Osteoprotegerin And MTHFR Gene Variations In Rheumatoid Arthritis: Association With Disease Susceptibility And Markers of Subclinical Atherosclerosis

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Abstract

Objective: To explore whether the osteoprotegerin (OPG) rs2073618 gene variant and the methylenetetrahydrofolate reductase (MTHFR) rs1801131 and rs1801133 polymorphisms contribute to rheumatoid arthritis (RA) susceptibility and RA related subclinical atherosclerosis.

Methods: 283 RA patients and 595 healthy controls (HC) were genotyped for OPG and MTHFR gene variants using PCR based assays. Clinical, laboratory parameters and markers of subclinical atherosclerosis (Carotid/Femoral intima media thickness/plaque formation) along with traditional risk factors for atherosclerosis were assessed in RA patients and 280HC.

Results: Increased prevalence of the rs2073618CC genotype was detected in RA patients vs HC ($p=0.04$), especially in RA patients with high serum titers of rheumatoid factor (RF) or anti-cyclic citrullinated peptide (anti-CCP) antibodies compared to HC (p -values: 0.0029 and 0.0077, respectively). This genotype was also associated with higher carotid-IMT scores ($p=0.01$) and marginally with higher rates of carotid plaque formation ($p=0.06$). MTHFR 1298CC genotype was more prevalent in the anti-CCP positive group compared to HC, with no associations detected with markers of subclinical atherosclerosis. Reduced rates of carotid/femoral plaque formation were detected among RA patients harboring the MTHFR 677TT genotype ($p=0.009$).

Conclusion: OPG and MTHFR gene variations seem to increase susceptibility for seropositive RA and potentially contribute to subclinical atherosclerosis linked to RA.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease, characterized by arthritis mainly affecting the peripheral joints, with considerable prevalence worldwide. It is well accepted that RA patients have increased morbidity and mortality due to accelerated cardiovascular disease (CVD) with respect to the general population, but the increased prevalence of traditional risk factors seem inadequate to fully account for the phenomenon [1–3]. Thus in recent years, great interest has been focused on the possible causal association between RA and CVD and the role of novel risk factors. Indeed, there is significant similarity between chronic inflammatory processes and the dysregulated immune response in RA and atheromatosis [2]. Moreover, recent studies have investigated the potential role of a few single nucleotide polymorphisms (SNPs) in various genes that are associated with CVD in the general population - such as *methylenetetrahydrofolate reductase (MTHFR)*, *TNFA*, and *ZC3HC1*, *NFKB* or other SNPs such as rs964184- on atherosclerosis in RA [4].

Osteoprotegerin (OPG) is a cytokine, member of the TNFR superfamily, that plays a role in the regulation of bone resorption [5, 6] as well as the development and progression of atherosclerosis, as OPG is actively involved in the development of vascular calcification [7]. We know from animal models that OPG-deficient mice develop, apart from early osteoporosis, arterial calcification and that OPG inactivation accelerates atherosclerotic lesion progression and calcification in ApoE^{-/-} mice [8, 9]. Along this side, numerous studies have designated that OPG represents an independent risk factor for progressive atherosclerosis and CVD, as OPG levels associate with increased carotid intima-media thickness (IMT) progression and coronary artery disease [10–12].

Several polymorphisms of the gene encoding OPG (*TNFRSF11B*) mainly at promoter sites, have been associated with atherosclerosis in different patient populations; however, results from different studies are diverse [13–15]. While the RANKL/RANK/OPG system may promote RA inflammation and bone erosion, studies exploring the role of OPG genetic variants in patients with RA have not shown so far to increase disease susceptibility [16]. However, the CC genotype of the rs2073618, which encodes an Asn3Lys missense change in the osteoprotegerin gene (*TNFRSF11B*), maybe associated with coronary artery atherosclerosis [17], while the GG genotype may be linked to reduced risk to develop cerebrovascular events in anti-cyclic citrullinated peptide (CCP) negative patients [18].

It is well known that elevated homocysteine levels represent an independent risk factor for CVD in the general population [19–21]. The putative role of hyperhomocysteinemia (HHcy) in the development of atherosclerosis in autoimmune disorders has also been a field of interest, as it could partly explain the increased CVD risk in these patients [22–24]. Genetic polymorphisms of the MTHFR enzyme are a main cause of HHcy and have been associated with CVD in the general population [25–27]. Regarding RA, data is very limited and controversial, however there could be a potential role of MTHFR polymorphisms in the acceleration of atherosclerosis [4, 28, 29].

In the present study, we aimed to explore whether the rs2073618 variant of the osteoprotegerin gene as well as the A1298C (rs1801131) and C677T (rs1801133) variant of the MTHFR gene contribute to RA related subclinical atherosclerosis.

