Atherosclerosis 285 (2019) 17–22

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

VEGFR2 and *OPG* genes modify the risk of subclinical coronary atherosclerosis in patients with familial hypercholesterolemia

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HIGHLIGHTS

- *OPG* rs2073618 and *VEGFR2* rs2071559 SNPs are associated with atherosclerotic cardiovascular disease.
- *VEGFR2* rs2071559 SNP decreases risk of developing coronary artery stenosis.
- *VEGFR2* rs2071559 SNP decreases risk of coronary artery calcium accumulation.
- *OPG* rs2073618 SNP increases the risk of the presence of coronary artery calcium.

ARTICLE INFO

Keywords:
Familial hypercholesterolemia
Polymorphism
VEGFR2
OPG
SAFEHEART
Coronary calcium and coronary stenosis

ABSTRACT

Background and aims: Heterozygous familial hypercholesterolemia (FH) is a genetic disorder characterized by high levels of low-density lipoprotein cholesterol (LDL-C). The magnitude of atherosclerotic cardiovascular disease (ASCVD) risk in FH patients is highly variable, and this can result from genetic factors. The aim of our study was to characterize whether polymorphisms in *VEGFR2* and *OPG* genes could influence the expression of ASCVD in FH patients.

Methods: We studied 318 FH patients from the SAFEHEART registry, without clinical diagnosis of ASCVD. A coronary tomographic angiography (CTA) was performed to determine and evaluate the presence of coronary stenosis and coronary artery calcium, as measured by coronary calcium score (CCS). Genotyping of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms was performed using TaqMan 5′-exonuclease allelic discrimination assays.

Results: Homozygous GG genotype and G allele of *VEGFR2* rs2071559 polymorphism were associated with decreased risk of developing coronary artery stenosis. In the analysis of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms, according to the presence of coronary artery calcium, we found significant differences in both polymorphisms. Homozygous GG genotype and G allele of *VEGFR2* rs2071559 polymorphism were associated

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https://doi.org/10.1016/j.atherosclerosis.2019.03.019
Received 9 November 2018; Received in revised form 18 March 2019; Accepted 29 March 2019
Available online 31 March 2019
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with decreased risk of accumulation of coronary artery calcium measured by CCS in CTA. Moreover, being a carrier of the GG genotype and G allele of the *OPG* rs2073618 polymorphism increased the risk of the presence of coronary artery calcium measured by CCS in CTA.
Conclusions: Polymorphisms in *VEGFR2* and *OPG* genes modify the risk of ASCVD in FH patients.

1. Introduction

Heterozygous familial hypercholesterolemia (FH) is a genetic disorder that is characterized by high levels of plasma low-density lipoprotein cholesterol (LDL-C) and early atherosclerotic cardiovascular disease (ASCVD) [1–3]. Most likely, the prevalence of FH may be as high as 0,4%. FH patients have a 3- to 13-fold greater risk of premature ASCVD than individuals without FH. Sudden death and acute ischaemic heart disease are the main causes of death among these subjects. The magnitude of ASCVD risk in FH patient is highly variable. In the expression of ASCVD, genetic factors, environmental, and metabolic risk factors could play an important role [4–9] and could explain the differences that are observed in clinical practice. To understand the development of atherosclerosis in FH patients, vascular imaging is a useful tool to define the atherosclerotic disease process; specifically, coronary computed tomographic angiography (CTA) is able to detect and quantify calcium in the coronary artery wall and luminal stenosis. Recently, we published the results of CTA findings in asymptomatic patients with FH [9]. The patients come from the SAFEHEART (Spanish familial hypercholesterolemia cohort study) [10].

Abnormal angiogenesis has been implicated in atherosclerosis and arterial diseases [11]. Vascular endothelial growth factor (VEGF) plays a crucial role in physiological and pathological angiogenesis. VEGF binds to VEGF-receptor 1 (VEGFR1) and to VEGF-receptor 2 (VEGFR2) to mediate angiogenesis and endothelial cell survival [12,13]. Therefore, variants of the *VEGFR2* gene have been associated with atherosclerosis and coronary heart disease [14,15]. Additionally, osteoprotegerin (OPG) has been associated with the coronary atherosclerosis process and with the presence of calcium deposits [16–18]. OPG protein is a member of the TNF receptor family and acts like a soluble receptor for receptor activator of nuclear factor KB ligand (RANKL). Initially, OPG protein was associated with bone resorption but, more recently, has been associated with the presence of atherosclerosis, coronary artery calcium and arterial inflammatory processes [19–21].

