# Inducible Nitric Oxide Synthase Polymorphism Is Associated with Susceptibility to Henoch-Schönlein Purpura in Northwestern Spain

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*ABSTRACT.* ***Objective.*** To assess the contribution of 2 polymorphisms within the inducible nitric oxide (*NOS2A*) promoter region to the susceptibility to Henoch-Schönlein purpura (HSP), and to determine if implications exist with severe systemic complications of HSP, in particular with severe renal involvement and permanent renal dysfunction (renal sequelae).

***Methods.*** Fifty-eight patients from Northwest Spain with primary cutaneous vasculitis classified as HSP were studied. All patients were required to have had at least 2 years' followup. Patients and ethnically matched controls (n = 251) were genotyped by PCR based techniques for a multiallelic $(CCTTT)_n$ and for the biallelic TAAA repeat in the promoter region of the *NOS2A* gene.

***Results.*** HSP patients exhibited a significantly increased frequency of the *NOS2A* short (8–11) $CCTTT_n$ alleles (OR 1.64, 95% CI 1.09–2.47, p = 0.017) and genotypes (OR 3.59, 95% CI 1.79–7.20, p = 0.0002) compared to controls, particularly when patients with nephritis were compared with controls. However, when the *NOS2A* TAAA repeat polymorphism was assessed, no differences were found.

***Conclusion.*** Significant differences in the *NOS2A* promoter polymorphism allele and genotype frequency between HSP patients and controls suggest a potential role for this gene in the susceptibility to HSP and in the development of nephritis. (J Rheumatol 2005;32:1081–5)

*Key Indexing Terms:*
HENOCH-SCHÖNLEIN PURPURA DISEASE SUSCEPTIBILITY RENAL INVOLVEMENT
RENAL SEQUELAE NITRIC OXIDE *NOS2A* POLYMORPHISMS

Henoch-Schönlein purpura (HSP) is the most common primary small blood vessel leukocytoclastic angiitis in children and is rare in adults[1]. Palpable purpura and joint and gastrointestinal (GI) manifestations are typical of this condition[2]. Renal manifestations, however, constitute the most serious complications, and longterm morbidity and mortality in HSP are mainly due to renal involvement[3].

Reports of well documented families of first-degree relatives with HSP support a genetic component in the pathogenesis of this vasculitis[1,3]. Susceptibility to HSP and associated clinical heterogeneity in HSP may be conferred by a number of genetic loci. Studies in people of Northwestern Spain have shown that different genes may influence the phenotype and the outcome of this condition[4-9].

Nitric oxide (NO) is the product of conversion of L-arginine to L-citrulline by a class of enzymes called NO synthases (NOS). NO is produced constitutively by endothelial (eNOS or NOS3) or neuronal synthases, or in higher concentrations by inducible (iNOS or NOS2) synthases after stimulation by proinflammatory cytokines[10].

Several functionally relevant polymorphisms in the *NOS2A* and eNOS genes have been identified, which have been associated with different vascular[11], autoimmune[12], and infectious diseases[13]. A highly polymorphic pentanucleotide $(CCTTT)_n$ repeat located at the *NOS2A* gene promoter region has been shown to be functionally important in the regulation of *NOS2A* transcription[12]. A trend of association of the $(CCTTT)_n$ repeat variations with rheumatoid arthritis (RA) has been reported[14]. Significant differences in this *NOS2A* promoter polymorphism genotype frequency between Spanish patients with RA and controls have been observed[15]. Also, a functional polymorphism in the proximal promoter involving the insertion or deletion of one unit of a TAAA repeat[16,17] has proved to be associated with

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increased risk of renal abnormalities and other complications of type 2 diabetes[18].

Although no association with 3 polymorphisms of the eNOS gene was found in Spanish HSP patients compared to controls[19], in this study we assessed whether polymorphisms in the *NOS2A* gene may be implicated in the susceptibility and clinical expression of cutaneous vasculitis that fulfilled classification criteria for HSP, in particular with severe renal involvement and permanent renal dysfunction (renal sequelae).