Results

Prevalence of osteoprotegerin (OPG) and MTHFR gene variants in RA patients vs controls

Suppl. Tables 1 and 2 summarize the demographics, disease related characteristics, clinical features and indices in subclinical atherosclerosis in study participants. As shown in Table 1, the CC genotype increased disease susceptibility (adjustment for age and gender in the dominant model CC vs CG/GG OR [95%CI]: 1.54 [1.03-2.27], p -value: 0.036). Further analysis revealed CG genotype of the rs2073618 OPG variant as a protective contributor against RA development in codominant and overdominant models (adjustment for age and gender ORs [95%CI]: 0.54 [0.35-0.84], p -value: 0.017 and 0.57 [0.38-0.84], p -value: 0.0048, respectively). While no statistically significant differences between the groups were detected for the MTHFR C677T, the CC genotype of the MTHFR A1298C variant was more frequent among RA patients (adjustment for age and gender in the recessive model genotype CC vs AA/AC: OR [95%CI]: 1.77 [1.05-2.98], p -value: 0.031).

Table 1

Prevalence of the OPG rs2073618 and the MTHFR rs1801133 and rs1801131 SNP genotypes in RA patients and HC, adjusted by gender and age. Genotypes, five genetic models (codominant, dominant, recessive, overdominant and additive) were estimated with SNPstats software (statistically significant if $p < 0.05$) MTHFR: methylene tetrahydrofolate reductase, OR: odds ratio, SNP: single nucleotide polymorphism, VS: versus, RA: rheumatoid arthritis, HC: healthy controls, map.

SNPs	Genotype	HAPMAP (European Database (%))	HC n (%)	RA n (%)	ORcodominant model [95%CI]	p- value	ORdominant model [95%CI]	p- value	ORrecessive model [95%CI]	p- value	ORover dominant model [95%CI]	p- value
OPG rs2073618					CC VS CG VS GG		CC VS (CG/GG)		(CC/CG) VS GG		CG VS (CC/GG)	
	CC	29	78 (33.3)	111 (42.7)	1.00	0.017	1.54 (1.03-2.27)	0.036	1.22 (0.77-1.93)	0.4	0.57 (0.38-0.84)	0.004
	CG	53	107 (45.7)	85 (32.7)	0.54 (0.35-0.84)							
	GG	18	49 (20.9)	64 (24.6)	0.89 (0.53-1.49)							
MTHFR rs1801133 (C677T)					CC VS CT VS TT		CC VS (CT/TT)		(CC/CT) VS TT		CT VS (CC/TT)	
	CC	46.9	141 (34.6)	106 (37.9)	1.00	0.47	0.82 (0.59-1.13)	0.23	0.87 (0.57-1.33)	0.52	0.90 (0.66-1.22)	0.49
	CT	44.2	200 (49.1)	131 (46.8)	0.83 (0.59-1.17)							
	TT	8.8	66 (16.2)	43 (15.8)	0.78 (0.49-1.25)							
MTHFR rs1801131 (A1298C)					AA VS AC VS CC		AA VS (AC/CC)		(AA/AC) VS CC		AC VS (AA/CC)	
	AA	43.4	198 (48.6)	128 (45.7)	1.00	0.096	1.14 (0.83-1.55)	0.41	1.77 (1.05-2.98)	0.031	0.93 (0.68-1.27)	0.64
	AC	45.1	176 (43.2)	118 (42.1)	1.03 (0.74-1.43)							
	CC	11.5	33 (8.1)	34 (12.1)	1.79 (1.04-3.09)							

Associations of OPG and MTHFR variants with disease related features and markers of subclinical atherosclerosis among RA patients

We next wished to explore whether OPG and MTHFR variants are associated with distinct clinical and serological characteristics, as well as with markers of subclinical atherosclerosis among RA patients. Thus, compared to RA patients sharing the CG/GG genotype, those with the CC genotype of the rs2073618 OPG variant were found to display increased rates of RF positivity and higher IMT values (69.9 vs 55.1, $p=0.02$ and 0.872 ± 0.264 vs 0.816 ± 0.284 , $p=0.01$, respectively). A trend towards higher DAS28 levels and rates of carotid atherosclerotic plaque formation was also observed among RA patients with the CC vs the CG/GG genotypes (3.5 ± 1.4 vs 3.2 ± 1.3 and 66% vs 54.1%, p -values: 0.06, for both comparisons). No other differences in disease related features or classical CVD risk factors between the CC and the GG/GC genotype group were detected (Table 2).

Table 2

Associations of osteoprotegerin (OPG) and methylene-tetrahydrofolate reductase (MTHFR) minor allele homozygous genotypes with demographics, disease related features, traditional CV risk factors and markers of subclinical atherosclerosis among rheumatoid arthritis (RA) patients.

