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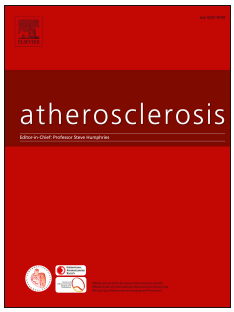

Gene variants and Asymmetric Dimethylarginine in patients with Rheumatoid Arthritis

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Relationship between Dimethylarginine Dimethylaminohydrolase Gene Variants and Asymmetric Dimethylarginine in Patients with Rheumatoid Arthritis

Clinical Research Paper

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Abstract

Objective: The aim of our study was to determine whether Dimethylarginine Dimethylaminohydrolase (DDAH) *1* and *2* gene polymorphisms – the main enzyme involved in ADMA degradation - are associated with high Asymmetric Dimethylarginine (ADMA) levels in Rheumatoid Arthritis (RA).

Methods: Serum ADMA levels were measured in 201 individuals with RA [155 females median age 67 (59 – 73)]. Four tag SNPs in DDAH*1* gene and 2 in the DDAH*2* gene were genotyped by using the LightCyclerTM System. ADMA was initially compared across the genetic variables using one-way ANOVA and then multivariate analysis examined each of the genes after adjustment for parameters of systemic inflammation and insulin resistance, namely erythrocyte sedimentation rate (ESR) and homeostatic model assessment (HOMA), which we have previously shown affect ADMA levels in RA.

Results: No significant relationship between DDAH genetic variables and ADMA levels was established in ANOVA analysis. Multivariate model adjusted for age, HOMA and ESR did not demonstrate any significant association between DDAH variants and ADMA

Conclusion: The results of our study give no evidence to suggest that increased ADMA levels in RA relate to DDAH genetic polymorphisms. Better understanding of disease-related factors and their interactions with traditional CV risk factors may represent mechanisms responsible for ADMA accumulation in this population.

Key words: Rheumatoid arthritis, ADMA, DDAH, CV disease

1. Introduction

In recent years there has been increasing interest in the cardiovascular (CV) complications of chronic systemic inflammatory conditions such as rheumatoid arthritis (RA). It is now well recognised that RA is closely associated with the development of premature atherosclerosis resulting in high CV mortality and morbidity [1,2]. Pathophysiological and epidemiological data suggest a clinically important relationship between RA and accelerated atherosclerosis as both conditions appear to be provoked by initial synovial/endothelial cell injury respectively [3]. However the mechanisms of vascular changes in RA remain partially understood with systemic inflammatory burden as well as classical cardiovascular disease (CVD) risk factors appearing to be pivotal in the initiation and progression of endothelial dysfunction [4,5]. Over the last years the implication of genetic factors in the CVD risk has been studied with several studies reporting associations between gene variants such as tumour necrosis factor promoter polymorphism and atherosclerosis in RA patients [6-8].

Endothelium-derived nitric oxide (NO) is a vasodilatory mediator with atheroprotective and anti-proliferative effects on the vascular wall. Constitutive production of NO is essential for the regulation of blood flow, the maintenance of vasorelaxation and the prevention of oxidative injury to the vascular endothelium [9]. Pro-inflammatory mediators and cytokines which are abundantly produced in RA exert numerous deleterious effects on the endothelial cells including a reduction in NO production, activation of endothelial cells and platelets, and derangement of fibrinolysis, all of which promote thrombosis and atherosclerosis.

Impairment of NO synthesis is multifactorial but a growing body of evidence suggests that circulating inhibitors of NO synthase play a crucial role [10]. Assymetric dimethylarginine (ADMA) is an endogenous guanido-substituted analogue of L-arginine and decreases the bioavailability of NO by competing with L-Arginine at the active site of all the three isoforms

