Nitric Oxide 92 (2019) 11–17

Contents lists available at ScienceDirect

Nitric Oxide

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

DDAH1 and *DDAH2* polymorphisms associate with asymmetrical dimethylarginine plasma levels in erectile dysfunction patients but not in healthy controls

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ARTICLE INFO

Keywords:
Asymmetrical dimethylarginine
Nitric oxide
Erectile dysfunction
DDAH1
DDAH2
Genetic polymorphisms

ABSTRACT

Erectile Dysfunction (ED) is one of the main complaints of aging male. A reduced production of Nitric Oxide (NO) may be involved in ED pathogenesis. NO is synthesized from L-Arginine, and asymmetrical dimethylarginine inhibits all NO synthases. *DDAH1* and *DDAH2* are genes that encode enzymes responsible for metabolizing ADMA. We aimed to assess whether: 1) ADMA and nitrite levels associated with ED risk and with symptoms intensity; and whether 2) DDAH1 and DDAH2 gene polymorphisms associate with changes in biochemical data, and with ED risk and symptoms intensity. In this study were included 98 healthy controls and 130 ED patients. ADMA levels were measured by ELISA and nitrite levels by Chemiluminescence. *DDAH1* and *DDAH2* polymorphisms were assessed by Taqman assays. We found that ED had increased nitrite levels and lower ADMA levels than Control group ($P < 0.05$). We found a significant correlation of ADMA with Nitrite levels only in ED ($B = -0.57$, $P < 0.001$). Genotypes and haplotypes of *DDAH1* were associated with ADMA levels in ED ($P < 0.05$), while haplotypes of *DDAH2* were associated with levels of nitrite in ED ($P < 0.05$). Erectile dysfunction patients show an association between *DDAH1* and *DDAH2* polymorphisms with ADMA levels, which in turn are negatively correlated with nitrite levels. This is not evident on healthy controls.

1. Introduction

Erectile dysfunction (ED) is a condition characterized by an erection that is insufficient for a successful sexual intercourse [1–3]. This is one of the main urologic complaints of aging male [2,3], and it is considered a marker for other cardiovascular-related complications [4,5]. The physiological mechanism of erection involves the production of Nitric Oxide (NO) [2,6,7]. NO will signal for smooth muscle to relax, allowing vasorelaxation and expansion of corpora cavernosa, which in turn will press venules against tunica albuginea, trapping blood within penis, allowing for full erection [6,7]. Disease state may lead to a reduction of NO production by endothelial dysfunction and may lead to NO scavenging and transformation into peroxynitrite, which is a toxic compound [8]. The progression of disease may result in permanent changes by tissue remodeling, making cavernosal tissue unresponsive to NO [9]. It is imperative to perform an early screening and treatment with both drugs that allow for a regular sexual life, and non-pharmacological approaches that may help to halt disease progression, reducing morbidity [10]. Among several factors that affect sexual life, nitric oxide bioavailability is one of the most important.

Asymmetrical dimethylarginine (ADMA) is a natural compound produced by our body after proteolysis of methylated proteins. This compound is very similar to L-Arginine that is used for NO synthesis and is able to attach to the substrate pocket of NO synthases. Therefore when ADMA interacts with this site, L-Arginine cannot bind to the enzyme, leading to reduced NO production [11,12]. High ADMA levels

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https://doi.org/10.1016/j.niox.2019.08.001
Received 6 May 2019; Received in revised form 24 July 2019; Accepted 3 August 2019
Available online 05 August 2019
1089-8603/

are associated with cardiovascular disease and with ED, as previously described [13,14]. Usually, ADMA levels do not accumulate, as there are enzymes called dimethylarginine dimethylaminohydrolase type 1 and 2 (DDAH1 and 2, respectively) that metabolize ADMA [12,15,16]. These enzymes are encoded by *DDAH1* and *DDAH2* genes, respectively, which have in their sequence important genetic polymorphisms that may alter the protein activity and expression [17–20]. Recently, we have shown that these polymorphisms have an impact on Sildenafil responsiveness in vascular ED [21], which leads us to believe these could also increase risk for developing ED.

Here we aim to assess whether: 1) ADMA levels reflects a NO marker (nitrite) on ED patients and healthy controls; 2) ADMA are associated with risk for ED and the magnitude of erectile function (assessed by the international index for erectile function); 3) genetic polymorphisms of *DDAH1* and *DDAH2* are associated with changes in ADMA levels, nitrite levels and ED risk and erectile function.

