



Analysis of S100A12 plasma levels in hyperlipidemic subjects with or without familial hypercholesterolemia

Roberto Scicali¹ · Antonino Di Pino¹ · Francesca Urbano¹ · Viviana Ferrara¹ · Simona Marchisello¹ · Stefania Di Mauro¹ · Alessandra Scamporrino¹ · Agnese Filippello¹ · Salvatore Piro¹ · Agata Maria Rabuazzo¹ · Francesco Purrello¹

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Abstract

Aims Inflammation is a key regulatory process that links hypercholesterolemia and immune mechanisms promoting atherosclerosis. Inflammatory biomarkers may be helpful to better define the atherosclerotic burden in patients with high cholesterol levels such as familial hypercholesterolemia (FH). Our aim was to evaluate the concentration of S100A12 protein in FH patients and its association with pulse wave velocity (PWV).

Methods We measured glucose and lipid profile, S100A12, sRAGE, esRAGE and PWV in 39 patients with a genetically confirmed diagnosis of FH and 39 hypercholesterolemic subjects without a clinical diagnosis of FH (Dutch score ≤ 3). All subjects were on statin treatment at the time of the enrollment.

Results No difference of glucose and lipid profile was found in the two groups. FH patients had higher S100A12 plasma levels than non-FH subjects (12.87 ± 4.82 vs. 8.57 ± 4.87 ng/mL, $p < 0.01$). No difference of hs-CRP, sRAGE and esRAGE was found between the two groups. Also, PWV was higher in FH patients than non-FH subjects (8.63 ± 0.92 vs. 6.68 ± 0.73 m/s, $p < 0.05$). Finally, S100A12 was independently correlated with age ($p < 0.01$), genetic mutation ($p < 0.01$) and PWV ($p < 0.001$).

Conclusions FH patients exhibited higher S100A12 levels than non-FH subjects. A novel vascular inflammation pathway, other than hs-CRP, might be useful to better characterize cardiovascular risk profile.

Keywords Familial hypercholesterolemia · S100A12 · Inflammation · Pulse wave velocity · Cardiovascular risk

Introduction

Atherosclerosis is a chronic and worsening process caused by various environmental and genetic factors [1]. Among these factors, an increased plasma concentration of low-density lipoprotein (LDL) cholesterol is causatively associated with atherosclerotic cardiovascular disease (ASCVD) [2].

However, despite changes in lifestyle and the use of pharmacologic treatments to reduce plasma cholesterol levels, ASCVD is still estimated to be the leading cause of death and loss of disability-adjusted life years [3]. Therefore, other processes are involved in the pathogenesis and progression of atherosclerosis together with LDL cholesterol.

Interestingly, in the last few years several studies have demonstrated the critical role of inflammation in the pathophysiology of atherosclerosis [4, 5]; in particular, inflammation seems to be a key regulatory process that links hypercholesterolemia and immune mechanisms promoting atherosclerosis [6]. In subjects with hypercholesterolemia, several studies showed that the deposition of lipids in the arterial wall activated an inflammatory cascade that promoted the migration of immune cells such as monocyte-derived macrophages and specific subtypes of T lymphocytes into the inflammatory lipidic lesion [7, 8]. In this context, novel inflammatory biomarkers may be helpful to

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✉ Francesco Purrello
fpurrell@unict.it

¹ Department of Clinical and Experimental Medicine, Internal Medicine, Garibaldi Hospital, University of Catania, Via Palermo, 636 95122 Catania, Italy

