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Atherosclerosis severity in patients with familial hypercholesterolemia: The role of T and B lymphocytes

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STRUCTURED ABSTRACT

Background and aims: Familial hypercholesterolemia (FH) is characterized by lifelong exposure to high LDL-c concentrations and premature atherosclerotic cardiovascular disease; nevertheless, disease severity can be heterogeneous.
We aimed at evaluating if the immune-inflammatory system could modulate atherosclerosis burden in FH.

Methods: From a cohort of subjects with confirmed FH (Dutch Lipid Clinic Network and genotype), 92 patients receiving high-intensity lipid-lowering therapy (statin ± ezetimibe) were included. The extension and severity of coronary atherosclerosis was assessed by standardized reporting systems (CAD-RADS) for coronary computed tomography angiography (CCTA) and coronary artery calcium (CAC) scores. Lipids, apolipoproteins, *anti*-oxLDL and anti-apolipoprotein B-D peptide (*anti*-ApoB-D) autoantibodies (IgM and IgG), lymphocytes subtypes, platelet, monocyte and endothelial microparticles (MP), IgM levels (circulating or produced by B1 cells) and cytokines in the supernatant of cultured cells were determined. Multiple linear regression models evaluated associations of these biomarkers with CAC and CAD-RADS scores.

Results: In univariate analysis CAC correlated with age, systolic blood pressure, TCD4+ cells, and titers of IgM *anti*-ApoB-D. In multiple linear regression [ANOVA F = 2.976; $p = 0.024$; $R^2 = 0.082$), CD4+T lymphocytes (B = 35.289; beta = 0.277; $p = 0.010$; 95%CI for B 8.727 to 61.851), was independently associated with CAC. CAD-RADS correlated with age, systolic blood pressure, titers of IgM *anti*-ApoB-D, and endothelial MP in univariate analysis. In multiple linear regression, [ANOVA F = 2.790; $p = 0.032$; $R^2 = 0.119$), only age (B = 0.027; beta = 0.234; $p = 0.049$; 95% CI for B 0.000 to 0.053) was independent predictor.

Conclusions: In subjects with FH, under high-intensity lipid-lowering therapy, age and CD4+T cells were associated to atherosclerosis burden.

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1. Introduction

Familial hypercholesterolemia (FH) is a major risk factor for premature atherosclerosis due to lifelong exposure to high LDL-cholesterol (LDL-c) levels [1]. Patients with FH are considered at high risk for coronary events and are usually treated by combined lipid-lowering agents [1]. However, part of these subjects presents only modest coronary atherosclerosis, that seems not explained by lower prevalence of classical risk factors.

The participation of the immune system in the development and progression of atherosclerosis has been widely recognized. LDL particle accumulation in the arterial wall triggers atherosclerotic process by activating the immune system, enhancing inflammatory responses [2]. Several mediators and cell types participate in this complex inflammatory network.

B lymphocytes subtypes have been studied in experimental atherosclerosis. B1 lymphocytes are considered atheroprotective, switching the pro-inflammatory phenotype of the plaque, by producing natural antibodies and interleukin 10 (IL-10), thus favoring the clearance of apoptotic cellular debris [3]. On the other hand, B2 cells or classic B lymphocytes (B naïve and B memory cells), are related to atherosclerosis progression, possibly by interaction with CD4+T lymphocytes [3].

Griffin et al. [4], proposed CD19 + CD20 + CD43 + $CD27^{+}$ lymphocyte cells as the human B1 phenotype. Human B1 cells spontaneously produce IgM and IL-10, and according to the absence or presence of the CD11b molecule on the surface of these cells, two different subtypes that differ, respectively, on the capacity of IgM production, and activation of CD4+T lymphocytes were established [5] (Fig. 1 and 2).

The differential role of lymphocyte subtypes can provide a pathway for understanding the pathophysiology of coronary heart disease in FH patients [6]. B1 lymphocytes, by producing IgM antibodies that interact with oxidized epitopes of apolipoprotein B, allowing their clearance, may have an important anti-atherogenic role [7–11]. Conversely, classical B2 lymphocytes may contribute to atherosclerosis progress favoring interactions of oxidized LDL with dendritic cells and subsequent T cells activation, including those of Th1 phenotype, thus contributing for the immune complex deposition in the intima layer [3,12].

Innate and adaptive immune responses against epitopes of apolipoprotein B (ApoB) can be measured by circulating levels of IgG and IgM antibodies to these oxidized or modified epitopes [7–11]. However, the myriad of epitopes, including neoepitopes that are recognized by antibodies against modified-LDL as well as differences in antibody isotypes has made the interpretation very difficult. Here we have taken advantage of a well-characterized peptide that can be recognized by both IgM and IgG antibodies. It is a 25-mer peptide and is located in the inner part of the ApoB, in between the two domains that constitute the receptor binding region [13]. This peptide is regarded as stable in solution and, therefore, suitable for ELISA determinations. It has been used extensively in both human clinical studies, as well as in animal models and can be considered an independent risk marker [14–16].

Even though the exact mechanisms involving antibodies remain unclear, the possibility that antibodies are protective by removing potentially bioactive products from the LDL degradation [17] or participate in the progression of the lesion by facilitating activated macrophages in the uptake of immune complexes, are two well accepted possibilities [18].

Coronary computed tomography angiography (CTA) imaging is a rapid noninvasive angiographic modality widely applied to evaluate chest pain, whereas coronary artery calcification (CAC) score has been tested most extensively for its use in estimating the cardiovascular prognosis. Research using these techniques has made clear that an increased plaque burden and increased CAC are present even among asymptomatic and long-term aggressively statin treated FH patients [19].

In subjects with FH, under the same statin background and similar exposure to LDL-c, the degree of atherosclerotic disease may vary. Statin treatment reduces LDL-c levels, but it is not known if harmful immune responses are reversed in statin-treated FH patients. In addition, the temporal relationship between LDL exposure and the likelihood, onset, and severity of atherosclerotic cardiovascular disease in FH patients is subject of interest [19].

