

ORIGINAL STUDY

Possible association between -954G/C iNOS polymorphism in nasal polyposis. A case-control study in a population group of Northern Romania

Andreea Catana¹, Alma Maniu², Doinel Radeanu², Radu A. Popp¹, Roxana F. Ilies¹, Iuliu V. Catana³

¹Molecular Department, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj Napoca, Romania

²Otorhinolaryngology Department, Emergency County Hospital, Cluj Napoca, Romania

³CMI Dr. Catana Iuliu, Cluj Napoca, Romania

ABSTRACT

BACKGROUND. Polymorphisms for genes encoding chemosensitive signalling proteins like NOS2 might contribute to the variability in individual susceptibility to nasal polyposis. NO produced by the inducible NO synthase enzyme NOS2A is generated at high levels in certain types of inflammation, so that the role of NOS2 might also be important in nasal polyposis etio-pathogeny.

MATERIAL AND METHODS. This is a cross-sectional, randomized, case-control study for the evaluation of the frequency of -954G/C NOS2A2 alleles among patients with nasal polyposis. The study included 91 cases of nasal polyposis diagnosed patients (nasal endoscopy and CT scan examination), and 117 healthy unrelated controls. NOS2 genotyping was carried out using PCR amplification of relevant gene fragment and it was followed by restriction enzyme digestion. Detection of the variant alleles was determined through analysis of resulting restriction fragment length polymorphism (RFLP) followed by gel electrophoresis.

RESULTS. Molecular analysis revealed an increased frequency of NOS2 variant allele in the study group compared to the control group ($p=0.019$, OR=1.991, CI=1.08-3.67). A statistically significant finding was highlighted among allergic and non-allergic patients with nasal polyposis ($p=0.046$, OR=0.449, CI=0.208-0.969) and a relationship between nasal polyposis patients with asthma and non-asthmatic patients ($p=0.119$, OR=1.825, CI=0.875-3.80).

CONCLUSION. The main finding of our study is that -954G/C polymorphism of NOS gene seems to be associated with an increased risk for nasal polyposis.

KEYWORDS: -954G/C iNOS, genetic polymorphism, nasal polyposis

INTRODUCTION

Nasal polyposis (NP) is a common pathology characterized by impaired regulation of nasal tissue growth, leading to the formation of semi translucent grapelike tumors in the upper airways. The etiology of NP remains unknown, but chronic inflammation appears to play a major pathogenic role, often involving other immune-linked statuses like allergy, aspirin intolerance and asthma¹.

Nitric oxide synthases (NOSs) are a family of catalyzing enzymes of nitric oxide (NO), an important signalling molecule². It has multiple biological roles,

modulating vascular and airway tone, insulin secretion, neural development and angiogenesis. There are three different isoforms of nitric oxide synthase (NOSs) responsible for the synthesis on endogenous NO: inducible nitric oxide synthase (iNOS, or NOS2), highly expressed in response to pro-inflammatory stimuli; and two other constitutive forms responsible for the synthesis of specific but decreased amounts of NO, endothelial nitric oxide synthase (eNOS) and neuronal oxide synthase (nNOS)³.

The inducible form of NOS (iNOS) is highlighted in an oxidative environment and regulates the immune defense mechanism by producing large amounts of NO

in response to cytokines, therefore considered a key factor in the infection response, tumor growth and autoimmune disorders. Human gene encoding for *iNOS* is located on chromosome 17q11.2-12 and was associated with a wide variety of disorders; it has 27 exons, the protein product is a catalytic enzyme with two different functional domains⁴. Mutations (deletions) of coding and regulatory regions are linked with decreased levels of NO and reported to play an important role in several different disorders. The gene encoding for NOS2 is extremely polymorphic; several microsatellites and single nucleotide polymorphisms (SNPs) were identified in relationship with defective NO synthesis.