of NO synthase (NOS). ADMA is generated during proteolysis of various proteins containing methylated arginine residues, a procedure catalyzed by a group of enzymes referred to as protein-arginine methyl transferase's (PRMT's) [11]. Over the last decade ADMA has emerged as a novel biochemical marker of endothelial dysfunction and CV risk in various disease settings associated with atherosclerosis such as peripheral and coronal artery disease, lipid disorders, diabetes mellitus, insulin resistance, hypertension, chronic heart and renal failure [12,13] as well as rheumatic diseases [14]. Several studies have demonstrated that plasma ADMA is an independent predictor for adverse cardiovascular events and death in patients with coronary artery disease [15] and in the general population [16]. Elevated ADMA levels have been reported in RA patients irrespective of the disease stage, the initiation and the type of treatment, the presence of clinical CVD or the detection of subclinical atherosclerosis in non-invasive assessments of endothelial function [17-20]. In addition a handful of studies have assessed the impact of biologic disease modifying drugs on ADMA levels [21,22].

Dimethylarginine dimethylaminohydrolase (DDAH) is the key enzyme for the degradation of ADMA into citrulline and dimethylamine [23]. Over 90% of endogenous ADMA is hydrolyzed by DDAH with the remainder renally extracted. DDAH exists in two isoforms (DDAH1, DDAH2) encoded by different genes, with DDAH1 being primarily an enzyme of epithelial cells whereas DDAH2 is present in the vasculature [24]. Recent insights indicate that reduced DDAH activity occurs in several pathological conditions accompanied by excess CV morbidity and it is considered one of the crucial mechanisms responsible for ADMA accumulation and endothelial dysfunction. Both deleting the DDAH-1 gene in mice and inhibiting its activity through DDAH-specific inhibitors resulted in structural and functional endothelial changes, increased systemic vascular resistance and abnormal systemic blood pressure via ADMA mediated dysregulation of NO production [25,26]. On the other hand

overexpression of DDAH in transgenic mice attenuates ADMA production and restores NO synthesis with favourable vascular outcomes such as reduced arterial stiffness, stabilisation of endothelial function and enhanced insulin sensitivity [27]. Functional variant of DDAH-2 gene is associated with chronic kidney disease and insulin sensitivity conditions linked with endothelial dysfunction and increased CVD risk [28,29]. These observations underlie the important role of DDAH in the regulation of vascular homeostasis. Despite experimental data suggesting that DDAH1 is primarily responsible for the degradation of methylarginines [30], the relative role of these isoforms in ADMA metabolism in humans remains unknown. Derangement of DDAH/ADMA pathway has recently been described to participate in the pathogenesis of RA in a collagen-induced arthritis animal model [31].

The aim of the present study was to determine whether DDAH1 and DDAH2 gene polymorphisms are associated with circulating ADMA in individuals with established RA.

2. Methods

2.1 Study population

Two-hundred and one consecutive RA patients were recruited from the rheumatology outpatient clinics of the Dudley Group NHS Foundation Trust, UK, between March 2011-March 2013. All patients met the retrospective application of the 1987 revised RA criteria of the American College of Rheumatology [32]. The study received local Research Ethics Committee approval and all participants gave their written informed consent according to the Declaration of Helsinki.

All participants underwent a thorough assessment including a detailed review of their medical history, hospital records, physical examination, and contemporary assessments of height, weight and body mass index. All medications were recorded, including disease

modifying anti-rheumatic drug (DMARD) use, oral prednisolone and anti-platelet agents. In addition, demographic information was collected by questionnaire. Insulin resistance was evaluated from fasting glucose and insulin using the Homeostasis Model Assessment of Insulin Resistance (HOMA) [33].

Blood was collected from the patient's antecubital vein using a 23G butterfly needle (Greiner Bio One GmbH, Austria). All tests were carried out in the routine and research laboratories of Russells Hall Hospital, Dudley Group NHS Foundation Trust, UK and were analysed for routine laboratory biochemistry, lipids, haematology, Westergren erythrocyte sedimentation rate (ESR), and C-reactive protein. ADMA levels were measured in serum samples by using a commercial enzyme immunoassay ELISA kit (Immundiagnostik, Bensheim, Germany) as previously described [17]. The intra-assay (n=26) standard deviation was 0.031% and the inter-assay (n=6) standard deviation was 0.037%.