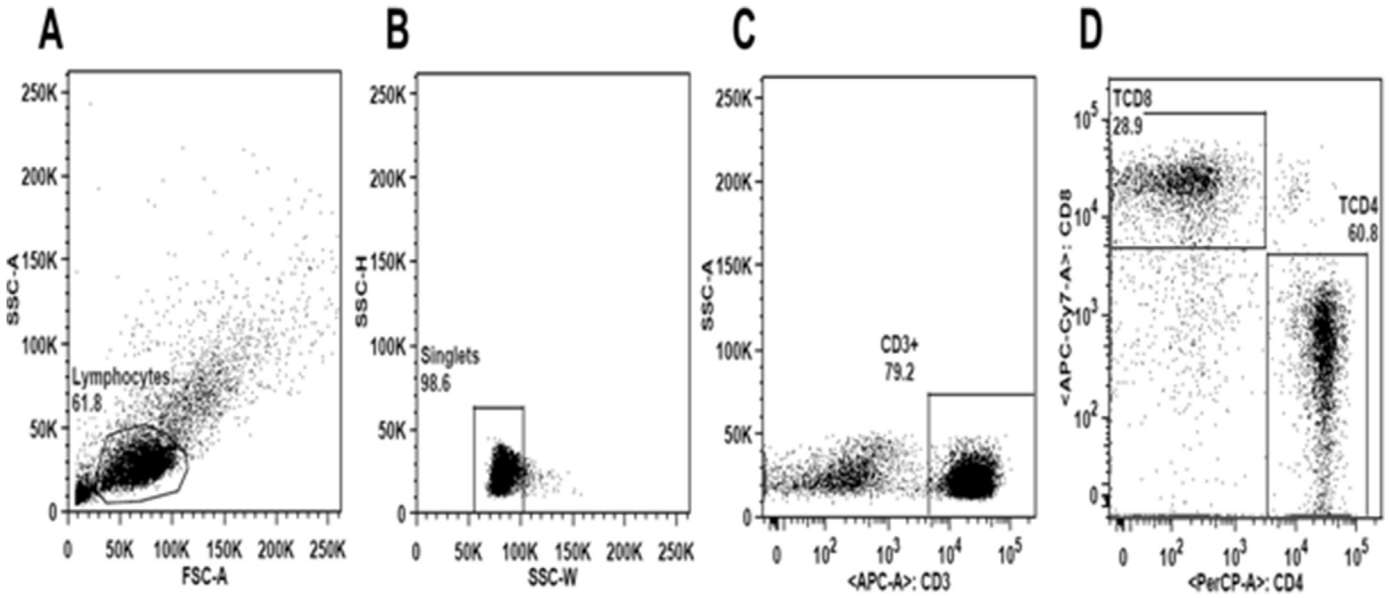

Fig. 1. Analysis of T lymphocytes from peripheral blood by flow cytometry.
Lymphocytes were defined in a graph of size and complexity (A, FSC-A x SSC-A) and analyzed as singlets (B, SSC-W x SSC-H). Among CD3+ cells (C, CD3 x SSC-A), cells were defined as exclusively CD4+ and CD8+ (D, CD4 x CD8). This scheme is representative of all analyzed samples. 50,000 CD3+ events were acquired.

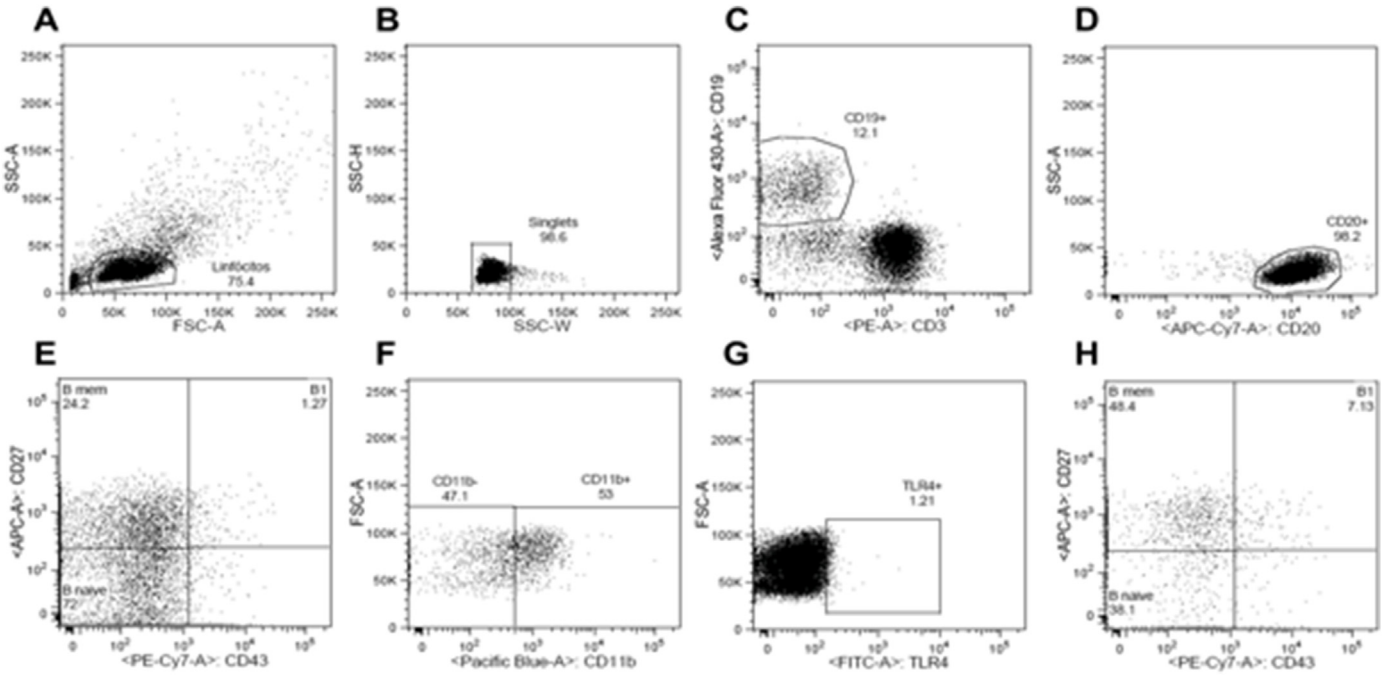

Fig. 2. Analysis and cell sorting of B lymphocytes from peripheral blood by flow cytometry.
Lymphocytes were defined in a graph of size and complexity (A, FSC-A x SSC-A) and analyzed as singlets (B, SSC-W x SSC-H). After excluding CD3+ cells and selecting CD19+ (C, CD3 x CD19) and CD20+ cells (D, CD20 x SSC), B cell subpopulations were defined (E, CD43 x CD27) as naïve (CD43- CD27-), memory (CD43- CD27+) and B1 cells (CD43+ CD27+). B1 cells were also evaluated for expression of the surface molecule CD11b (F, CD11b x FSC). Alternatively, B lymphocytes, defined in D, were also evaluated for TLR4 expression (G, TLR4 x FSC; H, CD43 x CD27). This scheme is representative of all analyzed samples. 2,000,000 total events were acquired, and the entire sample was submitted to cell sorting, and T lymphocytes (CD3+ CD19-, in C) as well as subpopulations of B lymphocytes (CD43- CD27-, CD43- CD27+, CD43+ CD27+, in E) were recovered.

