Influence of endothelial nitric oxide synthase polymorphisms in Psoriasis risk

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Abstract:

Nitric oxide (NO) is a potent regulator of keratinocyte growth and differentiation that has been implicated in the pathogenesis of psoriasis (Ps). The *NOS3* -786 T/C (SNP id rs2070744; www.ensembl.org), intron 4 variable number tandem repeat (VNTR), and Glu298Asp (SNP id rs1799983) polymorphisms, have been associated with differences in NO plasma concentrations and with the risk of hypertension (HT) and ischemic cardiac disease (IC). The aim of this study was to determine whether the above mentioned *NOS3* variants contributed to the risk of Ps, and were associated with the risk for HT and CAD in these patients. A total of 368 patients with chronic plaque Ps and 400 healthy controls were genotyped for the *NOS3* -786 T/C, intron 4 VNTR, and Glu298Asp polymorphisms. Carriers of the -786 C allele were significantly more frequent among the patients ($p<0.001$). Carriers of the 4-repeats allele (45+44 genotypes) were also more frequent a ($p<0.001$). No significant difference was found for the Glu298Asp polymorphism. None of the *NOS3* variants was associated with Ht and CAD in our population. In conclusion, *NOS3* gene polymorphism would be risk factors for developing Ps.

Introduction

Psoriasis (Ps) (Mendelian Inheritance in Man; MIM*177900) is a chronic inflammatory skin disease of unknown aetiology that affects approximately 2% of the general population [5]. Both, genetic/inherited and environmental/acquired risk factors contribute to the risk of developing Ps.

Hypotheses of Ps pathogenesis have changed over the years [21]. Nitric oxide (NO) is synthesised from L-arginine by three NO-synthases (NOS): NOS1 or neuronal, NOS2 or inducible, and NOS3 or endothelial [16]. NO has been involved in the maintenance of skin homeostasis, including the mediation of inflammation, regulation of vasodilatation, melanogenesis, wound healing, regulation of apoptosis following UV radiation, and is a potent regulator of keratinocyte growth and differentiation. [4]. The positive effect of the NOS on NO production is counteracted in part by arginase, an enzyme that competes with the NOS for arginine [3]. The three NOS are expressed by skin cell types [4,10], and keratinocytes express NOS2 in Ps and other inflammatory skin conditions [4,11,17,22,25].

Several studies have reported differences in NO-synthases expression and NO levels of tissue samples from Ps patients [3, 11, 22, 27, 30]. A few studies also determined the levels of nitrite and nitrate (two NO metabolites) in blood samples of Ps patients [9, 24, 28]. Nitrite/nitrate levels were significantly increased in plasma of Ps patients compared to healthy control and showed a positive correlation with PASI score [9], and a significant reduction of the metabolite levels after treatment with methotrexate has also been reported [28].

There is accumulating evidence that cardiovascular risk factors (CVRF) such as hypertension, diabetes, and hypercholesterolemia, and ischemic cardiac disease (IC) are common among Ps patients [7,8,13, 19, 20]. The *NOS3 -786 T/C, intron 4 variable*

number tandem repeat (VNTR), and Glu298Asp polymorphisms have been associated with differences in NO plasma concentrations [14, 29] and with the risk of cardiovascular diseases [12]. An association between the Glu298Asp polymorphism and Ps was also reported [26]. In this work, we analysed the association between three *NOS3* variants and Ps, and the effect of these polymorphisms on CVRF and IC among psoriatic patients.

Materials and Methods

The study included a total of 368 patients with chronic plaque Ps and 400 healthy controls. All the patients and controls were unrelated Caucasians from the region of Asturias (Northern Spain, total population 1 million). Written informed consent was obtained from each participant, under the protocols approved by the Ethical Committee of Hospital Universitario Central de Asturias.