2. Materials and methods

2.1. Subjects

This study was approved by our institutional Human Ethics Committee under the number CAAE: 51399015.3.0000.5393 and complied to the declaration of Helsinki. ED group was composed by 130 subjects followed at the Urology Outpatient Clinic at the University Hospital of Ribeirão Preto Medical School. Healthy controls (n = 98) were recruited from the Campus of the University of São Paulo at Ribeirão Preto, among faculty staff, passer-by subjects, or students. All subjects provided their written informed consent. Inclusion criteria for both groups was age between 40 and 80 years, while ED group had additionally medical diagnosis of ED and complaints about sexual activity. Exclusion criteria for both groups were anatomical abnormalities (i.e. Peyronie's disease), penile implants, neurogenic bladder dysfunction, hormonal diseases (hypogonadism, hypothyroidism), cardiovascular events (cardiac infarction, cerebrovascular accidents), central nervous system trauma psychiatric disorders, and diagnosis of prostate hyperplasia or cancer. Additionally, we also excluded subjects from Control group when International Index of Erectile Function (IIEF) score was below 26.

After informed consent was signed, subjects underwent physical and medical examination. Afterwards, blood pressure was measured after a 5-min resting time, followed by application of the IIEF questionnaire [22], and blood collection by antecubital venipuncture. Blood was immediately centrifuged at 1000 g for 5 min to obtain plasma samples, which were stored at −80 °C. Whole blood samples were used for DNA extraction, which was stored at −20 °C.

2.2. Laboratory analysis

Lipid profile, glucose, testosterone, urea and creatinine were measured by commercially available kits and following manufacturer's instructions. Low-density lipoprotein was calculated using Friedewald's formula.

2.3. Asymmetrical dimethylarginine and nitrite quantification

ADMA levels were measured by Competitive Enzyme Linked Immunoassay (ELISA) using a commercially available kit (Elabscience, cat# E-EL-0042, Wuhan, China). Absorbance at 450 nm was measured using the μQuant microplate spectrophotometer (BioTek, Winooski, USA), and 4-parameter fitting was performed using a freely available online tool (www.elisaanalysis.com). Nitrite quantification was performed as previously described [23] using an ozone-based chemiluminescent method. This method uses an acid triiodide solution purged with nitrogen gas. When samples are added to the solution, nitrite is reduced to NO and the nitrogen stream takes it into the equipment reaction chamber (Nitric Oxide analyzer - Sievers 280, Boulder, CO, USA). There, NO reacts with ozone producing light, that is measured by the equipment.

2.4. DDAH1 and DDAH2 genetic analysis

We genotyped two single nucleotide polymorphisms (SNPs) of *DDAH1*, rs1554597 and rs18582, and two of *DDAH2,* rs805304 and rs805305, following procedures previously described [21]. Haplotype estimation was performed using PHASE v2.1 software (http://stephenslab.uchicago.edu/phase/download.html). The possible haplotypes were TG, TA, CG, CA (rs1554597 T > C and rs18582 G > A) and CC, CG, AC, AG (rs805304 C > A and rs805305 C > G). Linkage analysis was performed using Haploview [24] version 4.2 (available at www.broadinstitute.org/haploview/haploview).

2.5. Statistical analysis

Here we performed unadjusted tests, comprising chi-squared tests (categorical variables), Student's T test or ANOVA followed by Tukey's post hoc test (parametric data), or Mann-Whitney and Kruskal-Wallis followed by Dunn's post-hoc test (non-parametric data). Besides this, we also constructed logistic and linear multivariate models accounting for confounding factors. Independent variables were age, smoking status, diabetes and genetic data, while dependent variables were group (multivariate logistic regression), or ADMA or Nitrite levels (multivariate linear regression).

3. Results

Clinical data of subjects included in Control and ED groups are presented in Table 1. There were some differences between groups,

Table 1
Clinical features of studied subjects.