better define the atherosclerotic burden in the general population and, in particular, in subjects with high levels of LDL cholesterol and high risk of cardiovascular event such as familial hypercholesterolemia (FH) [9, 10]. The receptor for advanced glycation end products (RAGE) has been considered a central regulator for vascular inflammation and subsequent atherosclerosis [11, 12]. As a multi-ligand receptor expressed on the cell surface, it is able to bind various kinds of ligands such as S100A12, a member of the S100 protein family [13]. S100A12 is secreted from activated myeloid cells and endogenously expressed in different cells correlated with vascular disease [14]. The binding of S100A12 with RAGE promotes the activation of transcription factors for inflammatory events and induces the adhesion of molecules in vascular endothelial cells [15]. Several authors have previously shown the central role of S100A12 as an important inflammatory biomarker in different chronic morbidities at high cardiovascular risk such as diabetes, rheumatoid arthritis and chronic kidney disease [16–18]. No data exist regarding S100A12 plasma levels in subjects who have a genetic or environmental condition of high LDL cholesterol. RAGE has a secretory isoform, which is termed soluble RAGE (sRAGE). sRAGE is primarily formed by the proteolytic cleavage of membrane-bound RAGE and, secondarily, by a secreted, non-membrane-bound form of the receptor resulting from alternative splicing of the RAGE gene, which is known as endogenously secreted RAGE (esRAGE) [19]. esRAGE may contribute to the removal/neutralization of circulating ligands such as S100A12, thus functioning as a decoy by competing with cell-surface RAGE for ligand binding [20].

To better define the link between the axis RAGE/RAGE ligands and a chronic exposure of high LDL cholesterol levels, in this study we aimed to investigate the plasma concentration of the inflammatory markers S100A12, sRAGE and esRAGE in subjects with or without familial hypercholesterolemia. Moreover, we evaluated the association of S100A12 with pulse wave velocity (PWV), an instrumental parameter of early atherosclerosis largely utilized in clinical practice for CV risk assessment [21].

Methods

Study design and population

This was a case–control study involving patients with a diagnosis of FH previously confirmed by genetic analysis [22] and subjects with an LDL cholesterol ≥ 160 mg/dL and triglycerides (TG) < 200 mg/dL before statin therapy without a clinical diagnosis of FH (Dutch Lipid Clinic Network score ≤ 3 [23]). All participants were enrolled from the University Hospital of Catania, Italy from January 2017 to June

2018. This is a tertiary center for the screening, diagnosis and management of familial dyslipidemias. All participants were aged between 18 and 70 years, and they were on statin therapy at the time of the enrollment.

All participants underwent a physical examination and review of their clinical history. After a 12-h fasting, all participants had standard hematological and clinical biochemistry parameters measured. Body weight and height were measured, and body mass index (BMI) was calculated as weight divided by the squared value of height (kg/m^2). Arterial hypertension was defined as brachial blood pressure (BP) ≥ 140 mm Hg (systolic) and/or 90 mm Hg (diastolic) on at least two different occasions, or if the subject was on antihypertensive therapy. Statin therapy was defined as a daily intake of statins. Duration of statin therapy was defined as the numbers of years on statin therapy. According to intensity of drug, statin therapy was classified as low-intensity (fluvastatin 20–40 mg, lovastatin 20 mg, pravastatin 20 mg, simvastatin 10 mg) moderate-intensity (fluvastatin XL 80 mg, lovastatin 40 mg, pravastatin 40 mg, simvastatin 20–40 mg, atorvastatin 10–20 mg, rosuvastatin 5–10 mg) and high-intensity statin therapy (atorvastatin 40–80 mg, rosuvastatin 20–40 mg) [24]. Diabetes mellitus was defined as fasting plasma glucose ≥ 126 mg/dL on two consecutive readings and/or glycated hemoglobin (HbA1c) $\geq 6.5\%$ or the use of anti-diabetic medications. Advanced renal disease was defined as glomerular filtration rate (GFR) < 30 mL/min. Smoking habits were divided into either current smoking (defined as any cigarette in the last month [25]) or not. ASCVD was defined as documented previous myocardial infarction, acute coronary syndrome, coronary revascularization (percutaneous coronary intervention or coronary artery bypass graft surgery) or other arterial revascularization procedures, stroke or transient ischemic attack, or peripheral arterial disease. Exclusion criteria were: secondary hypercholesterolemia, previous history of diabetes, ASCVD or clinical evidence of advanced renal disease.

