identify CAD risk in asymptomatic adults, which add to the prognostic value provided by standard risk factors. While alternate strategies that go beyond measuring traditional risk factors are needed, they must also be better than existing non-invasive strategies (imaging for carotid intimal medial thickness and coronary artery calcium) and have a wide variability, which does not correlate with traditional risk factors. The strengths of the present study, however, include the well characterised treated patient groups and the extreme selection of CAD endpoints at a relatively young age.

In conclusion, the present study has identified six protein biomarkers that are associated with atherosclerotic disease progression and subsequent coronary events in treated patients with FH. As these are a group of individuals already at elevated risk of developing CAD, this offers a novel tool for more accurate prediction of risk and therefore commencement of early aggressive therapy to prevent future coronary events. Although the present study is hypothesis generating due to its cross-sectional design and needs to be tested in larger populations with prospective follow-up, this may be possible through international collaborations that utilise the power of well-characterised registry data.

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Conflicts of Interest

None declared.

Author Contributions

All authors have approved the final version of this article. Individual author contributions are as follows: conception and design of the study (NW, GW, ES), acquisition, analysis and interpretation of data (MP, NW, GW, ES, SB, AV), drafting and critical appraisal of the manuscript (NW, MP, GW, ES, SB).

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8

CHAPTER 9

Summary and Discussion

SUMMARY

Since the introduction of statin therapy as cholesterol-lowering medication in the 1990s, the life expectancy of patients with familial hypercholesterolemia (FH) has improved significantly. However, despite the wide-spread use of statins among FH patients, some still do develop CVD (1). The aim of this thesis was to develop an approach to identify the residual cardiovascular disease (CVD) risk in statin-treated FH patients. Therefore, in part 1 I investigated the value of applying imaging techniques such as carotid ultrasonography and computed tomography coronary angiography (CTCA) (chapter 2,3 and 4), and in part 2 measurement of non-traditional and traditional risk factors such as lipoprotein (a) [Lp(a)] levels in statin treated FH patients (chapter 5, 6) to distinguish who are high-risk FH patients and who not. In Chapter 7 I discussed the different treatment options of high Lp(a) levels. In Chapter 8 I concluded by using a proteomics technique to identify novel proteins that are associated with cardiovascular disease and coronary calcification in statin treated FH patients.

Part 1: Cardiovascular imaging and residual cardiovascular risk in FH patients.
Subclinical atherosclerosis can be measured by carotid ultrasonography. As it is a non-invasive measurement and relatively easy to measure it is often used in clinical studies to determine cardiovascular risk.

First, in chapter 2 I showed that the carotid ultrasonography technique that was used in our studies, is reliable and reproducible by performing an intra- and inter-observer validation between a traditional and semi-automatic ultrasound device.

Subsequently, I studied in chapter 3 whether carotid imaging results by ultrasonography, reflected by the prevalence of carotid plaques and carotid intima-media-thickness (C-IMT), are suitable for determining residual risk. Can we distinguish FH patients who use long-term statin treatment from non-FH subjects? Therefore, I compared carotid plaque prevalence and C-IMT between FH patients using long-term statin treatment and healthy non-FH controls. I showed that these outcomes were similar between the groups. In a subset of FH patients of whom a CTCA was available, I showed that carotid plaques presence was associated with coronary calcification determined by CTCA , implying that not C-IMT but carotid plaques could be of interest to determine residual risk in statin-treated FH patients.

Patients with homozygous FH patients have a high prevalence of aortic valve disease (AoVC) and this is, in addition to coronary artery disease (CAD), the major cause of premature death in this group. On the other hand, whether AoVC is more common in heterozygous FH patients (heFH) compared to non-FH individuals is unknown. In **chapter 4** I show that AoVC is more prevalent in asymptomatic FH patients than in non-FH controls. Among heFH patients, the prevalence of AoVC was highest in FH patients with a LDL-receptor negative mutation with the highest untreated LDL-C levels compared to FH patients with a LDL-receptor defective mutation. This suggests a causal role of LDL-C in the early onset of aortic valve pathology. Therefore, I established that the health of heFH patients is threatened beyond "classical" atherosclerotic CVD through the accelerated development of AoVC. While statin therapy can significantly diminish the risk of CVD, statins do not affect the course of AoVC and stenosis once this is established. This study implies: 1) to start with statin therapy at a young age not only to prevent CVD but also to prevent AVC; 2) that regularly screening heFH patients for AoVC might be needed.

Part 2: Non-traditional risk factors and residual cardiovascular risk in FH patients.

Lp(a) is a genetically determined atherogenic lipoprotein which is currently not part of the traditional lipid panel. Lp(a) is not only an independent risk factor for CVD, but has also been associated with aortic valve stenosis with SNPs in the *LPA* gene as well as plasma Lp(a) levels (2). In Chapter 5 I show that Lp(a) levels are associated with AoVC determined by CTCA in asymptomatic FH patients. In Chapter 6 I investigated whether Lp(a) levels were associated with atherosclerosis depicted as C-IMT and carotid plaques measured by carotid ultrasonography in statin-treated HeFH patients. I found no association between Lp(a) levels and carotid ultrasonography outcomes. In Chapter 7 I discuss the latest developments in the treatment of Lp(a). Both statins, the most widely used lipid lowering agents, and lifestyle intervention have no effect on Lp(a) levels. The new protein subtilisin/kexin type 9 inhibitors lower Lp(a) by 30-50%, but also lower LDL-C. It is unknown whether specifically lowering of Lp(a) can reduce CVD risk. However, recently an antisense oligonucleotide directed to apolipoprotein (a) has been developed which can specifically lower Lp(a) levels up to 90%. Trials with this new drug shall reveal whether reduction of Lp(a) levels can decrease CVD risk. In Chapter 8 I identified six novel proteins associated with atherosclerosis and CVD events in heFH patients. For this purpose I used the isobaric tag for relative and absolute quantification

