Subjects with familial hypercholesterolemia have lower aortic valve area and higher levels of inflammatory biomarkers.

Running head; Lower aortic valve area in FH

Anders Hovland MD, PhD[1,2*†], Ingunn Narverud PhD[3,4*], Linn Kristin Lie Øyri MSc[3,], Martin Prøven Bogsrud MD, PhD[4,6], Inger Aagnes MD[1], Thor Ueland , PhD[7,8,9], Monique Mulder , PhD[10], Frank Leijten, PhD[10], Gisle Langslet MD, PhD[3,5], Cecilie Wium MD, PhD[5,8], Arne Svilaas MD, PhD[5], Kjell Erik Arnesen MD[5], Jeanine Roeters van Lennep MD, PhD[10], Pål Aukrust MD, PhD[7,8,11], Bente Halvorsen , PhD[7,8], Kjetil Retterstøl MD, PhD[3,5] and Kirsten B Holven PhD[3,4]

[1]Division of Internal Medicine, Nordland Hospital, Norway,

[2]Department of Clinical Medicine, University of Tromsø, Norway

[3]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway

[4]Norwegian National Advisory Unit on Familial Hypercholesterolemia, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, Norway,

[5]Lipid Clinic, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, Norway,

[6]Unit for Cardiac and Cardiovascular Genetics, Oslo University Hospital, Norway

[7]Research Institute for Internal Medicine, Oslo University Hospital, Norway,

[8]Institute of Clinical Medicine, University of Oslo, Norway,

[9]K.G. Jebsen Thrombosis Research and Expertise Centre, University of Tromsø, Norway

[10]Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands,

[11]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Norway

*Contributed equally

[†]Corresponding author: Anders Hovland

Anders Hovland

Division of Internal Medicine

NLSH Bodø

8092 Bodø

Email: anders.w.hovland@gmail.com

Telephone: 004775534000

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Abstract

Background

Reduction of the aortic valve area (AVA) may lead to aortic valve stenosis with considerable impact on morbidity and mortality if not identified and treated. Lipoprotein (a) [Lp(a)] and also inflammatory biomarkers, including platelet derived biomarkers, have been considered risk factor for aortic stenosis, however, the association between Lp(a), inflammatory biomarkers and AVA among patients with familial hypercholesterolemia (FH) is not clear.

Objective

We aimed to investigate the relation between concentration of Lp(a), measurements of the aortic valve including velocities and valve area and circulating inflammatory biomarkers in adult FH subjects and controls.

Methods

In this cross-sectional study aortic valve measures were examined by cardiac ultrasound and inflammatory markers were analyzed in non-fasting blood samples. The study participants were 64 FH subjects with high (n=29) or low (n=35) Lp(a), and 14 healthy controls.

Results

Aortic valve peak velocity was higher (p=0.02), and AVA was lower (p=0.04) in the FH patients compared to controls, when performing multivariable linear regression, there were no significant differences. Furthermore, there was no significant differences between the high and low FH Lp(a) groups regarding the aortic valve. FH subjects had higher levels of several platelet-derived markers CD40L, PF4, NAP2 and RANTES compared to controls ($0.003 \leq P \leq 0.03$). This result persisted after multiple linear regression.

Conclusions

Middle-aged, intensively treated FH subjects have higher aortic valve velocity, lower AVA, and higher levels of the platelet-derived markers CD40L, PF4, NAP2 and RANTES compared to healthy control subjects. The aortic valve findings were not significant after multiple linear regression, whereas the higher levels of platelet-derived markers were maintained.

Word count abstract: 257

Introduction

Gradual reduction of the aortic valve area (AVA) may ultimately lead to aortic valve stenosis (AS), a common cardiovascular disease (CVD) [1] with considerable impact on morbidity and mortality if not identified and treated [2]. Low density lipoprotein-cholesterol (LDL-C) and inflammation have been suggested to play an important role in the pathophysiology of AS consisting of both an *initiation* phase resembling atherosclerosis, including lipid infiltration, oxidation and inflammation, and a *propagation* phase characterized by fibrosis and calcification [3]. Lipid-lowering therapy, however, has not been able to halt the progression of AS once it has been diagnosed, suggesting that intervention against other targets than LDL-C could be beneficial in this phase [4]. Lipoprotein (a) [Lp(a)] has been considered a risk factor for AS, however, whereas the relation between LDL-C and inflammation is well established, the relationship between Lp(a) and inflammation is not fully elucidated.