Biochemical analysis

Fasting plasma glucose (FPG) was measured with the glucose oxidase method. Serum total cholesterol, TG, high-density lipoprotein (HDL) cholesterol, and hs-CRP, were assessed by available enzymatic methods. Apolipoprotein B (ApoB) and Apolipoprotein A1 (ApoA1) were evaluated with a nephelometer assay (Siemens AG Healthcare Sector, Erlangen, Germany). Levels of lipoprotein (a) [Lp(a)] were measured with the latex agglutination immunoassay. LDL cholesterol was calculated using the Friedewald formula. Glycated hemoglobin (HbA1c) was measured with high-performance liquid chromatography using a National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial assay

Ref. [26]. Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.; normal range 4.25–5.9% [23–41 mmol/mol]). To quantify the plasma concentration of sRAGE (Human sRAGE ELISA; Biovendor, Brno, Czech Republic), esRAGE (B-Bridge esRAGE ELISA Kit, Cupertino, USA), S100A12 (Cloud-clone corp., ELISA Kit for S100A12, Houston, USA) fasting blood samples were collected and assessed as previously described [27].

Pulse wave velocity evaluation

The SphygmoCor CVMS (AtCor Medical, Sydney, Australia) system was used for the determination of the PWV. This system uses a tonometer and 2 different pressure waves obtained at the common carotid artery (proximal recording site) and at the femoral artery (distal recording site). The distance between the recording sites and the suprasternal notch was measured using a tape measure. An electrocardiogram was used to determine the start of the pulse wave. The PWV was determined as the difference in interval time of the pulse wave between the 2 different recording sites and the heart, divided by the travel distance of the pulse waveform. The PWV was calculated on the mean of 10 consecutive pressure waveforms to cover a complete respiratory cycle.

Statistical analysis

We based the power calculation on previous studies examining differences of S100A12 plasma levels in subjects with chronic metabolic disorders [16, 28, 29]. We used a level of significance (α) set to 5% and a power (1- β) set to 80%; thus, the estimated sample size was 35 subjects per group. The distributional characteristics of each variable, including normality, were assessed by the Kolmogorov–Smirnov test. Data are reported as mean $\pm$ standard deviation (SD) for continuous parametric and median (interquartile range-IQR) for continuous nonparametric variables and as frequency (percentage) for categorical variables. When necessary, continuous nonparametric variables (TG, duration of statin therapy and hs-CRP) were logarithmically transformed for statistical analysis to reduce skewness. The χ^2 test was used for categorical variables. To test differences in clinical and biochemical characteristics between the groups, we used Student's *t* test. To identify variables independently associated with variations of S100A12 and PWV, we performed three multiple regression models with several metabolic, genetic and cardiovascular variables who may modify S100A12 and PWV in clinical practice. The first model included several cardiovascular risk factors (age, sex, smoking, BMI, systolic and diastolic BP, FPG, HbA1c, and genetic mutation). Subsequently, variables reaching significance were inserted into a second multivariate regression model that included

lipid parameters (HDL cholesterol, TG, LDL cholesterol, Lp(a), duration and intensity of statin therapy). Finally, significant variables were added to a third multiple regression model including inflammatory markers (sRAGE, esRAGE, S100A12, and hs-CRP). The variance inflation factor (VIF) was used to check for the problem of multicollinearity in multivariate analysis [30]. For this reason total cholesterol, ApoB and ApoAI were excluded. All statistical analyses were performed using IBM SPSS Statistics for Windows version 23. For all tests, $p < 0.05$ was considered significant.

The study was approved by the local ethic committee in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent was obtained from each subjects included in the study.

Results

In total, 170 hypercholesterolemic individuals (98 FH patients and 72 subjects without FH) referred to our outpatient clinic were evaluated. Of these, 149 individuals (85 FH patients and 64 subjects without FH) satisfied the inclusion criteria; after matching for age, sex, BMI and LDL cholesterol levels 78 individuals (39 FH patients and 39 subjects without FH) participated in the study (Fig. 1). The study population was divided into the following two groups based on FH diagnosis: 39 FH patients (FH group) and 39 subjects without FH (non-FH group).

Among FH patients (Table 1), the most frequent genetic variants were LDL receptor mutations (97.4%); also, the majority of mutations caused an amino acid change (74.4%), whereas the percentage of null allele was 25.6%. All patients were heterozygous FH.