	<i>OPG rs2073618</i>			<i>MTHFR rs1801133 (C677T)</i>			<i>MTHFR rs1801131 (A1298C)</i>		
	CC (n=111)	CG/GG (n=151)	p-value	TT (n=43)	TC/CC (n=239)	p-value	CC (n=35)	AC/AA (n=247)	p-value
Age (Mean± SD)	60.4±12	61.1±11.6	ns	61.6±10.5	62±12.2	ns	59.3±11	62.3±12	ns
Gender (male)(%)	12.6	18.5	ns	16.3	14.6	ns	85.7	85	ns
BMI (kgs/m ²)	28.5±5.8	27.8±5.1	ns	30.2±6.4	27.8±5.2	0.02	27.3±4.5	28.2±5.6	ns
Disease duration	14.2±10.2	13.2±10.0	ns	13.4±9.6	13.8±10.3	ns	16±12.6	13.4±9.8	ns
RF positivity (%)	69.9	55.1	0.02	57.9	62.8	ns	61.3	62.2	ns
Anti-CCP positivity (%)	65.1	54.4	ns	63.2	58.1	ns	63.3	58.3	ns
ESR (mean± SD)	26.5±23.3	24.1±19.7	ns	21.2±18.7	27±22.4	ns	24.5±16.6	26.3±22.6	ns
DAS 28 (mean± SD)	3.5±1.4	3.2±1.3	0.06	3.0±1.2	3.5±1.5	ns	3.4±1.1	3.5±1.5	ns
Diabetes (%)	9.9	4	ns	4.7	7.5	ns	5.7	7.3	ns
Hypertension (%)	46.9	42	ns	44.2	43.5	ns	40	44.1	ns
Current Smokers (%)	21.6	30.7	ns	9.3	29.7	0.005	34.3	25.5	ns
Total Cholesterol (mg/dl)	201.1±35.6	204.5±37.8	ns	204.3±33.8	202.4±36.8	ns	212.4±39.5	201.5±35.8	ns
LDL (mg/dl)	119.9±30.6	120.9±30.7	ns	120.3±27.2	120.4±31.1	ns	127.7±35	119.4±29.8	ns
Carotid IMT (mean± SD)	0.872±0.264	0.816±0.284	0.01	0.803±0.294	0.872±0.333	ns	0.780±0.270	0.874±0.334	0.049
Plaques									
Carotid and/or femoral	72	65.5	ns	52.4	72.7	0.009	60	70.9	ns
Carotid	66	54.1	0.06	47.6	63.4	0.05	48.6	62.8	ns
Femoral	59	51.4	ns	42.9	57.7	0.08	48.6	56.4	ns

Following stratification of RA patients according to RF status and adjustment for age and sex, a significantly increased frequency of the CC genotype was detected in the RF positive RA group compared to both HC control group (Table 3) and RF negative RA subset (Table 4) [48.6% vs 33.3%, OR [95%CI]:1.89 [1.25-2.86], p-value:0.0029 and 2.0 [1.16-3.45], p-value:0.012, respectively, in dominant models). Moreover, decreased frequency of the CG genotype in the RF positive RA group compared to both HC (Table 3) and RF negative patients (Table 4) [27.7% vs 45.7% vs 40.9%, p-values:0.0004 and 0.032, respectively, in the overdominant model]. No significant differences were found between HC and RF negative groups (Suppl. Table 3).

Table 3

Prevalence of the OPG rs2073618 and the MTHFR rs1801133 and rs1801131 SNP genotypes in RA RF positive patients and HC, adjusted by gender and age. (for the five genetic models (codominant, dominant, recessive, overdominant and additive) were estimated with SNPstats software (statistically significant). OPG: osteoprotegerin, MTHFR: methylene tetrahydrofolate reductase, OR: odds ratio, SNP: single nucleotide polymorphism, VS: versus, RA: rheumatoid arthritis, HRF: high resolution factor, HAPMAP: haplotype map.

SNPs	Genotype	HAPMAP (European Database (%))	HC n (%)	RF positive RA n (%)	OR codominant model [95%CI]	p- value	OR dominant model [95%CI]	p- value	OR recessive model [95%CI]	p- value	OR overdominant model [95%CI]	p- value
OPG rs2073618					CC VS CG VS GG		(CG/GG) VS CC		(CC/CG) VS GG		CG VS (CC/GG)	
	CC	29	78 (33.3)	72 (48.6)	1.00	0.0011	1.89 (1.25- 2.86)	0.0029	1.17 (0.71- 1.91)	0.53	0.45 (0.29-0.71)	0.000
	CG	53	107 (45.7)	41 (27.7)	0.42 (0.26- 0.67)							
	GG	18	49 (20.9)	35 (23.6)	0.77 (0.45- 1.33)							
MTHFR rs1801133 (C677T)					CC VS CT VS TT		CC VS (CT/TT)		(CC/CT) VS TT		CT VS (CC/TT)	
	CC	46.9	141 (34.6)	56 (34.6)	1.00	0.5	0.93 (0.63- 1.38)	0.71	0.73 (0.43- 1.24)	0.24	1.10 (0.76-1.60)	0.61
	CT	44.2	200 (49.1)	84 (51.9)	1.00 (0.66- 1.51)							
	TT	8.8	66 (16.2)	22 (13.6)	0.73 (0.41- 1.31)							
MTHFR rs1801131 (A1298C)					AA VS AC VS CC		AA VS (AC/CC)		(AA/AC) VS CC		AC VS (AA/CC)	
	AA	43.4	198 (48.6)	75 (46.3)	1.00	0.18	1.11 (0.77- 1.61)	0.58	1.81 (0.98- 3.37)	0.064	0.91 (0.62-1.32)	0.61
	AC	45.1	176 (43.2)	68 (42)	1.00 (0.67- 1.48)							
	CC	11.5	33 (8.1)	19 (11.7)	1.81 (0.95- 3.46)							

Prevalence of the OPG rs2073618 and the MTHFR rs1801133 and rs1801131 SNP genotypes in RA RF negative and RF positive patients, adjusted by gender ; and p-value for the five genetic models (codominant, dominant, recessive, overdominant and additive) were estimated with SNPstats software (statistically significant). OPG: osteoprotegerin, MTHFR: methylene tetrahydrofolate reductase, OR: odds ratio, SNP: single nucleotide polymorphism, VS: versus, RA: rheumatoid arthritis, F HAPMAP: haplotype map.