The aim of our study was to characterize whether polymorphisms in *VEGFR2* and *OPG* genes could influence in the expression of ASCVD in FH patients without clinical diagnosis of ASCVD, using the SAFEHEART registry, in which CTA was previously performed. Therefore, in this study we have analysed the *OPG* rs2073618, a missense polymorphism that produces an amino acid change (p. Asn3Lys), and *VEGFR2* rs2071559, a polymorphism in the gene promoter region. We evaluate the role of these variants in modulating ASCVD risk in FH patients.

2. Patients and methods

2.1. Patients

The SAFEHEART study is a multicentre, nationwide, long-term prospective cohort study in a molecularly defined population of patients with heterozygous FH in Spain. For this study, 318 SAFEHEART individuals (aged 20–70 years) without clinical cardiovascular disease, and consecutively enrolled in the registry in 6 university hospitals, voluntarily underwent a coronary CTA between January 2013 and December 2016. Patients with contraindications for coronary CTA were excluded. All patients were managed according to the indications of their treating physician, pharmacological treatment were statins and ezetimibe at the maximum tolerated dose to reach the therapeutic goal established individually for each patient in primary prevention and followed-up on a yearly basis through a standardized protocol. Clinical and analytical variables such as sex, age at enrolment, blood pressure, smoking, body mass index, total cholesterol, LDL-c, HDL-c, triglycerides, apolipoprotein AI, apolipoprotein B and lipoprotein(a) were collected from each patient [10,22].

The experimental protocol was in accordance with the Declaration of Helsinki (2008) of the World Medical Association, approved by the University Hospital of Salamanca Ethics Committee and in compliance with the Spanish data protection law (LO 15/1999) and specifications (RD 1720/2007). All who accepted to participate in the study signed a written consent.

2.2. Coronary computed tomography

A coronary tomographic angiography (CTA) was performed in a subgroup of FH patients without clinical diagnosis of ASCVD to determine and evaluate the presence of coronary stenosis and coronary artery calcium measured by coronary calcium score (CCS). Coronary CTA was performed as previously was described [9] using 64–detector row scanners or higher with prospective or retrospective electrocardiographic gating. Eighty to 100 ml of intravenous contrast, followed by 50–80 ml of saline, was administered at a rate of 5 ml/s via a power injector through an antecubital vein. Scanning parameters included heart-rate–dependent pitch (0.20–0.45), 330 ms gantry rotation time, 100 kVp or 120 kVp tube voltage, and 350–800 mA tube current. Coronary CTAs were reconstructed using the following parameters: 0.5- to 0.75-mm slice thickness, 0.3-mm slice increment, 160- to 250-mm field of view, 512 × 512 matrix, and a standard kernel. Optimal phase reconstruction was assessed by comparison of different phases, if available, and the phase with the least amount of coronary artery motion was chosen for analysis. Multiple phases were utilized for image interpretation if minimal coronary artery motion differed among the various arteries. Every coronary CTA was analysed by two independent experienced readers, blinded to the characteristics of the subjects, in a central lab. In case of discrepancy, a third reader was consulted. Coronary CT analysis was performed on dedicated workstations (Philips Extended Brilliance TM Workspace 4.5 equipped with the software Comprehensive Cardiac Analysis; Philips Medical Systems Nederland). CTAs were evaluated by using different techniques, including axial, multiplanar reformat, maximum intensity projection, and cross-sectional views.

2.3. Cardiovascular risk estimation

For each patient, 5-year cardiovascular risk was estimated according to the SAFEHEART-risk equation (SAFEHEART-RE). SAFEHEART-RE is a robust risk prediction equation, developed and published by our working group to predict the risk of incident ASCVD in FH patients using clinical and laboratory parameters including age, gender, familial history of ASCVD, blood pressure, body mass index, smoking, low-density lipoprotein-cholesterol and lipoprotein(a) levels [22].

2.4. DNA isolation and polymorphism genotyping

Genomic DNA was extracted from peripheral blood leukocytes by a standard phenol/chloroform procedure. Genotyping of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms was performed using TaqMan 5′-exonuclease allelic discrimination assays that contain sequence-specific forward and reverse primers to amplify the polymorphic sequences and two probes labelled with VIC and FAM dyes to detect both alleles of each polymorphism [23]. PCR reactions were carried out using TaqMan

Table 1
Characteristics of FH patients included in the study.