Clinical features	Control (n = 98)	ED (n = 130)
Age (years)	47 ± 9	55 ± 11*
Ethnicity (whites/non-whites)	52/46	68/62
BMI (Kg/m^2)	27.7 ± 4.3	28.1 ± 4.8
Physical Exercise Practitioners (n)	59	72
Smoking status (n)		
Smokers	15	19
Ex-smokers	23	62*
Non-smokers	60	49
Ethanol Consumption (%)	11	10
SBP (mmHg)	130 ± 19	138 ± 19*
DBP (mmHg)	88 ± 13	89 ± 13
Total Cholesterol (mg/dL)	201 ± 42	181 ± 38*
HDL Cholesterol (mg/dL)	36 ± 11	41 ± 9*
LDL Cholesterol (mg/dL)	127 ± 38	112 ± 35*
Triglycerides (mg/dL)	175 ± 106	161 ± 111
Glucose (mg/dL)	100 ± 41	128 ± 55*
Urea (mg/dL)	35 ± 9	33 ± 12
Creatinine (mg/dL)	1.0 ± 0.3	1.1 ± 0.3*
Hypertensive (n)	0	62*
Diabetic (n)	5	52*
EF Score	29 ± 1	10 ± 7*
ADMA (μM)	0.90 ± 0.33 (n = 87)	0.81 ± 0.32 (n = 84)*
Nitrite (μM)	134 ± 73 (n = 92)	192 ± 172 (n = 112)*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; EF Score, score obtained at the erectile function domain from the International Index of Erectile Function.

[a] Ethanol consumption was defined as taking a dose of ethanol higher than 30 g per day.

Data expressed as means ± standard deviation, absolute numbers (n) or relative frequencies (%). *P < 0.05 *versus* Control.

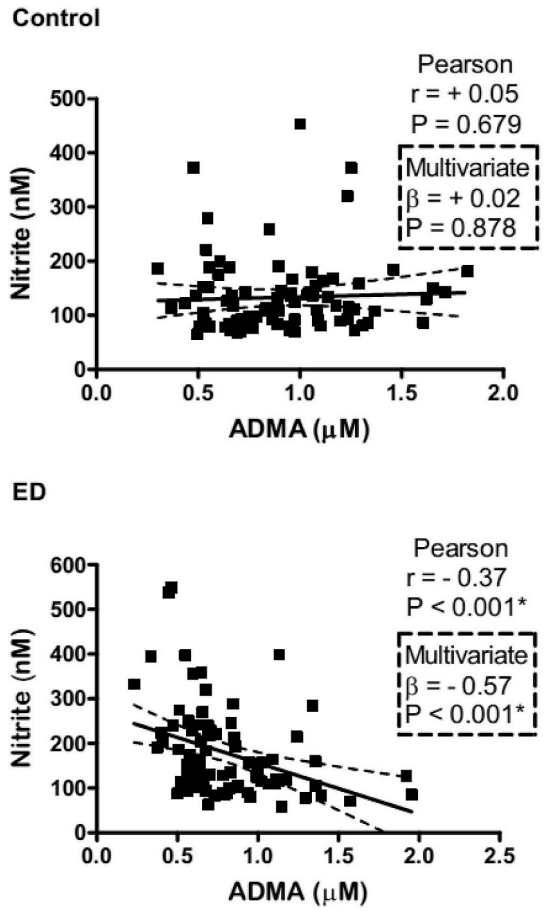

Fig. 1. Correlation between ADMA and nitrite levels on plasma in Controls and ED groups.

Dot plots represent crude data. Data was analyzed by Pearson's correlation or by Multivariate Linear Regression analysis ($R^2 = 0.03$ for Control group and $R^2 = 0.23$ for ED group). Continuous line represents linear regression and dotted liner represent 95% confidence interval.

*: statistically significant.

such as age, smoking status, systolic blood pressure, lipidogram, creatinine, hypertension, diabetes and IIEF score. All these parameters have been considered when building multivariate linear and logistic models. Besides that, we found that ED patients had increased levels of Nitrite and reduced levels of ADMA when compared to Control group (Table 1). Besides that, while in patients there was a negative correlation between nitrite and ADMA levels, in Control group there was no correlation (Fig. 1). These results were confirmed by multivariate analysis.

It was also found a negative correlation between nitrite levels and erectile function assessed by IIEF (Fig. 2) on ED group, which was absent in Control group. This, however, did not resist after multivariate analysis. ADMA levels did not correlate with IIEF score.