After RA patients were stratified according to anti-CCP status and adjustment for age and sex, genotype analysis revealed a statistically increased prevalence of the CC genotype of rs2073618 OPG SNP in anti-CCP positive RA patients compared to HC (Table 5) (OR [95%CI]: 1.89 [1.18-3.03], p-value:0.0077, in the dominant model). Moreover, anti-CCP positive RA group displayed decreased frequency of the CG genotype compared to HC [32.6% vs 45.7%, p-value:0.018, in the overdominant model] (Table 5), as well as of the GG genotype compared to anti-CCP negative counterparts (19.9 vs 31.6, p-value:0.04 in the recessive model, Table 6). Decreased frequency of the CG genotype in anti-CCP negative RA patients compared to HC [31.6% vs 45.7%, p-value:0.013, in the overdominant model] was also found (Suppl. Table 4).

Table 5

Prevalence of the OPG rs2073618 and the MTHFR rs1801133 and rs1801131 SNP genotypes in RA anti-CCP positive patients and HC, adjusted by gender and p-value for the five genetic models (codominant, dominant, recessive, overdominant and additive) were estimated with SNPstats software (statistically significant). OPG: osteoprotegerin, MTHFR: methylene tetrahydrofolate reductase, OR: odds ratio, SNP: single nucleotide polymorphism, VS: versus, RA: rheumatoid arthritis, anti-CCP: anti-cyclic citrullinated peptide, HAPMAP: haplotype map.

SNPs	Genotype	HAPMAP (European) Database (%)	HC n (%)	RA anti- CCP positive n (%)	OR codominant model [95%CI]	p- value	OR dominant model [95%CI]	p- value	OR recessive model [95%CI]	p- value	OR overdominant model [95%CI]	p- value
OPG rs2073618					CC VS CG VS GG		CC VS (CG/GG)		(CC/CG) VS GG		CG VS (CG/GG)	
	CC	29	78 (33.3)	67 (47.5)	1.00	0.021	1.89 (1.18- 3.03)	0.0077	0.90 (0.51- 1.58)	0.71	0.57 (0.35-0.91)	0.018
	CG	53	107 (45.7)	46 (32.6)	0.49(0.29- 0.81)							
	GG	18	49 (20.9)	28 (19.9)	0.63(0.34- 1.17)							
MTHFR rs1801133 (C677T)					CC VS CT VS TT		CC VS (CT/TT)		(CC/CT) VS TT		CT VS (CC/TT)	
	CC	46.9	141 (34.6)	59 (38.6)	1.00	0.57	0.81 (0.55- 1.20)	0.29	0.89 (0.53- 1.49)	0.65	0.88 (0.60-1.28)	0.50
	CT	44.2	200 (49.1)	70 (45.8)	0.82(0.54- 1.24)							
	TT	8.8	66 (16.2)	24 (15.7)	0.79(0.45- 1.10)							
MTHFR rs1801131 (A1298C)					AA VS AC VS CC		AA VS (AC/CC)		(AA/AC) VS CC		AC VS (AA/CC)	
	AA	43.4	198 (48.6)	72 (47.1)	1.00	0.14	1.07 (0.74- 1.57)	0.71	1.87 (1.01- 3.47)	0.05	0.86 (0.58-1.26)	0.43
	AC	45.1	176 (43.2)	62 (40.5)	0.95 (0.63- 1.42)							
	CC	11.5	33 (8.1)	19 (12.4)	1.83 (0.96- 3.48)							

Table 6

Prevalence of the OPG rs2073618 and the MTHFR rs1801133 and rs1801131 SNP genotypes in RA anti-CCP negative and anti-CCP positive patients, adjusted Genotypes, OR and p-value for the five genetic models (codominant, dominant, recessive, overdominant and additive) were estimated with SNPstats software ($p < 0.05$). OPG: osteoprotegerin, MTHFR: methylene tetrahydrofolate reductase, OR: odds ratio, SNP: single nucleotide polymorphism, VS: versus, RA: rheumatoid arthritis, cyclic citrullinated peptide, HAPMAP: haplotype map.