Familial hypercholesterolemia (FH) is a prevalent genetic disorder characterized by high LDL-C levels, thus leading to premature coronary heart disease (CHD) [5]. Furthermore, FH patients are characterized by increased inflammation [6,7], and we have recently shown that the prevalence of CHD was doubled in FH subjects with elevated Lp(a) levels compared to FH subjects with relatively low Lp(a) [8]. Moreover, studies have suggested that Lp(a) could be a risk factor for calcific aortic valve disease in heterozygous FH [9,10]. Thus, FH could represent a model disease for studying the relation between LDL-C, Lp(a) and other biomarkers with regard to the aortic valve function. The aim of our study was to investigate the relation between Lp(a) and other circulating biomarkers with measurements of the aortic valve including velocities, gradients and AVA in adult FH subjects and controls.

Material and Methods

Subjects and study design

We consecutively recruited heterozygous subjects with FH above 18 years of age with and without high Lp(a) from the outpatient Lipid Clinic, Oslo University Hospital, Norway, using a cross-sectional design. Participants with definite FH either genetically verified or a Dutch lipid clinic network score >8, and willingness to give a blood sample, were recruited. Exclusion criteria were diabetes mellitus type 1, uncontrolled hypertension, pregnancy or lactation.

At the study visits we performed cardiac ultrasound, measured height, weight and blood pressure, and obtained a non-fasting blood sample. Age- and sex-matched (by percentage) healthy controls were recruited among employees and friends of employees at the Department of Nutrition, University of Oslo, Norway and Oslo University Hospital, Norway. Exclusion criteria for the controls were: Lp(a) levels ≥75 nmol/L, cardiovascular or metabolic disease, use of lipid-lowering therapy, severe illness such as cancer the last 5 years, pregnancy or breastfeeding. The recruitment period was September 2016-September 2017. All participants gave informed consent. The Regional Committee of Medical and Health Research Ethics, South-East Norway (no. 2015/1577) and the Oslo University Hospital Data Protection Officer approved the study protocol. We conducted the study in accordance with the principles of the Declaration of Helsinki.

Ultrasound examination and measurements

The ultrasound examination was performed by means of GE Vivid E95 scanners and GE M5Sc transducers for transthoracic examination (GE Healthcare, Milwaukee, WI). The examiners were two experienced cardiologists blinded to clinical information. Measurements

were made “off-line” by means of GE Echopac version 202 (GE Healthcare, Milwaukee, WI) and all the measurements were performed by both the examiners for all of the examinations. For aortic valve measurements, the patients were examined in the left decubitus position. The velocity in the left ventricle (LV) outflow tract (LVOT) velocity was obtained with pulsed wave doppler with the sample volume placed just below the aortic valve (average of three measurements). LVOT diameter was measured at the level of the aortic valve annulus using zoom mode (average of three measurements). Peak aortic valve jet velocity was obtained with continuous wave doppler (GE M5Sc probe) from multiple acoustic windows to yield the highest velocity signal. Peak and mean aortic valve gradients were derived from peak velocity. Stroke volume was calculated as $\pi \times (\text{LVOT diameter}/2)^2 \times$ velocity-time integral of pulsed-wave LVOT velocity. AVA was defined as stroke volume divided by the velocity-time integral of the peak aortic jet velocity and indexed to body surface area (cm^2/m^2). An average of the two raters was used for the final statistical analyses.

Biochemical analyses

Standard biochemical analyses, fibrinogen, D-dimer, troponin T, C-reactive protein (CRP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) were analyzed by standard methods at an accredited medical laboratory at Oslo University Hospital, Norway, using plasma or serum. The immunoturbidimetric method by Roche Diagnostics was used to analyze Lp(a). Roche uses immunoturbidimetric assays with five-point calibration curves constructed with five lyophilized standards which were reconstituted separately. This method is one of several validated methods for quantifying Lp(a) [11]. The cut-off values for high Lp(a) in our laboratory system is set to 75 nmol/L, and hence we have defined high Lp(a) above 75nmol/L, and low Lp(a) to below 75 nmol/L. Lp(a) levels were also measured in

mg/dL with an automatic nephlometric assay, N latex Lp(a) from Dade Behring, using a Behring Nephlometer-II.