In this study we assessed lymphocytes subtypes and autoantibodies to autoantigens involved in the severity of coronary atherosclerosis in FH subjects, measured by CTA and CAC.

2. Patients and methods

This is a cross-sectional study including a sample population (N = 92) from a prospective cohort of individuals of both sexes, age ≥18 years old, with definite or probable criteria of FH, by the Dutch Lipid Clinic Network (DLCN) [20].

The study was conducted at the Lipids, Atherosclerosis and Vascular Biology outpatient clinic, Cardiology Division, Universidade Federal de São Paulo, São Paulo, SP, Brazil. The ethical aspects followed the principles of the 1975 Declaration of Helsinki. The study protocol was approved by our local ethics committee (Comitê de Ética em Pesquisa da Universidade Federal de São Paulo, CAAE 46378415.3.0000.5505, # 1207461), and all participants were included after signing the written informed consent form.

Participants were taking maximally tolerated doses of lipid-lowering therapy (atorvastatin 40–80 mg or rosuvastatin 20–40 mg, with or without ezetimibe 10 mg). The intensity of the lipid-lowering treatment was classified as high-intensity statin (LDL-C reduction >50%), moderate-intensity (LDL-C reductions between 30 and 50%), and low-intensity (reductions <30%) [21].

Patients with secondary causes of hypercholesterolemia, malignancies, inflammatory diseases, using immunosuppressant drugs, unable or unwilling to participate were excluded.

2.1. Clinical assessment

Demographic data, medical history, concomitant medication, family history of dyslipidemia, and family or personal history of atherosclerotic cardiovascular disease (ASCVD) were recorded. Clinical examination included assessment of anthropometric parameters, vital signs (systolic and diastolic blood pressure, and heart rate), and presence of xanthomas, corneal arcus, or xanthelasmas. Criteria for FH was based on DLCN score [20]. To confirm the diagnosis of FH, 84 patients were submitted to genetic study that included NGS of FH-related genes (*LDLR*, *APOB*, *PCSK9*, and *LDLRAP1*), analyzed in Torrent Suite, and with confirmation by Sanger sequencing. In some patients, with undetected mutations MLPA was also performed. The method is fully described in Supplemental methods.

2.2. Samples

Venous blood samples (20 mL) were obtained in heparin tubes for complete blood count and biochemistry, in Central Laboratory of Hospital São Paulo, and for *in vitro* assays in the Department of Microbiology, Immunology and Parasitology, at Universidade Federal de São Paulo, UNIFESP.

2.3. Biochemistry assays

Overnight fasting blood samples were used for biochemistry analyses. All analyses were performed at the Central Laboratory, using commercial kits and automated assays. Lipid profile and LDL-c-years score were assessed [22]. See Supplemental Methods for details.

2.4. Blood samples

Heparinized blood samples were centrifuged at 448 g for 10 min at room temperature. Plasma aliquots were obtained and stored at −20 °C until use for total IgM titer determination. For immunologic tests, blood samples were collected and promptly examined by flow cytometry and *in vitro* assays. See Supplemental Methods for details.

2.5. Flow cytometry

The detailed method for detection of lymphocytes subtypes is described in Supplemental Methods.

2.6. Enzyme-linked immunospot (ELISpot) assay

The enzyme-linked immune absorbent spot was used to determine the frequency of spontaneously secreting IgM cells [4] (Fig. 3). See Supplemental Methods for details.

2.7. Evaluation of total IgM titer in plasma

Determination of IgM titers in plasma was described in Supplemental methods.

2.8. Cytometric bead array

Detection of cytokines (IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2) in T lymphocytes, in B1, B naïve, and B memory cells isolated by cell sorting is described in Supplemental methods.

2.9. Circulating microparticles

Circulating endothelium-, monocyte- and platelet-derived microparticles were determined as previously reported [23–25]. See Supplemental Methods for details.

2.10. Determination of anti-OxLDL and anti-apolipoprotein B-peptide derived autoantibodies

To determine the autoantibodies to copper-oxidized LDL, we used an enzyme-linked immunosorbent assay (ELISA) method as previously described [26,27]. Quantification of anti-apolipoprotein B (ApoB) autoantibodies (Abs) was assessed in total plasma by ELISA as previously reported and similar to *anti*-oxLDL ELISA procedures [10,28]. See Supplemental Methods for details.

2.11. Coronary computed tomography angiography (CTA) and coronary artery calcification (CAC)

To investigate atherosclerosis degree and calcium deposition related to evolution of coronary disease, tomographic slices were performed with helical system in equipment with multidetectors