Going further, we analyzed whether genotypes for *DDAH1* and *DDAH2* genes would be associated with disease risk, with erectile function and with changes in biochemical data. All genotypes distributions were in Hardy-Weinberg equilibrium (data not shown) and rs805304 and rs805305 were in high linkage disequilibrium (Supplementary Fig. 1). We found that both *DDAH1* polymorphisms were associated with ADMA levels only in ED group (multivariate analysis, additive models; Table 2). Besides, we found both in unadjusted and adjusted analysis that *DDAH1* TG and CA haplotypes were associated with increases and decreases of ADMA levels, respectively. On the other hand, *DDAH2* CC and AG haplotypes associate with reduced and increased Nitrite levels, respectively only on multivariate models (Tables 4 and 5 for adjusted analysis; Fig. 3 and Supplementary Fig. 2 for unadjusted analysis). Although genotypes were associated with changes in biochemical data, there was no association between genotypes and disease risk (Supplementary Table 1), nor with changes in IIEF score (Supplementary Fig. 3).

4. Discussion

Our novel findings reported here: 1) ADMA, nitrite and genetic markers seem to be significantly associated, especially when disease is present; 2) ADMA plasma levels are not associated with IIEF score; 3) Nitrite and EF correlate negatively in ED. In addition, previously published associations were reproduced here: 1) ADMA and Nitrite correlate negatively in ED; 2) DDAH1 polymorphisms were associated with changes in ADMA levels in ED; 3) DDAH2 polymorphisms were related to changes in nitrite levels in ED.

NO is the main vasorelaxation agent and has pivotal importance in erectile function [1]. Its main metabolites are nitrite and nitrate, however, nitrite serves as a marker for recent NOS activity, since its plasma half-life is very short (around 5 min) [8]. Therefore, nitrite levels could help to predict the health of endothelial cells [8]. Here we found a result that may seem paradoxical at a first glance: increased levels of nitrite in ED patients. However, our control group has levels of nitrite that are within average levels of the literature [8]. We believe that this increase in nitrite levels may involve an activation of inducible nitric oxide synthase (iNOS) due to inflammatory processes, that actually have been shown in the literature [8]. This is consistent with previous results showing that Sildenafil responsiveness was better in patients with reduced nitrite levels [21,25,26].

ADMA is an endogenous inhibitor of all NO synthases [11,12,16] and has been suggested to directly disturb endothelial function [14]. ADMA could inhibit NO synthases when present on cytoplasm [16], and could also reduce L-Arginine inflow to endothelial cells by interacting with specific amino acid transporters, leading to a compartmental substrate exhaustion [15], and reduced NO due to low L-Arginine inside endothelial cell. All these processes are difficult to assess clinically, since we only have easy access to plasma. Here we found reduced levels of ADMA on disease. This is paradoxical, since high ADMA levels have been implicated in different cardiovascular diseases [13]. Furthermore, increased inflammation is known to reduce *DDAH1* expression [27] and higher ADMA levels were observed in ED [14]. On the other hand, reduced ADMA in ED will implicate in increased NO production, for instance by iNOS enzyme and thus more tissue damage elicited by peroxynitrite under oxidative stress conditions. Interestingly, this is consistent with reports showing that metabolic syndrome and diabetes patients have higher plasma levels of nitrite or nitrite + nitrate (NOx) [28–30]. Future studies comparing levels of nitrite, methylated forms of Arginine and inflammatory markers in ED with and without concurrent diabetes and metabolic syndrome are warranted to clarify this.

Although associated with ED group, both nitrite and ADMA levels did not correlate with the intensity of ED symptoms given by IIEF Score (Fig. 2). This is interesting and may explain the lack of association of the genetic markers with ED risk (Supplementary Table 1).

The main enzyme involved in ADMA clearance from plasma is *DDAH1* [31,32]. We clearly found that genotypes and haplotypes in this gene are associated with changes in ADMA plasma levels (Tables 2 and 4, Fig. 3). The association we found of rs18582 with plasma levels is consistent with previous results, where GG carriers had higher while A allele carriers had lower ADMA levels [21]. This is also consistent with a GWAS data with a very robust sample size [18]. The association of *DDAH1* haplotypes with ADMA levels (Fig. 3) was not found previously [21]. Possibly, a higher number of participants here allowed us to observe this association. Although associated with biochemical variability, these genetic markers did not increased risk to develop ED (Supplementary Table 1), nor associated with symptoms intensity (Supplementary Fig. 3).

