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Lipoprotein-associated phospholipase A_2 activity is increased in patients with definite familial hypercholesterolemia compared with other forms of hypercholesterolemia.

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SHORT TITLE

Lp-PLA$_2$ activity in definite FH and non-definite FH hypercholesterolemic subjects

ABSTRACT

Background: Lipoprotein-associated phospholipase A_2 (Lp-PLA$_2$) plays a key role in atherosclerosis development. It is considered a marker of increased risk of cardiovascular disease (CVD) and plaque vulnerability. Familial hypercholesterolemia (FH) is a genetic disorder characterized by elevated plasma levels of low-density lipoprotein cholesterol and a higher prevalence of early CVD.

Objective: Our aim was to evaluate the differences in Lp-PLA$_2$ activity in a population of hypercholesterolemic patients with and without definite FH.

Methods: Hypercholesterolemic patients were consecutively recruited. Definite FH was defined according to Dutch Lipid Clinic Network criteria ≥ 8. All patients underwent routine clinical examination and biological assessments and Lp-PLA$_2$ activity was measured in blood samples.

Results: Among 469 patients, 118 had a definite diagnosis of FH. Lp-PLA$_2$ activity was significantly higher in definite FH patients compared to non-definite FH patients (206.5 ± 54.5 vs. 180.8 ± 48.4 nmol/min/mL, p <0.0001). Lp-PLA$_2$ positively correlated with total cholesterol, LDL-C and apolipoprotein B and negatively with HDL-C and apolipoprotein A-1. In multivariate analysis, definite FH diagnosis, LDL-C, HDL-C and statin treatment remained correlates of Lp-PLA$_2$ independently of systolic blood pressure.

Conclusions: Lp-PLA$_2$ activity was higher in definite FH than in non-definite FH patients independently of LDL-C levels and statin treatment. These results highlight the particular phenotype of FH subjects among hypercholesterolemic patients. As increased Lp-PLA$_2$ activity suggests, FH patients exhibit higher arterial inflammation that may contribute to their high cardiovascular risk. Our results reinforce the potential beneficial role of statins pleiotropic effects and the need for proper identification and treatment of FH patients.

KEYWORDS: lipoprotein-associated phospholipase A_2, familial hypercholesterolemia, dyslipidemia, cardiovascular disease, plaque vulnerability, vascular inflammation, cardiovascular risk, statin treatment, high density lipoprotein, low density lipoprotein.

INTRODUCTION

Lipoprotein-associated phospholipase A_2

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a calcium-independent enzyme mainly produced by macrophages. Lp-PLA_2 has been shown to play a key role in the development of atherosclerosis due to its pro-inflammatory and pro-oxidative effects[1]. Lp-PLA_2 is synthesized within the atherosclerotic plaque and circulates bound to LDL-C particles[2]. By the hydrolysis of oxidized LDL-C particles, Lp-PLA_2 generates bioactive lipid products (lysophosphatidylcholine and oxidized free fatty acids) which can activate and sustain the atherosclerosis process[3,4]. Lp-PLA_2 is also implicated in endothelial dysfunction[5], plaque vulnerability[6,7,8] and has been associated with inflammation within the atherosclerotic plaque[9]. Furthermore, elevated Lp-PLA_2 levels were related to increased risk of coronary disease, stroke, and vascular mortality in several large scale prospective studies[10].

However, little data exists in the specific population of patients with familial hypercholesterolemia (FH) which is an autosomal dominant lipoprotein metabolism disorder characterized by elevated plasma levels of low-density lipoprotein cholesterol (LDL-C)[11]. FH results from genetic heterozygous (heFH) or homozygous (hoFH) mutation in the low density lipoprotein receptor gene (*LDLR*), apolipoprotein B-100 gene (*APOB*) or proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*)[11]. FH patients exhibit early atherosclerotic lesions[12,13]with premature CVD compared to non-FH patients[14,15]. Prevalence of CVD among heFH has been estimated to be about 33%[16] with a CVD increased risk by 8 to 17- fold vs. non-FH subjects. In patients untreated with statins, angina and acute myocardial infarctions can be observed early in life, even before 40 years old[17].