2.2 DDAH single nucleotide polymorphism (SNP) Genotyping

2.2.1 DNA Extraction

DNA was extracted from whole blood using the QuickGene-810 system. Blood was collected in EDTA-containing tubes, placed in an isolation vessel and the red cells were lysed. The white blood cells were then captured in a filter matrix and lysed so that the DNA was physically entrapped around the fibers. Isolated DNA was released from the matrix and eluted into a collection vessel in the enclosed environment of the QuickGene 810 system. Resulting DNA samples were stored at 4^{o}C until analysis. Quality control with each batch of extractions was performed by running a blank tube through the whole process, omitting only the addition of blood. If any DNA was found in the blank, the whole DNA batch was rejected.

2.2.2 Polymorphisms

DDAH1/2 Polymorphisms were assessed in all subjects by using the LightCycler™ 480 System (Idaho Technology Inc. Salt Lake City, Utah, USA), as previously described [34]. Rs2474123 (DDAH1), rs669173 (DDAH1), rs13373844 (DDAH1), rs7521189 (DDAH1) and rs3131383 (DDAH2) were identified using LightSNip probes and primers (TIB Molbiol GmbH, Berlin, Germany). Cycle conditions were as follows: denaturation of the template DNA for 1 cycle of 95ºC for 10 minutes, programmed transition rate of 4.4ºC/s; amplification of the target DNA for 45 cycles of 95ºC for 10 sec, 60ºC for 10 sec and 72°C for 15 sec, each with a temperature transition rate of 4.4/2.2/4.4°C/sec; melting curve analysis for 1 cycle of 95°C for 30 sec and 40°C for 2 minutes, each with a transition temperature rate of 4.4/1.5°C/sec, and then ramping to 75°C continuous.

The temperatures for DDAH2 (rs3131383) melting peaks were 63.5^{o}C for the CC and 69.5^{o}C for the AA genotype and heterozygous (CA) there was a peak at 63.5^{o}C and another at 69.5^{o}C.

DDAH1 (rs2474123) melting peaks were GG: -64.5°C, AA: – 69.5^{o}C and GA: -63.5 and 69.5^{o}C. DDAH1 (rs669173) were TT: -58^{o}C, CC: –66^{o}C and CT: 58 and 66^{o}C. DDAH1 (rs13373844) were AA: -62^{o}C, CC: -70 and AC: -62 and 70^{o}C. DDAH1 (rs7521189) were AA: -56 ^{o}C, GG: -63^{o}C and AG: 56 and 63^{o}C.

2.3 Statistical analysis

Initially, ADMA levels were compared across the different classifications of the DDAH genes, namely minor allele, carrier and major allele, using one-way ANOVA models. Multivariable general linear models were then used to account for potential confounding effects of factors which have previously been found to be associated with ADMA. These

factors were included in the model, alongside DDAH status, as continuous covariates, with log-transformations applied to those with skewed distributions. Analysis was repeated after splitting patients in tertiles based on ADMA values. All analysis was performed using IBM SPSS 19, with $p<0.05$ deemed to be indicative of statistical significance.

Post-hoc power calculations were performed for the comparisons of ADMA across the categories of each of the genes considered. As in the main analysis, separate calculations were performed using all of the data, and with the outliers excluded. In each case, pairwise comparisons were made between each combination of categories, based on independent samples t-tests. The alpha value was Bonferroni adjusted for 3 comparisons, hence a value of 1.67% was used. The minimal detectable differences for 80% power for each comparison were quoted (Supplementary material).

3. Results

3.1 Participant Characteristics

The demographic and disease-related characteristics for the RA patients are presented in Table 1. One-hundred and twenty-two (61%) patients were receiving non-biologic DMARDS, fifty-one (25%) oral steroids, sixty (30%) biologic DMARDS, twenty nine (14%) non-steroidal anti-inflammatory drugs, seventy nine (39%) anti-hypertensive treatment and seventy four (37%) cholesterol-lowering agents.

3.2 Outliers

Three cases were identified as having unusually high ADMA measurements (>1.1 µmol/l), which became highly influential outliers in the statistical models. In order to ensure that the analysis was robust, the models were reproduced with these outliers excluded. The difference

between the models was generally small for the significant factors, but both models are quoted for each outcome for completeness.