Characteristics	FH patients
Sex (male), n (%)	152 (47,8)
Age at enrolment, mean ± SD	46,93 ± 10,32
High blood pressure, n (%)	24 (7,5%)
Active tobacco smoker, n (%)	93 (29,2%)
Ex-smoker, n (%)	69 (21,7%)
BMI (kg/m^2), mean ± SD	25,83 ± 4,31
Total cholesterol (mg/dL), mean ± SD	252, 04 ± 67,17
LDL-C (mg/dL), mean ± SD	181,93 ± 61,53
HDL-C (mg/dL), mean ± SD	49,80 ± 12,21
TG, mg/dL (mean) ± SD	101,08 ± 60,00
ApoAI (mg/dL), mean ± SD	138,81 ± 27,25
ApoB (mg/dL), mean ± SD	117,87 ± 39,78
SAFEHEART-RE value, mean ± SD	0,97 ± 0,90
Coronary artery stenosis, n (%)	150 (47,2%)
Coronary artery calcium, n (%)	196 (61,6%)
Non CAS, non CAC, n (%)	118 (37,10)

SD: standard deviation; FH: familial hypercholesterolemia; BMI: body mass index; LDL-C: low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TG: triglycerides; ApoAI: apolipoprotein AI; ApoB: apolipoprotein B; Lp(a): lipoprotein(a); SAFEHEART-RE: Spanish familial hypercholesterolemia cohort study-risk equation; CAS: coronary artery stenosis; CAC: coronary artery calcium.

universal PCR Master Mix, following instructions, in a StepOnePlus Real-time PCR system. To assess reproducibility, a random selection of 5% of the samples were re-genotyped, and all of these genotypes matched with the genotypes initially designated.

We selected *VEGFR2* rs2071559 and *OPG* rs2073618 polymorphisms because they have a population frequency of the minor allele higher than 10% in Caucasian population and are located in sequences highly conserved throughout the evolution. Moreover, the two selected polymorphisms were previously reported in the literature. The rs2071559 polymorphism is located in the promoter region of the *VEGFR2* gene (−604A > G) and alters transcription factor E2F binding, which causes a downregulation of VEGFR2 expression [24]. Wang et al. described that the GG genotype of the *VEGFR2* rs2071559 polymorphism is associated with a 68% decrease in transcriptional activity, resulting in reduced levels of VEGFR2 protein [15]. *Chung* et al. published that GG genotype of *OPG* rs2073618 polymorphism, that encodes an Asn3Lys missense substitution, was significantly associated with the presence of coronary artery calcium in patients with rheumatoid arthritis [25].

2.5. Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated for each polymorphic variant using unconditional logistic regression models to evaluate the association with coronary artery stenosis and coronary artery calcium risk. These statistical analyses were performed using SPSS 21.0 software. For the analysis, differences with a p-value < 0.05 were considered statistically significant. p-values were adjusted with the SAFEHEART-RE value.

3. Results

A total of 318 FH patients without clinical diagnosis of ASCVD from the SAFEHEART registry were analysed. The individuals included in the study had a balanced distribution between men and women (47.8% of the men), with an average age of 46.93 years (± 10.32 years), typical of a study in primary prevention. To perform an analysis of coronary CTA results, they were grouped into coronary artery stenosis (CAS): 150 (47.2%), coronary artery calcium (CAC): 196 (61.6%), and non CAS, non CAC: 118 (37,10%). The clinical and laboratory parameters of the patients included in the study are summarized in Table 1.

The genotypic and allelic frequencies of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms and the results of the association analysis according to the presence of coronary artery stenosis in FH patients are summarized in Table 2. We found statistically significant differences in genotypic and allelic distribution of the *VEGFR2* rs2071559 polymorphism. Homozygous GG genotype and G allele of *VEGFR2* rs2071559 polymorphism were associated with decreased risk of developing coronary artery stenosis (Table 2). When we analysed *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms according to coronary artery calcium, we found significant differences with both polymorphisms. Homozygous GG genotype and G allele of *VEGFR2* rs2071559 polymorphism were associated with decreased risk of

Table 2
Genotypic and allelic frequencies of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms among FH patients with coronary artery stenosis and FH patients without coronary artery stenosis.