The pretreated lipid values of the study population are presented in Supplemental Table 1. As expected, FH group had a greater amount of total, LDL and non-HDL cholesterol and a lower amount of TG than non-FH group.

The general characteristics of the study population are presented in Table 2. No difference of glucose and lipid profile was found in the two groups. Concerning risk factors, similar values of systolic and diastolic BP were found between the two groups; also, the percentage of smokers was similar in FH and non-FH group. Concerning treatment, the FH group had a longer duration of statin treatment compared with the non-FH group (3.5 [2.5–7.5] vs. 2 [1.5–3] years, $p < 0.01$). Moreover, the percentage of subjects on ezetimibe was higher in FH group than non-FH group (41% vs. 7.7%, $p < 0.01$). Few patients were on antihypertensive treatment. Concerning intensity of statin therapy, no FH patients were on low-intensity statin. While the majority

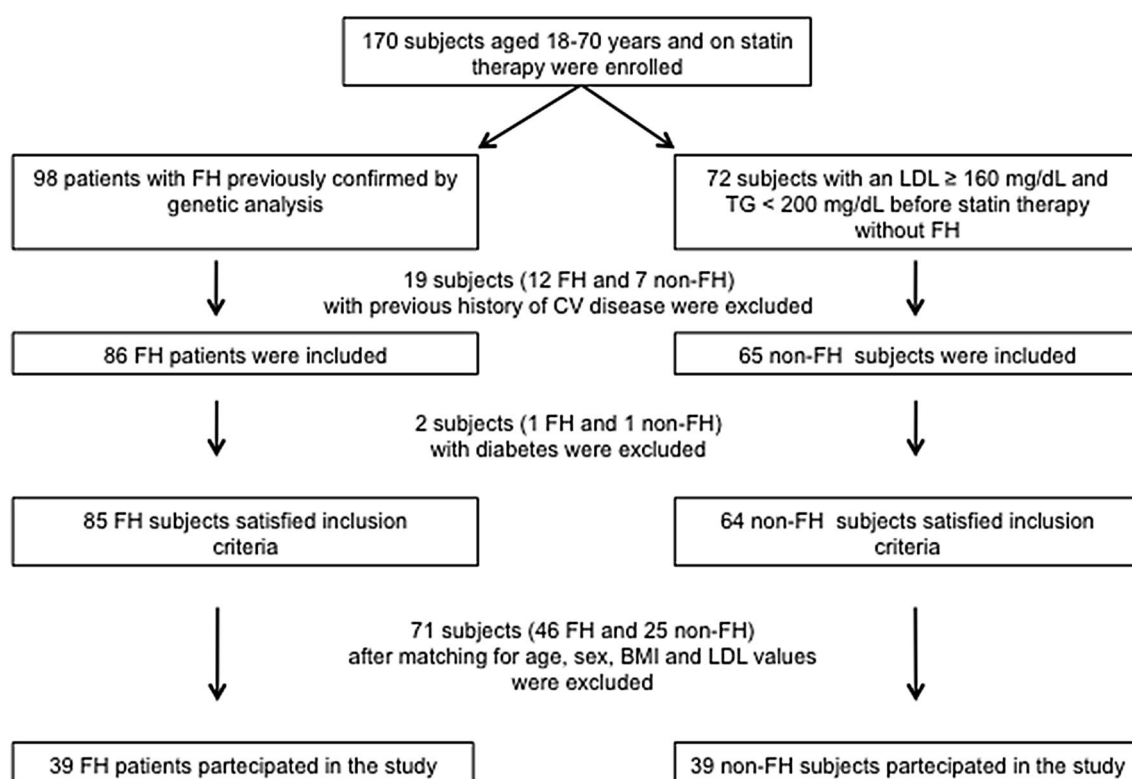


Fig. 1 Enrollment of the study population. *FH* familial hypercholesterolemia, *LDL* low-density lipoprotein, *TG* triglycerides, *CV* cardiovascular, *BMI* body mass index

Table 1 Genetic and phenotypic characteristics of FH patients ($n=39$)