## MATERIALS AND METHODS

*Patients and controls.* Patients with primary cutaneous vasculitis were recruited from the Divisions of Pediatrics and Rheumatology of Hospital Xeral-Calde, Lugo, Spain; they fulfilled the 1990 American College of Rheumatology classification criteria for hypersensitivity vasculitis or HSP[20,21], and they were differentiated using the criteria proposed by Michel, *et al*[22]. They were classified as having HSP if they fulfilled at least 3 of the 6 criteria: (1) palpable purpura, (2) bowel angina, (3) GI bleeding, (4) macroscopic or microscopic hematuria, (5) age at disease onset ≤ 20 years, and (6) no history of medication taken before onset of disease. Patients who met fewer than 3 criteria were excluded.

As described[4], in adults a skin biopsy showing leukocytoclastic vasculitis was always required. In children, a diagnosis of cutaneous vasculitis was considered if they had typical nonthrombocytopenic symmetric palpable purpura involving the lower extremities and other conditions such as connective tissue disease and infections had been excluded. For the purpose of examining the outcome of HSP, in this study only patients with at least 2 years' followup were included.

The main epidemiological and clinical data of the 58 patients with HSP in this study have been reported[7]. Briefly, all patients presented palpable purpura; 40 had arthralgias and/or arthritis; 47 had severe GI manifestations (GI bleeding and/or bowel angina). Hematuria with or without proteinuria was observed in 38 patients. However, after a minimum of 2 years' followup (median 8 yrs), only 12 of the 58 patients had persistent renal involvement (renal sequelae), mainly hematuria without renal insufficiency.

Ethnically matched controls (n = 251) were also recruited from the Lugo area.

*TAAAn and CCTTTn genotyping.* DNA was isolated from anticoagulated peripheral blood mononuclear cells using standard methods. We determined the TAAAn and CCTTTn genotypes by a polymerase chain reaction (PCR) based method as described[23,24].

Forward and reverse primers were 5' TGC CAC TCC GCT CCA G 3' and 5' GGC CTC TGA GAT GTT GGT CTT 3' for TAAAn; and 5' ACC CCT GGA AGC CTA CAA CTG CAT 3' and 5' GCC ACT GCA CCC TAG CCT GTC TCA 3' for CCTTTn. The forward primers were 5' labeled with the fluorescent dye 6-FAM. PCR aliquots of 0.5 µl were added to 3 µl of formamide and 0.5 µl of internal size standard. Samples were analyzed in denaturing gels (6% acrylamide/7 M urea) and sized using Genescan 672 software (Applied Biosystems, Foster City, CA, USA). For TAAAn repeats, sequencing revealed that the longer PCR fragment (224 base pairs) contained 5 TAAA repeats (denoted *NOS2A*+ allele) whereas the shorter one, 220 bp, contained 4 repeats (*NOS2A*– allele). Regarding CCTTTn repeats, the sizes of the PCR products ranged from 171 to 216 bp, depending on the number of pentanucleotide repeat units.

*Statistical analysis.* Strength of association between patient groups and controls and alleles or genotypes of the *NOS2A* polymorphisms was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher exact analysis. Statistical significance was defined as $p \leq 0.05$. Corrected p values ($p_{corr}$) were calculated by the Bonferroni method. Calculations were performed with the Stata v6 statistical package.

## RESULTS

*NOS2A promoter CCTTT repeat microsatellite polymorphisms in HSP.* $CCTTT_n$ allele and genotype frequencies were examined in HSP patients and controls. Increased frequency of the 9-repeat single allele (181 bp) was observed in HSP patients compared to controls (OR 3.28, 95% CI 1.54–6.99, p = 0.0014, $p_{corr}$ = 0.013). The frequency of other individual short alleles (shorter than 12 repeats) was also increased in patients, although this did not reach statistical significance (Table 1). However, when we stratified the *NOS2A* alleles into short (8–11) and long (12–16) repeats, significant differences were observed between HSP patients and controls (OR 1.64, 95% CI 1.09–2.47, p = 0.017, $p_{corr}$ = 0.033; Table 2). Of note, a statistically significantly increased frequency of individuals carrying 2 alleles with fewer than 12 repeats (less than 196 bp) was also found among HSP patients (29.3%) compared to the controls (10.4%) (Table 3). In this regard, although no evidence of departure from Hardy-Weinberg equilibrium was observed in controls, homozygosity for short genotypes (+/+ vs +/– and –/–) was significantly increased in HSP patients compared to controls (OR 3.59, 95% CI 1.79–7.20, p = 0.0002, $p_{corr}$ = 0.0004; Table 3).