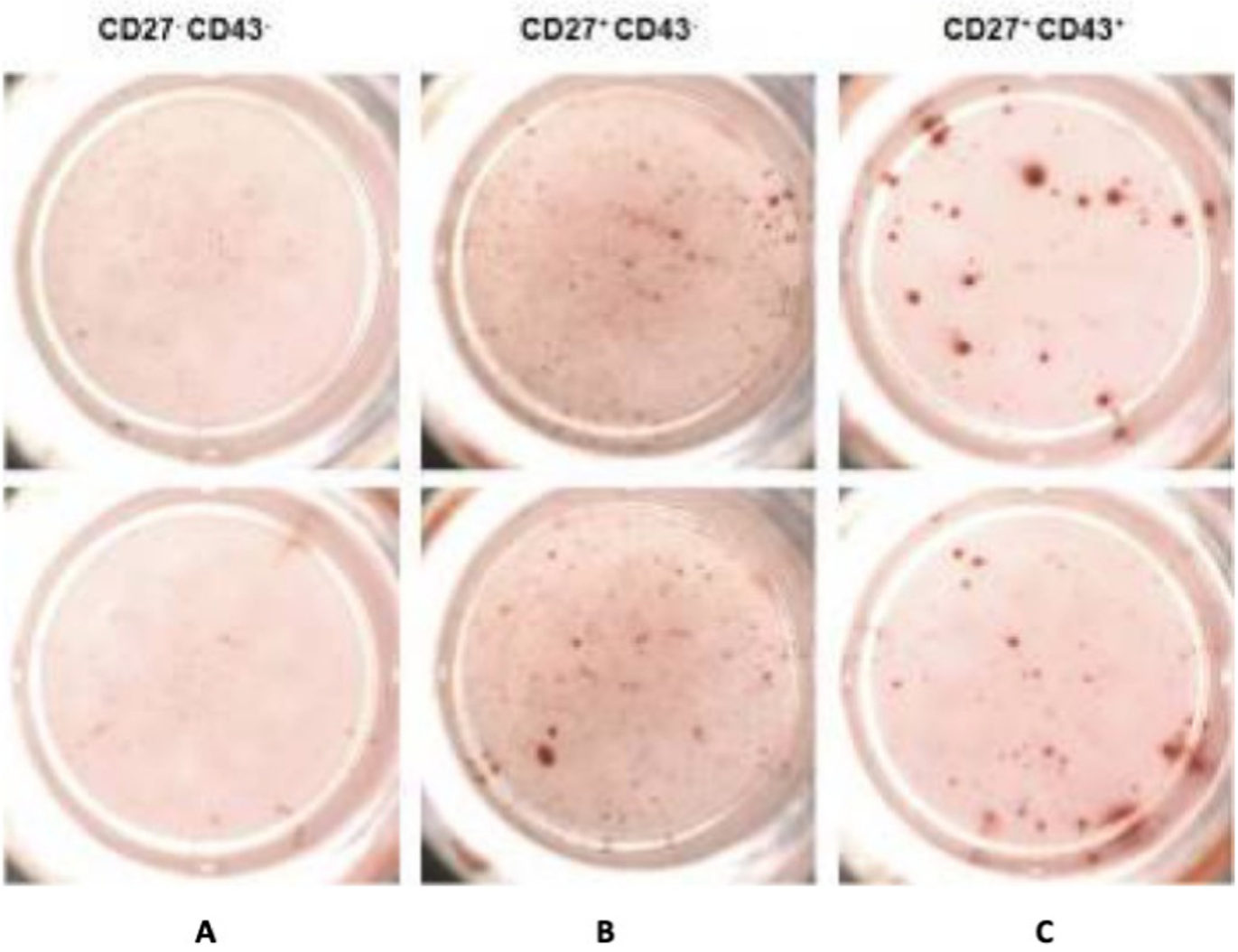

Fig. 3. Replicates of B cells subpopulations showing IgM secreting cells.
Replicates are B naïve (CD27- CD43-), B memory (CD27+ CD43-) and B-1 (CD27+ CD43+) cells, plated as 4,000 cells per well.

(Phillips Brilliance 64), synchronized with ECG waves. See Supplemental Methods for full description of this method [29–31].

2.12. Statistical analysis

Categorical variables are described as frequencies (percentages) and continuous variables are presented as mean (standard deviation), or median and interquartile range (IQR). Normality was tested by Kolmogorov-Smirnoff test. Statistical analyses were performed using the Chi-square or Fisher's exact test to compare categories, and independent samples *t*-test or Mann-Whitney test for continuous variables. CAC was categorized as zero or >0, and CAD-RADS score < or ≥ median. Correlation coefficients were tested by Pearson's or Spearman's correlation coefficient tests.

To identify the independent factors associated with the degree of atherosclerosis in subjects with FH, crude and multiple linear regression models were performed using CAC or CAD-RADS as dependent variable, and selected variables as independent determinants of the extent of atherosclerosis. In the adjusted model, variables were included based on clinical and physiological plausibility, following a *p*-value cutoff of 0.05 in crude analysis for entry. The magnitude of the associations was estimated by regression coefficient beta (β) and 95% CI. The variance inflation factor (VIF) was computed for the variables included in the model.

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) version 22. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of study population

Our patient population was constituted of middle aged (48–65 years old) patients with FH, predominantly women (70%), 50% with hypertension, 14% with diabetes, 31% had a positive history of ASCVD. CHD (17%) was the most common manifestation of ASCVD, followed by stroke (6%), and PAD (3%). CAC median values were 4 AU (IQR 0–99 AU), and the median CAD-RADS score was 1 (IQR 0–2). Fifty-two patients (57%) with FH presented CAC>0, and 40 (43%) had CAC = 0. For CAD-RADS score, 38 (42%) had a score < median, whereas in 53 (58%) the CAD-RADS was ≥ median (Table 1). Patients with CAC >0, were older ($p < 0.0001$), with higher prevalence of family history of CHD ($p = 0.047$), and higher SBP ($p = 0.013$) than those with CAC = 0 (Table 1). Patients with CAD-RADS ≥ median were older ($p = 0.004$), with higher percentages of males ($p = 0.047$), higher prevalence of family history of CHD ($p = 0.047$), smoking habit ($p = 0.033$), with higher body weight ($p = 0.026$), BMI ($p = 0.045$), and SBP ($p = 0.006$), as

Table 1
Demography and clinical characteristics of patients with FH, according to the degree of coronary calcification and the extension of coronary atherosclerosis.