The inflammatory markers interleukin (IL)-1β, IL-6, IL-8/CXCL8, IL-10, tumor necrosis factor (TNF), interferon (IFN)-γ (Catalogue number [Cat#] for all: K151A0H-1) and transforming growth factor (TGF)-β (Cat#, K151XWK-1) were analyzed using commercial kits from Meso Scale Discovery by the commercial laboratory Vitas Analytical Services. The ratio between TNF and IL-10 was determined as molecular mass of [17×3] kDa for TNF [trimer] and 18.6kDa for IL-10. Platelet factor 4 (PF4)/CXCL4, neutrophil activating peptide 2 (NAP2)/CXCL7, *r*egulated on **a**ctivation *n*ormal **T** cell **e**xpressed and **s**ecreted (RANTES)/CCL5 and cluster of differentiation 40 ligand (CD40L) were analysed by enzyme-linked immunosorbent assay from R&D Systems catalogue number (catalogue # DY795, DY393, DY278 and DY617, respectively, RnD Systems, Minneapolis, USA).

Kringle IV (KIV) isoform 2 size

The Apo(a) KIV repeat number was determined by immunoblotting, as previously described [9,12]. In case two distinct Apo(a) isoforms were present, the band representing the smaller isoform, showing the strongest intensity in most cases, was used as a continuous variable.

Statistics

Characteristics are presented as mean (standard deviation, SD) or median (25th -75th percentile, IQR) for continuous variables and frequency (%) for categorical variables. Independent samples T test or Mann-Whitney U test were used for normally and not normally distributed continuous variables, respectively, and Chi-Square test was used for categorical data. In the main analyses, linear regression analyses were performed for all circulating CVD markers and cardiac ultrasound measures, except calcification in valves. The assumption of

normally distributed residuals was checked. Variables were $\log_e$ transformed when model assumptions were not fulfilled. We summarized the results by presenting univariable p-values in a table and multivariable p-values and estimated regression coefficients with 95% confidence interval (CI) in forest plots. The categorical variable calcification in valves were analyzed by logistic regression analyses. Use of proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitor were included in models comparing FH subjects with and without elevated Lp(a). Use of PCSK9 inhibitor, BMI and mean arterial blood pressure (MAP) were included in models comparing FH and healthy subjects. Estimated regression coefficients (log-odds ratios from logistic regression analyses) with 95% confidence intervals (CIs), and P-values are presented in supplementary tables. We conducted a sensitivity analysis excluding subjects using PCSK9 inhibitor in the model comparing FH subjects with and without elevated Lp(a). Furthermore, we conducted another sensitivity analysis in the model comparing FH to healthy subjects with additional adjustment for age, sex and smoking. Finally, we used Spearman's rho when correlating circulating CVD markers with cardiac ultrasound measures. Spearman's rho and P-values are presented in a supplementary heatmap and table. We used IBM SPSS Statistics 24.ink and R version 3.6.1 with RStudio IDE to perform statistical analyses. P-values (two-tailed) < 0.05 were considered significant.

Results

Characteristics

The study population consisted of a total of 64 FH subjects with high (n=29) and low Lp(a) (n=35) respectively, and 14 healthy controls (Table 1). The median (25^{th}-75^{th} percentile) Lp(a) concentration was 191 nmol/L (157-280), 7 nmol/L (7-9) and 10 nmol/L (7-28) in the three groups, respectively. When using Lp(a) measurements from a different Lp(a) assay, the Lp(a) concentrations were 89.0 mg/dL (74.7-138.0), 3.1 mg/dL (1.8-6.3) and 5.4 mg/dL (3.2-17.9) for the three groups respectively. The Lp(a) values measured with the two assays correlated highly ($r=0.95$, $p<0.001$). Body mass index (BMI) and blood pressure were significantly higher in the total FH group compared to healthy controls (Table 1). Coronary heart disease was present in 9 (14.1%) FH subjects with elevated Lp(a), 4 (11.4%) FH subjects without elevated Lp(a) and none of the healthy subjects. Ninety-one percent of the subjects with FH were using statins, ezetimibe, resins, PCSK9-inhibitors or combinations thereof (Table 2). FH patients with elevated Lp(a) used PCSK9-inhibitors more often than FH patients with low Lp(a) levels ($p=0.03$). As expected, KIV-2 repeat number was lower in FH subjects with elevated Lp(a) compared to FH subjects without elevated Lp(a) ($p=0.003$, Figure 1).

Cardiac ultrasound measures

LVOT diameter, the indexed and non-indexed AVA were lower, and peak velocity in the aortic valve was higher in the FH subjects compared to controls ($0.003 \leq P \leq 0.03$), while there was a trend for increased aortic valve peak and middle gradients (Table 3). When performing multivariable linear regression, correcting for blood pressure, BMI and use of PCSK9-inhibitor, there were no significant differences between the FH and control subjects, except for the LVOT diameter (Figure 2 and Supplementary Table 1). Also, we could not

demonstrate any significant differences between the high and low Lp(a) groups (Table 3 and Supplementary Table 2).