3.3 Associations between ADMA and DDAH polymorphism

All DDAH-1 and DDAH-2 SNPs tested were in Hardy-Weinberg equilibrium (Chi-square test p values 0.237, 0.587, 0.246, 0.274, 0.824 for rs13373844, rs2474123, rs669173, rs 7521189 and rs 3131383 respectively). All DDAH-1 SNPs studied were in strong linkage disequilibrium with each other as demonstrated by D' values (LD analysis using Haploview) (Figure 1) [35].

One-way ANOVA analysis did not reveal any statistically significant associations between serum ADMA concentrations and DDAH gene variants in our populations. These findings were similar in the analysis which excluded the three outliers (See Table 2). The data was also analyzed with ADMA concentrations divided into approximate tertiles (<0.5, 0.5-0.6 and >0/6 µmol/l). This gave results consistent with the ANOVA analysis, with no evidence of significant associations between gene categories and ADMA. (Table 3)

3.4 Multivariable Analysis

We investigated whether parameters of inflammation and insulin resistance, which have previously been linked with ADMA levels in RA, could have had a deleterious effect on the relationship between ADMA and gene expression. Multivariable general linear models were produced, which accounted for the effects of age, HOMA and ESR, in addition to gene expression, on ADMA. As with the previous analysis, separate models were produced for all patients, and with the outliers excluded (Table 4).

ESR was consistently found to be associated with ADMA (p=0.001). However, after accounting for this relationship, as well as for the other potentially confounding factors in the model, the associations between the genotypes and ADMA remained non-significant.

4. Discussion

In this study we did not establish any significant association between circulating ADMA and SNPs in the DDAH1 and DDAH2 genes in patients with RA. DDAH genetic variants were not found to influence ADMA levels after adjustment for age, disease-related inflammation and insulin resistance parameters which have been previously linked with raised ADMA in this population. This is the first study of this kind to explore genetic variations in DDAH genes and their relationship with ADMA in RA patients.

A handful of studies have assessed the role of genetic variations of the DDAH1 and DDAH2 genes polymorphism and ADMA levels in humans [28,29,36,37]. In diabetes mellitus, significant correlations between DDAH genes polymorphisms and ADMA levels were reported in patients with type 2 [38] but not type 1 [39]. Diabetes mellitus represents the prototypic disease carrying excessive risk for future CV events and similarly to RA, classical CVD risk factors are not sufficient to explain the increased incidence of CVD. In addition, clinical presentation and outcomes of coronary artery disease follow the same pattern in both conditions and currently the magnitude of CV risk in RA is considered equivalent and comparable to that of diabetes [40]. ADMA is elevated in patients with diabetes [41] and is associated with insulin resistance [42] while overexpression of DDAH appears to normalise hyperglycaemic profile in rats by enhancing insulin sensitivity [43]. It is worth noting that insulin resistance has been described as an independent predictor of high ADMA in patients with RA [44] and hypertension [13], providing further support for the link between

impairment of NO/ADMA/DDAH pathway, endothelial injury and abnormal glucose metabolism. However, in our multivariate model, DDAH gene polymorphisms were not found to affect ADMA after correction for HOMA – an indicator of insulin resistance.

Evidence for ADMA accumulation in RA is wide ranging and its role in mediating endothelial dysfunction and accelerated atherosclerosis has been the focus of several publications. ADMA has been associated with morphological and functional parameters of subclinical vascular disease in some [18] but not all the studies assessing vascular morphology [45], coronary microvascular perfusion [46] and *in vivo* endothelium-dependent and -independent microvascular and macrovascular function [17]. Higher mortality rates in RA in comparison with the general population are largely attributable to CVD, predominantly coronary atherosclerosis. Disruption of NO synthesis is crucial in the pathogenesis of endothelial dysfunction, so the recognition of the regulators of NO activity will help to provide a better understanding of the precise mechanisms involved and may also indicate potential therapeutic targets. As a growing amount of evidence suggests that ADMA levels predict future CV events and outcomes in retrospective cohort studies in the general population [16] and patients with established CVD risk factors [47] - which are highly prevalent in RA - endogenous mechanisms that regulate ADMA and NO bioavailability in RA are of great interest.