SNPs	Genotype	HAPMAP (European Database (%))	RA anti- CCP negative n (%)	RA anti- CCP positive n (%)	OR codominant model [95%CI]	p- value	OR dominant model [95%CI]	p- value	OR recessive model [95%CI]	p- value	OR overdominant model [95%CI]	p- value
OPG rs2073618					CC VS CG VS GG		CC VS (CG/GG)		GG VS (CC/CG)		CG VS (CC/GG)	
	CC	29	36 (36.7)	67 (47.5)	1.00	0.093	0.64 (0.38- 1.09)	0.10	0.54 (0.30-0.97)	0.04	1.05 (0.60-1.82)	0.86
	CG	53	31 (31.6)	46 (32.6)	0.80(0.43- 1.47)							
	GG	18	31 (31.6)	28 (19.9)	0.49(0.25- 0.93)							
MTHFR rs1801133 (C677T)					CC VS CT VS TT		CC VS (CT/TT)		(CC/CT) VS TT		CT VS (CC/TT)	
	CC	46.9	38 (35.5)	59 (38.6)	1.00	0.63	0.87 (0.52- 1.45)	0.59	1.24 (0.61-2.52)	0.55	0.79 (0.48-1.30)	0.35
	CT	44.2	55 (51.4)	70 (45.8)	0.81(0.47- 1.39)							
	TT	8.8	14 (13.1)	24 (15.7)	1.10(0.51- 2.39)							
MTHFR rs1801131 (A1298C)					AA VS AC VS CC		AA VS (AC/CC)		(AA/AC) VS CC		AC VS (AA/CC)	
	AA	43.4	47 (43.9)	72 (47.1)	1.00	0.65	0.88 (0.53- 1.44)	0.60	1.25 (0.57-2.75)	0.58	0.80 (0.48-1.32)	0.38
	AC	45.1	49 (45.8)	62 (40.5)	0.82(0.48- 1.39)							
	CC	11.5	11 (10.3)	19 (12.4)	1.13(0.49- 2.60)							

As for the MTHFR polymorphisms, anti-CCP positive, but not anti-CCP negative RA patients following adjustment for age and gender, depicted increased frequency of the MTHFR 1298 CC genotype compared to HC (OR [95%CI]: 1.87 [1.01-3.47], p-value: 0.05, recessive model, Table 5 and Suppl. Table 4). No differences in MTHFR variants between anti-CCP positive and negative RA groups were detected (Table 6). Moreover, no significant associations between MTHFR C677T variants with disease susceptibility or related features were detected (Tables 1 and 2).

Of interest, RA individuals carrying the TT genotype of the C677T variant were less likely to smoke (9.3 vs 29.7, p-value: 0.005), had higher BMI levels (30.2±6.4 vs 27.8±5.2, p-value: 0.02) and reduced rates of carotid/femoral plaque formation (52.4 vs 72.7, p-value: 0.009, respectively, Table 2). This association remained significant following adjustment for classical CVD risk factors (OR [95%CI]: 0.364 [0.173-0.765], p-value: 0.008). Moreover, the A1298CC genotype was found to be associated with reduced carotid IMT values (0.780 ± 0.270 vs 0.874 ± 0.334 mm, p-value: 0.049). The latter associations were not retained following adjustment for classical CVD contributors (data not shown).

Discussion

In the present study we focused on the possible implication of genotypes of the osteoprotegerin gene and the MTHFR gene on the acceleration of atherosclerosis in RA. We found that seropositive RA patients (either for RF or anti-CCP) display increased prevalence of the CC genotype of the rs2073618 variant of the OPG gene compared to HC. Of interest, this genotype turned to be associated with higher carotid IMT scores and marginally with carotid plaque formation. Moreover, the MTHFR 1298CC genotype was more prevalent only in the anti-CCP positive group compared to HC and negatively associated with indices of subclinical CVD. These associations were not retained following adjustment for traditional CVD predictors. In contrast, a negative association between MTHFR TT and plaque formation was detected, following adjustment for CVD risk factors.

It is well established that the RANKL/RANK/OPG system plays a role in RA disease pathogenesis and that RA patients have significantly higher circulating OPG levels compared to controls in association with disease activity [30–32]. Moreover, increased OPG levels cause artery calcification and atherosclerosis and thus associate with CVD in the general population and in RA patients [10, 12, 33, 34]. However, only specific variants of the OPG gene have been associated with atherosclerosis and CVD in non-rheumatic patients in few studies [35–37]. In RA, only two studies have examined the association of different variants of the OPG gene and atherosclerosis and results are somewhat inconsistent. In accordance with our results, Chung et al found that the CC genotype

of the rs2073618 was associated with coronary artery calcium, whereas another study failed to associate any of the examined variants with the risk of CV events and designated a possible protective effect of the OPG CGA haplotype on CV risk in the anti-CCP negative RA subset [17, 18].