Genetic mutation, n (%)	39 (100.0)
LDL receptor, n (%)	38 (97.4)
ApoB, n (%)	1 (2.6)
Mutation class, n (%)	39 (100.0)
Amino acid change, n (%)	29 (74.4)
Null allele, n (%)	10 (25.6)
FH phenotype, n (%)	39 (100.0)
Heterozygous FH, n (%)	39 (100.0)

Data are presented as percentages

FH=familial hypercholesterolemia, LDL=low-density lipoprotein, ApoB=apolipoprotein B

of non-FH subjects were on moderate-intensity statin, all patients on high-intensity statin were FH.

Table 3 reports the evaluation of inflammation and atherosclerotic biomarkers in the two groups. No difference of hs-CRP was found between the two groups; also, FH group had similar levels of sRAGE and eRAGE compared with the non-FH group. FH group had higher levels of S100A12 than non-FH group (12.87 ± 4.82 vs. 8.57 ± 4.87 ng/mL, $p < 0.01$). Also, FH group had higher PWV compared with the non-FH group (8.63 ± 0.92 vs. 6.68 ± 0.73 m/s, $p < 0.05$).

To estimate the independent contributions of cardiovascular risk factors to S100A12 levels, we performed multiple regression analysis using two models. The first model showed a significant correlation between S100A12 and age ($p < 0.01$) and genetic mutation ($p < 0.001$). In the second model, the same variables remained significantly associated with S100A12 ($p < 0.01$ for age and $p < 0.01$ for genetic mutation) (Table 4). Moreover, to evaluate the independent contributions of cardiovascular risk factors and inflammation markers to PWV, we performed multiple regression analysis using three models. The first model showed a significant correlation between PWV and genetic mutation ($p < 0.05$). This association was also confirmed in the second model ($p < 0.05$); finally, the third model showed a significant association between PWV and S100A12 ($p < 0.001$) and hs-CRP ($p < 0.05$) (Table 5).

Discussion

Over the last few years, thanks to a better definition of atherosclerosis, increasing attention has been given to the role of inflammation in the atherosclerotic process. In this study, we investigated the role of S100A12 in individuals with or without FH; to our knowledge, this is the first study exploring

Table 2 General characteristics of the study population

	FH group (<i>n</i> = 39)	Non-FH group (<i>n</i> = 39)	<i>p</i> value between two groups
Demographic characteristics			
<i>N</i>	39	39	
Age, years	48.87 ± 12.29	50.57 ± 11.16	0.36
Men, <i>n</i> (%)	21 (53.8)	20 (51.3)	0.84
Body mass index, kg/m ²	25.27 ± 3.13	25.8 ± 3.69	0.58
Glucose values			
FPG, mg/dL	86.81 ± 6.66	88.83 ± 7.22	0.36
HbA1c, %	5.54 ± 0.26	5.61 ± 0.26	0.28
Lipid values			
Total cholesterol, mg/dL	209.77 ± 13.20	206.14 ± 13.14	0.26
HDL cholesterol, mg/dL	52.79 ± 10.8	55.77 ± 12.14	0.11
Triglycerides, mg/dL	92 (74.5–110.5)	106 (82.75–129.5)	0.06
LDL cholesterol, mg/dL	126.75 ± 12.05	124.36 ± 11.08	0.45
Non-HDL cholesterol, mg/dL	152.21 ± 13.62	148.34 ± 13.89	0.16
ApoB, mg/dL	103.9 ± 12.57	101.03 ± 10.17	0.51
ApoAI, mg/dL	136.06 ± 19.48	140.92 ± 20.31	0.09
ApoB to ApoAI ratio	0.76 ± 0.18	0.72 ± 0.18	0.22
Lp(a), nmol/L	62.87 ± 58.07	56.86 ± 52.47	0.64
Risk factors			
Systolic BP, mmHg	114.7 ± 12.31	115.8 ± 10.67	0.65
Diastolic BP, mmHg	69.9 ± 10.21	71.36 ± 9.17	0.49
Smoking, <i>n</i> (%)	19 (48.8)	18 (46.2)	0.68
Treatment			
Duration of Statin therapy, years	3.5 (2.5–7.5)	2 (1.5–3)	<0.01
Ezetimibe, <i>n</i> (%)	16 (41.0)	3 (7.7)	<0.01
Antihypertensive therapy, <i>n</i> (%)	5 (12.8)	6 (15.4)	0.75
Intensity of statin therapy			
Low, <i>n</i> (%)	–	6 (15.4)	–
Moderate, <i>n</i> (%)	20 (51.3)	33 (84.6)	<0.05
High, <i>n</i> (%)	19 (48.7)	–	–