(iTRAQ) proteomics technique in 60 specifically selected heFH patients, and discovered plasma proteins previously not related to atherosclerosis. These proteins were, as expected, part of the coagulation pathway, the inflammation pathway, and the lipid metabolism pathway. Further research is required to confirm the importance of these proteins and if they are suitable as biomarkers or even potentially targets for novel therapeutic interventions to reduce CVD risk.

DISCUSSION

The introduction of statin treatment has had great impact in the survival of FH patients by increasing the life expectancy similar to that of the general population. However, some FH patients still develop CVD despite statin treatment. In this thesis I investigated the role of cardiovascular imaging and non-traditional risk factors to discriminate between FH patients who are at high risk of developing CVD despite long-term statin treatment and those with low risk of CVD. Furthermore, I investigated the prevalence of aortic valve calcifications (AoVC) in FH patients, since this is another entity of CVD which has not been studied extensively yet.

Emerging lipid-lowering medication in FH patients

Identifying FH patients with high residual risk is very relevant in light of the development of novel therapeutic agents. The most promising, and recently approved therapeutic agents are proprotein convertase substillin/kexin type 9 (PCSK-9) inhibitors. The proposed mechanism of the PCSK-9 protein is binding to the LDL-receptor and subsequently degradation of the LDL-receptor after internalisation in the cell. When PCSK-9 cannot bind to the LDL-receptor, the receptor will be recycled and re-emerge on the cell surface where it can bind a new LDL-cholesterol particle. PCSK-9 inhibition with monoclonal anti-bodies lowers circulating PCSK-9 and prevents LDL-receptor degradation. These monoclonal anti-bodies have been shown to greatly reduce LDL-Cholesterol levels, also in addition to maximum statin therapy, and seem to be well tolerated (3-6). The PCSK-9 inhibitors Alirocumab and Evolocumab have been recently approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as an "adjunct to diet and maximally tolerated statin therapy for the treatment of adults with heFH or clinical atherosclerotic cardiovascular disease, who require additional lowering of LDL [low-density lipoprotein]-cholesterol (7). Additionally, the first CVD outcome

study (FOURIER) showed a reduction of CVD events when the PCSK9 inhibitor was used on top of high dose statin therapy (8). This new therapy grands new possibilities in lowering LDL-C and subsequently CVD risk reduction. However, the costs of these novel agents are high. In the Unites States the launched list price of Alirocumab and Evolocumab were $14,600 and $14,100 per patient per year respectively (9). Recently Schulman et al. questioned the cost effectiveness of these drugs in hyperlipidaemia patients (9). Since the costs are so high and the absolute CVD risk prevention in these statin treated patients will probably be quite low, the economic benefits seem to be limited. Although, these were: 1) not the formal economic evaluations; 2) the exact reduction in CVD events is currently unknown, and 3) the price will probably be reduced when available in the Netherlands. Given the high costs of these agents it certainly emphasizes the benefit of determining residual risk not only from a health perspective but also in reducing healthcare costs.

Cardiovascular imaging

In the general population C-IMT and the presence of carotid artery plaques in particular, are significant predictors of CVD (10-13). Because of this association in the general population many studies including drug trials, have used C-IMT as a surrogate marker for atherosclerotic disease to determine the effectiveness of novel agents (14-16). An important factor to consider when investigating and comparing carotid ultrasonography outcomes in multicentre studies and meta-analysis is the use of different devices, measurement techniques, measurement software and inter-observer variability might yield very different results. I showed in **chapter 2** that the outcomes between the devices and different observers used for the studies in this thesis was within acceptable range. One of the landmark trials using C-IMT as endpoint was the ENHANCE trial which randomized FH patients using simvastatin to addition of placebo or ezetimibe. This trial showed that the addition of ezetimibe in these patients did not result in difference in changes in C-IMT between the 2 groups. Later the IMPROVED trial showed that the addition of ezetimibe compared to placebo on top of statin therapy led to a decrease in CVD events. A proposed explanation for the lack of effect of ezetimibe in the ENHANCE trial was that the statin-treated FH patients included in this trial had normalized C-IMT values at baseline. In **chapter 3** I showed indeed a normalization in C-IMT values in statin treated FH patients compared to healthy controls.