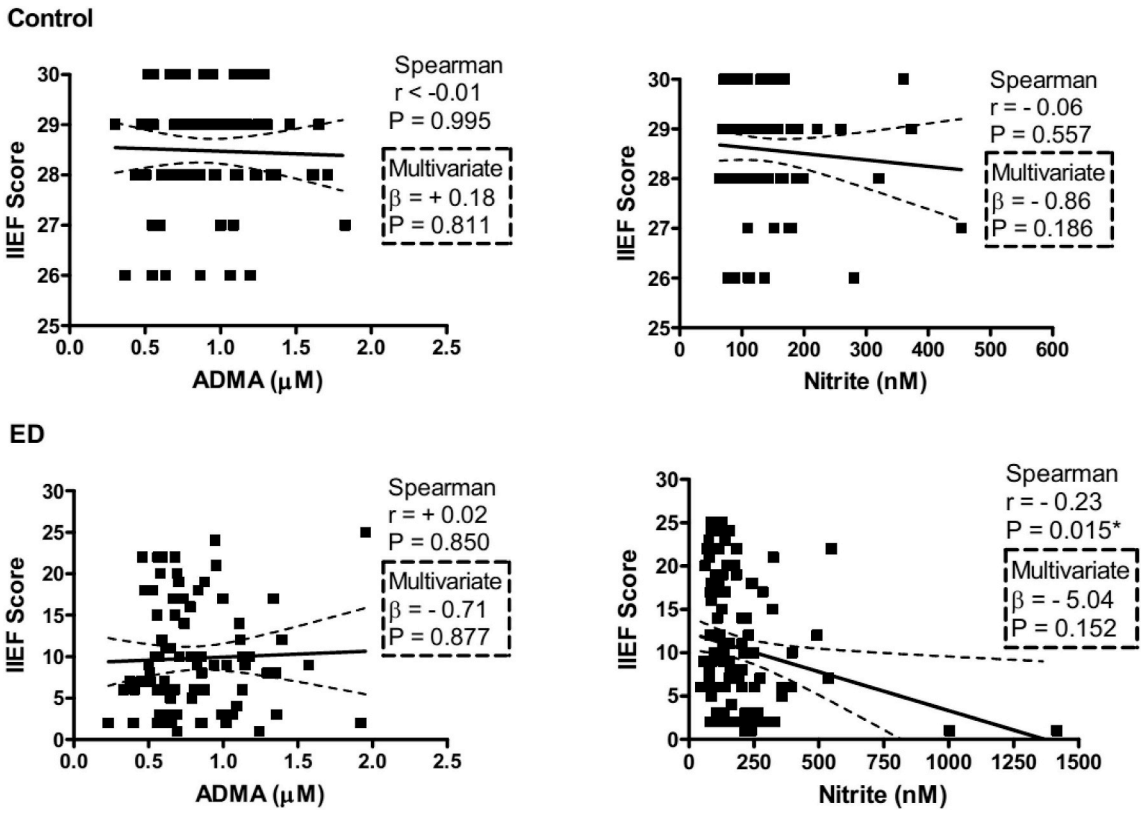

Fig. 2. Correlation between erectile function (IIEF score) and ADMA or nitrite levels on plasma in Controls and ED groups.
Dot plots represent crude data. Crude data was analyzed by Spearman's correlation or log-transformed data was analyzed by a multivariate linear regression model (R^2 = 0.12 for Control group; R^2 = 0.16 for ED group). Continuous line represents linear regression and dotted liner represent 95% confidence interval.
*: statistically significant.

DDAH2, on the other hand, is mainly expressed in other tissues, including endothelial cells [31,32]. We found here only in controls an association of *DDAH2* genotypes of rs18582 with nitrite levels (Table 3), while in patients we found that *DDAH2* haplotypes associated with nitrite levels (Table 5). Interestingly, the CC haplotype associated with lower nitrite combines alleles of two polymorphisms that were previously associated with increased ADMA [19,20]. These subtle effects of genetic markers are not always observed, as *DDAH1* haplotypes

Table 2
Multivariate linear regression analysis showing the association of studied polymorphisms with changes in ADMA levels on Patients and Controls in additive, dominant and recessive models.