Several pathways incorporating increased formation and diminished hydrolysis are involved in the high circulating levels of ADMA in RA. The activation of the PRMT's and the augmentation of protein arginine type I N-methyltransferase by increased release of reactive oxygen species in inflamed synovium result in upregulation of ADMA synthesis [48]. However it appears that the most important parameter in ADMA accumulation is DDAH dysfunction [49]. This enzyme seems to be extremely sensitive to oxidative stress and high-levels of NO production following overexpression of inducible NOS, nitrosates DDAH and

inhibits activity [50]. The ensuing elevation in ADMA not only reduces vascular NO bioavailability but potentially evokes endothelial NOS uncoupling switching it to a superoxidate synthase [51]. By this way ADMA acts in a multiplicative manner in promoting endothelial dysfunction. This vicious circle results in the progression of vascular damage and may be of higher importance in chronic, systemic inflammatory conditions such as RA compared to other vascular disorders. It is tempting to speculate that cumulative inflammatory burden in this population may have a more significant impact on impaired ADMA metabolism, overshadowing the potential effect of DDAH gene polymorphism.

In addition ADMA concentration can induce inflammatory vascular reaction by activation of adhesion molecules and cytokine production [52]. In collagen-induced arthritis animal model the culture of fibroblast-like synoviocytes with Tumour Necrosis Factor-alpha resulted in elevation of ADMA levels accompanied with increase in interleukin-1 and -6 and downregulation of DDAH2 expression [31]. The inflammatory effects were reversible with DDAH2 overexpression suggesting that ADMA may have a role as a pro-inflammatory mediator. However, the association between endothelial dysfunction and particularly ADMA with disease-related inflammation in RA remains to be determined and more research is required to answer whether disruption of DDAH/ADMA pathway contributes to both inflammatory process and vascular damage in RA

High circulating ADMA levels predict adverse outcomes specifically vascular events and death in patients with different CVD settings [47,53,54]. Strong causal relationships, however, are yet to be established and it is still unknown whether elevated ADMA levels in these conditions are the cause or the result of endothelial dysfunction. Such associations are much more complex in RA in which interplays between classical CVD risk factors, immune activation, chronic cumulative inflammatory load and cardiotoxicity of anti-rheumatic treatment form a complicated puzzle which remains unresolved [1]. Since ADMA correlates

with traditional and non-traditional CVD risk factors as well as autoantibodies in RA [55], it may represent an attractive marker for CVD risk stratification in this population. We performed a genetic association study to assess the potential contribution of polymorphic variants of DDAH genes to elevated ADMA levels in RA which yielded negative results. Although we cannot exclude the possibilities that untyped SNPs in the current study may have an impact on ADMA levels or that none of the SNPs assessed are of functional importance for ADMA metabolism, it appears that ADMA concentration in RA is dependent on a variety of parameters other than DDAH activity which are involved in endothelial dysfunction in RA.

We performed our study in a well-defined, real-life RA population with detailed clinical and laboratory characterisization. We acknowledge that the major limitation of our study is the relatively small size of our cohort which cannot draw definite conclusions regarding the genetic basis of enhanced ADMA production in RA. However out study was sufficiently powered as according to post hoc power calculations the minimal detectable differences in ADMA levels between groups were consistently less than 0.1 μmol/l. It is worth noting that three patients with unusually high ADMA levels had similar genetic profile with reduced expression for rs2474123 SNP in DDAH1 gene and increased expression for the other polymorphism. The inclusion of these patients in the analysis did not change the results, but this observation may suggest that genetic control of ADMA may occur in patients with more severe disruption of endothelial function.

In conclusion, no evidence was found to suggest that genetic variation in DDAH1 and DDAH2 genes is significantly associated with serum ADMA levels in patients with RA. There is still inadequate understanding of the production of ADMA in RA and its role in promoting atherosclerosis and contributing to CV morbidity and mortality in this population. The complexity of CVD and the risk factor interactions requires further studies to investigate

whether DDAH sequence variation influences DDAH activity and ADMA levels in individuals with RA which will provide better insights in the role of DDAH/ADMA pathway in the dysregulation of NO metabolism and endothelial dysfunction in RA.