Our aim was to evaluate Lp-PLA_2 activity in a population of hypercholesterolemic patients with and without a clinical confirmed definite FH.

MATERIAL AND METHODS

Study population

Patients were consecutively recruited at our outpatient clinic (Cardiovascular Prevention Unit, Institute of Cardiometabolism and Nutrition, La Pitié-Salpêtrière-Charles Foix University Hospital, APHP, Paris, France) between June 2014 and June 2015. Only subjects with hypercholesterolemia were eligible for enrolment in this study. Hypercholesterolemia was

defined according to patient's records as: LDL-C >4.14 mmol/L or/and treatment with statin. FH groups and Dutch Lipid Clinic Score were determined using patient's files. Patients were included and divided into two subgroups:

- Definite FH (DFH) group defined according to the Dutch Lipid Clinic Network criteria[18,19]with a score ≥8;
- Non-definite FH (NDFH) group of patients with Dutch Lipid Clinic Network criteria score <8.

Exclusion criteria were: LDL-C ≤1.3 mmol/L without lipid lowering treatment, high-density lipoprotein cholesterol (HDL-C) ≥2.6 mmol/L or ≤ 0.39 mmol/L; triglycerides (TG) ≥11.4 mmol/L; fasting glucose ≥10 mmol/L; history or treatment of thyroid disease; FH homozygous status. The aim for those exclusions was to define a homogenous population of patients with hypercholesterolemia.

All patients underwent routine clinical examination and biological assessments. In addition, Lp-PLA_2 activity was assessed. Arterial hypertension (AH) was defined as arterial blood pressure (BP) >140/90 mmHg and/or antihypertensive treatment; type 2 diabetes (T2D) as fasting plasma glucose >6.94 mmol/L and/or glucose lowering treatment. Cigarette smoking (yes/no), body mass index (BMI) (weight in kilograms divided by the square of height in meters) and waist circumference were also evaluated. The study was carried out according to the principles outlined in the Declaration of Helsinki. Approval of the local Ethics Committee was obtained and informed consent was signed by all participants.

Laboratory methods

Lipid analysis

Fresh venous blood was collected in gel-containing Vacutainer® tubes (Becton-Dickinson, Plymouth, UK), then centrifuged at 4,500 rpm at 4°C for 10 minutes.

Fresh serum lipids were analysed by using routine methods on a Konelab 30i analyser (Thermo Electron Corporation). TG were measured by using an enzymatic method with colorimetric detection (Diasys France Condom)[20]. Total cholesterol (TC), direct LDL-C and direct HDL-C were determined using automated enzymatic methods (Konelab Thermo Fisher Scientifics, Asnières sur Seine, France)[21,22,23]. LDL-C was calculated by the Friedewald

formula when TG <3.9 mmol/L[24] and directly dosed when TG were between 3.9 and 11.4 mmol/L.

ApoA-I, apoB and Lp(a) were measured by immunonephelometry using anti-ApoA-I, anti-ApoB and anti-apo(a) antisera and a BN II nephelometer analyser from Siemens (Siemens Healthcare Diagnostics S.A.S, Saint Denis France; inter- and intra-assay coefficients of variation <4%)[25].

Measurement of lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activity

Measurement of Lp-PLA_2 activity was performed with automated colorimetric method on Konelab 30i analyser, by using a rate reaction assay with 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine as substrate (Diadexus Inc. South San Francisco. CA. Eurobio, France). Units were expressed as nmol platelet activating factor (PAF) hydrolysed per minute per mL of serum (nmol/min/mL)[26]. All assays were performed in the Department of Metabolic Biochemistry of Pitié-Salpêtrière - Charles Foix Hospital.