		Non CAS	CAS	p-value	OR (95% CI)
***VEGFR2* rs2071559**	Genotype				
	AA	24 (20,3%)	36 (24,0%)	/	1,00
	AG	55 (46,6%)	88 (58,7%)	0,837	1,06 (0,57–1,97)
	GG	39 (33,1%)	26 (17,3%)	0,026	0,44 (0,21–0,91)
	AA + AG	79 (66,9%)	124 (82,7%)	/	1,00
	GG	39 (33,1%)	26 (17,3%)	0,003	0,42 (0,24–0,75)
	AA	24 (20,3%)	36 (24,0%)		
	AG + GG	94 (79,7%)	114 (76,0%)	0,475	
	Allele				
	A	103 (43,6%)	160 (53,3%)	/	1,00
	G	133 (56,4%)	140 (46,7%)	0,026	0,67 (0,48–0,95)
***OPG* rs2073618**	Genotype				
	CC	47 (39,8%)	57 (38,0%)		
	CG	48 (40,7%)	55 (36,7%)	0,516	
	GG	23 (19,5%)	38 (25,3%)		
	CC + CG	95 (80,5%)	112 (74,7%)		
	GG	23 (19,5%)	38 (25,3%)	0,258	
	CC	47 (39,8%)	57 (38,0%)		
	CG + GG	71 (60,2%)	93 (62,0%)	0,760	
	Allele				
	C	142 (60,25)	169 (56,3%)		
	G	94 (39,8%)	131 (43,7%)	0,372	

FH: familial hypercholesterolemia; CAS: coronary artery stenosis; OR: odds ratio; *VEGFR2*: vascular endothelial growth factor-receptor 2; *OPG*: osteoprotegerin. p-values were adjusted by SAFEHEART-RE value.

Table 3
Genotypic and allelic frequencies of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms according with the presence of coronary artery calcium.

		Non CAC	CAC	*p*-value	OR (95% CI)
***VEGFR2* rs2071559**	Genotype				
	AA	24 (20,3%)	52 (26,5%)	/	1,00
	AG	55 (46,6%)	112 (57,1%)	0,834	0,94 (0,52–1,68)
	GG	39 (33,1%)	32 (16,3%)	0,005	0,37 (0,19–0,74)
	AA + AG	79 (66,9%)	164 (83,7%)	/	1,00
	GG	39 (33,1%)	32 (16,3%)	0,001	0,39 (0,23–0,67)
	AA	24 (20,3%)	52 (26,5%)		
	AG + GG	94 (79,7%)	144 (73,5%)	0,215	
	Allele				
	A	103 (43,6%)	216 (55,1%)	/	1,00
	G	133 (56,4%)	176 (44,9%)	0,006	0,63 (0,45–0,87)
***OPG* rs2073618**	Genotype				
	CC	47 (39,8%)	69 (35,2%)		
	CG	48 (40,7%)	66 (33,7%)	0,076	
	GG	23 (19,5%)	61 (31,1%)		
	CC + CG	95 (80,5%)	135 (68,9%)	/	1,00
	GG	23 (19,5%)	61 (31,1%)	0,025	1,86 [1,08-3,22]
	CC	47 (39,8%)	69 (35,2%)		
	CG + GG	71 (60,2%)	127 (64,8%)	0,411	
	Allele				
	C	142 (60,2%)	204 (52,0%)	/	1,00
	G	94 (39,8%)	188 (48,0%)	0,048	1,39 (1,11–1,93)

FH: familial hypercholesterolemia; CAS: coronary artery stenosis; OR: odds ratio; *VEGFR2*: vascular endothelial growth factor-receptor 2; *OPG*: osteoprotegerin. *p*-values were adjusted by SAFEHEART-RE value.

coronary artery calcium measured by coronary calcium score (CCS). In the case of *OPG* rs2073618 polymorphism, being a carrier of GG genotype and G allele increased the risk of coronary artery calcium (Table 3). *p*-values were adjusted by the SAFEHEART-RE value.

4. Discussion

This study shows the relationship between polymorphisms in the *VEGFR2* and *OPG* genes and ASCVD in patients with FH. We have found a relation between the homozygous GG genotype and the G allele of the *VEGFR2* rs2071559 polymorphism with a decreased risk of developing. In the analysis of *OPG* rs2073618 and *VEGFR2* rs2071559 polymorphisms according to CAC, we found significant differences with both polymorphisms. The homozygous GG genotype and the G allele of the *VEGFR2* rs2071559 polymorphism were associated with decreased risk of CAC, as measured by coronary calcium score (CCS) ($p = 0{,}005$ and $p = 0{,}006$). In the case of the *OPG* rs2073618 polymorphism, being a carrier of the GG genotype and G allele increased the risk of CAC. These results show, for the first time, the relationship between the genes we have studied and atherosclerosis in patients with FH. The findings allow us to better understand the development of atheromatous plaques in patients with FH.