Interestingly plaque presence, measured by ultrasonography, was associated with the severity of coronary atherosclerosis as depicted by CTCA in a subgroup of statin-treated

FH patients for whom CTCA results were available. This suggests that carotid plaque presence might distinguish low residual CVD risk FH patients from those with high residual risk. In our study the prevalence of carotid plaques was 31% in asymptomatic statin-treated FH patients at the age of 46±15 years, with 95% of carotid plaques present in those of 40 years or older. In a study in asymptomatic FH patients older than 40 years it was concluded that carotid plaques, measured by ultrasonography, were not significantly associated with coronary calcifications on CTCA (17). An explanation of this lacking association was the high prevalence of carotid plaques (93%) in this study. The results presented in that study and **Chapter 3** emphasize the importance of studying the correlation between ultrasonography findings and coronary calcifications in subjects younger than 40 years (17). Currently CT coronary angiography scans, are usually not performed below the age of 40 years in asymptomatic individuals due to radiation exposure and the associated elevated long-term risk of cancer. With improvement in CT scanning techniques radiation exposure will further reduced, and in the future these studies might be possible in younger individuals. These studies might lead to a screening program among FH patients below the age of 40 years to identify those FH patients with the highest residual CVD risk.

Another imaging finding reported in this thesis is the presence of AoVC measured by CTCA as was used in **Chapter 4** and **Chapter 5.** I concluded that AoVC is more prevalent and severer in statin-treated heFH than in controls, and that the severity of AoVC was associated with higher untreated LDL-C levels which is related to LDL-receptor negative mutations as compared to carriers of LDL-receptor- defective mutations. This suggests a causal role of LDL-C in the development of AoVC. However previous clinical trials such as the SALTIRE, ASTRONOMER, and SEAS showed that statins cannot delay the progression of aortic valve sclerosis towards aortic valve stenosis (18-20). An explanation might be that the initiation and progression of AoVC are two different entities with separate pathophysiological mechanisms. The initiation of AoVC seems to be effected by the classical risk factors of atherosclerosis, including as LDL-C. These risk factors might be responsible for the differentiation of interstitial aortic valve cells to an osteogenic phenotype. These osteogenic cells cause the progressing of calcification of the valve and cannot be delayed by statins. This theory emphasizes the need of early LDL-C reduction in FH patients, to prevent early differentiation towards osteogenic cells and thereby progression to AoVC. Routine screening for aortic valve pathology by cardiac ultrasound, which is currently advised by guidelines for homozygous FH patients, could be considered in patients with heterozygous FH, especially in those with an LDL-

receptor negative mutation or those who started with statin therapy in adulthood. Additionally, more research is needed to evaluate whether novel therapeutic agents like PSCK-9 inhibitors can inhibit the progression towards aortic valve stenosis.

Novel biomarkers

In **chapter 5** I show that plasma Lp(a) levels were independently associated with AoVC but not with coronary atherosclerosis in asymptomatic heterozygous FH patients. A possible explanation for the lack of association of Lp(a) levels with coronary atherosclerosis is that statins reduce coronary atherosclerosis so effectively that additional risk factors like Lp(a) no longer play a role. If Lp(a) is important in the initiation of AoVC like LDL-C, it is likely that the effect of high Lp(a) levels on AoVC also remains detectable. Currently, elevated Lp(a) levels cannot be specifically treated (as discussed in **Chapter 7**) and risk reduction in patients with high L(a) levels (>0,5 g/l) should be achieved by optimizing other risk factors such as blood pressure, BMI and LDL-C. PCSK-9 inhibitors, do not only lower LDL-C, but also lower Lp(a) levels by 30% (3). The mechanism of Lp(a) lowering of these agents is currently unknown and it unclear whether this effect ads to CVD risk reduction on top of the LDL-C lowering properties. Whether specific Lp(a) lowering can reduce CVD events and stop progression of aortic valve stenosis should be evaluated in future studies, that will be made possible by the specific antisense Lp(a) agents that are currently being developed (21).

In **chapter 8** I chose another approach in risk prediction in FH patients by using the iTRAQ proteomic technique to identify possible proteins that are associated with subclinical atherosclerosis or atherosclerotic cardiac events. I found six novel proteins whose levels were negatively associated with atherosclerotic disease progression and subsequent coronary events in patients. These six proteins; leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG) appear to mainly have functions in the coagulation pathway, arterial compliance, and inflammation. These pathways are historically involved in the development of CVD risk and therefore these results seem promising. This study was conducted in FH patients and therefore it is possible that there are additional underlying factors, unique to FH and its associated mutations, which manifest differently to a patient with CAD not associated with FH. However, it seems worth investigating whether the levels of these novel discovered proteins differ

between patients with CVD and healthy controls in the general population. The exact role of these novel proteins also needs to be further investigated. They might be suitable in predicting CVD risk as markers or might even be causal in CVD disease progression.

Future perspectives

Over a third of statin-treated FH patients still develop CVD events, it is relevant and important to identify these patients to intensify treatment, for example by initiating PCSK-9 inhibitor therapy (1). Optimal risk prediction among FH patients should be used to differentiate between those who are likely to remain asymptomatic using statin treatment and those who will develop CVD events or who have experienced a CVD event and are at an increased risk of experiencing subsequent CVD events despite maximum lipid lowering therapy. It is essential to, at least to attempt, to make this differentiation because of the high costs of the PCSK-9 inhibitors. To identify these patients at risk I showed that the asymptomatic statin-treated FH patients who have carotid plaques also have more coronary atherosclerosis whereas in these patients C-IMT is unlikely to add to further risk prediction. Whether carotid ultrasonography outcomes are related to CVD events in statin-treated patients is thus far unknown and should be clarified in the future. Moreover I showed that AoVC prevalence is high in heFH patients. Since statins do not reduce AoVC once established, but are associated with a reduction in mortality in FH patients, aortic valve disease might become a growing health threat for the aging FH patients. Further research is needed to clarify whether starting statins at a young age reduces the risk of developing AoVC. It is also important to study whether the current aging heFH patient would benefit from routinely cardiac ultrasound monitoring for valve diseases as is advised by guidelines for hoFH patients.