Circulating biomarkers

The total FH group had significantly higher levels of the inflammatory platelet markers CD40L, PF4, NAP2 and RANTES, and lower of IL10 compared to controls ($0.001 \leq P \leq 0.02$, Table 3). When performing multivariable linear regression, correcting for blood pressure, BMI and use of PCSK9-inhibitor, these differences were still significant (Figure 3 and Supplementary Table 1). There were no significant differences in circulating cardiovascular markers between the high or low Lp(a) groups (Table 3 and Supplementary Table 2).

Supplementary calculations are added including correlation between circulating cardiovascular markers and cardiac ultrasound measures in FH subjects (Supplementary Figure 1 and Table 3).

Table 1. Characteristics of the participants

	FH subjects					
	High Lp(a) n=29	**Low Lp(a) n=35**	**All n=64**	**Healthy subjects n=14**	**P[1]**	**P[2]**
Descriptives						
Age, years	49 (15)	49 (15)	49 (15)	44 (12)	0.82	0.21
Female, n(%)	17 (59)	18 (51)	35 (55)	8 (57)	0.75	1.00
BMI, kg/m^2	23 (22-27)	25 (23-29)	25 (22-28)	21 (19-22)	0.18	**<0.001**
SBP, mmHg	128 (15)[‖]	128 (18)	128 (17)[#]	115 (10)	0.94	**0.005**
DBP, mmHg	76 (9)[‖]	76 (10)	76 (9)[#]	70 (8)	0.93	**0.047**
Current smoking, n(%)	3 (10)	5 (14)	8 (13)	0	0.92	0.36
Coronary heart disease, n(%)	9 (14.1)	4 (11.4)	13 (20.3)	0	0.10	0.15
Blood biochemistry						
Total cholesterol, mmol/L	4.4 (3.9-5.3)	4.9 (4.1-5.6)	4.7 (4.0-5.5)	5.0 (4.5-5.3)	0.28	0.33
HDL-C, mmol/L	1.5 (1.2-1.8)	1.6 (1.3-1.8)	1.5 (1.3-1.8)	1.6 (1.3-2.1)	0.44	0.39
LDL-C, mmol/L	2.6 (2.1-3.0)	2.9 (2.3-3.5)	2.8 (2.2-3.2)	3.0 (2.4-3.4)	0.30	0.42
Triglycerides, mmol/L	0.9 (0.8-1.2)	0.9 (0.7-1.4)	0.9 (0.7-1.3)	1.0 (0.8-1.5)	0.94	0.43
ApoA1, g/L	1.5 (1.3-1.7)	1.5 (1.3-1.7)	1.5 (1.3-1.7)	1.5 (1.4-1.7)	0.93	0.57
ApoB, g/L	0.9 (0.7-1.1)	1.0 (0.9-1.1)	0.9 (0.8-1.1)	0.8 (0.7-1.0)	0.57	0.05
Lp(a), nmol/L	191 (157-280)	7 (7-9)	20 (7-188)	10 (7-28)	**<0.001**	0.10
Lp(a), mg/dL	89.0 (74.7-138.0)	3.1 (1.8-6.3)	12.7 (2.9-87.5)	5.4 (3.2-17.9)	**<0.001**	0.12
Glucose, mmol/L	5.3 (4.9-5.5)	5.2 (5.1-5.5)	5.3 (5.0-5.5)	5.1 (4.6-5.5)	0.92	0.16

Data are presented as median (25th-75th percentile) or mean (standard deviation) for continuous variables, and frequency (%) for categorical variables. P-values from independent samples T-test, Mann-Whitney U test, or Chi-Square test.

[1]High vs low Lp(a)

[2]FH all vs healthy subjects

Apo, apolipoprotein; BMI, body mass index; DBP, diastolic blood pressure; FH, familial hypercholesterolemia; g/L, grams per Litre; HDL-C, high density lipoprotein cholesterol; kg, kilograms; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein (a); m, meters; mg/L, milligrams per Litre; mmHg, millimeter mercury; mmol/L, millimoles per Litre; nmol/L, nanomoles per Litre; SBP, systolic blood pressure.