Variable	Total (N = 92)	CAC = 0 (N = 40)	CAC>0 (N = 52)	p-value	CAD-RADS < median (N = 38)	CAD-RADS ≥ median (N = 53)	p-value
Age, years	59 (48–65)	49 (35–63)	62 (52–66)	<0.0001	53 (36–63)	61 (51–66)	0.004
Male/female, %	28 (30)/64 (70)	10 (25)/30 (75)	18 (35)/34 (64)	0.320	7 (18)/31 (82)	20 (38)/33 (62)	0.047
Hypertension, %	46 (50)	23 (44)	29 (56)	0.207	15 (39)	31 (59)	0.074
Criteria for FH using DLCN, %							
Definite	90 (97.8)	39 (97.5)	51 (98.1)	0.683	37 (97.4)	52 (98.1)	0.663
Probable	2 (2.2)	1 (2.5)	1 (1.9)		1 (2.6)	1 (1.9)	
Mutation, %							
Pathogenic or likely pathogenic	28 (30)	9 (23)	19 (37)	0.345	9 (24)	18 (34)	0.563
VUS	17 (19)	8 (20)	9 (17)		8 (21)	9 (17)	
No mutation, benign or not performed	47 (51)	23 (57)	24 (46)		21 (55)	26 (49)	
Diabetes, %	13 (14)	5 (13)	8 (15)	0.618	5 (13)	9 (17)	0.618
CHD, %	16 (17)	4 (10)	12 (23)	0.101	4 (11)	12 (23)	0.134
Stroke, %	6 (6)	2 (5)	4 (8)	0.604	2 (5)	4 (7)	0.665
PAD, %	3 (3)	0 (0)	3 (6)	0.122	0 (0)	3 (6)	0.136
Family history of premature CHD, %	29 (31)	17 (43)	12 (23)	0.047	16 (42)	12 (23)	0.047
Smoking, %							
Never	67 (73)	33 (83)	34 (65)	0.164	33 (87)	33 (62)	0.033
Past	12 (13)	4 (10)	8 (15)		2 (5)	10 (19)	
Current	13 (14)	3 (7)	10 (20)		3 (8)	10 (19)	
CAC, AU	4 (0–99)	0 (0–0)	99 (24–499)	NA	0 (0–0)	99 (16–499)	NA
CAD-RADS score, %							
0	38 (42)	37 (92)	1 (2)	NA	38 (100)	0 (0)	NA
1	23 (25)	2 (5)	21 (40)		0 (0)	23 (43)	
2	11 (12)	1 (3)	10 (20)		0 (0)	11 (21)	
3	6 (7)	0 (0)	6 (12)		0 (0)	6 (11)	
4	6 (7)	0 (0)	6 (12)		0 (0)	6 (11)	
5	7 (8)	0 (0)	7 (14)		0 (0)	7 (14)	
Lipid-lowering therapy[a], %	76 (83)	32 (80)	44 (86)	0.391	31 (82)	44 (85)	0.576
High-intensity	1 (2)	0 (0)	1 (2)		0 (0)	1 (2)	
Moderate-intensity	14 (15)	8 (20)	6 (12)		7 (18)	7 (13)	
Low-intensity/no therapy							
Body weight, kg	70 (62–81)	70 (61–78)	72 (74–83)	0.279	69 (10)	76 (17)	0.026
BMI, kg/m^2	27 (25–31)	27 (24–30)	27 (26–32)	0.133	27 (24–30)	27 (26–32)	0.045
SBP, mmHg	120 (110–133)	110 (110–130)	128 (112–140)	0.013	110 (109–130)	127 (114–140)	0.006
DBP, mmHg	75 (70–80)	70 (67–80)	77 (70–80)	0.102	70 (65–80)	77 (70–80)	0.076
Heart rate, bpm	70 (67–76)	72 (70–80)	70 (66–74)	0.034	73 (70–80)	70 (65–73)	0.010

Categorical variables are expressed as frequencies (%); numerical variables expressed as median (IQR). Categorical variables compared by Pearson's chi-square test; numerical variables compared by Mann-Whitney test. AU, Agatston units; BMI, body mass index; CAC, coronary artery calcification score; CAD-RADS, Coronary Artery Disease – Reporting and Data System; CHD, coronary heart disease; hs-CRP, high-sensitivity *C*-reactive protein; DBP, diastolic blood pressure; IQR, interquartile range; PAD, peripheral arterial disease; DLCN, Dutch Lipid Clinic Network; SBP, systolic blood pressure; SD, standard deviation; VUS, variant of uncertain significance.

[a] Statin intensity: < 30%, low-intensity; 30–50%, moderate-intensity; >50%, high-intensity.

compared with CAD-RADS < median (Table 1). Lower heart rate observed in those with CAC >0 or CAD-RADS ≥ median may reflect use of betablocker agents.

Ninety patients (97.8%) were diagnosed as definite, and 2 (2.2%) as probable FH, by DLCN criteria. In addition, of the 84 subjects with genetic test, 28 (30%) had pathogenic or likely pathogenic mutations, and 17 (19%) presented variants of unknown significance (VUS). The remaining had no mutation or presented benign variants.

Regarding lipid-lowering therapy, 83% were taking high-intensity statins, 2% moderate-intensity, and 15% were taking low-intensity, or were not taking statins at enrollment. Current smokers were 14%, and 13% were past smokers (Table 1).

3.2. Laboratory parameters

In FH patients, total cholesterol was 233 (186–296) mg/dL, HDL-c 54 (43–65) mg/dL, LDL-c 141 (104–202) mg/dL, non-HDL-c 173 (131–237) mg/dL, and triglycerides 135 (96–176) mg/dL; ApoB levels were 114 (88–142) mg/dL, Lp(a) 23 (8–53) mg/dL, glucose 93 (85–106) mg/dL, HbA1c 5.8 (5.5–6.1) %, and hs-CRP was 1.60 (0.78–3.60) mg/L. There were no differences between treated FH patients with or without coronary artery calcification regarding total cholesterol, LDL-c, non-HDL-c, HDL-c, triglycerides, Apo A1, Apo-B, Lp(a) serum levels, fasting glucose, HbA1c, and high-sensitivity *C*-reactive protein. In addition, subjects with CAD-RADS ≥ median did not differ from those with CAD-RADS < median regarding most laboratory variables. The LDL-c-years-score in the total cohort was 14,342 (10,131–20,604) mg, higher in those with CAC >0 [14,762 (11,520–21,830 mg vs. 11,954 (7,760–16,810) mg, $p = 0.011$] or CAD-RADS ≥ median [14,758 (10,721–21,750) mg/dL vs. 12,938 (7,920–17,030) mg/dL, $p = 0.038$]. Total serum IgM levels did not differ among CAC categories. (Table 2). TSH levels were higher in subjects with CAC >0 and in CAD-RADS ≥ median, but these values were in the normal range.

3.3. Circulating immune cells

Distribution of total leukocytes, lymphocytes and lymphocyte subtypes ($CD4^+$ and $CD8^+$ T cells; B naïve, B memory, and B1 cells) was similar in patients with FH with or without coronary artery calcification, considered as frequencies or converted to absolute number (cells/mL), and also according to CAD-RADS score (Supplemental Table 1).

3.4. Circulating B cells, according to expression of CD11b + or TLR4+

Distribution of CD11b+ and CD11b- B1 cells did not differ in subjects with FH according to CAC categories; in addition, TLR4 expression in lymphocyte subtypes (B naïve, B memory, and B1 cells) was similar in patients with FH with or without coronary artery calcification (Supplemental Table 2). The same distribution was seen for CD11b and TLR4 expression in circulating B cells according to CAD-RADS categories (Supplemental Table 2).

3.5. ELIspot assay

The number of IgM producing spots was higher in FH patients with CAC = 0, $p = 0.031$. Conversely, no differences were seen when patients were categorized by CAD-RADS score (Supplemental Table 3 and Supplemental Fig. 1).

3.6. Cytokines production by immune cells

The expression levels of the cytokines interferon-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 (pg/mL) by cultured T cells, B memory and B naïve cells was similar among CAC categories. However, when compared by the extension of atherosclerosis, using the CAD-RADS score, titers of interferon-γ were higher in those with CAD-RADS score < median ($p = 0.032$) (Supplemental Table 4).