I showed that circulating Lp(a) levels in statin treated patients are associated with AoVC. Future studies will tell if specific lowering of Lp(a) levels can lower the occurrence of CVD events. Because of the association of Lp(a) levels with AoVC, it will be interesting to find out if reducing Lp(a) levels will also slow down aortic valve disease progression.

Finally, I found that lower protein levels of leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG) were associated with a higher abundance of subclinical atherosclerosis and previous CVD events in heFH patients. Whether these proteins can be used as potential biomarkers or are causally related to CVD outcome needs to be further investigated.

What was already known

- Carotid plaques are a better cardiovascular disease (CVD) risk predictor than carotid intima-media thickness (C-IMT) in the general population (13,22,23)
- Carotid plaque progression and C-IMT are reduced by high dose statin treatment (24,25)
- Premature aortic valve disease is highly present in patients with homozygous Familial Hypercholesterolemia (FH) (26-28)
- Lipoprotein (a) is an independent risk factor of CVD and Aortic valve calcification (AoVC) (2,29,30)

What this thesis adds

- The prevalence of carotid plaques and the C-IMT are similar in long-term statin-treated FH patients and healthy controls (Chapter 3)
- The prevalence and extent of AoVC are increased in statin-treated heterozygous FH patients compared to controls (Chapter 4)
- The dose response relation found in the prevalence of AoVC in non-FH patients, compared to FH patients with LDLR-defective mutations compared to FH patients with LDLR-negative mutations which reflects increasing LDL-C levels, suggests a causal role for cholesterol in the initiation of AoVC (chapter 4)
- Lp(a) levels are independently associated with AoVC in statin-treated FH patients (Chapter 5)
- Possible residual risk of Lp(a) is not detected via carotid ultrasonography outcomes (C-IMT and carotid plaque) in FH patients (Chapter 6)
- Novel plasma proteins: leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG) associated with previous CVD events and coronary atherosclerosis in FH patients (Chapter 8)

Proposed research

- The predictive value of carotid plaques and intima media thickness on CVD events in long-term statin-treated FH patients
- Prevalence of clinical relevant aortic valve pathology in aging heterozygous FH patients
- Cost-effectiveness of routine cardiac ultrasound in the aging heterozygous FH patient to detect aortic valve pathology
- Value of specific Lp(a) lowering medication for CVD prevention and to prevent progression of aortic valve sclerosis to clinical aortic valve stenosis
- Further investigation of the value of novel proteins: "leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG)" as suitable biomarkers or CVD risk factors in FH and non-FH patients, and to study the potential mechanisms by which these proteins contribute in atherosclerotic disease.

OVERALL CONCLUSION OF THE THESIS

In conclusion I found that imaging carotid ultrasonography outcomes were similar between asymptomatic statin-treated familial hypercholesterolemia (FH) patients and healthy controls, and that carotid plaque presence was associated with coronary calcification in these FH patients. I also showed that the prevalence and extent of aortic valve calcification (AoVC) was twice as high in heterozygous FH patients compared to non-FH controls, and that this effect is strongest in those with a LDL-receptor negative mutation and highest untreated LDL-cholesterol levels. This suggest a causal relationship between AoVC and LDL-cholesterol. Additionally, AoVC is also independently associated with plasma Lipoprotein (a) [Lp(a)] levels, but I did not see an effect of Lp(a) on carotid ultrasonography outcomes in asymptomatic statin-treated FH patients. Finally, I found that the absence of plasma protein levels of leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14) and histidine-rich glycoprotein (HRG) proteins were associated with subclinical atherosclerosis and previous CVD events in heterozygous FH patients.

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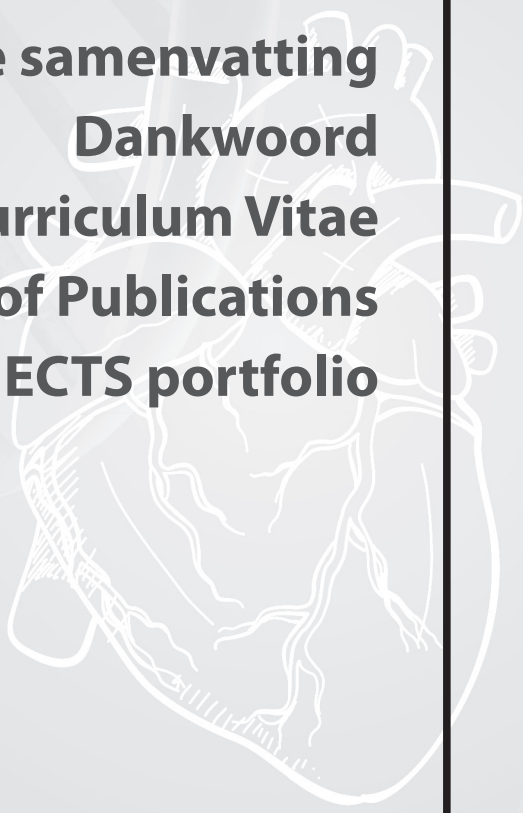
APPENDICES