Statistical analysis

All continuous variables were described by their mean ± standard deviation (SD) and proportions (%) for categorical variables. The differences between groups were evaluated by Student's t test for continuous variables and χ^2 test for categorical variables. Correlations between Lp-PLA_2 and lipid parameters were assessed using Pearson's correlation coefficient except for Lp(a) and TG where Spearman's rank correlation coefficients were provided. Multivariate linear regression analysis was used to assess the independent contribution of the variables. A p value <0.05 was considered significant. JMP® Statistical Software, Version 11, SAS Institute Inc., Cary, NC, was used.

RESULTS

Our population consisted of 469 subjects. Among them, 118 (25.1%) were classified into the definite FH group. Among DFH subjects, 51 had a known heterozygous mutation of *LDLR*, *APOB* or *PCSK9* gene. The clinical and biochemical characteristics of the whole population, as well as in DFH and in NDFH subjects are shown in Table 1. The mean Lp-PLA_2 activity of the whole population was 187.3 ± 51.2 nmol/min/mL, with a significant difference between

males and females (195.9 ± 48.5 vs. 178.0 ± 52.4 nmol/min/mL respectively, p 0.0001, data not shown). No difference in Lp-PLA_2 levels was found in the DFH group among subjects with known mutations and subjects without known mutations (210.1 ± 50.5 and 203.8 ± 57.5 nmol/min/mL respectively, p 0.5330, Additional Figure 1).

Lp-PLA_2 activity was significantly higher in DFH subjects than it was in NDFH subjects (206.5 ± 54.5 vs 180.8 ± 48.4 nmol/min/mL, p <0.0001). DFH subjects were significantly younger than NDFH and less likely to suffer from diabetes or hypertension. Moreover, DFH subjects also exhibited lower HbA1c, TG, ApoA-1 levels, blood fasting glucose and blood pressure (BP) levels than NDFH. In opposite, they had higher TC, LDL-C and ApoB levels than NDFH subjects. Values of HDL-C and Lp(a) as well as the percentage of patients under statin treatment were identical in both groups.

Table 1. Whole population, definite familial hypercholesterolemia (DFH) and non-definite FH (NDFH) subgroups characteristics.

Characteristics	**All subjects**	**DFH**	**NDFH**	**p[a]**
n	469	118	351	
Age, years	59 ± 14	50 ± 16	62 ± 11	**<0.0001**
Sex, men (%)	243 (52)	60 (50.9)	183 (52.1)	0.8084
Lp-PLA_2 activity, nmol/min/mL	187.3 ± 51.2	206.5 ± 54.5	180.8 ± 48.4	**<0.0001**
Cardiovascular risk factors				
Current smoking, n (%)	88 (19)	20 (17.0)	68 (19.4)	0.5096
Type 2 Diabetes, n (%)	74 (16)	6 (5.1)	68 (19.4)	**<0.0001**
HbA1c, %	5.9 ± 0.60	5.68 ± 0.55	5.96 ± 0.60	**<0.05**
Arterial Hypertension, n (%)	195 (42)	21 (17.8)	174 (49.7)	**<0.0001**
Systolic BP, mmHg	116.7 ± 12.8	112.7 ± 12.2	118 ± 12.8	**<0.0001**
Treatment				
Antihypertensive treatment, n (%)	186 (40)	19 (16)	167 (48)	**<0.0001**
Statins, n (%)	332 (71.0)	90 (76.3)	242 (69.0)	0.1301
Ezetimibe, n (%)	75 (16)	31 (26.3)	44 (12.5)	**< 0.001**
Lipid profile				
Total Cholesterol, mmol/L	5.89 ± 1.58	6.34 ± 1.96	5.75 ± 1.41	**<0.001**
LDL-Cholesterol, mmol/L	3.77 ± 1.5	4.36 ± 1.92	3.60 ± 1.26	**<0.0001**
HDL-Cholesterol, mmol/L	1.37 ± 0.44	1.42 ± 0.43	1.37 ± 0.45	0.2830
Triglycerides, mmol/L	1.65 ± 1.32	1.25 ± 0.75	1.81 ± 1.45	**<0.0001**
Apolipoprotein A-1, mg/dL	159.3 ± 26.7	155.1 ± 27.4	160.7 ± 26.3	**< 0.05**
Apolipoprotein B, mg/dL	112.4 ± 32.6	121.4 ± 40.9	109.4 ± 28.7	**<0.001**
Lp(a), mg/dL	43.2 ± 46.0	40.82 ± 44.0	44.0 ± 46.7	0.5237