[‖]n=27, [#]n=62

Table 2. Medical treatment of subjects with FH

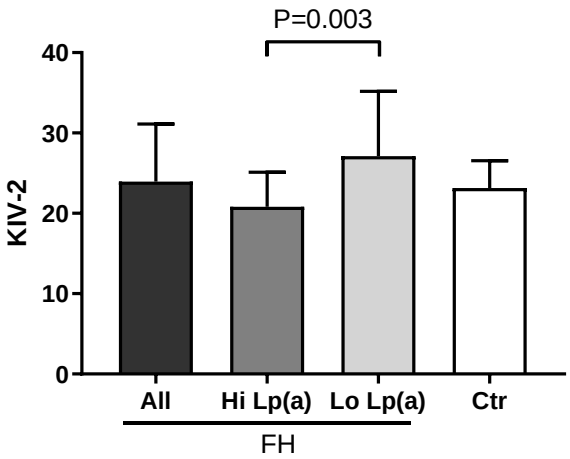
	High Lp(a), n=29	Low Lp(a), n=35	P[1]
Medicament			
Statins	26 (90)	30 (86)	0.72
PCSK9-inhibitor	10 (35)	4 (11)	**0.03**
Colesevelam	6 (21)	4 (11)	0.49
Ezetimibe	20 (69)	21 (60)	0.46
Acetylsalicylic acid	13 (45)	9 (26)	0.11

Data are presented as frequency (%).

[1]Chi Square test between the two groups.

FH, familial hypercholesterolemia; Lp(a), lipoprotein a; PCSK9, proprotein convertase subtilisin/kexin type 9.

Figure 1.



Kringle IV isoform 2 molecular weight in all FH patients (n=58), FH subjects with (n=29) and without (n=29) elevated Lp(a) and healthy controls (n=14) measured by immunoblotting. Data are presented as median and interquartile range. FH familial hypercholesterolemia; Lp(a), lipoprotein (a).

Table 3. Cardiac ultrasound measures and circulating CVD markers in the FH and healthy subjects.

	FH subjects					
	High Lp(a) n=29	**Low Lp(a) n=35**	**All n=64**	**Healthy subjects n=14**	**P[1]**	**P[2]**
LVOT diameter, mm	21.3 (1.6)	21.7 (1.7)	21.5 (1.7)	22.6 (2.4)	0.31	**0.03**
LVOT V max, m/s	1.0 (0.1)	1.0 (0.2)	1.0 (0.2)	0.9 (0.1)	0.52	**0.03**
AV V peak, m/s	1.3 (1.1-1.5)	1.2 (1.1-1.5)	1.2 (1.1-1.5)	1.0 (1.0-1.1)	0.65	**0.02**
AV peak grad, mm Hg	7.7 (5.7-9.3)	6.4 (5.1-9.5)	7.0 (5.3-9.5)	4.8 (4.3-5.8)	0.73	0.05
AV middle grad, mm Hg	4.3 (3.3-5.4)	3.8 (2.9-5.5)	3.9 (3.0-5.5)	2.9 (2.6-3.3)	0.64	0.05
AVA, cm^2	2.4 (0.6)	2.5 (0.5)	2.5 (0.6)	2.8 (0.6)	0.30	**0.04**
AVA indexed, cm^2/m^2	1.3 (0.3)	1.3 (0.3)	1.3 (0.3)	1.6 (0.3)	0.36	**0.003**
Calcification valve	8 (27.6)	9 (25.7)	17 (26.6)	0	0.80	0.99
CD40L, ng/mL	0.4 (0.3-0.4)	0.4 (0.3-1.0)	0.4 (0.3-0.6)	0.2 (0.1-0.3)	0.06	**0.02**
PF4, μg/mL	0.9 (0.7-1.1)	1.0 (0.8-1.2)	1.0 (0.7-1.2)	0.6 (0.4-0.7)	0.99	**<0.001**
NAP2, ng/mL	0.3 (0.2-0.3)	0.3 (0.2-0.3)	0.3 (0.2-0.3)	0.2 (0.1-0.2)	0.96	**<0.001**
RANTES, ng/mL	1.6 (1.4-1.8)	1.6 (1.5-1.7)	1.6 (1.5-1.7)	1.1 (0.8-1.3)	0.62	**<0.001**
Fibrinogen, g/L	3.1 (2.9-3.5)	3.2 (2.8-3.5)	3.2 (2.8-3.5)	2.9 (2.7-3.1)	0.47	0.27
Troponin T, ng/L	5.0 (5.0-7.0)	6.0 (5.0-8.5)	6.0 (5.0-8.5)	5.5 (5.0-7.0)	0.39	0.69
CRP, mg/L	0.6 (0.6-0.6)	0.6 (0.6-0.8)	0.6 (0.6-0.7)	0.6 (0.6-0.8)	0.34	0.74
NT-proBNP, ng/L	49.1 (34.7-88.4)	55.8 (32.2-99.5)	50.0 (32.7-99.5)	49.7 (30.0-131)	0.58	0.54
TGF-β1, ng/mL	2.8 (1.7-3.8)	2.6 (1.9-3.4)	2.7 (1.8-3.6)	2.4 (1.4-3.3)	0.69	0.46
IFN-γ, pg/mL	3.5 (2.6-4.8)	4.0 (3.3-6.3)	3.7 (2.6-6.2)	5.9 (3.5-6.8)	0.80	0.14
IL6, pg/mL	0.3 (0.2-0.6)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	0.3 (0.2-0.4)	0.42	0.14
IL8, pg/mL	3.1 (2.9-4.3)	3.3 (2.7-4.6)	3.3 (2.7-4.5)	2.8 (2.3-3.6)	0.74	0.44
IL10, pg/mL	0.2 (0.2-0.3)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	0.4 (0.2-0.7)	0.59	**0.001**
TNF, pg/mL	1.8 (0.7)	2.0 (0.7)	1.9 (0.7)	2.2 (0.5)	0.25	0.21
TNF/IL10 ratio	2.7 (1.7-3.2)	2.3 (1.8-3.9)	2.6 (1.8-3.7)	2.3 (1.5-2.8)	0.62	0.16