NEDERLANDSE SAMENVATTING

Sinds de introductie van statines als cholesterolverlagende medicatie in de jaren negentig, is de levensverwachting van patiënten met familiare hypercholesterolemie (FH) aanzienlijk verbeterd. Ondanks het gebruik van statines, ontwikkelen sommige FH-patiënten toch nog steeds hart- en vaatziekten (HVZ) (1). Het doel in dit proefschrift was om het resterende, oftewel residuale, risico op HVZ te identificeren in FH patienten die worden behandeld met lipidenverlagende medicatie zoals statines. In Deel 1 heb ik onderzocht wat de bijdrage is van beeldvormende technieken zoals echografie van de carotiden (hoofdstuk2,3), en coronaire angiografie (CTCA) hoofdstuk 4) in het bepalen van het risico op HVZ bij FH patiënten die worden behandeld met lipidenverlagende medicatie. In Deel 2 onderzocht ik of niet-traditionele risicofactoren zoals het atherosclerose veroorzakende lipoproteïne (a) [Lp (a)] (hoofdstuk 5, 6, 7) en "proteomics", een techniek waarmee grootschalig eiwitten en hun biologische functie kunnen worden bestudeerd, (hoofstuk 8) de diagnostiek en behandeling van FH patiënten kunnen verbeteren.

Deel 1: Cardiovasculaire beeldvorming en resterend hart- en vaatrisico bij FH-patiënten.

Subklinische atherosclerose kan worden gemeten door middel van een echo van de halsslagaders ofwel carotiden. Met deze meting kan de afstand tussen de binnenste laag (intima) en middelste laag (media) worden gemeten, de "intima-media thickness" afgkort IMT. Tevens kan worden bepaald of er atherosclerotisch plaques aanwezig zijn in de carotiden. Omdat het een niet-invasieve meting is die relatief makkelijk te verrichten is en in een korte tijd te meten, wordt de halsslagader echo vaak gebruikt in klinische studies om cardiovasculaire risico's te bepalen.

In hoofdstuk 2 heb ik aangetoond dat het echoapparaat dat ik in dit proefschrift beschreven onderzoeken heb gebruikt voor echografie van de carotiden, betrouwbaar en reproduceerbaar is. Dit heb ik gedaan door middel van het bepalen van de variatie tussen verschillende metingen van dezelfde patiënt door 1 onderzoeker op hetzelfde echoapparaat te meten (intra-observer variatie). Daarnaast werd de variatie tussen metingen van dezelfde patiënt tussen twee verschillende onderzoekers op hetzelfde echoapparaat gemeten (inter-observer variatie). En ten slotte heb ik onderzocht of er een verschil was in metingen van dezelfde patiënt door dezelfde onderzoeker met verschillende echoapparaten; een traditioneel echografie apparaat en het moderne

echoapparaat wat wij gebruikten in onze onderzoeken (inter-device variatie). De belangrijkste bevindingen waren dat de intra-observer, inter-observer en intra-device variatie klein was en dat de resultaten van het door ons gebruikte echoapparaat reproduceerbaar en daarmee betrouwbaar zijn.

Vervolgens heb ik in hoofdstuk 3 onderzocht of beeldvorming van de carotiden middels echografie, door het meten van de aanwezigheid van carotis plaques en de IMT van de carotiden (C-IMT), geschikt is om in met lipidenverlagende medicatie behandelde FH patiënten te bepalen welke patiënten een hoger risico op HVZ hebben. Dit heb ik gedaan door de echografie uitslagen van FH-patiënten die langdurig behandeld werden met lipidenverlagende medicatie te vergelijken met gezonde controles. Hieruit bleek dat de er geen verschil bestond tussen de groep FH patiënten en de gezonde controles in de C-IMT en percentage mensen met carotis plaques. In een aantal van de FH patiënten was er tevens een CTCA beschikbaar. De uitslagen van de echografie resultaten en de CTCA lieten zien dat de aanwezigheid van carotis plaques, maar niet C-IMT verband, hield met de hoeveelheid coronair calcificatie op CTCA. Deze uitkomsten impliceren dat 1) de behandeling met lipidenverlagende medicatie in FH patiënten zo succesvol is dat er qua halsslagader echo geen verschil meer is tussen gezonde mensen zonder FH en 2) dat van de halsslagader metingen niet C-IMT maar plaques in de carotiden van belang kunnen zijn om het resterende risico in statine-behandelde FH patiënten te bepalen.

Bijna alle patiënten met homozygote FH ontwikkelen versneld aortaklepsclerose (AoVC) en dit is naast coronarialijden de belangrijkste oorzaak van voortijdige dood in deze groep. Bij heterozygote FH-patiënten (heFH) is het echter niet bekend of AoVC vaker voorkomt in vergelijking met niet-FH-patiënten. In hoofdstuk 4 laten we zien dat AoVC meer voorkomt bij asymptomatische FH patiënten dan in controles die geen FH hebben. Bij heFH patiënten was de prevalentie van AoVC het hoogst bij FH patiënten met een LDL-receptor negatieve mutatie (zonder LDL-receptor restfunctie) die de hoogste onbehandelde LDL-C waardes hebben in vergelijking met FH patiënten met een LDL-receptor defecte mutatie die nog enige LDL-receptor restfunctie hebben. Dit suggereert een causale rol van LDL-C bij het ontstaan van aortaklep verkalking. Hiermee heb ik vastgesteld dat de gezondheid van heFH-patiënten niet alleen bedreigd wordt door 'klassieke' atherosclerotische HVZ maar ook zeker door de versnelde ontwikkeling van AoVC. Klinisch is dit belangrijk omdat statine therapie het risico op HVZ aanzienlijk kan verminderen, maar geen effect meer lijkt te hebben als er al AoVC is opgetreden en dit zich verder kan ontwikkelen naar aortaklep stenose, wat een levensbedreigende aandoening kan zijn. Deze resultaten geven aan 1) het beter is om op jonge leeftijd al te