Conflict of interest

The authors declare no conflict of interest

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Table 1: Demographic and clinical characteristics of RA patients

	RA Patients (n = 201)
General characteristics	
Age (years)	67 (59 – 73)
Sex: female	155 (77%)
Disease characteristics	
Disease duration (years)	16 (11 – 25)
Rheumatoid factor positive	148 (74%)
Anti-CCP positive	123 (61%)
DAS28	3.1 (2.5 – 4.0)
C-reactive protein (mg/l)	3 (3 – 9)
Erythrocyte sedimentation rate (mmhr)	12 (5 – 23)
Health assessment questionnaire	1.6 ± 0.9
Cardiovascular Disease Risk Factors	
Hypertension	130 (65%)
Dyslipidemia	158 (79%)
Insulin resistance	53 (26%)
Diabetes	21 (10%)
Current Smokers	23 (11%)
ADMA (µmol/l)	0.55 (0.48 – 0.64)

Data reported as median (25^{th} – 75^{th} percentile), number (percentage) or mean and standard deviation as appropriate. ADMA = asymmetric dimethylarginine; CCP = cyclic citrullinated peptides; DAS28 = disease activity score in 28 joints.

Table 2: Comparison of ADMA across genotypes for each of the genes

		Minor Allele	Carrier	Major Allele	p-Value
DDAH1 – RS2474123	All Patients	0.60 (0.03)	0.57 (0.01)	0.55 (0.02)	0.219
	Outliers Excluded	*0.55 (0.01)*	*0.57 (0.01)*	*0.55 (0.02)*	*0.463*
DDAH1 – RS669173	All Patients	0.57 (0.02)	0.56 (0.01)	0.59 (0.02)	0.371
	Outliers Excluded	*0.57 (0.02)*	*0.56 (0.01)*	*0.56 (0.01)*	*0.973*
DDAH1 – RS13373844	All Patients	0.58 (0.03)	0.57 (0.01)	0.57 (0.02)	0.998
	Outliers Excluded	*0.58 (0.03)*	*0.57 (0.01)*	*0.55 (0.01)*	*0.456*
DDAH1 – RS7521189	All Patients	0.56 (0.02)	0.57 (0.01)	0.59 (0.02)	0.497
	Outliers Excluded	*0.56 (0.02)*	*0.57 (0.01)*	*0.56 (0.01)*	*0.865*
DDAH2 – RS3131383	All Patients	0.58 (0.09)	0.57 (0.02)	0.57 (0.01)	0.998
	Outliers Excluded	*0.58 (0.09)*	*0.57 (0.02)*	*0.56 (0.01)*	*0.669*

Data reported as: "Mean (SEM)", with p-values from One-Way ANOVA. DDAH = Dimethylarginine dimethylaminohydrolase

Table 3: Results after splitting of patients in tertiles

	ADMA µmol/l			
	< 0.5	**0.5 - 0.6**	**>0.6**	**p-Value**
DDAH1 - RS2474123				***0.559***
minor allele	10 (25%)	17 (43%)	13 (33%)	
carrier	26 (28%)	33 (36%)	33 (36%)	
major allele	22 (36%)	24 (39%)	15 (25%)	
DDAH1 - RS669173				***0.312***
minor allele	11 (38%)	10 (34%)	8 (28%)	
carrier	34 (34%)	35 (35%)	31 (31%)	
major allele	13 (20%)	29 (45%)	22 (34%)	
DDAH1 - RS13373844				***0.981***
minor allele	4 (33%)	4 (33%)	4 (33%)	
carrier	27 (31%)	32 (37%)	27 (31%)	
major allele	27 (28%)	38 (40%)	30 (32%)	
DDAH1 - RS7521189				***0.600***
minor allele	13 (39%)	12 (36%)	8 (24%)	
carrier	31 (30%)	37 (36%)	34 (33%)	
major allele	14 (24%)	25 (43%)	19 (33%)	
DDAH2 - RS3131383				***0.989***
minor allele	1 (25%)	2 (50%)	1 (25%)	
carrier	14 (28%)	20 (40%)	16 (32%)	
major allele	43 (31%)	52 (37%)	44 (32%)	