To determine whether *NOS2A* promoter CCTTT repeat microsatellite polymorphism was also implicated in disease severity, we stratified the patients according to the presence of nephritis, renal sequelae at last followup, and severe GI manifestations. Following this procedure we found a significantly increased frequency of short alleles (8–11 repeats) in HSP patients with nephritis compared to controls (OR 1.73, 95% CI 1.06–2.81, p = 0.02, $p_{corr}$ = 0.05; Table 2). In addition, homozygosity for short genotypes (+/+ vs +/– and –/–) was significantly increased in HSP patients with nephritis compared to controls (34.2% vs 10.4%; OR 4.50, 95% CI 2.06–9.86, p = 0.0001, $p_{corr}$ = 0.0002; Table 3). This was also the case when homozygosity for short genotypes (+/+ vs +/– and –/–) in HSP patients with renal sequelae was compared with controls (41.7% vs 10.4%; OR 6.18, 95% CI 1.83–20.88, p = 0.001, $p_{corr}$ = 0.002; Table 3). However, no significant differences in the frequency were observed when patients were stratified by the presence of severe GI manifestations (data not shown).

*NOS2A promoter TAAA repeat polymorphism in HSP.* Although the frequency of *NOS2A* 224 (*NOS2A*+) allele was increased in HSP patients (13.8%) compared to controls (9.4%), no statistically significant allele or genotype differences between HSP patients and controls for this *NOS2A* polymorphism were found (Table 4). Similarly, HSP patients with nephritis or severe GI manifestations did not exhibit a different allele or genotype distribution compared to those without these complications or controls (Table 4). This was also the case when the influence of the *NOS2A* promoter TAAA repeat polymorphism in the susceptibility to renal sequelae was assessed (data not shown).

*Table 1.* Allele frequencies of *NOS2A* $(CCTTT)_n$ gene polymorphism in Henoch-Schönlein purpura (HSP) and controls from Northwest Spain.

| Repeat No. | Size (base pair) | HSP, n = 116 (%) | Controls, n = 502 (%) | OR | 95% CI | p |
|---|---|---|---|---|---|---|
| 8 | 176 | 2 (1.7) | 5 (1.0) | 2.44 | 0.45–13.15 | 0.29 |
| 9 | 181 | 14 (12.1) | 26 (5.2) | 3.28 | 1.54–6.99 | 0.0014* |
| 10 | 186 | 11 (9.5) | 44 (8.8) | 1.53 | 0.71–3.28 | 0.28 |
| 11 | 191 | 28 (24.1) | 103 (20.5) | 1.66 | 0.94–2.93 | 0.08 |
| 12 | 196 | 30 (25.9) | 183 (36.5) | 1 (reference) | — | — |
| 13 | 201 | 19 (16.4) | 75 (14.9) | 1.54 | 0.82–2.91 | 0.18 |
| 14 | 206 | 7 (6.0) | 48 (9.6) | 0.89 | 0.37–2.15 | 0.79 |
| 15 | 211 | 4 (3.4) | 12 (2.4) | 2.03 | 0.62–6.72 | 0.24 |
| 16 | 216 | 1 (0.9) | 6 (1.2) | 1.01 | 0.12–8.74 | 0.99 |

* $p_{corr} = 0.013$.