[a] Student's t test p value: DFH vs. NDFH groups. DFH: definite familial hypercholesterolemia; NDFH: non-definite familial hypercholesterolemia; Lp-PLA_2: Lipoprotein-associated phospholipase A_2. BP: blood pressure; LDL: low density lipoprotein; HDL: high density lipoprotein.

Table 2 displays Lp-PLA$_2$ univariate correlations between Lp-PLA$_2$ activity and lipid biomarkers. Lp-PLA$_2$ activity was strongly positively correlated with TC, LDL-C and ApoB and negatively with HDL-C and ApoA-1. No correlation was found with TG and Lp(a).

In DFH patients on statins, Lp-PLA$_2$ activity levels were different from non-definite FH patients on statins (202.3 ± 53.8 vs 168.1 ± 43.6 nmol/min/mL, p <0.0001, Additional Figure 2).

Table 2. Univariate correlations between Lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) activity and lipid profile in the whole hypercholesterolemic population.

	Lp-PLA$_2$ activity	
	r	**p**
Total Cholesterol, mmol/L	0.44	<0.0001
LDL-Cholesterol, mmol/L	0.57	<0.0001
HDL-Cholesterol, mmol/L	-0.31	<0.0001
Triglycerides, mmol/L	-0.05[a]	NS
Apolipoprotein A-1, mg/dl	-0.43	<0.0001
Apolipoprotein B, mg/dl	0.57	<0.0001
Lp(a), mg/dl	0.04[a]	NS

LDL: low density lipoprotein; HDL: high density lipoprotein; NS: not significant.
[a]by Spearman's rank correlation coefficients.

Partial correlation of Lp-PLA$_2$ with ApoB and LDL showed that LDL-C and ApoB remains associated with Lp-PLA$_2$ (r adj ApoB = 0.12, p <0.01; r adj LDL-C = 0.12, p <0.01). Non-HDL-C did not remain correlated with Lp-PLA$_2$ when adjusted with LDL-C (r adj LDL-C = -0.03, p 0.86).

In a multivariate analysis, definite definite FH diagnosis as well as LDL-C, HDL-C and statin treatment remained correlated with Lp-PLA$_2$ independently of systolic BP. Inclusion of ApoB in the model in replacement of LDL-C did not change the outcome of the analysis.

Sex was independently related with Lp-PLA$_2$ (Table 3). Separate multivariate analysis in DFH and NDFH patients showed minor differences in Lp-PLA$_2$ determinants but LDL-C and HDL-C remained significantly associated to Lp-PLA$_2$ (Additional Tables 1 and 2).

Table 3. Variables independently associated with Lipoprotein-associated phospholipase A_2 (Lp-PLA_2). Beta coefficient ± standard error is shown.

	Lp-PLA_2		
	R^2	**β**	**p**
Overall Model	0.55		<0.0001
Age, years		-0.33 ± 0.15	**0.0240**
Sex (men = 1)		19.44 ± 3.91	**<0.0001**
Definite FH diagnosis (yes = 1)		9.36 ± 4.48	**0.0373**
LDL-Cholesterol, mmol/L		17.93 ± 1.43	**<0.0001**
HDL-Cholesterol, mmol/L		-29.48 ± 4.34	**<0.0001**
Statins (yes = 1)		-9.30 ± 4.50	**0.0394**
Systolic BP, mmHg		-0.21 ± 0.14	0.1406

FH: Familial Hypercholesterolemia; LDL: low density lipoprotein; HDL: high density lipoprotein; BP: blood pressure.