starten met lipidenverlagende medicatie, niet alleen ter preventie van atherosclerotische HVZ, maar ook om AoVC te voorkomen; 2) dat regelmatige screening van oudere heFH patiënten voor AoVC nuttig zou kunnen zijn.

Deel 2: Niet-traditionele risicofactoren en onverklaard cardiovasculair risico bij FH patiënten

Lp (a) is een genetisch bepaald atherogeen lipoproteïne. Lp (a) is niet alleen een onafhankelijke risicofactor voor HVZ, maar SNP's in het LPA gen en plasma Lp (a) waardes (2), zijn ook geassocieerd met aortaklep stenose. Lp(a) is een niet traditionele risicofactor omdat het momenteel geen deel uitmaakt van het traditionele lipidenpanel wat bestaat uit totaal cholesterol, HDL-C, LDL-C, triglyceriden en in sommige klinieken apoB bepaling. In hoofdstuk 5 laat ik zien dat in asymptomatische FH patiënten plasma Lp(a) concentraties geassocieerd zijn met AoVC bepaald door CTCA. In hoofdstuk 6 onderzocht ik in HeFH-patiënten die behandeld werden met lipidenverlagende medicatie of plasma Lp (a) concentraties geassocieerd waren met atherosclerose weergegeven als C-IMT en carotis plaques gemeten door middel van halsslagaderechografie. In deze studie werd geen associatie aangetoond tussen plasma Lp(a) waardes en de uitkomsten van de echografie. In hoofdstuk 7 bespreek ik de nieuwste ontwikkelingen in de behandeling van Lp (a). Zowel leefstijl interventie en statines, de meest gebruikte lipidenverlagende medicatie, hebben geen effect op plasma Lp (a) concentratie. De nieuwe proteïne subtilisine / kexine type 9 (PCSK-9) remmers verlagen Lp (a) concentraties met 30-50%, maar verlagen ook het LDL-C. Ondanks dat PCSK-9 remmers het risico op HVZ verlagen is het onbekend of specifieke verlaging van de Lp (a) waardes het risico op HVZ kan verminderen. Onlangs is er een antisense oligonucleotide gericht op apolipoproteïne (a) ontwikkeld die specifiek Lp (a) concentraties tot 90% kan verlagen. Studies met dit nieuwe geneesmiddel zullen aantonen of verlaging van Lp (a) concentraties het risico op HVZ kan verminderen. In hoofdstuk 8 identificeerden ik zes nieuwe eiwitten in die verband houden met atherosclerose en HVZ in HeFH-patiënten. Hiervoor gebruikten we de isobarische tag voor relatieve en absolute kwantificering (iTRAQ) proteomics techniek in 60 specifiek geselecteerde heFH patiënten. In deze groep ontdekte ik plasmaeiwitten die eerder niet gerelateerd waren aan atherosclerose. Deze eiwitten waren, zoals verwacht, onderdeel van stollings- ontstekings- en lipide metabolisme. Verder onderzoek is nodig om het belang van deze eiwitten te bevestigen en om uit te zoeken of ze geschikt zijn als biomarkers of zelfs potentiële doelen voor nieuwe therapeutische interventies om het risico op HVZ te verminderen.

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En dan ten slotte het meest gelezen en minst aangepaste gedeelte van dit proefschrift. Ik wil iedereen in het algemeen bedanken die heeft meegeholpen met het tot stand brengen van dit boekwerk. Er zijn zo veel mensen te bedanken dat ik vrees niet iedereen te kunnen noemen, maar ook als je naam hieronder niet apart genoemd staat toch heel veel dank voor alle hulp!

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Onmisbaar voor mijn proefschrift is ook de hulp van Edith Padberg geweest. Edith, echt ontzettend veel dank voor het regelen van alle afspraken en op het einde het uiteindelijk toch voor elkaar te krijgen om de promotiecommissie op één dag bij elkaar te krijgen. En uiteraard voor alle moeite die je afgelopen jaren hebt gedaan om alles in goede banen te lijden.

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Ook wil ik graag kort Erna Egelie nog bedanken voor haar werk als secretaresse van de COEUR. Erna, veel dank voor het op orde houden van ieders studiepunten en dat je van je pensioen mag genieten!

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Ook onmisbaar voor elk onderzoek zijn de analisten. Graag wil ik alle analisten in ons lab bedanken waaronder in het bijzonder Leonie en Jeanette. Graag wil ik jullie speciaal bedanken voor het afdraaien en organiseren voor de ongelooflijke hoeveelheid bloed die van controle patiënten is afgenomen. En uiteraard voor alle metingen die nodig zij geweest voor de database en uiteindelijk alle data voor de studies. Evelien Jäger, Evelien graag wil ik je bedanken met het alfabetiseren en structureren van mijn informed consent formulieren en het elektronisch registreren hiervan. Het was een enorme klus maar je hebt het fantastisch gedaan! Verder ook veel dank voor alle overige hulp en de gezelligheid!