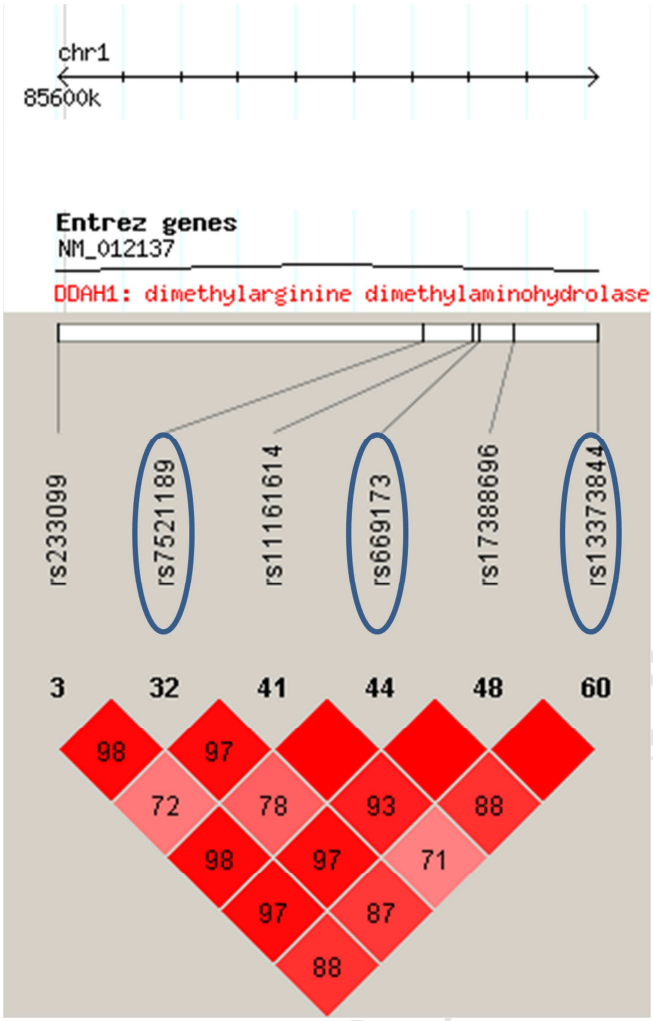
Data reported as: "N (%)", with p-values from Fisher's exact tests