Table 2
Laboratory characteristics of patients with FH, according to the degree of coronary calcification and the extension of coronary atherosclerosis.

Variable	Total (N = 92)	CAC = 0 (N = 40)	CAC>0 (N = 52)	p-value	CAD-RADS < median (N = 38)	CAD-RADS ≥ median (N = 53)	p-value
Total cholesterol, mg/dL	233 (186–296)	241 (193–300)	233 (182–292)	0.720	242 (64)	240 (68)	0.935
HDL-c, mg/dL	54 (43–65)	52 (43–65)	55 (43–66)	0.595	53 (43–65)	55 (43–66)	0.878
LDL-c, mg/dL	141 (104–202)	133 (105–205)	145 (104–202)	0.978	133 (104–200)	144 (107–202)	0.699
Non-HDL-c, mg/dL	173 (131–237)	177 (133–249)	177 (127–237)	0.711	185 (65)	185 (66)	0.968
Triglycerides, mg/dL	135 (96–176)	175 (127–237)	143 (95–179)	0.587	145 (68)	142 (63)	0.819
Apo A1, mg/dL	149 (129–151)	141 (126–171)	149 (129–170)	0.511	145 (131–173)	145 (127–170)	0.949
Apo B, mg/dL	114 (88–142)	119 (91–138)	107 (84–144)	0.741	119 (93–140)	107 (84–143)	0.629
Lp(a), mg/dL	23 (8–53)	23 (8–56)	23 (8–51)	0.606	22 (9–61)	24 (7–53)	0.678
Fasting glucose, mg/dL	93 (85–106)	93 (85–106)	95 (87–108)	0.322	93 (85–103)	94 (87–107)	0.387
HbA1c, %	5.8 (5.5–6.1)	5.7 (5.4–6.1)	5.8 (5.6–6.0)	0.760	5.8 (5.5–6.1)	5.8 (5.6–6.0)	0.984
AST (U/L)	20 (16–26)	21 (17–27)	19 (15–24)	0.119	21 (17–27)	19 (15–24)	0.253
ALT (U/L)	21 (15–26)	22 (15–31)	21 (15–25)	0.452	22 (15–31)	21 (15–25)	0.518
CK (U/L)	135 (110–241)	154 (108–181)	135 (110–252)	0.619	149 (107–179)	135 (110–252)	0.486
TSH (μIU/L)	2.14 (1.63–3.43)	1.93 (1.63–2.82)	2.56 (1.65–4.18)	0.030	1.93 (1.63–2.66)	2.56 (1.66–4.02)	0.038
Creatinine (mg/dL)	0.91 (0.73–1.10)	0.87 (0.73–1.08)	1.00 (0.73–1.20)	0.339	0.85 (0.72–1.07)	1.00 (0.73–1.14)	0.229
hs-CRP (mg/L)	1.60 (0.78–3.60)	1.23 (0.66–3.13)	1.67 (0.78–4.15)	0.434	1.23 (0.69–3.30)	1.67 (0.81–4.07)	0.463
Serum IgM (dilution)	51,200 (51,200–102,400)	102,400 (51,200–102,400)	51,200 (51,200–102,400)	0.156	102,400 (51,200–102,400)	51,200 (51,200–102,400)	0.106
Serum IgM (LN_{10})	4.71 (4.71–5.01)	5.01 (4.71–5.01)	4.71 (4.71–5.01)	0.156	5.01 (4.71–5.01)	4.71 (4.71–5.01)	0.106
LDL-c-year-score, mg	14,342 (10,131–20,604)	11,954 (7,760–16,810)	14,762 (11,520–21,830)	0.011	12,938 (7,920–17,030)	14,758 (10,721–21,750)	0.038

Numerical variables expressed as median (IQR) and compared by Mann-Whitney test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAC, coronary artery calcification score; CAD-RADS, Coronary Artery Disease – Reporting and Data System; CK, creatine phosphokinase; HDL, high-density lipoprotein; hs-CRP, high-sensitivity *C*-reactive protein; IgM, immunoglobulin M; IQR, interquartile range; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); TSH, thyroid-stimulating hormone.

Table 3
Titers of auto-antibodies of IgG and IgM type *anti*-OxLDL and *anti*-ApoB-D, from participants with familial hypercholesterolemia, according to the degree of coronary calcification and the extension of coronary atherosclerosis.

Variable	Total (N = 92)	CAC = 0 (N = 40)	CAC>0 (N = 52)	p-value	CAD-RADS < median (N = 38)	CAD-RADS ≥ median (N = 53)	p-value
Serum IgM *anti*-OxLDL (RI)	0.50 (0.10)	0.51 (0.10)	0.49 (0.09)	0.393	0.51 (0.11)	0.49 (0.09)	0.419
Serum IgG *anti*-OxLDL (RI)	0.90 (0.86–0.93)	0.88 (0.85–0.92)	0.91 (0.88–0.93)	0.034	0.89 (0.05)	0.90 (0.04)	0.304
Serum IgM *anti*-ApoB-D (RI)	1.03 (0.63–1.47)	1.23 (0.82–1.75)	0.97 (0.55–1.35)	0.030	1.25 (0.93–1.75)	0.97 (0.55–1.34)	0.018
Serum IgG *anti*-ApoB-D (RI)	0.19 (0.13–0.27)	0.17 (0.12–0.23)	0.21 (0.12–0.29)	0.311	0.18 (0.13–0.24)	0.20 (0.12–0.29)	0.538

Numerical variables expressed as median (IQR) and compared by Mann-Whitney test. Apo, apolipoprotein; CAC, coronary artery calcification score; CAD-RADS, Coronary Artery Disease – Reporting and Data System; Ig, immunoglobulin; IQR, interquartile range; Ox, oxidized; RI, reactivity index.

3.7. Titers of auto-antibodies anti-OxLDL and anti-ApoB-D

Higher titers of IgG *anti*-OxLDL were found in FH subjects with CAC >0 ($p = 0.034$). For *anti*-ApoB-D peptide, IgM titers were higher in those FH patients with CAC = 0 ($p = 0.030$) and in those with CAD-RADS < median ($p = 0.018$) (Table 3).

3.8. Platelet, endothelial and monocyte microparticles

The distribution of platelet, endothelial and monocyte microparticles counts in participants with FH did not differ according to CAC categories. However, endothelial microparticles were higher in those with CAD-RADS < median ($p = 0.029$) (Supplemental Table 5).