The rs2071559 polymorphism is located in the promoter region of the *VEGFR2* gene (- % decrease in transcriptional activity, resulting in reduced levels of VEGFR2 protein [15]. The VEGF-VEGFR2 pathway has been implicated in atherosclerosis and arterial diseases [11,26]. It has been reported that upregulation of VEGF signalling is linked to neovascularization within the plaque and that downregulation of *VEGFR2* gene expression could decrease the plaque growth [24,27,28]. Several studies have associated the *VEGFR2* rs2071559 polymorphism with a reduced susceptibility to atherosclerosis processes due to reduction of VEGFR2 expression [14,15,24]. Our results showed that the GG genotype and G allele of the *VEGFR2* rs2071559 polymorphism are associated with both decreased risk of developing coronary artery stenosis and decreased risk of accumulation of coronary artery calcium measured by CCS; therefore, they could be associated with a reduced risk of expression of ASCVD.

The RANK/RANKL/OPG pathway has well-established regulatory effects on bone metabolism. The OPG protein is a soluble receptor for RANKL causing the inhibition of the nuclear factor KB (NF-KB) pathway, which prevents osteoclast differentiation [29]. Moreover, OPG protein has been proposed as the link between osteoporosis and atherosclerosis [20]. Bucay et al. showed that $Opg^{-/-}$ mice develop severe osteoporosis and arterial calcification of the aorta and renal arteries [30]. Studies performed on patients with coronary artery disease showed strong association between plasma levels of OPG protein and the presence of arterial calcium deposits and atherosclerosis [16–18,31,32]. These findings suggest that OPG protein could play an important role not only in bone metabolism but also in the progression of vascular disease. A shift of calcium from the skeleton towards the arterial wall could explain the relationship between OPG protein and calcification of arteries, but the underlying molecular mechanisms that operate in bone metabolism and vascular calcification are unclear [17,18]. Significant differences were found in genotypic and allelic distribution of the *OPG* rs2073618 polymorphism according to the coronary artery calcium. Being a carrier of the GG genotype and the G allele of the *OPG* rs2073618 polymorphism increased the risk of the presence of coronary artery calcium in FH patients measured by CCS. Our results are in concordance with the results reported by Chung et al. showing that the GG genotype of the *OPG* rs2073618 polymorphism was significantly associated with the presence of coronary artery calcium in patients with rheumatoid arthritis [25].

The main strengths of our work are the cohort of patients drawn from a follow-up study such as SAFEHEART; the study is enhanced with patients with a coronary CT image in primary prevention. The functional polymorphisms that have been associated in this study have a broad physiopathological base. Another strength is the lack of studies of this type in patients with FH with a follow-up as broad as ours and including diagnostic imaging. All these factors make our study novel. However, one limitation of our study could be the size of the cohort; Another limitation is the lack of an experimental model to clarify the calcification of the endothelial wall. Further follow-up study in this population will analyse to see if our findings are associated with the development of clinical heart disease over time and not just changes in the atheroma plaque.

In conclusion, our results show that polymorphisms in the *VEGFR2* and *OPG* genes could modify the risk of expression of atherosclerotic cardiovascular disease in patients with FH. These polymorphisms could

increase the risk to develop coronary artery calcium measured by CCS in CTA. Our study lays the foundation for future research to define the role of these polymorphisms in high risk-patients with familial hypercholesterolemia.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

José Pablo Miramontes-González: study concept and design, data acquisition, critical review of the manuscript throughout the editorial process, and approval of the final manuscript draft submitted for publication.

Ricardo Usategui-Martín: samples genotyping, data interpretation, critical review of the manuscript throughout the editorial process.

Leopoldo Pérez de Isla: study concept and design, data acquisition, TC data interpretation.

Rodrigo Alonso: data acquisition.

Ovidio Muñiz-Grijalvo: data acquisition.

José Luis Díaz-Díaz: data acquisition.

Daniel Zambón: data acquisition.

Francisco Fuentes Jiménez: data acquisition.

Javier Martín-Vallejo: statistical analysis.

Ana Elisa Rodríguez Gude: samples genotyping.

David León Jiménez: critical review of the manuscript throughout the editorial process, and approval of the final manuscript draft submitted for publication.

Teresa Padró: DNA collection.

Rogelio González Sarmiento: study concept and design, critical review of the manuscript throughout the editorial process, and approval of the final manuscript draft submitted for publication.

Pedro Mata: study concept and design, critical review of the manuscript throughout the editorial process, and approval of the final manuscript draft submitted for publication.

All authors agree to be accountable for all aspects of the work, ensuring the accuracy and integrity of the publication.

Financial support

This work was supported by Fundación Hipercolesterolemia Familiar. Grant G03/181 and FIS PI12/01289 from Instituto de Salud Carlos III (ISCIII), Grant 08-2008 Centro Nacional de Investigaciones Cardiovasculares (CNIC) and the grant from Instituto de Salud Carlos III (Ministry of Economy and Competitiveness) (ISC IIII-FEDER: PI16/01920).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.03.019.

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