The association between the MTHFR gene variants -mainly C677T but also A1298C- and CVD has been widely investigated in the general population and it is thought that individuals with the MTHFR677TT genotype had a significantly higher risk of CVD [26, 38–40]. This association has also been a field of interest for patients with autoimmune disorders, especially systemic lupus erythematosus, where the MTHFR677TT genotype emerged as an independent predictor for both plaque formation and arterial wall thickening [41]. In the setting of RA, HHcy could partially account for the heightened CVD risk, as there are higher Hcy concentrations in RA patients with a history of CVD compared to controls or RA patients without CVD in association with atherosclerosis in RA [24, 42]. However, since methotrexate treatment and folic acid supplementation could alter Hcy levels, the contribution of the MTHFR gene polymorphisms in HHcy and the development of CVD is yet unclear [43]. In two studies, RA patients with the 677TT genotype had higher plasma homocysteine levels than those with 677CC genotype, and this was also associated with increased cIMT [28, 44]. Moreover, TT genotype and T allele were also linked to susceptibility to develop RA, probably indicating a genetic interaction between RA and CVD [28]. However, another study failed to show an association between the C677T variant and CV events or subclinical atherosclerosis, whereas the MTHFR 1298 allele C frequency was increased in patients with CV events, and patients carrying the MTHFR 1298 AC and CC genotypes had a significantly decreased flow-mediated endothelium-dependent vasodilatation than those with the 1298AA genotype [45]. In contrast, Davis et al. supported there is no association of MTHFR polymorphisms with CV events in RA, and that CV events are associated with traditional risk factors and methotrexate use [29]. Interestingly, a recent study showed that the expression of the MTHFR gene is down-regulated in patients with RA compared to controls, especially those with ischaemic heart disease, which could suggest a potential implication of the transcriptional regulation of MTHFR in the pathogenesis of RA [46].

While we focused on only these two specific genes and their association with CVD in RA, many other single nucleotide polymorphisms (SNPs) –that have been associated with CVD in the general population- have been investigated in recent years for their possible impact on the progression of atherosclerosis in RA. A recent review by López-Mejías et al. summarized all knowledge on the genetic influence in the risk of subclinical atherosclerosis and CV disease in RA through 2016, classifying genes according to their role or “product” [4]. Overall, specific SNPs were associated in some studies with acceleration of atherosclerosis in RA and the authors proposed the use of genetic and serological biomarkers, along with non-invasive clinical surrogates, for estimation of CVD risk, as current CV algorithms seem to underestimate this risk in RA patients.

Since then, few other studies have enriched our knowledge on the contribution of variants of specific genes, apart from MTHFR and OPG, in acceleration of CVD in RA.

Paraoxonase-1 (PON1) is an enzyme involved in the antioxidant and anti-atherosclerotic role of the High-density lipoprotein (HDL) and PON1 gene has been associated with impaired PON1 activity and CVD [47]. Likewise, in RA, the low activity of PON1 is related to a more atherogenic lipid in these patients and the implication of some polymorphisms of the PON1 gene has been described recently with apparently contradictory results [4]. A recent study examining three polymorphisms of the PON1 genes (-108C>T, L55M, and Q192R), associated the haplotype TLQ with reduced PON1 activity and PON1 levels, and thus with a more atherogenic lipid profile in RA [48]. In accordance with previous studies that have linked QQ genotype with reduced PON activity and higher prevalence of CV events, a Spanish study confirmed that the Q192R is involved in reduced enzymatic activity, however that does not apply for the L155M [49–51]. Moreover, Atwa et al. showed that the QQ genotyping was associated with increased cIMT & plaques, while another study linked the RR genotype with reduced CV risk, as previously supported for carotid plaques [52–54].

Apolipoprotein E is a lipoprotein that plays a role in lipid metabolism and CVD, with some variants of the ApoE gene being more accountable [55]. We retrieved three studies examining the link between apolipoprotein E (ApoE) polymorphisms and CVD in RA. In all three, ApoE genotypes distribution was similar between RA patients and HC and all reported an association between the ϵ 4 allele to a worse, more atherogenic lipid profile. However, no ApoE genotype was associated with CVD or the presence of plaques or carotid IMT in previous studies [56, 57], whereas a recent study by Chen et al. claimed that patients with the ϵ 3 ϵ 4 genotype presented with higher CVD risk [58].

Nitric oxide (NO) is an important molecule that has a direct effect on the endothelium of blood vessels causing vasodilation. Moreover, in physiological conditions, NO has an anti-inflammatory effect and mediates many aspects of inflammatory responses. Nitric oxide synthases (NOS) are enzymes catalyzing the production of NO and some NOS genes polymorphisms, by reducing NOS expression and endothelial NO synthesis and availability in the vessel, have been associated with endothelial dysfunction and pathogenesis of atherosclerosis [59, 60]. In addition, some variants of the NOS genes have been associated with RA susceptibility [61–63], however it is not clear whether these polymorphisms have a direct impact on CVD risk in RA [64]. Gonzalez-Gay et al. designated a possible interaction between HLA-DRB1*0404 allele and the NOS3 (-786) TT genotype, that could increase the risk of CV events in RA patients [65]. On the other hand, a recent study by Luo et al showed - in accordance with previous associating the C allele to reduced NO plasma levels and coronary artery disease in the general population- that patients with the CC genotype had lower flow-mediated dilation and that the down-expression of -786T>C is associated with an increased risk of endothelial dysfunction in RA [66]. Finally, Dimitroulas et al, in the same concept of reduced NO plasma levels and consequent impaired vascular homeostasis, investigated the effect of Alanine-glyoxylate aminotransferase 2 (AGTX2) gene polymorphisms on the levels of Asymmetric (ADMA) and symmetric (SDMA) dimethylarginines in patients with RA. The AGTX2 is an alternative pathway of dimethylarginine catabolism, which are inhibitors of nitric oxide (NO) synthase and have emerged as novel, independent biomarkers of CVD in the general population, and are correlated with active inflammation and oxidative stress in RA patients [4, 67]. However, the researchers found no association between serum concentrations of dimethylarginines and genetic variants of the AGTX2 gene [68].