		Control (n = 87)						ED (n = 84)					
Model		Additive R^2 = 0.12 RMSE = 0.16		Dominant R^2 = 0.10 RMSE = 0.16		Recessive R^2 = 0.11 RMSE = 0.16		Additive R^2 = 0.21 RMSE = 0.16		Dominant R^2 = 0.04 RMSE = 0.17		Recessive R^2 = 0.09 RMSE = 0.16	
		β	P	β	P	β	P	β	P	β	P	β	P
Age (years)		-0.01	0.080	-0.01	0.119	-0.01	0.081	-0.01	0.538	-0.01	0.754	-0.01	0.518
Smoking Status													
Smokers		-0.07	0.032	-0.07	0.030*	-0.07	0.034*	-0.03	0.401	-0.02	0.568	-0.03	0.338
Ex-smokers		+0.06	0.072	+0.05	0.086	+0.05	0.081	-0.01	0.657	-0.01	0.608	-0.01	0.969
Non-smokers		+0.02	0.378	+0.02	0.326	+0.02	0.386	+0.04	0.153	+0.03	0.243	+0.03	0.237
Diabetes (yes)		+0.03	0.404	+0.03	0.371	+0.03	0.410	+0.01	0.475	+0.01	0.752	+0.01	0.527
DDAH1													
rs1554597	TT	-0.01	0.750	+0.01	0.613	+0.01	0.583	**+0.13**	**0.008***	-0.01	0.664	+0.05	0.082
	TC	+0.02	0.613	-0.01	0.613			**+0.09**	**0.016***	+0.01	0.664		
	CC	-0.01	0.943			-0.01	0.583	**-0.22**	**< 0.001***			-0.05	0.082
		Global P = 0.850						**Global P = 0.002***					
rs18582	GG	-0.04	0.438	-0.02	0.401	-0.02	0.218	**+0.22**	**< 0.001***	+0.03	0.326	**+0.04**	**0.032***
	GA	-0.01	0.690	+0.02	0.401			**-0.06**	**0.097**	-0.03	0.326		
	AA	+0.05	0.214			+0.02	0.218	**-0.16**	**0.001***			**-0.04**	**0.032***
		Global P = 0.446						**Global P = 0.002***					
DDAH2													
rs805304	CC	-0.01	0.670	-0.01	0.817	-0.01	0.693	-0.01	0.958	+0.01	0.897	+0.01	0.709
	CA	-0.01	0.948	+0.01	0.817			+0.01	0.804	-0.01	0.897		
	AA	+0.01	0.650			+0.01	0.693	-0.01	0.882			-0.01	0.709
		Global P = 0.891						Global P = 0.968					
rs805305		-		-		-	-	-		-	-	-	-

R^2, proportion of the variability explained by our model; RMSE, root mean square error. ADMA levels were log-transformed for this statistical analysis. Since in our sample rs805304 and rs805305 were in strong linkage disequilibrium (see Supplementary Fig. 3 in Supplementary Materials file), we could not consider both as independent variables. Therefore we inserted in our model only rs805304, and we considered these genotypes marking for the presence of rs805305 genotypes: CC/CC, CA/CG and AA/GG. Gray shades indicate the genotypes that were combined in dominant and recessive models. *P < 0.05 statistically significant.

Table 3
Multivariate linear regression analysis showing the association of studied polymorphisms with changes in Nitrite levels on Patients and Controls in additive, dominant and recessive models.