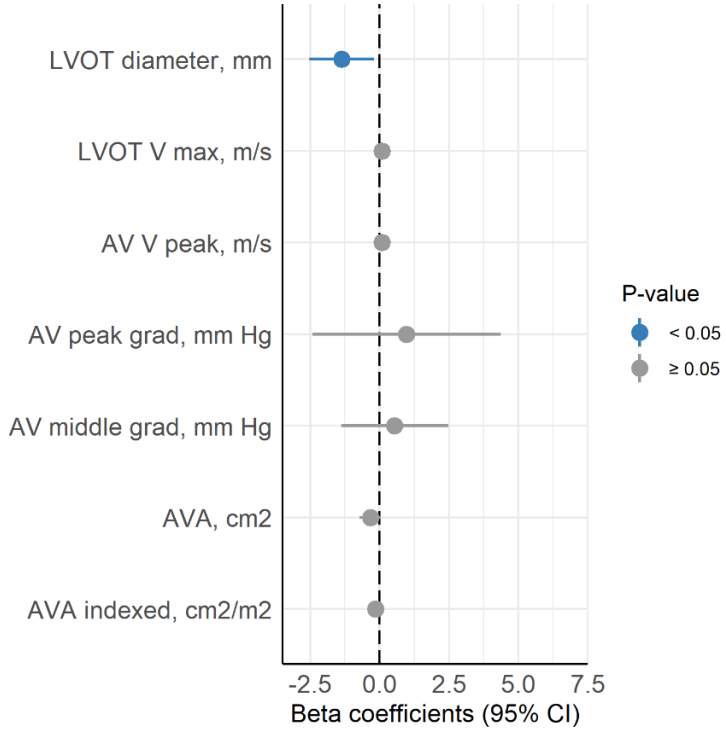
Data are presented as mean (standard deviation) or median (25^{th}-75^{th} percentile). P-values from univariable linear regression analyses for all variables except valve calcification where logistic regression analyses were used. For C-reactive protein (CRP) values <0.6 are set to 0.6.

[1]High vs low Lp(a)