Data are presented as mean ± standard deviation, percentages, or median (interquartile range)

FH familial hypercholesterolemia, *FPG* fasting plasma glucose, *HbA1c* glycated hemoglobin, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ApoB* apolipoprotein B, *ApoAI* apolipoprotein AI, *Lp(a)* lipoprotein (a), *BP* blood pressure

Table 3 Evaluation of inflammation and early atherosclerotic biomarkers in the study population

	FH group (<i>n</i> = 39)	Non-FH group (<i>n</i> = 39)	<i>p</i> value between two groups
Inflammatory markers			
hs-CRP, mg/dL	0.10 (0.05–0.16)	0.08 (0.04–0.14)	0.23
sRAGE, ng/mL	1.23 ± 0.42	1.08 ± 0.37	0.21
esRAGE, ng/mL	0.34 ± 0.18	0.31 ± 0.17	0.35
S100A12, ng/mL	12.87 ± 4.82	8.57 ± 4.87	<0.01
Early atherosclerotic biomarker			
PWV, m/s	8.63 ± 0.92	6.68 ± 0.73	<0.05

Data are presented as mean ± standard deviation, percentages, or median (interquartile range)

FH familial hypercholesterolemia, *hs-CRP* high sensitivity C-reactive protein, *sRAGE* soluble receptor for advanced glycation end products, *esRAGE* endogenously secreted receptor for advanced glycation end products, *PWV* pulse wave velocity

Table 4 Multiple regression analysis evaluating S100A12 as a dependent variable

Independent variables	Coefficient β	<i>p</i> value
Model 1*		
Age, years	0.382	<0.01
Genetic mutation, for presence = 1	0.438	<0.001
Model 2**		
Age, years	0.330	<0.01
Genetic mutation, for presence = 1	0.347	<0.01

*Model 1 was adjusted for age, sex, smoking status, systolic and diastolic blood pressure, fasting plasma glucose, glycated hemoglobin, body mass index and genetic mutation

**Model 2 was adjusted for HDL cholesterol, triglycerides, LDL cholesterol, lipoprotein (a), duration and intensity of statin therapy

Table 5 Multiple regression analysis evaluating PWV as a dependent variable

Independent variables	Coefficient β	<i>p</i> value
Model 1*		
Genetic mutation, for presence = 1	0.212	<0.05
Model 2**		
Genetic mutation, for presence = 1	0.217	<0.05
Model 3***		
S100A12, ng/mL	0.422	<0.001
hs-CRP, mg/dL	0.245	<0.05

PWV pulse wave velocity, *hs-CRP* high sensitivity C-reactive protein, *sRAGE* soluble receptor for advanced glycation end products, *esRAGE* endogenously secreted receptor for advanced glycation end products

*Model 1 was adjusted for age, sex, smoking status, systolic and diastolic blood pressure, fasting plasma glucose, glycated hemoglobin, body mass index and genetic mutation

**Model 2 was adjusted for HDL cholesterol, triglycerides, LDL cholesterol, lipoprotein (a), duration and intensity of statin therapy

***Model 3 was adjusted for hs-CRP, sRAGE, esRAGE, and S100A12

S100A12 plasma levels in hypercholesterolemic subjects with or without a genetic condition. We showed that the FH group had higher plasma levels of S100A12 compared with the non-FH group; also, we found a significant association between S100A12 plasma levels and genetic mutation. The link between inflammation and hypercholesterolemia has been explained by the formation of the fatty streak that is the first atherosclerotic lesion in the arterial wall. In fact, the accumulation of lipids in the arterial wall promotes the deposition of monocyte-derived macrophages and T lymphocytes; thus, the secretion of S100A12 is enhanced with a resulting pure inflammatory lesion [31]. In this context, it is possible that S100A12 plasma levels may be increased in a genetic condition of high LDL cholesterol levels such as familial hypercholesterolemia. Interestingly, Menini et al.