Patients were assessed by a dermatologist and the diagnosis of Ps was based on clinical findings. The *Psoriasis Area and Severity Index* (PASI) was calculated for each patient, and the disease was considered as severe if the patients had a PASI score ≥ 10 at any time of the disease. Patients were considered to have “*early onset*” Ps if the onset was <40 years. Patients were considered to have “*familial*” Ps if they had at least one first/second-degree relative also affected by the disease. Patients with psoriatic arthritis (PsA) were assessed by a rheumatologist according to the criteria of Moll and Wright [15] or according to the more recent CASPAR criteria. Patients were asked if they had other comorbid conditions and their medical records were revised to obtain information about hypertension (HT), dyslipemia (DL), diabetes mellitus type 2 (DM), and ischemic cardiopathy (IC).

Controls were from the same Caucasian population and were recruited through the blood bank. They were matched to patients for age and gender, and none was using lipid-lowering, oral antidiabetics, insulin, or antihypertensive drugs. None of them had been diagnosed with CVD, autoimmune, or neurodegenerative processes.

The *NOS3* Glu298Asp (SNP rs1799983; c.893 T>G, Ensembl transcript ENST00000297494; www.ensembl.org) was genotyped with a custom Taqman assay (assay id [C_3219460_20](https://www.appliedbiosystems.com); www.appliedbiosystems.com) in a 7500 real time PCR system (Applied Biosystems, Ca, USA). The -786 T/C (SNP rs2070744) and intron 4 VNRT polymorphisms were genotyped in patients and controls as previously reported [2]. To determine the accuracy of the genotyping methods we amplified and sequenced 5 individuals with each of the three genotypes.

A $p < 0.05$ was considered as significant for all the statistical comparisons. Allele and genotype frequencies were compared between patients and controls. We also compared the allele and genotype frequencies for the three *NOS3* polymorphisms in the patients according to the family history of Ps, disease severity, age of onset, presence of arthritis, smoking status, HT, DM, DL and IC. Data management and statistical analysis were carried out using the R-project for statistical computing (<http://www.r-project.org/SPSS>). The differences of the allele frequencies between patients and controls or between patients according to the clinical form of Ps or the smoking status were assessed using the χ^2 test. Fisher exact test was preferred when an expected cell was less than 5. Odds ratios (OR) and their 95% confidence intervals (CI) and significant values were also calculated. Logistic regression was used to determine the association between the *NOS3* variants and Ps after adjusting by age and gender. The

statistical power was calculated online (<http://statpages.org/proppowr.html>). To detect a difference of 10% in the three polymorphisms studied, with $\alpha=0.05$ and $1-\beta=0.80$, at least 344 patients and 378 controls should be necessary.

The frequencies for the haplotypes defined by the three *NOS3* polymorphisms were calculated online (Cubex programm, www.oege.org/software/cubex). This software estimates the haplotype frequencies, the normalised linkage disequilibrium (LD) parameter (D'), and the LD correlation coefficient between two loci (r^2).

Results

Baseline characteristics of psoriatic patients and controls are summarized in **Table 1**. The genotype frequencies did not deviate from those of the Hardy-Weinberg equilibrium in both, patients and controls, suggesting that the observed frequencies were representative of the general population. Carriers of the -786 C allele were significantly more frequent among the patients (72% vs 59%; $p<0.001$; OR:1.70, 95% CI=1.24-2.33), suggesting a dominant effect for this allele on Ps-risk (**Table 2**). Compared to TT, the CC genotype showed a higher OR than the TC genotype (OR=2.08 vs. 1.12). This suggested a risk-dosing for the C allele. The -786C allele was more frequent in the late-onset form of Ps than in the early onset group (79 vs 69; $p=0.047$).

The 4-repeats allele (45+44 genotypes) were also more frequent among the patients (32% vs 21%; $p<0.001$), and there was also a dose effect of this allele: compared to 55-homozygotes, the 44 genotype showed a higher OR value than the 45 genotype (4.74 vs. 1.63) (**Table 2**). No significant association between the Glu298Asp polymorphism and the risk for Ps was found, and that SNP did not influence the age of onset, family

history, and severity of Ps. We found a trend toward a higher frequency of GT+TT in patients with PsA (70% vs 57%; $p=0.04$) .