DISCUSSION

In this study, we found that Lp-PLA_2 activity in hypercholesterolemic subjects was independently higher in subjects with a clinically confirmed definite FH than in subjects with a non-definite FH according to Dutch Lipid Clinic Network criteria.

Our results extend the results of Tsimihodimos et al.[27] who found higher Lp-PLA_2 activity in FH patients (carrying hetero and homozygous mutations) compared to non-FH hypercholesterolemic subjects and controls in a smaller population. Moreover, in our study, definite FH status and LDL-C levels both independently correlated to Lp-PLA_2 activity.

As observed here as well as in some previous studies[5,27], Lp-PLA_2 activity correlates strongly and positively with LDL-C while negatively with HDL-C. The positive relationship with LDL-C may be explained by the fact that Lp-PLA_2 is mainly associated with apoB-containing lipoproteins (primarily with LDL), i.e. 70-75% when expressed in mass, and 90-95% when expressed in activity[28,29,30]. This difference in assay procedure could also explain why we found a negative correlation with HDL-C despite about 30% of Lp-PLA_2 being carried by HDL particles[31]. Several lines of evidence suggest that the Lp-PLA_2 roles in atherosclerosis pathophysiology may depend on the type of lipoprotein particles with which it is associated to[32]. It seems that only LDL-associated Lp-PLA_2 exerts pro-inflammatory activity while HDL-associated Lp-PLA_2 seems to have an anti-atherogenic role[33]. HDL prevents or inhibits the formation of LDL-derived oxidized phospholipids[34] by the action of at least four specific enzymes present on its surface, including HDL-associated Lp-PLA_2. Since oxidized

phospholipids are the target of hydrolysis by Lp-PLA_2, its activity can be reduced by increasing HDL concentration.

The inverse relationship between Lp-PLA_2 activity and HbA1c was also observed in previous studies[35,36]. It may be explained by the distribution of Lp-PLA_2 among lipoprotein particles. In diabetic subjects[37]in particular, Lp-PLA_2 is associated mainly with HDL particles and the Lp-PLA_2 glycosylation process leads to a greater activity of HDL-associated Lp-PLA_2[38].

While mendelian randomization studies may suggest otherwise[39], there are some evidence for an active role of Lp-PLA_2 in atherosclerosis[40]. As Lp-PLA_2 hydrolyses oxidized phospholipids in LDL-C particles to release oxidized fatty acids at the sn-2 position and lysophosphatidylcholine[9] its activity can enhance plaque inflammation[9]. Hence, upregulation of Lp-PLA_2 has been demonstrated within the plaque necrotic core and in macrophages prone to rupture plaques[41]. Inflamed vulnerable plaques have been associated with a higher prevalence of coronary heart disease[42,43] in the general population and increased circulating Lp-PLA_2 levels have been associated with higher plaque vulnerability[8]. Concerning FH, Caballero et al.[44] found an increased prevalence of lipid-rich atherosclerotic plaques in FH using magnetic resonance imaging. Moreover, Van Den Oord et al.[45] found that FH subjects more frequently exhibited atherosclerotic plaques neovascularization, a sign of plaque vulnerability. Along with the early and lifelong exposure to elevated LDL-C levels, those observations of more vulnerable plaques in FH patients could partially explain why FH patients develop premature symptomatic atherosclerosis. Our study suggests that the increased CVD risk in patients with FH could be partly explained by the arterial wall inflammation associated with plaque vulnerability and witnessed by high circulating Lp-PLA_2 levels. This hypothesis may be hampered by the STABILITY trial[46] failure to prove that pharmacological Lp-PLA_2 activity lowering could decrease cardiovascular events in a high risk population of patients. However, no specific data on FH patients within the STABILITY trial were published. Also, in another trial[51], while the darapladib molecule inhibited necrotic core growth of human coronary plaques, it failed to prevent cardiovascular events in a phase III trial.