		Control (n = 92)						ED (n = 112)					
Model		Additive R^2 = 0.13 RMSE = 0.18		Dominant R^2 = 0.12 RMSE = 0.18		Recessive R^2 = 0.06 RMSE = 0.19		Additive R^2 = 0.09 RMSE = 0.25		Dominant R^2 = 0.08 RMSE = 0.25		Recessive R^2 = 0.08 RMSE = 0.25	
		β	P	β	P	β	P	B	P	β	P	β	P
Age (years)		-0.00	0.426	-0.00	0.384	-0.00	0.566	+0.00	0.382	+0.00	0.298	+0.00	0.437
Smoking Status													
Smokers		-0.02	0.686	-0.02	0.530	-0.03	0.469	+0.04	0.410	+0.04	0.406	+0.03	0.516
Ex-smokers		-0.01	0.765	-0.01	0.886	-0.00	1.000	+0.04	0.326	+0.03	0.370	+0.04	0.270
Non-smokers		+0.03	0.355	+0.03	0.301	+0.03	0.321	-0.07	0.051	-0.07	0.061	-0.07	0.066
Diabetes (yes)		-0.05	0.285	-0.05	0.295	-0.05	0.301	+0.01	0.816	+0.01	0.800	+0.00	0.884
DDAH1													
rs1554597	TT	+0.04	0.390	+0.00	0.902	-0.03	0.410	-0.01	0.879	+0.02	0.415	-0.03	0.450
	TC	+0.02	0.532	-0.00	0.902			-0.05	0.338	-0.02	0.415		
	CC	-0.06	0.272			+0.03	0.410	+0.06	0.449			+0.03	0.450
		Global P = 0.544						Global P = 0.593					
rs18582	GG	**+0.15**	**0.009***	**+0.08**	**0.009***	+0.02	0.479	-0.04	0.561	+0.01	0.872	-0.03	0.297
	GA	-0.07	0.080	**-0.08**	**0.009***			+0.01	0.769	-0.01	0.872		
	AA	-0.09	0.061			-0.02	0.479	+0.03	0.651			+0.03	0.297
		Global P = 0.031*						Global P = 0.844					
DDAH2													
rs805304	CC	-0.00	0.933	+0.00	0.915	-0.01	0.546	-0.06	0.151	-0.04	0.169	-0.04	0.165
	CA	-0.01	0.750	-0.00	0.915			+0.00	0.905	+0.04	0.169		
	AA	+0.01	0.717			+0.01	0.546	+0.05	0.166			+0.04	0.165
		Global P = 0.920						Global P = 0.294					
rs805305		-		-		-		-		-		-	

R^2, proportion of the variability explained by our model; RMSE, root mean square error. Nitrite levels were log-transformed for this statistical analysis. Since in our sample rs805304 and rs805305 were in strong linkage disequilibrium (see Supplementary Fig. 3 in Supplementary Materials file), we could not consider both as independent variables. Therefore we inserted in our model only rs805304, and we considered these genotypes marking for the presence of rs805305 genotypes: CC/CC, CA/CG and AA/GG. Gray shades indicate the genotypes that were combined in dominant and recessive models. *P < 0.05 statistically significant.

Table 4
Multivariate linear regression analysis showing the association of *DDAH1* and *DDAH2* haplotypes with changes in ADMA levels on Patients and Controls.

Model	Control (n = 174)		ED (n = 168)	
	R^2 = 0.11	RMSE = 0.15	R^2 = 0.09	RMSE = 0.16
	β	P	β	P
Age (years)	−0.01	0.012	−0.01	0.638
Smoking Status				
Smokers	−0.07	0.001	−0.02	0.236
Ex-smokers	+0.05	0.009	−0.01	0.575
Non-smokers	+0.02	0.017	+0.04	0.056
Diabetes (yes)	+0.04	0.158	+0.01	0.487
DDAH1				
TG	−0.01	0.768	**+0.12**	**0.016***
TA	+0.01	0.866	+0.01	0.594
CG	−0.04	0.089	+0.05	0.082
CA	+0.05	0.230	**−0.18**	**0.002***
	Global P = 0.240		**Global P = 0.008***	
DDAH2				
CC	−0.01	0.442	+0.01	0.401
CG	-	-	-	-
AC	-	-	-	-
AG	+0.01	0.442	−0.01	0.401

R^2, proportion of the variability explained by our model; RMSE, root mean square error.
ADMA levels were log-transformed for this statistical analysis. Haplotypes CG and AC of *DDAH2* gene were excluded from statistical analysis since its frequencies were below 2%.
*P < 0.05 statistically significant.

Table 5
Multivariate linear regression analysis showing the association of *DDAH1* and *DDAH2* haplotypes with changes in nitrite levels on Patients and Controls.

Model	Control (n = 182)		ED (n = 224)	
	R^2 = 0.06	RMSE = 0.18	R^2 = 0.08	RMSE = 0.25
	B	P	B	P
Age (years)	−0.00	0.515	+0.00	0.181
Smoking Status				
Smokers	−0.02	0.482	+0.03	0.385
Ex-smokers	−0.01	0.751	+0.04	0.116
Non-smokers	+0.03	0.171	−0.06	0.011*
Diabetes (yes)	−0.06	0.093	+0.01	0.745
DDAH1				
TG	+0.06	0.272	−0.05	0.438
TA	−0.01	0.833	+0.06	0.120
CG	+0.04	0.189	+0.02	0.556
CA	−0.09	0.071	−0.03	0.705
	Global P = 0.179		Global P = 0.454	
DDAH2				
CC	−0.00	0.858	**−0.03**	**0.045***
CG	-	-	-	-
AC	-	-	-	-
AG	+0.00	0.858	**+0.03**	**0.045***