*Table 2.* Association of shorter forms of *NOS2A* $(CCTTT)_n$ microsatellite repeats with Henoch-Schönlein purpura (HSP).

| Alleles | HSP, n = 116 (%) | Controls, n = 502 (%) |
|---|---|---|
| Short (8–11 repeats) | 55 (47.4)* | 178 (35.5)* |
| Long (12–16 repeats) | 61 (52.6) | 324 (64.5) |

* The frequency of short alleles (8–11 repeats) was significantly increased in HSP patients compared to controls (OR 1.64, 95% CI 1.09–2.47, p = 0.017, $p_{corr} = 0.033$).

| | HSP with Nephritis | | HSP with Renal Sequelae | | |
|---|---|---|---|---|---|
| Alleles | Yes n = 76 (%) | No n = 40 (%) | Yes n = 24 (%) | No n = 92 (%) | Controls, n = 502 (%) |
| Short (8–11 repeats) | 37 (48.7)† | 18 (45.0) | 13 (54.2) | 42 (45.7) | 178 (34.5)† |
| Long (12–16 repeats) | 39 (51.3) | 22 (55.0) | 11 (45.8) | 50 (54.3) | 324 (64.5) |

† The frequency of short alleles (8–11 repeats) was significantly increased in HSP patients with nephritis compared to controls (OR 1.73, 95% CI 1.06–2.81, p = 0.02, $p_{corr} = 0.05$).

## DISCUSSION

Our study constitutes the first attempt to establish the potential implication of 2 polymorphisms in the *NOS2A* gene in susceptibility to primary cutaneous vasculitis. The results support a potential role of *NOS2A* promoter CCTTT repeat microsatellite polymorphism in the susceptibility to nephritis and persistent renal dysfunction, in particular in those patients who carried 2 alleles shorter than 12 repeats.

The etiology of HSP remains unknown. Infections, in particular those from the upper respiratory tract, were reported to be a precipitating factor in at least 50% of cases[3,25]. Cytokines produced during antigenic challenge may play a role in the susceptibility to and severity of HSP. Genetic factors may determine the immunological and inflammatory response to unknown antigens in patients with this condition. The polymorphisms of cytokine genes may influence the level of expression of cytokines. Interleukin 1ß (IL-1ß) gene participates in the regulation of IL-1 receptor antagonist (IL-1ra) production. Polymorphisms in these genes may mediate an abnormal inflammatory response that may lead to the development of severe renal involvement and renal sequelae in HSP. In this regard, in Spaniards a biallelic (–511 C/T) polymorphism in the IL-1ß gene was directly implicated in the severity and outcome, but not the susceptibility, of unselected patients with cutaneous vasculitis[9]. Association between an IL-1ra variable number of tandem-repeat gene polymorphisms with the development of severe renal manifestations and renal sequelae in HSP has been reported in the same population[7].

Abnormal expression of proinflammatory cytokines may mediate the proliferation and differentiation of lymphoid cells. It may lead to an increased number of circulating IgA-secreting cells in patients with HSP[26]. Infectious agents may also promote the production of NO through the direct effect of proinflammatory cytokines. Since NO is an important regulator of Th1/Th2 balance limiting the Th1 response, a possible explanation for this association with disease susceptibility and especially severity in patients with cutaneous vasculitis may be that individuals carrying 2 alleles with fewer than 12 CCTTT repeats (low NO producers)[12] may be susceptible to an increased predisposition to autoimmune disease.

*Table 3.* Genotype distribution according to the presence of short (8–11 repeats) or large *NOS2A* $(CCTTT)_n$ microsatellite repeats in Henoch-Schönlein purpura (HSP) patients and controls.

| Genotype | HSP, n (%) | Controls, n (%) | OR | 95% CI | p |
|---|---|---|---|---|---|
| Short (8–11 repeats) | | | | | |
| –/– | 20 (34.5) | 99 (39.4) | 1 | — | — |
| +/– | 21 (36.2) | 126 (50.2) | 0.83 | 0.42–1.61 | 0.57 |
| +/+ | 17 (29.3)* | 26 (10.4)* | 3.24 | 1.49–7.04 | 0.002 |