Table 4: Comparison of ADMA across genotypes for each of the genes

	All Patients		Outliers Excluded	
	Coefficient	**p-value**	**Coefficient**	**p-value**
DDAH1 – RS2474123				
Gene Category		0.307		0.641
Minor	*0.043 (-0.013, 0.098)*	*0.130*	*-0.005 (-0.052, 0.042)*	*0.844*
Carrier	*0.012 (-0.034, 0.058)*	*0.610*	*0.014 (-0.024, 0.052)*	*0.470*
Major	-	-	-	-
Age	0.000 (-0.002, 0.002)	0.835	-0.001 (-0.002, 0.001)	0.427
HOMA[#]	0.010 (-0.009, 0.029)	0.310	0.012 (-0.004, 0.028)	0.142
ESR[#]	0.023 (0.009, 0.036)	0.001*	0.020 (0.009, 0.031)	0.001*
DDAH1 – RS669173				
Gene Category		0.346		0.981
Minor	*-0.036 (-0.098, 0.025)*	*0.244*	*-0.004 (-0.056, 0.047)*	*0.872*
Carrier	*-0.029 (-0.073, 0.015)*	*0.198*	*0.000 (-0.037, 0.038)*	*0.979*
Major	-	-	-	-
Age	0.000 (-0.002, 0.002)	0.782	0.000 (-0.002, 0.001)	0.519
HOMA[#]	0.010 (-0.009, 0.030)	0.293	0.011 (-0.005, 0.028)	0.170
ESR[#]	0.024 (0.010, 0.037)	0.001*	0.020 (0.009, 0.032)	0.001*
DDAH1 – RS13373844				
Gene Category		0.921		0.285
Minor	*-0.005 (-0.089, 0.079)*	*0.904*	*0.017 (-0.052, 0.086)*	*0.621*
Carrier	*0.007 (-0.034, 0.049)*	*0.727*	*0.027 (-0.007, 0.062)*	*0.115*
Major	-	-	-	-
Age	0.000 (-0.002, 0.002)	0.733	-0.001 (-0.002, 0.001)	0.471
HOMA[#]	0.011 (-0.009, 0.030)	0.277	0.012 (-0.004, 0.028)	0.145
ESR[#]	0.023 (0.010, 0.037)	0.001*	0.021 (0.009, 0.032)	<0.001*
DDAH1 – RS7521189				
Gene Category		0.407		0.971
Minor	*-0.028 (-0.088, 0.033)*	*0.367*	*0.006 (-0.044, 0.057)*	*0.812*
Carrier	*-0.030 (-0.075, 0.015)*	*0.194*	*0.003 (-0.035, 0.041)*	*0.870*
Major	-	-	-	-
Age	0.000 (-0.002, 0.002)	0.814	-0.001 (-0.002, 0.001)	0.495
HOMA[#]	0.011 (-0.009, 0.030)	0.272	0.012 (-0.005, 0.028)	0.161
ESR[#]	0.023 (0.010, 0.037)	0.001*	0.020 (0.009, 0.032)	<0.001*
DDAH2 – RS3131383				
Gene Category		0.928		0.537
Minor	*0.024 (-0.114, 0.162)*	*0.735*	*0.036 (-0.077, 0.150)*	*0.530*
Carrier	*0.005 (-0.041, 0.051)*	*0.829*	*0.019 (-0.019, 0.057)*	*0.329*
Major	-	-	-	-
Age	0.000 (-0.002, 0.002)	0.764	0.000 (-0.002, 0.001)	0.597
HOMA[#]	0.011 (-0.008, 0.031)	0.262	0.012 (-0.004, 0.028)	0.138
ESR[#]	0.023 (0.010, 0.037)	0.001*	0.020 (0.009, 0.031)	<0.001*

*Results from multivariable general linear models. Data reported as: "Coefficient (95% Confidence Interval)". [#]Factor was log_2-transformed, hence the coefficient represents the increase in ADMA for a two-fold increase in the factor. *Significant at p<0.05*
ESR = erythrocyte sedimentation rate; DDAH = Dimethylarginine dimethylaminohydrolase; HOMA = homeostasis model assessment

Figure 1: Linkage Disequilibrium plot of DDAH-1 genes polymorphisms.



- ADMA is an endogenous inhibitor of NO and an emerging risk factor for endothelial dysfunction
- ADMA levels are increased in RA but the mechanisms responsible for this remain to be determined
- DDAH genes variants investigated in this study do not appear to influence ADMA levels in our population of RA patients.

Supplementary table – Power calculation: minimal detectable differences

		Minor Allele vs. Carrier	Minor vs. Major Allele	Carrier vs. Major Allele
DDAH1 – RS2474123	All Patients	0.09	0.10	0.07
	Outliers Excluded	*0.07*	*0.07*	*0.07*
DDAH1 – RS669173	All Patients	0.09	0.11	0.07
	Outliers Excluded	*0.09*	*0.08*	*0.06*
DDAH1 – RS13373844	All Patients	0.13	0.15	0.07
	Outliers Excluded	*0.13*	*0.10*	*0.06*
DDAH1 – RS7521189	All Patients	0.08	0.11	0.08
	Outliers Excluded	*0.08*	*0.08*	*0.06*
DDAH2 – RS3131383	All Patients	0.22	0.24	0.07
	Outliers Excluded	*0.22*	*0.19*	*0.06*

Quoted values are minimal detectable differences between groups from post-hoc power calculations, based on independent samples t-tests, with 80% power and 1.67% alpha.