3.9. Correlations

We performed correlations taking CAC as dependent variable. Age, CAD-RADS score, systolic blood pressure, and LDL-c-year-score were positively correlated, whereas heart rate and IgM *anti*-ApoB-D were negatively correlated with CAC (Supplemental Table 6).

Using CAD-RADS score as dependent variable, age, CAC score, body weight, systolic blood pressure, and LDL-c-year-score were positively correlated, whereas heart rate, IgM *anti*-ApoB-D and endothelial MPs were negatively correlated with CAD-RADS score (Supplemental Table 7).

In our study, LDL-years-score and age were significantly correlated [Pearson (r2) 0.378; $p < 0.0001$ and Spearman (rho) = 0.288; $p = 0.004$], therefore, we did not include this variable in the regression models.

3.10. Crude and adjusted linear regression

To identify independent variables related to the extension of atherosclerosis in subjects with FH, multiple linear regression models were performed, taking CAC or CAD-RADS scores as dependent variables. In univariate analysis CAC correlated with age, systolic blood pressure, TCD4+ cells, and titers of IgM *anti*-ApoB-D. In multiple linear regression [ANOVA F = 2.976; $p = 0.024$; $R^2 = 0.082$), CD4+T lymphocytes (B = 35.289; beta = 0.277; $p = 0.010$; 95%CI for B 8.727 to 61.851), was independently associated with CAC (Table 4). CAD-RADS score was correlated with age, systolic blood pressure, titers of IgM *anti*-ApoB-D, and endothelial MP in univariate analysis. In multiple linear regression, [ANOVA F = 2.790; $p = 0.032$; $R^2 = 0.119$), only age (B = 0.027; beta = 0.234; $p = 0.049$; 95% CI for B 0.000 to 0.053) was independent predictor (Table 4).

4. Discussion

In this study with FH participants, we detected heterogeneous distribution of CAC and CAD-RADS scores, with patients without any sign of atherosclerosis, and others with pronounced disease. In fact, ~58% of participants in this study presented CAC >0 and/or CAD-RADS score above the median. However, the distribution of lipid variables and use of high intensity lipid-lowering therapy was similar among groups.

With similar exposure to LDL-c levels and under lipid-lowering therapy, we investigated if clinical and laboratory determinants, inflammatory markers, such as lymphocytes subtypes, expression of TLR4 and CD11b, production of cytokines, microparticles release, circulating autoantibodies to OxLDL and ApoB-D peptide could differentiate those with higher atherosclerosis burden in the FH setting.

Our findings have shown that age, SBP, heart rate, LDL-c-year-score, $CD4^+$ T lymphocytes, and IgM-anti ApoB-D were correlated with CAC score. However, in multiple linear regression model, only CD4+T lymphocytes was independently associated with CAC. Evaluating the extension of coronary atherosclerosis, age, SBP, heart rate, body weight, LDL-c-year-score, IgM-anti ApoB-D, and endothelial MP were correlated with CAD-RADS score, but only age was independently associated with the extension and severity of coronary atherosclerosis. In a pilot study, we tested 27 healthy controls and found that the percentage of B classic, B naïve and B1 cells expressing TLR4 was lower in non-FH subjects. In addition, CD11b-B1 cells, serum IgM levels, and titers of IL-2 in the supernatant of B naive cells were lower in non-FH individuals. (Supplemental Table 8). However, in FH, the distribution of these markers was similar regardless the degree of calcification or atherosclerosis.

There is evidence from recent studies that similar to the heterogeneous manifestations of ASCVD, the presence and distribution of coronary artery calcium (CAC), is variable in subjects with FH [32–35]. Vascular calcification comprises a complex biological phenomenon, and not only a passive dystrophic process. Conventional computed tomography calcium scoring measures visible calcium deposition in the coronary arteries, known as macrocalcification, that identifies a high-risk vulnerable patient. Instead, microcalcification consists of micro-deposits of calcium (<50 μm), not detectable by traditional CTA, represents early stages of the process and may indicate plaque vulnerability. There is also the "spotty calcification", a transition from micro-to macrocalcification, consisting of small discrete nodules of calcium (up to 3 mm) [36]. Pathological studies have shown that sheet calcification is highly prevalent in stable plaques, while microcalcifications, punctate, and fragmented calcifications are more frequent in unstable lesions [37].

Mechanisms of vascular calcification include differentiation into osteogenic-like cells that initiate calcium deposition [38], generation of apoptotic bodies, and release of extracellular vesicles, related to the priming of the initial site of hydroxyapatite crystal nucleation [39,40]. Innate and adaptive immunity are triggers of vascular calcification, with OxLDL being recognized by membrane TLR, favoring vascular mineralization; TLR4 amplifies the immune response with release of numerous cytokines in atherosclerotic plaques including TNF-α, IL-1 beta, and IL-6, implicated in arterial calcification [41].

Table 4
Crude and adjusted linear regression models with CAC or CAD-RADS as dependent variable in participants with familial hypercholesterolemia.