Numerous studies have also focused on the potential impact of genes related to specific cytokines on the progression of CVD in RA [4]. In addition to them, we retrieved some other studies that provide further insight. A recent study associated an IL-19 risk allele, rs17581834(T), with stroke/myocardial infarction in patients with SLE and RA, but not HC, implying a shared immune pathway in the pathogenesis of immune diseases and CVD [69]. Another study examining

the role of IL-32 genes, found that, while the distribution of alleles was similar between RA patients and controls, subjects homozygous for the C allele had higher levels of high density lipoprotein cholesterol (HDLc), suggesting a protective role against CVD. On contrary, the CC-genotype was associated with elevated low density lipoprotein cholesterol (LDLc) and total cholesterol (TC) in individuals with plaques [70]. Farias et al. found no association between two polymorphisms (-607C/A and -137G/C) of the IL-18 gene and RA nor risk factors for CVD; similarly no influence was found of the rs1058587 SNP within GDF15(MIC1) gene on the development of CVD [71, 72]. Although discrepant with a previous report [73], Agca et al. examined the effects of the Interferon regulatory factor 5 (IRF5) gene polymorphisms (rs2004640 and rs4728142) and found that some genotypes were associated with cIMT and cIMT progression, but not CV events in RA patients, implicating a role of the IRF5 transcription pathway in atherosclerosis [74].

Given the association of CRP levels and CVD in the general population, as well as in patients with RA, some studies evaluated the impact of CPR gene polymorphisms on CV risk. Evidence from studies in non-rheumatic patients shows that some SNPs influence the blood CRP levels, and some variants correlate with cIMT or CV events, however results are diverse [75–81]. Regarding RA, Ibrahim et al. examined three SNPs (rs1205, rs1800947 and rs3091244) and found no association between any of the haplotypes investigated and all cause or CVD mortality [82]. Similarly, a recent study by Lopez-Merijas et al. found no association between evaluated genes, that influence CRP levels, and the presence of CV events, carotid plaques or cIMT [83]. In contrast, another study evaluating the genetic variants NLRP3-Q705K and CARD8-C10X related to the NLRP3 inflammasome, showed that the NLRP3-Q705K minor allele was associated with an increased risk of stroke/transient ischemic attack, but not myocardial infarction (MI)/angina pectoris, while CARD8-C10X was not associated with any type of CV event [84].

Regarding other genes, a recent genome-wide association study on almost 3000 RA patients suggested that the minor allele G of the rs116199914 variant in the RARB (Retinoic Acid Receptor Beta) gene is associated with cIMT and possibly contributes to the development of subclinical atherosclerosis in patients with RA [85]. Another large study evaluating 12 SNPs in RA patients, identified novel associations between subclinical atherosclerosis and variants in SLC17A2 (rs17526722), PPCDC (rs1867148), COL4A1 (rs496916) and SLCA13 (rs515291) genes, that may constitute new candidate risk loci for CVD in RA [86]. Evaluation of variants of the platelet endothelial cell adhesion molecule-1 (PECAM-1) gene showed that, while the frequencies of 373C (rs668) and 1688A (rs12953) alleles were higher in RA patients than controls, there was no association between genotype and atherosclerotic complications, as opposed to patients with SLE [87]. Finally, two other studies failed to designate any role of the Asp299Gly Toll-like receptor 4 polymorphism on endothelial dysfunction, or an association of the rs10116277 or rs1537375 SNPs of the 9p21.3 genomic region with cIMT or carotid plaques [88, 89].

Interestingly, few studies, while not directly evaluating CVD in RA, associated polymorphisms of specific genes with increased prevalence of classical CVD risk factors. Regarding dyslipidemia, Davis et al. examined SNPs associated with RA susceptibility with lipid levels in RA and found that the REL SNP rs9309331 homozygous minor allele was associated with higher LDL levels [90]. On the antipode, alleles of SNPs modulating low-density lipoprotein (LDL) cholesterol were associated with disease risk, activity and severity, thus implying the presence of common genetic mechanisms [91]. In respect to blood pressure (BP) levels and the prevalence of hypertension, Panoulas et al. showed that RA patients who were CC homozygotes for the galectin-2 (LGALS2) 3279 C/T single nucleotide polymorphism (SNP) had higher diastolic BP than the TT homozygotes. The same researchers examined the TGFB1 869T/C (rs1982073) gene polymorphism and two endothelin-1 (ET-1) gene locus (EDN1) polymorphisms (rs1800541 and rs5370) in RA. While they found no difference in the distribution of haplotypes or genotypes between patients and controls, the T allele of the TGFB1 869T/C and the rs1800541-rs5370 T-T EDN1 haplotype were associated with increased risk of hypertension [92–94]. Finally, given the effect of MTX therapy on BP levels, a recent study investigated the association between the ATP-binding cassette efflux transporter gene *ABCG2* (rs2231142) SNP and BP and arterial stiffness in RA and found that rs2231142 heterozygotes (AC) had significantly lower age-adjusted clinical systolic BP levels when compared to the CC group, however there was no difference in Augmentation Index (AIx) or Pulse Wave Velocity (PWV) [95].