R^2, proportion of the variability explained by our model; RMSE, root mean square error.
Nitrite levels were log-transformed for this statistical analysis. Haplotypes CG and AC of *DDAH2* gene were excluded from statistical analysis since its frequencies were below 2%.
*P < 0.05 statistically significant.

associated with changes in ADMA on plasma did not reflect on changes of nitrite in plasma in our work. It is possible that ADMA within endothelial cells compartment may be more important in determining NO production than circulating ADMA. More studies are needed to address this.

This study has some strengths, such as the well characterized phenotype of patients. Here we excluded any psychological, endocrine, neurological and anatomical causes for ED. Thus we enriched our Patients sample with vasculogenic ED. We cannot affirm this is complete, since we did not exclude diabetic patients. These have both vasculogenic and neurogenic alterations that occur concurrently. Besides, different articles associated diabetes and metabolic syndrome

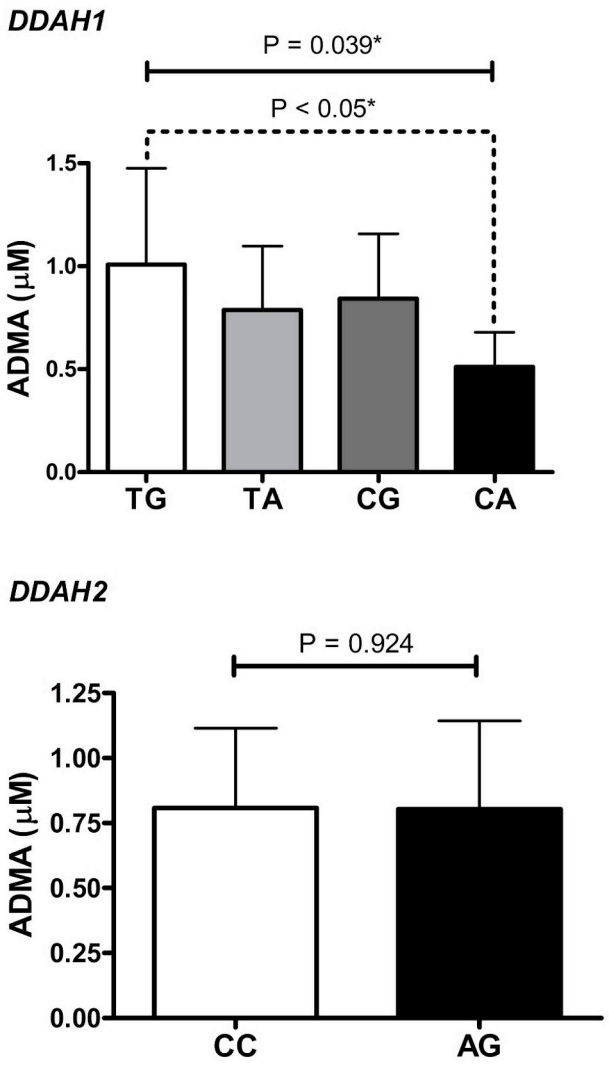

Fig. 3. Association analysis of *DDAH1* and *DDAH2* haplotypes with ADMA levels on ED.
ADMA levels expressed as means ± standard deviation.
Haplotypes CG and AC of *DDAH2* gene were excluded from statistical analysis since its frequencies were below 2%*P < 0.05 statistically significant.

with higher levels of nitrite or NOx [28–30]. This may be a limitation of our study, as the increased levels of nitrite we show in ED patients may have been driven by a higher proportion of diabetes patients in that group than in Control group. Besides that, we analyzed a small (although not underpowered) sample size, and therefore we may have missed very subtle associations or risk increases.

We conclude here that *DDAH1* and *DDAH2* genotypes are associated with changes in ADMA or Nitrite, and that changes in these markers appear only in the pathological condition of erectile dysfunction.

Declaration of interest

The authors report no conflict of interest.

Acknowledgements

This work was supported by the FAPESP (Grant # 2016/04449-0), CNPq (Grant # 302241/2017-5); CAPES (student's scholarships).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.08.001.

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