[2]FH all vs healthy subjects

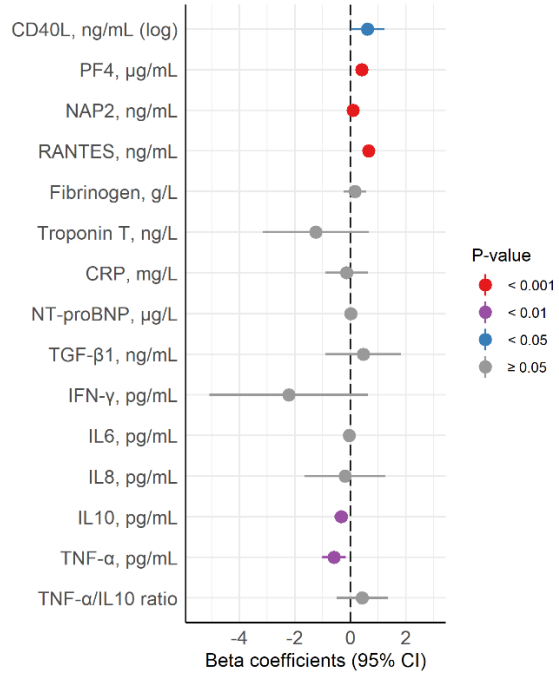
AV, aortic valve; AVA, aortic valve area; CD40L, cluster of differentiation 40 ligand; FH, familial hypercholesterolemia; IFNγ, interferon γ; IL, interleukin; Lp(a), lipoprotein (a); LVOT, left ventricular outlet tract; NT-proBNP, N-terminal pro-Brain Natriuretisk Peptid; NAP2, neutrophil activating peptide 2; Peak grad, peak gradient; PF4, platelet factor 4; RANTES, Regulated on Activation Normal T Cell Expressed and Secreted; TGF-β1, transforming growth factor β1, TNF, tumor necrosis factor; V, velocity.

Figure 2. Forest plot of estimated regression coefficients from multivariable linear regression analyses of cardiac ultrasound measures in FH (n=64) compared to healthy (n=14) subjects.



Results are presented as estimated regression coefficients with 95% confidence intervals (CIs), and P-values for FH compared healthy subjects (vertical zero-line). AV, aortic valve; AVA, aortic valve area; CI, confidence interval; FH, familial hypercholesterolemia; LVOT, left ventricular outlet tract; Peak grad, peak gradient; V, velocity.

Figure 3. Forest plot of estimated regression coefficients from multivariable linear regression analyses of circulating cardiovascular markers in FH (n=64) compared healthy (n=14) subjects.



Results are presented as estimated regression coefficients with 95% confidence intervals (CIs), and P-values for FH compared healthy subjects (vertical zero-line). CI, confidence interval; FH, familial hypercholesterolemia; CD40L, cluster of differentiation 40 ligand; CRP, c-reactive protein; IFNγ, interferon γ; IL, interleukin; Lp(a), lipoprotein (a); NT-proBNP, N-terminal pro-Brain Natriuretisk Peptid; NAP2, neutrophil activating peptide 2; PF4, platelet factor 4; RANTES, Regulated on Activation Normal T Cell Expressed and Secreted; TGF-β1, transforming growth factor β1, TNF-α, tumor necrosis factor α.

Discussion

The risk of aortic valve calcification and AS is increased in individuals with FH [13,14]. Furthermore, emerging data link increased levels of Lp(a) with the development of AS and also indicate the possibility of reducing Lp(a) as a mean to reduce the progression of AS [15–18].

To our knowledge, this is the first study to show an increased aortic valve peak velocity and a decreased adjusted an unadjusted AVA in FH patients compared to controls even though the difference was not significant using multiple linear regression. We could not demonstrate any difference between FH patients with high and low Lp(a) in this small group of patients, but other studies have pointed out that the Lp(a) effect on cardiovascular endpoints are modulated by IL-1 genotypes [19,20]. In the present study we have no information on the IL-1 genotype.

Our findings are interesting in several aspects. Firstly, the study participants are relatively young, with a mean age just below 50 years. Finding an increased aortic valve peak velocity and reduced AVA in this relatively young patient group is interesting, as AS is considered to be a disease mainly of older persons. An epidemiological study from Norway demonstrated that AS had a prevalence of 0.2% in the 50-59 years cohort, 1.3% in the 60-69 years cohort and 3.9% in the 70-79 years cohort [21], which is in line with international findings [22,23]. Secondly, the subjects with FH were quite well-treated, with a median LDL-C of 2.8 mmol/L (2.6 and 2.9 mmol/L in the group with and without elevated Lp(a) respectively), only slightly above current recommendations [24], but better than “real-life” clinical data of 3.5 mmol/L in normal-risk FH [8]. Thus, our finding of increased peak aortic valve velocity and reduced AVA in these middle-aged FH subjects, may reflect an early phase of aortic valve area reduction, which may lead to AS later on.