Our study has some limitations. Firstly, it is not a single-center study and ultrasounds evaluating subclinical atherosclerosis were not performed by one technician. Secondly, we did not measure homocysteine and OPG levels in our patients, however, we believe that the association of the SNPs under study and the levels of Hcy and OPG are well described and accepted. We acknowledge that Hcy levels could have been affected by MTX use and folic acid intake, though there was no direct association between MTX use and cIMT or plaques.

In conclusion, there appear to be some significant associations between specific SNPs and the progression of atherosclerosis and CVD in RA. It remains unclear whether in RA these associations reflect a common genetic pathway implicated in the pathogenesis of both RA and atherosclerosis, or whether these genes, due to their genetic proximity or function, result- though gene-environment interaction, in the acceleration of CVD in RA.

Methods

Study population

In this prospective study, we enrolled 283 consecutive patients with RA (aged 60.8±11.9 years, 16% men), who met the 1987 revised criteria of the American College of Rheumatology [96] and attended the Rheumatology Outpatient Clinics of Laikon General Hospital and General Hospital of Athens “G.Gennimatas” and 595 healthy controls (HC) (aged 67.7±12.4 years, 20% men). Patients and HC were at least 18 years old, did not have history of clinical CVD, malignancy, chronic renal failure, or other concomitant chronic or acute inflammatory disease.

The study was approved by the Institutional Body Review and all subjects provided informed consent according to the Declaration of Helsinki.

Clinical, Laboratory And Subclinical Atherosclerosis Assessment

All 283 RA patients and 280 HC were comprehensively studied for (a) preclinical atheromatosis defined by the presence of carotid and/or femoral artery plaques in the far and near wall of eight arterial sites (left and right common, internal carotid arteries and carotid bulb, and common femoral arteries) [3, 97] and (b) arterial hypertrophy of the common carotid arteries by IMT. Plaques were defined as local increase of the IMT of >50% compared with the surrounding vessel wall, an IMT >1.5 mm, or local thickening >0.5 mm. All ultrasound measurements were performed using high-resolution B-mode ultrasound.

The presence of classical CV risk factors, as well as blood test measurements and other clinical parameters (DAS28) were identified from each patient's file.

Osteoprotegerin And Mthfr Genotyping

Peripheral blood DNA samples from 262 RA patients and 234 HC were genotyped for the detection of the OPG rs2073618 variant. Additionally, 282 RA patients and 407 HC were studied for MTHFR A1298C and C677T gene polymorphisms. Genomic DNA was extracted from blood samples, collected in EDTA tubes, using the Nucleospin Blood QuickPure kit (Macherey-Nagel GmbH & Co, Germany), according to the manufacturer's instructions. DNA concentration was spectrophotometrically measured with Biospec-Nano (Shimadzu, Japan).

The rs2073618 polymorphism of the OPG gene and the A1298C (rs1801131) and C677T (rs1801133) polymorphisms of the MTHFR gene were analyzed by polymerase chain reaction (PCR) of genomic DNA, as previously described [37, 98].

Statistical analysis

Normality of sample distribution was examined by the Kolmogorov Smirnov test. Continuous variables are presented as mean (S.D.) when sample had a normal distribution, or median and 25th and 75th percentile values [interquartile range (IQR)] for non-normally distributed samples. Categorical variables are presented as percentiles and are compared by Fisher's exact tests. For normal or non-parametric distribution, continuous variables were compared by t-test or Mann Whitney test, respectively. Allele and genotype frequencies in RA patients and HC were determined for each SNP by SNPStats software [99]. Genotypic frequencies in control subjects for each SNP were tested for departure from Hardy-Weinberg equilibrium. Genotype frequencies for each SNP of RA anti-CCP positive patients were compared to both anti-CCP negative patients and HC using the χ^2 test. ORs and corresponding 95% CIs were estimated by unconditional logistic regression adjusting for the effects of age and gender. The five genetic models (codominant, dominant, recessive, overdominant and additive) were also determined [99]. To explore independent predictors for high IMT carotid scores, multivariate logistic regression models were implemented, taking into account CVD risk factors (BMI, smoking, hypertension, lipid levels). SPSS version 26 and Stata version 12 (StataCorp, College Station, TX, USA) were used for analyses and $p < 0.05$ was considered as the level of statistical significance in all cases.

Declarations

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Ethics Statement

The study was reviewed and approved by Laikon General Hospital of Athens and "G. Gennimatas" General Hospital of Athens Institutional Body Review. All subjects gave informed consent in accordance with the Declaration of Helsinki.

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Author contributions

CPM and PPS designed the study. All authors contributed to data collection. AA, AN, IP and CPM analyzed the data. AA, AN, and CPM critically interpreted the results and drafted the first version of the manuscript. All coauthors discussed the findings together, critically reviewed the manuscript and approved its final version.

Data sharing statement

All data relevant to the study are included in the article or uploaded as supplementary information.

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