There was a significant linkage disequilibrium (LD) between the -786 T/C and intron 4 VNTR loci in patients ($D' = 0.61$) and controls ($D' = 0.72$), with the -786 C/4-repeats and the -786 T/5-repeats having frequencies higher than expected under an independent segregation. In agreement with this LD and the putative risk effect for the -786 T and 4-repeat alleles, we found a higher frequency of carriers of -786 C + 4-repeats among the patients (25% vs. 19%). The LD values for the -786 T/C and Glu298Asp loci were 0.37 in patients and 0.42 in controls; and for the VNTR and Glu298Asp, -0.87 in patients and -0.89 in controls. This suggested that the three loci defined two major haplotype blocks (-786 T/C + VNTR, and VNTR + Glu298Asp), with a significant association between Ps and the -786 / VNTR haplotypes.

The most frequent CVRF in our patients was cigarette smoking (38%), followed by HT (20%), dyslipemia (16%), and DM2 (9%). Six per cent of the patients had had IC episodes. We did not find significant association between the *NOS3* allele/genotypes and the CVRF or the risk for IC (data not shown).

Discussion

The potential role of the NO in the pathogenesis of Ps remains unclear. On the one hand, an increased expression of NOS2 has been found in psoriatic plaques and this could lead to high local concentrations of NO [11]. On the other hand, over expression of Arginase 1 and the calcitonin gene-related peptide in the psoriatic skin could act in a compensatory manner, leading to a significant consumption of arginine and a decrease in NO production in the psoriatic plaque [1, 4]. Some authors have reported improvement of the Ps plaque with the topical application of NO-donors [1] while others found the opposite effect [23]. Significantly increased nitrite/nitrate levels were found in plasma of patients with Ps and, furthermore, these levels showed a positive correlation with PASI score [9].

The *NOS3* -786 C and intron 4 VNTR alleles were significantly associated with Ps in our population. The Glu298Asp polymorphism had been associated with Ps-risk in at least one study [26]. However, we failed to confirm this association in our population.

The increased frequency of the -786 C and the 4-repeats allele among our patients could be a consequence of a higher frequency of the -786C/4-repeats haplotype. Several studies supported a direct role of the two polymorphisms on NOS3 protein expression. The -786 C allele was linked to significant reduction in NOS3 promoter activity and reduced mRNA and protein expression compared to -786 T [6,18]. The intron 4 repeat polymorphism is a source of a 27 nt small RNA that inhibits NOS3 expression through a mechanism that involved histone acetylation and DNA methylation in the 27 bp repeat region and the promoter. Cells containing five 27-bp repeats produced higher levels of 27 nt RNA and lower levels of eNOS mRNA than the cells with four repeats [31,32]. The C-4 repeats haplotype would thus contain signals for reduced (-786 C) and increased (4-repeats) NOS3 expression. However, this haplotype was associated

with lower plasma nitrite concentrations compared to the other haplotypes [14]. This suggested that the -786 T/C was more relevant for NO production than the VTR, and the association with the intron 4 polymorphism could be a consequence of its linkage disequilibrium with the promoter polymorphism.

Finally, we are well aware that our study has several limitations. Although our sample size was enough to reach a power of 95 for the -786 and intron 4 variants, the study was conducted in a single population and with a limited number of patients and controls. Our results would thus require further replication by others. If this association is finally confirmed, the NO/NOS3 pathway could become a putative pharmacological target for Ps.

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Table 1. Baseline characteristics of Ps patients and healthy controls.

	Patients, n=368	Controls, n=400
Gender (Male:Female)	199 (54):169 F(46)	220(55):180(45)
Mean Age	46.48±14.80	45.20±11.30
Smokers	129(35)	124(31)
TC (mg/dL)	206± 42	205±40
HDL(mg/dL)	54±15	54±14
TG(mg/dL)	128 ± 81	126±82

Numbers are presented as mean ± standard deviation. (%). No significant differences between two groups were observed after adjustment by age and sex.