We also found that FH subjects had higher levels of the inflammation markers CD40L, PF4, NAP2 and RANTES compared to controls, and these differences persisted also after multiple linear regression. Activated platelets have been considered important in driving calcification and progression of AS which has been demonstrated in a study utilizing explanted aortic valves [25]. Thus, it is remarkable that all cytokines that were elevated in the FH group compared with healthy controls were platelet-derived cytokines (i.e., CD40L, PF4, NAP2 and RANTES). This may suggest that platelet-mediated inflammation could be of particular interest when developing new treatment strategies in FH subjects on optimal lipid lowering therapy [26]. The relationship between platelet-derived cytokines and FH is not fully clarified, however cross talk between LDL-C regulation and the PF4 gene has been implicated [27].

Although Lp(a) has been associated with inflammation *in vitro* [28], few studies have investigated this relationship in humans. Interestingly, in these FH patients we could not demonstrate a relationship between Lp(a) and any of the circulating inflammatory markers. This is in contrast to previous human studies showing correlations between CRP and Lp(a) concentration [29,30].

Major strengths of the present study are the ultrasound measurements and the relatively large number of circulating markers measured in genetically verified FH subjects. A limitation is the low number of subjects included in the study, in particular control subjects. This increases the probability of type 1 and 2 errors, and this study cannot exclude a relationship between Lp(a) and development of aortic valve area reduction in FH patients due to the low number of participants. Also, in the control group, the subjects were leaner and had lower blood

pressure, which may have affected the results. However, the main reason for including the control group was to compare the measurements to a reference population as many of the measured markers do not have established cut-off values. A higher proportion of the FH patients in the high Lp(a) group were on PCSK-9 inhibitors which may have attenuated the median Lp(a) values in this group and according to a prespecified analysis from the Fourier trial, the Lp(a) values are reduced with 27% (median values) by evolocumab compared to placebo [31]. Recently, the use of AVA alone for classifying aortic stenosis is questioned, especially due to the fact that the LVOT diameter can be difficult to quantify [32], and indeed our results indicate a small difference in LVOT diameter that could be important if just relying on AVA measurements alone, especially as the LVOT diameter is squared in the formula estimating AVA. Both American and European guidelines endorse the use of several parameters for evaluating aortic stenosis including velocities and gradients, and used in a clinical context [33–35]. Furthermore, other imaging techniques including Cardiac CT could add information regarding the aortic valve [36,37].

In conclusion, middle-aged, intensively treated FH subjects have higher aortic valve velocity lower AVA, and higher levels of the platelet-derived markers CD40L, PF4, NAP2 and RANTES compared to healthy control subjects. The aortic valve findings were not significant after multiple linear regression, whereas the higher levels of platelet-derived markers were maintained. Nonetheless, as aortic stenosis is considered to be a disease mainly of older persons, therefore our finding of increased peak aortic valve velocity and reduced AVA in these middle-aged FH subjects, may reflect an early phase of aortic valve area reduction, which may lead to AS later on.

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Conflict of Interest

Dr. Bogsrud has received research grants and/or personal fees from Amgen, Sanofi, MSD, Boehringer Ingelheim, Mills DA and Kaneka, none of which are related to the content of this manuscript. Dr. Retterstøl has received research grants and/or personal fees from Amgen, Mills DA, The Directorate for Health in Norway, The Norwegian Medical Association, Sanofi, Chiesi, Takeda, Bayer, MSD, none of which are related to the content of this manuscript. Dr. Ulven has received research grants and/or personal fees from Mills DA, Tine BA, and Rimfrost, none of which are related to the content of this manuscript. Dr Roeters van Lennep reports honoraria from Akcea and grants from Aegerion/Amryt none of which are related to the content of this manuscript. Dr. Holven reports grants and/or personal fees from Tine SA, Mills DA, Olympic Seafood, Amgen, Sanofi, Kaneka and Pronova, none of which are related to the content of this manuscript. Dr Wium has received grants and/or personal fees from Amgen, Sanofi, MSD, Novo Nordisk, Boehringer Ingelheim, Lilly and

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Authorship

Authors' contribution: I.N., M.P.B., and K.B.H. conceived and designed research; A.H., I.N., M.P.B., I.Aa., T.U., M.M., F.L., and K.B.H. conducted research; I.N and L.K.L.Ø. performed statistical analyses; A.H., I.N., L.K.L.Ø., M.P.B., I.Aa., T.U., M.M., F.L., G.L., K.R., C.W., A.S., K.E.A., J.R.v.L., P.A., B.H., S.M.U., and K.B.H., interpreted results; A.H, I.N., and K.B.H were responsible for drafting the manuscript; A.H., I.N., L.K.L.Ø., and K.B.H. were responsible for final content; all authors read, critically revised, and approved the final manuscript.

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