Zonder wie mijn proefschrift ook nooit zo compleet had kunnen zijn is Martijne Duvekot. Martijne, ik ben enorm blij dat je gekozen hebt om je master onderzoek bij ons

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Annette Galema, Annette inmiddels ben je al gepromoveerd, proficiat! In het begin van mijn traject heb je me enorm geholpen bij de logistiek van de poli en bij het bepalen van het beleid voor de mensen met FH op de poli. Daarvoor heel veel dank

Verder ook veel dank aan alle medewerkers van de polikliniek Interne geneeskunde voor jullie ondersteuning en hulp.

Het cardiovasculair researchteam Sint-Fransciscus Gasthuis. Anho Liem, Anho graag wil ik jou en natuurlijk de research verpleegkundigen Marja, Ingrid Margreet en Irene bedanken voor jullie samenwerking met de PCSK-9 trials. Al met al hebben we een mooie groep patiënten bij jullie kunnen includeren in de studies. Daarvoor dank en uiteraard ook voor de gezelligheid.

Gerald Watts, Gerald thank you for giving me the opportunity to work on the proteomics project in the Royal Perth Hospital, and also showing and emphasising the importance of clinical work. I really enjoyed the clinical rounds, the outpatient clinic and the meetings with the general practitioners on improving FH care in Western Australia.

Natalie Ward, Natalie it was a real pleasure working with you in Perth and you were great as a supervisor. I am really proud of the proteomics project, and wish you all the best in your further work.

I also want to thank all the other people I have met during my project in Perth. Danie, Esther, Dick, Michael, Helen , many thanks all for the wonderful time.

Damon and Katja Bell, Damon it was great meeting you in Perth, and I cannot thank you enough for all the help from you and Katja showing us around Perth and Subi. The most memorable part has to be the purple bike! Noa really loved playing with your kids and we were thrilled to be invited to your daughter's birthday with a classic Aussie Barbie! Once more many thanks also from Marjan.

Familie Schoonderwoerd; Henri, Gonnie, Marcel en Erik; dertien jaar geleden alweer kwam ik binnengewandeld als het vriendje van Marjan. Jullie als schoonfamilie hebben me altijd behandeld als een van jullie, bedankt daarvoor. Inmiddels hoor ik nu echt wel bij het meubilair en zijn we al vaak samen op vakantie geweest. Jullie zijn echt mijn tweede familie geworden en ik ben dan ook heel blij dat jullie er tijdens mijn promotie bij zijn.

Familie Bos; Peter Bos, Pa, graag wil ik je bedanken omdat ik altijd bij je terecht kan voor hulp en dat de deur altijd open staat. Vind het fijn om te zien hoe trots je altijd op al je kinderen bent en ben ontzettend blij dat je er bij bent.

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Berth en Conny, Berth, 21 jaar geleden kwam je bij mij en mama wonen , ik was eerst uiteraard wat sceptisch maar ben uiteindelijk ontzettend blij dat het zo is gelopen. Mama, graag wil ik je enorm bedanken voor alle liefde die je me altijd hebt gegeven en dat je vanaf dat ik een klein manneke was al bezig was om alles voor mij mogelijk te maken in het leven. Jullie zeiden al vroeg dat ik geneeskunde moest gaan studeren, maar het duurde even voordat het landde. Laten we het op mijn eigenwijze inslag houden. Ik ben in ieder geval ontzettend dankbaar dat jullie altijd in mij hebben gelooft en dat jullie het voor mij mogelijk hebben gemaakt dat ik kon studeren. Ik ben blij dat jullie bij mijn promotie zijn en trots dat jullie me hebben gemaakt tot de man die ik vandaag de dag ben.

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het laatste jaar van mijn promotie samen een huis te kopen, van top tot teen te verven, net op tijd voor de geboorte van onze prachtige zoon. Als laatst wil ik zeggen dat je een geweldige vrouw bent, een fantastische partner en moeder en daarom ontzettend bedankt dat je me altijd steunt ook in deze tropenjaren en dat we nog heel lang van elkaar mogen genieten.

CURRICULUM VITAE

Personalia

Last Name	Bos
First Name	Sven
Work address	's Gravendijkwal 230
Zip code	3015 CE
City	Rotterdam
Nationality	Dutch
Year of birth	1987
E-mail address	s.bos@erasmusmc.nl / svenbos87@hotmail.com

Training

Sept – 2012	Medical degree at Erasmus MC in Rotterdam
2006 – 2012	Medicine at Erasmus Universiteit in Rotterdam
2006 – 2007	Propedeuse Erasmus Universiteit in Rotterdam for Medicine.
1999 – 2006	Athenaeum with profile Nature en techniques + Nature and Health at the Walburg College in Zwijndrecht. Graduated in 2006.