Models for CAC	Unstandardized coefficients		Standardized coefficients	t	Sig.	95% CI for B		Statistics of collinearity	
	B	Standard model	Beta			Lower-bound	Upper-bound	Tolerance	VIF
1 (Constant)	−110.027	156.726		−0.702	0.485	−412.537	201.483		
Age	5.078	2.780	0.192	1.827	0.071	−0.448	10.603	1.000	1.000
2 (Constant)	−130.247	268.476		−0.515	0.608	−671.659	395.465		
Age	4.952	2.958	0.187	1.674	0.098	−0.929	10.833	0.893	1.120
SBP	0.288	2.219	0.015	0.130	0.897	−4.124	4.700	0.893	1.120
3 (Constant)	58.752	319.781		0.183	0.855	−577.237	694.382		
Age	3.862	3.107	0.146	1.243	0.217	−2.316	10.041	0.807	1.239
SBP	−0.136	2.247	−0.007	−0.060	0.952	−4.604	4.333	0.868	1.152
IgM-anti ApoB-D	−72.718	64.428	−0.130	−1.129	0.262	−200.819	55.383	0.839	1.192
4 (Constant)	−128.488	322.279		−0.566	0.573	−823.375	458.399		
Age	5.088	3.039	0.193	1.674	0.098	−0.956	11.131	0.788	1.269
SBP	−0.660	2.181	−0.033	−0,303	0.763	−4.997	3.678	0.861	1.161
IgM-anti ApoB-D	−93.692	62.779	−0.168	−1.492	0.139	−218.535	31.151	0.825	1.212
CD4+T lymphocytes	35.289	13.357	0.277	2.642	0.010	8.727	61.851	0.945	1.058
Models for CAD-RADS score	**Unstandardized coefficients**		**Standardized coefficients**	**t**	**Sig.**	**95% CI for B**		**Statistics of collinearity**	
	B	Standard model	Beta			Lower-bound	Upper-bound	Tolerance	VIF
1 (Constant)	−0.462	0.669		−0.690	0.492	−1.792	0.868		
Age	0.033	0.012	0.290	2.815	0.006	0.010	0.057	1.000	1.000
2 (Constant)	−0.903	1.118		−0.807	0.422	−3.125	1.320		
Age	0.031	0.013	0.271	2.454	0.016	0.006	0.056	0.878	1.138
SBP	0.005	0.009	0.055	0.493	0.623	−0.014	0.023	0.878	1.138
3 (Constant)	−0.022	1.359		−0.016	0.987	−2.724	2.680		
Age	0.026	0.013	0.227	1.940	0.056	−0.001	0.052	0.781	1.280
SBP	0.003	0.009	0.033	0.290	0.772	−0.016	0.021	0.852	1.173
IgM-anti ApoB-D	−0.317	−0.279	−0.130	−1.137	0.259	−0.872	0.238	0.816	1.226
4 (Constant)	0.290	1.375		0.211	0.834	−2.444	3.024		
Age	0.027	0.013	0.234	2.001	0.049	0.000	0.053	0.780	1.283
SBP	0.001	0.009	0.017	0.153	0.879	−0.017	0.020	0.843	1.187
IgM-anti ApoB-D	−0.291	0.279	−0.120	−1.045	0.299	−0.846	0.263	0.811	1.232
Endothelial MP	−1.766	1.367	−0.135	−1.292	0.200	−4.484	0.952	0.980	1.012

Apo, apolipoprotein; CAC, coronary artery calcification score; CAD-RADS, Coronary Artery Disease – Reporting and Data System; CD, cluster differentiation; Ig, immunoglobulin; SBP, systolic blood pressure; VIF, variance inflation factor.

The role of T and B lymphocytes in atherosclerosis is a matter of debate. T cells originate from bone marrow progenitor cells, migrate to the thymus, where they undergo maturation and selection. After gene chain rearrangements, these cells express co-receptors CD4 and CD8. These double-positive thymocytes undergo a selection process that terminates in downregulation of one of the co-receptors and mature into single $CD4^+$ or $CD8^+$ T cells, which are finally released into the bloodstream. A distinct subset of $CD4^+$ T cells, natural regulatory T cells (nTregs), also originates from the thymus, comprises 5–10% of all peripheral $CD4^+$ T cells and are already differentiated, acting as key protectors from autoimmunity. These activated $CD4^+$ T cells can react to epitopes derived from unmodified apolipoprotein B (ApoB) and may either promote or limit the immune response [42] (Graphical abstract).

Conversely, B cells have two main types of immune responses, a T-Independent immune response, in which B cells can respond directly to the antigen, secrete germline-encoded IgM antibodies, produced without previous infection or immunization, and a T-dependent immune response, where B cells need assistance from T cells in order to respond. These activated B cells (B2) differentiate into IgG secreting plasma cells that target a specific antigen [42].

In our study, IgM *anti*-ApoB-D peptide was higher in FH with CAD-RADS < median, and IgG *anti*-OxLDL was higher in those with CAC>0, suggesting a protective innate response to ApoB and a harmful adaptive response to oxidized LDL. The IgG autoantibodies *anti*-ApoB-D, have shown an association with markers of inflammation and blood pressure [10,11], and also with coronary angioplasty [16], assessed by different methods. In atherosclerotic plaques, B1-derived IgM is known to be atheroprotective and B2-derived IgG recognizing OxLDL or ApoB is pro-atherogenic [42].

Other immune-inflammatory cells, and the expression of CD11b, TLR4, or hs-CRP levels did not discriminate FH individuals with subclinical ASCVD. CRP is not considered causal to atherosclerosis [43], but a marker for cardiovascular disease. IL-6 can be released by T lymphocytes [44], and activate IL-6R pathway. Based on genetic evidence, a Mendelian randomization analysis showed IL-6R with a causal role in ASCVD [45].

Our finding that IFN-γ levels were higher in patients with CAC = 0 may denote its dual role on atherogenesis. Usually known as pro-atherogenic, IFN-γ can also upregulate inducible nitric oxide synthases (iNOS) gene expression in macrophages, considered an anti-atherogenic effect [46].

In our study, the LDL-c-years-score was positively correlated with both CAC and CAD-RADS. The cumulative traditional cardiovascular risk factors are currently used to categorize heterozygous FH (HeFH) patients into more or less severe forms [47,48]. In the SAFEHEART study [35] LDL-c-years-score was independently associated with CAC, and in the REFERCHOL cohort [49], LDL-c-years score and the SAFEHEART risk equation were predictors of cardiovascular events. We did not use this score in our regression model because it includes age, which itself is a robust predictor.

4.1. Strengths and limitations

This study addressed many steps of the innate and adaptive immune cascade in this special population with FH and the relationship with atherosclerosis. The cross-sectional nature of our study, the relatively small number of participants, and the use of

statins appear a limitation due to the immunomodulatory effects of these drugs. In addition, we did not perform subtyping of CD4+T cells into Th1, Th2, Th17, and Tregs.

4.2. Conclusion

In subjects with FH, under high-intensity lipid-lowering therapy, age and CD4+T cells were associated to atherosclerosis burden. Future larger studies are needed to confirm these results.

Declaration of competing interests

MCI reports having received modest grants from Amgen, Amryt Pharma, AstraZeneca, Sanofi-Aventis, Novartis, NovoNordisk, Servier, PTC outside the submitted work. FAHF reports having received modest grants from Amgen, AstraZeneca, Sanofi-Aventis, Novartis, NovoNordisk, Servier, outside the submitted work. Otherwise, the authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CRediT authors contribution statement

WTF participated in the study design, performed data collection, participated in data analyses and interpretation, and drafted and approved the final version of the manuscript. MCI and FAHF participated in the study conception and design, participated in data analysis and interpretation, and drafted, revised, and approved the final version of the manuscript. AAEY, DT, MEI, MEC, HARF, CNF, VARS participated in data collection and interpretation and revised and approved the final version of the manuscript. HTB, ILM, MG, CAF participated in the study conception, data interpretation, and approved the final version of the manuscript. JBP, RLM, CAB performed genetic tests, data interpretation, and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2022.03.002.

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