Table 2. Genotype frequencies of the *NOS3* -786 T/C SNP (rs2070744), intron 4 VNTR and Glu298Asp (rs1799983) polymorphisms in patients and controls.

	786 T/C			intron 4 VNTR			Glu298Asp		
	TT	TC	CC	55	45	44	GG	GT	TT
Patients, n=368	103(0.28)	199(0.54)	66(0.18)	250(0.68)	103(0.28)	15(0.04)	151(0.41)	173(0.47)	44(0.12)
Controls, n=400	156(0.39)	188(0.47)	48(0.12)	316(0.79)	80(0.20)	4(0.01)	156(0.39)	200(0.50)	44(0.11)
	p¹: 0.0006; OR:1.70(1.24-2.33)*			p²: 0.0005; OR:1.78(1.27-2.49)*			p³:0.67; OR:0.91(0.57-1.45)		
Familial, n=180(0.49)	51(0.28)	92(0.51)	38(0.21)	128(0.71)	43(0.24)	4(0.02)	70(0.39)	86(0.48)	24(0.13)
Sporadic, n=188(0.51)	52(0.28)	107(0.57)	28(0.15)	128(0.68)	54(0.29)	4(0.02)	77(0.41)	83(0.44)	28(0.15)
	p¹:0.94;OR:0.98(0.61-1.59)			p²:0.36;OR:0.81(0.50-1.31)			p³:0.69; OR:1.09(0.70-1.69)		
Non-severe, n=228(0.62)	61(0.27)	122(0.53)	45(0.20)	160(0.70)	59(0.26)	5(0.02)	98(0.43)	103(0.45)	27(0.12)
Severe, n=140(0.38)	42(0.30)	77(0.55)	21(0.15)	97(0.69)	38(0.27)	3(0.02)	53(0.38)	70(0.49)	17(0.12)
	p¹:0.50;OR:0.85(0.52-1.39)			p²:0.81;OR:1.06(0.65-1.73)			p³:0.33; OR:1.24(0.79-1.95)		
Early-onset, n=254(0.69)	79(0.31)	132(0.52)	43(0.17)	177(0.70)	66(0.26)	5(0.02)	102(0.40)	117(0.46)	35(0.14)
Late-onset, n=114(0.31)	24(0.21)	67(0.59)	23(0.20)	76(0.67)	32(0.28)	3(0.03)	44(0.39)	54(0.47)	16(0.14)
	p¹ : 0.047; OR:1.69(0.97-2.96)*			p²:0.57;OR:0.87(0.52-1.46)			p³:0.78; OR:0.94(0.58-1.51)		
Arthritis+, n=77 (0.21)	20(0.26)	47(0.61)	10(0.13)	54(0.70)	22(0.29)	0(0.00)	23(0.30)	45(0.58)	9(0.12)
Arthritis-, n=291 (0.79)	83(0.29)	152(0.52)	56(0.19)	201(0.69)	76(0.26)	9(0.03)	125(0.43)	125(0.43)	41(0.14)
	p¹:0.66;OR:1.14(0.62-2.09)			p²:0.89;OR:0.96(0.53-1.74)			p³:0.04; OR:1.77(1.00-3.15)*		
Cw6-, n= 228(0.62)	61(0.26)	122(0.53)	48(0.21)	153(0.67)	62(0.27)	7(0.03)	86(0.41)	99(0.47)	25(0.12)
Cw6+, n= 140(0.38)	45(0.32)	77(0.55)	18(0.13)	105(0.75)	34(0.24)	3(0.02)	50(0.39)	57(0.43)	22(0.18)
	p¹ 0.23;0.76(0.47-1.23)			p²:0.30;OR:0.78(0.47-1.28)			p³:0.68; OR:0.91(0.57-1.46)		

Frequencies are presented as *n* (%). *p*¹ *value* calculated with Chi-square test TC+CC vs. TT; *p*² *value* calculated with Chi-square test 45+44 vs. 55; *p*³ calculated with Chi-square test GG vs GT+TT; Rare 3 and 6 repeat alleles with less than 5 cases were excluded from the analysis.