* Homozygosity for short genotypes (+/+ versus +/– and –/–) was significantly increased in HSP patients compared to controls (OR 3.59, 95% CI 1.79–7.20, p = 0.0002, $p_{corr}$ = 0.0004).

| | HSP with Nephritis | | HSP with Renal Sequelae | | |
|---|---|---|---|---|---|
| | Yes | No | Yes | No | Controls, |
| Genotype | n (%) | n (%) | n (%) | n (%) | n (%) |
| Short (8–11 repeats) | | | | | |
| –/– | 14 (36.8) | 6 (30.0) | 4 (33.3) | 16 (34.8) | 99 (39.4) |
| +/– | 11 (29.0) | 10 (50.0) | 3 (25.0) | 18 (39.1) | 126 (50.2) |
| +/+ | 13 (34.2)[#] | 4 (20.0) | 5 (41.7)[†] | 12 (26.1) | 26 (10.4)[#†] |

[#] Homozygosity for short genotypes (+/+ vs +/– and –/–) was significantly increased in HSP patients with nephritis compared to controls (OR 4.50, 95% CI 2.06–9.86, p = 0.0001, $p_{corr}$ = 0.0002). [†] Homozygosity for short genotypes was also significantly increased in HSP patients with renal sequelae compared to controls (OR 6.18, 95% CI 1.83–20.88, p = 0.001, $p_{corr}$ = 0.002).

*Table 4.* Allele frequencies and genotype distribution of *NOS2A* TAAA polymorphism in a series of patients with Henoch-Schönlein purpura (HSP) and controls*. Data are No. (%).

| | | | Renal Involvement | | GI Manifestations** | |
|---|---|---|---|---|---|---|
| | Controls | HSP | Yes | No | Yes | No |
| Allele (2N) | | | | | | |
| 220 | 455 (90.6) | 100 (86.2) | 65 (85.5) | 35 (87.5) | 80 (85.1) | 20 (90.9) |
| 224 | 47 (9.4) | 16 (13.8) | 11 (14.5) | 5 (12.5) | 14 (14.9) | 2 (9.1) |
| Genotype | | | | | | |
| 220/220 | 208 (82.9) | 43 (74.1) | 28 (73.7) | 15 (75.0) | 34 (72.3) | 9 (81.8) |
| 220/224 | 39 (15.5) | 14 (24.1) | 9 (23.7) | 5 (25.0) | 12 (25.5) | 2 (18.2) |
| 224/224 | 4 (1.6) | 1 (1.7) | 1 (2.6) | 0 (0.0) | 1 (2.1) | 0 (0.0) |

* No statistically significant differences were found between patients and controls and between patients with or without nephritis or GI manifestations. ** GI bleeding and/or bowel angina.

Some studies have shown an association of the *NOS2A* CCTTT repeat promoter polymorphism with severe clinical manifestations of insulin-dependent diabetes mellitus[12,27]. A significantly increased frequency of short-repeat alleles was also observed in Spanish patients with RA compared with controls[15]. In this population a double risk of developing RA was observed in individuals carrying 2 alleles with fewer than 12 repeats[15].

It is possible, however, that the effect of different polymorphisms on HSP susceptibility and severity might be due to the interdependent effects of more than one polymorphism rather than to the effect of individual polymorphisms. Since *NOS2A* gene has been mapped on chromosome 17q, and a region on this chromosome has been shown to contain a RA susceptibility allele[28], we cannot exclude that the potential role of short-repeat $CCTTT_n$ alleles in the susceptibility to RA and HSP in Spaniards may be due to other polymorphisms in linkage disequilibrium with these $CCTTT_n$ alleles located on chromosome 17q.

Our observations may contribute to the knowledge of stratification of patients with primary cutaneous vasculitis. Studies in other populations and in particular on larger numbers of patients are required to confirm the association between *NOS2A* gene polymorphisms and HSP, its relationship with other genes implicated in susceptibility to primary cutaneous vasculitis, and the development of severe renal manifestations and renal sequelae in HSP.

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