Work experience

2012 – December 2015	M.D. researcher vascular internal medicine (Phd-student)
01-01-2016 – present	Specialist registrar Internal Medicine, Albert Schweitzer ziekenhuis Dordrecht

Experience Medical Trials

Sub-investigator in randomized controlled trials:

- FOURIER (PCSK-9 inhibitors, monoclonal antibodies)
- TAUSSIG (PCSK-9 inhibitors, monoclonal antibodies)
- Odyssey choice-II (PCSK-9 inhibitors, monoclonal antibodies)
- SPIRE (PCSK-9 inhibitors, monoclonal antibodies)
- GAUSS-3 (PCSK-9 inhibitors, monoclonal antibodies)

Study Coördinator on site:

- Lomitapide registry (MTP inhibitor)

LIST OF PUBLICATIONS

Latest developments in the treatment of lipoprotein (a)
Current Opinion Lipidology. 2014 Dec;25(6):452-60

Increased aortic valve calcification in familial hypercholesterolemia: Prevalence, extent and associated risk factors in a case-control study
J Am Coll Cardiol 2015;66:2687–95

Lipoprotein(a) levels are associated with aortic valve calcification in asymptomatic patients with familial hypercholesterolaemia.
J Intern Med. 2015 Aug;278(2):166-73

Lipoprotein (a) levels are not associated with carotid plaques and carotid intima media thickness in statin-treated patients with familial hypercholesterolemia.
Atherosclerosis. 2015 Sep;242(1):226-9.

Health Status and Psychological Distress in Patients with Non-compaction Cardiomyopathy: The Role of Burden Related to Symptoms and Genetic Vulnerability.
Int J Behav Med. 2015 Dec;22(6):717-25.

Carotid artery plaques and intima medial thickness in familial hypercholesteraemic patients on long-term statin therapy: A case control study
Atherosclerosis 2016 Dec;256: 62-66

Novel protein biomarkers associated with coronary artery disease in statin-treated patients with familial hypercholesterolemia.
J Clin Lipidol. 2017 May - Jun;11(3):682-693

Greater preclinical atherosclerosis in treated monogenic familial hypercholesterolemia vs. polygenic hypercholesterolemia.
Atherosclerosis. 2017 Aug;263:405-411

Soluble LR11 associates with aortic root calcification in asymptomatic treated male patients with familial hypercholesterolemia.
Atherosclerosis. 2017 Oct;265:299-304

Systemic mastocytosis associates with cardiovascular events despite lower plasma lipid levels
Atherosclerosis. 2018 Jan;268:152-156.

PORTFOLIO

Summary of PhD training and teaching activities

Name PhD student: S. Bos
Promoter: prof. dr. E.J.G. Sijbrands
Institution: Erasmus MC
Co-promotors: dr. J.E. Roeters van Lennep, dr. M.T. Mulder
Research school: COEUR

General Courses	**ECTS**
BROK (good clinical practice) + exam	1,8
Statistical course (ESP01) en (ESP03)	2,0
Biomedical English writing (By David Alexander)	3,0

General Seminars	
Human Smooth Muscle Cell Heterogeneity-from bedside to CALM(odulin)	0,1
Lecture LP(a) latest developments	0,1
Lecture wine ponyphenols and Health	0,1
Lecture The HDL story (so far) Jay Heinecke	0,1
Attending the PhD day 2013	0,1
Speaker at patients information evening FH 2013 and 2015 (novel therapies)	2,0
Netherlands Lipid Conference + presentation	1,0
Attending the PhD day 2014	0,1

COEUR courses / seminars	
COEUR seminar:	
The cardiovascular system in women: a relation with female hormones	0,4
Clinical cardiovascular epidemiology (COEUR course)	1,5
Peripheral and intracranial obstructive vascular disease (COEUR-course)	1,5
COEUR seminar:	
Glucose metabolism and vascular disease.	0,4
COEUR seminiar: Translational Electrophysiology	0,4
COUER PhD day + oral presentation 2013	0,5
Cardiovascular Pharmacology (COEUR course)	1,5
COEUR Research seminar Gender differences in CVD	0,4
COEUR seminar: Coronary and Cranial Thrombosis	0,4
COEUR course cardiovascular medicine	1,5
COUER debate on cardiovascular controversies	0,4
COEUR course molecular medicine	1,5

Conferences	
Wetenschapsdagen antwerpen 2013 and 2014 + Poster presentations	1,8
Annual Scandinavian Atherosclerosis Socieity Meeting (Denmark) 2013 + poster presentation	1,5
ESC annual meeting in Amsterdam, 2013 + poster	1,8
Annual Scandinavian Atherosclerosis Socieity Meeting (Denmark) + poster presentation 2014	1,5
EAS 2014 (Madrid) + poster presentation	1,5
ESC annual meeting in Barcelona 2014 + poster presentation	1,8
Annual Scandinavian Atherosclerosis Socieity Meeting (Denmark) + poster presentation	1,5
International society of atherosclerosis meeting, Amsterdam, 2015, 1 moderated poster session, 2 additional posters	3,0
Teaching activities	
Onderwijs vet, koolhydraat en aminozuur metabolisme (Met Adrie Verhoeven) 2013 en 2015	0,6
Onderwijs: klinisch redeneren (Master studenten geneeskunde)	0,3
Teaching intima media thickness measurements (45 weeks, 4 students)	1,25
Part of lab-day committee 2014	0,25
Junior Med school students, presentation + quiz 2013 and 2015	0,6
Peer reviewer of a manuscript for Atherosclerosis	0,1
Medical trial Meetings	
FOURIER investigator's meeting Woerden 2013 + 2015	0,6
AMGEN hyperlipidemia Acadamy Berlin	0,6
Total ECTS	**39.8**