In our study, statin treatment was negatively correlated to Lp-PLA_2 levels independently of LDL-C and definite FH status. This is coherent with the well-known effect of statins which decrease Lp-PLA_2 in general populations [47] but also in selected FH population[48,49] Our study extend those results in a larger population of real life definite FH patients. Beyond their LDL-C lowering effect, statins exhibit pleiotropic effects. One of them is to stabilize atherosclerotic

plaques by reducing foam cell formation and inflammatory process and by inhibiting the macrophages matrix metalloproteinases production[50]. High Lp-PLA_2 levels observed in our population may also be explained by the fact that only three quarters of definite FH subjects were under statin treatment. Our results further reinforce the concept that cardiovascular protection with statins may be partially explained by anti-inflammatory effects, perhaps on Lp-PLA_2 especially.

Our study exhibits some limitations. First, it is a cross sectional study and therefore we cannot determine causality. Then, we did not analyse the different lipoproteins particles in our study. Also, we did not measure Lp-PLA_2 mass, but Lp-PLA_2 activity has several technical advantages[30] including in the pre-analytic phase that makes it more easy to use in clinical practice. Another limit is that without the certainty of genetic diagnosis, it can be assumed that a variable percentage of FH patients were attributed to the NDFH group. This however did not prevent us from finding statistical differences between the groups.

CONCLUSION

In a population of hypercholesterolemic patients, we found that Lp-PLA_2 activity was higher in patients with a clinically confirmed definite FH independently of LDL-C levels and statin treatment in comparison with subjects with a non-definite FH according to Dutch Lipid Clinic Network criteria. These results highlight the particular phenotype of FH within the vast population of patients with hypercholesterolemia. In addition to early and lifelong elevated LDL-C, the high levels of arterial inflammation may also be involved in the premature onset of CVD in FH patients. Their high cardiovascular risk can be prevented by adequate therapeutic strategies using statin anti-inflammatory pleiotropic effects. Our results also reinforce the need for proper identification of this disease which affects 1/200 people in Western countries.

ACKNOWLEDGEMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

AM: study conception, study realization, statistics and manuscript writing; DR: study conception, study supervision, statistics and manuscript supervision; RB: data collection and analysis, manuscript reviewing and scientific supervision; DBR: manuscript reviewing and scientific supervision; DN: statistics and manuscript supervision; MA: manuscript reviewing; EB: manuscript reviewing; PG: manuscript reviewing and supervision.

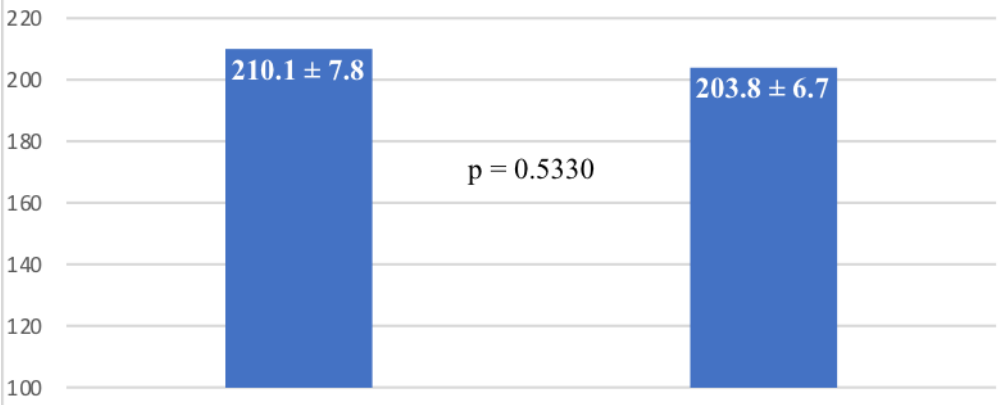
All authors have approved the final article.

ACRONYMS

Adj: adjusted. AH: arterial hypertension. *APOB*: apolipoprotein B-100 gene. BMI: body mass index. BP: blood pressure. CVD: cardiovascular disease. DFH: definite familial hypercholesterolemia (group). FH: familial hypercholesterolemia. HDL-C: high-density lipoprotein cholesterol. LDL-C: low-density lipoprotein cholesterol. *LDLR*: low density lipoprotein receptor gene. Lp-PLA_2: lipoprotein-associated phospholipase A_2. NDFH: non-definite familial hypercholesterolemia (group). *PCSK9*: proprotein convertase subtilisin/kexin type 9 gene. T2D: type 2 diabetes. TC: total cholesterol. TG: triglycerides.