[32] showed that RAGE is present in atherosclerotic plaque and the complex RAGE-ligand enhanced plaque inflammation and promoted its rupture; thus, high S100A12 plasma levels may reflect an increased plaque inflammation. In this context, the continuous cholesterol burden of FH patients may accelerate fatty streak formation, enhance S100A12 secretion and promote the progression of the fatty streak into an ‘inflammation streak’ [33].

FH patients have a high risk of cardiovascular events, and statin treatment has significantly reduced it [34]. However, despite statin therapy FH patients have a higher cardiovascular risk than non-FH subjects on statin therapy [35]; in line with this consideration, in our study we found that FH patients had higher PWV than non-FH subjects. Also, in our population we showed a positive association of S100A12 and PWV; so, S100A12 may promote the increase in cardiovascular risk and the progression of atherosclerosis together with LDL cholesterol in FH patients.

Several studies have shown that S100A12 is a good predictor for future cardiovascular events: Ligthart et al. found that S100A12 better predicted coronary events in the general population, beyond the traditional cardiovascular risk factors and other inflammatory markers such as high sensitivity C-reactive protein (hs-CRP) or interleukins [36]; moreover, Nowak et al. have recently found that S100A12 was one of the most important biomarkers for prediction of cardiovascular events in a large cohort of diabetic subjects [37]. In this context, further studies are needed to evaluate the progression of atherosclerotic burden and its possible association with S100A12 in a large cohort of FH patients.

We found no difference in sRAGE and esRAGE plasma levels in the FH group compared with the non-FH group. This result is not surprising, indeed, sRAGE and esRAGE are strongly associated with glucose homeostasis [38] and in our population no differences in glucose parameters were found between the two groups. Moreover, common oral agents such as statins are known to stimulate the production of sRAGE and esRAGE [39] and in this study all subjects were on statin therapy. Statin treatment may also explain the similar levels of hs-CRP in the two groups; the effect of statins on hs-CRP reduction has been reported in previous studies in subjects without ASCVD [40, 41]. Conversely, according to our data, it is possible to hypothesize that S100A12 is not modulated by statins; other studies are needed to evaluate other possible pathophysiological pathways of S100A12.

There are several limitations to our study. First, because of its cross-sectional design, causal relationship and temporality cannot be established between statin therapy and possible changes in S100A12. Population size was relatively small; however, we were still able to show a significant difference of S100A12 in the two groups and an independent association of S100A12 and age, genetic mutation and PWV was

found. Finally, other parameters regarding the evaluation of cardiovascular risk in FH patients such as cholesterol burden were not available and, thus, were not taken into consideration.

In conclusion, the FH group exhibited higher levels of S100A12 compared with the non-FH group. Moreover, S100A12 was significantly associated with age, genetic mutation and PWV. A novel vascular inflammation pathway, other than hs-CRP, might be useful to better characterize CV risk profile. Further studies are needed to evaluate a possible correlation of S100A12 plasma levels with advanced markers of atherosclerosis such as coronary artery calcium; moreover, it may be interesting to evaluate if novel lipid lowering therapies could modulate S100A12 plasma levels.

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Authors' contributions RS contributed to the study design, researched data, contributed to the discussion, and wrote the article. ADP contributed to the study design, researched data, contributed to the discussion, and reviewed and edited the article. FU, VF, SDM, SM, AS, AF, researched data, contributed to the discussion, and reviewed and edited the article. SP and AMR contributed to the study design and discussion, and reviewed and edited the article. FP designed the study, researched data, contributed to the discussion, and reviewed and edited the article.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

Ethical approval This study has been approved by the local ethic committee in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from each participants included in the study.

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