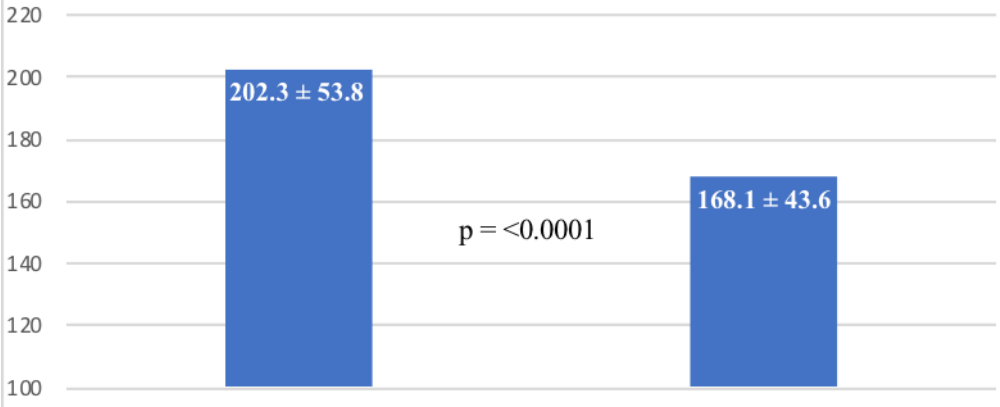
APPENDIX

Additional Figure 1. Lp-PLA$_2$ (nmol/min/mL) in the DFH group among subjects with genetic diagnosis and subjects with only clinical diagnosis



Lp-PLA$_2$: Lipoprotein-associated phospholipase A$_2$;
DFH: definite familial hypercholesterolemia.

Additional Figure 2. Lp-PLA$_2$ (nmol/min/mL) among hypercholesterolemic subjects in statin treatment with and without definite FH.



Lp-PLA$_2$: Lipoprotein-associated phospholipase A$_2$;
DFH: definite familial hypercholesterolemia;
NDFH: non-definite familial hypercholesterolemia.

Additional Table 1. Variables independently associated with Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) in non- definite FH group. Beta coefficient ± standard error is shown.

	Lp-PLA_2		
	R^2	**β**	**p**
Overall Model	0.55		<0.0001
Age, years		-0.09 ± 0.18	0.6175
Sex (men = 1)		21.63 ± 4.30	**<0.0001**
LDL-Cholesterol, mmol/L		21.38 ± 1.98	**<0.0001**
HDL-Cholesterol, mmol/L		-28.27 ± 4.80	**<0.0001**
Statins (yes = 1)		-10.26 ± 5.40	0.0586
Systolic BP, mmHg		0.003 ± 0.15	0.9826

FH: Familial Hypercholesterolemia; LDL: high density lipoprotein; HDL: high density lipoprotein; BP: blood pressure.

Additional Table 2. Variables independently associated with Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) in definite FH group. Beta coefficient ± standard error is shown.

	Lp-PLA_2		
	R^2	**β**	**p**
Overall Model	0.55		<0.0001
Age, years		-0.56 ± 0.26	**0.0292**
Sex (men = 1)		9.61 ± 8.49	0.2601
LDL-Cholesterol, mmol/L		14.02 ± 2.22	**<0.0001**
HDL-Cholesterol, mmol/L		-41.98 ± 9.52	**<0.0001**
Statins (yes = 1)		-4.56 ± 9.35	0.6267
Systolic BP, mmHg		-0.69 ± 0.32	**0.0347**

FH: Familial Hypercholesterolemia; LDL: high density lipoprotein; HDL: high density lipoprotein; BP: blood pressure.

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