(CCTTT)_n polymorphic pentanucleotide microsatellite has an important role in the regulation of NOS2A gene transcription, and has been linked to a number of infectious and immunological diseases including nasal polyposis⁵⁻⁷. Another polymorphism of the proximal promoter of NOS2A gene, characteristic by a deletion or insertion of the TAAA unit repeat, was also associated with inflammatory disorders⁸⁻¹¹. A polymorphism of the promoter region of NOS2a gene, -954G/C (rs2297518), characterized by a missense mutation leading to substitution (TCG ⇒ TTG) and therefore an amino acid exchange S [Ser] ⇒ L [Leu] was studied in relation to a wide range of infectious and immune disorders (cancer, malaria, rheumatoid arthritis, asthma)^{6,12-15}.

To our knowledge, there is no other study for evaluating -954G/C polymorphism of NOS2 gene in nasal polyposis, therefore we considered it would be a proper study subject.

MATERIAL AND METHODS

Study population

This is a cross-sectional, randomized case-control study consisting of 91 cases with nasal polyposis and 117 healthy unrelated controls. Patients with nasal polyposis were recruited between 2013 and 2015, from the ENT Department of the Emergency County Hospital, Cluj Napoca, Romania. Randomly selected controls were accrued from the Internal Medicine Department of the Emergency Hospital, Cluj. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy, Cluj Napoca, Romania; all of the subjects signed a written informed consent and each participant was personally interviewed by specialist doctors to obtain detailed information on lifetime and family history, associated pathology, tobacco use, toxic and allergy exposure.

Selection and evaluation of patients

Nasal polyposis was confirmed in all cases after nasal endoscopic and clinical examination. All pa-

tients included in the study were diagnosed with de novo or recurrent nasal polyposis. Associated pathology like allergic rhinosinusitis or asthma was well documented and based upon valid allergic tests (skin prick test and intradermal dilution testing) and para-clinical lung assessment by spirometry.

Olfactory dysfunction was assessed using the extended version of the Sniffin' Sticks test (Heinrich Burghart GMBh, Wedel, Germany)¹⁶. Pathological olfactory function was indicated by a TDI score of 30.5 or less, with the separation of hyposmia from functional anosmia at a TDI score of 15.5.

A sample of 2 ml of venous blood was then collected from all patients and controls in order to perform the genotyping analysis.

Genotyping

DNA samples were extracted from 300µl peripheral blood, using a commercial Wizard Genomic DNA Purification Kit (Promega, Madison, USA).

The -954G/C NOS2 polymorphism was detected using a PCR-RFLP method. The PCR primers were synthesized by Eurogentec (Belgium):

- Forward primer 5'- CATATGTATGGGAATACTGTATTTTCAG - 3'
- Reverse primer 5'- TCTGAACTAGTCACTTGAGG - 3'

For DNA amplification, a total amount of 100 ng of genomic DNA was amplified in a volume of 25µl reaction mixture containing reaction buffer of 1,5nM MgCl₂, 20pmol of each primer, 200µm of each dNTPs and 0,5 units of Taq polymerase. Thermocycling conditions were carried out as follows: initial denaturation at 94 °C for 1 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s, primer extension at 72 °C for 75s and then a final extension at 72 °C for 10 min. The amplification products were digested with 4 units of BsaI enzyme (Fermentas, Thermo Scientific Biosciences GmbH, Germany) for 8 hours and separated on a 2 % agarose gel (MetaPhor®, FMC Bio Products, Rockland, ME, USA), allowing detection by ethidium bromide staining. In order to validate the veracity of the results, positive and negative polymorphic controls were used for all PCR-RFLP probes. There are 3 possible genotypes, each defined by 3 distinct banding patterns: GG (437 and 136 bp fragments), GC (573, 437 and 136 bp fragments) and CC (573 bp fragment).

Statistical analysis

For statistical analysis we used SPSS 18.0 for Windows. (SPSS, Inc., Chicago, IL). Hardy-Weinberg equilibrium test and Ors were calculated to assess the relationship between -954G/C NOS2 polymorphism and nasal polyposis. For genotype-phenotype polyposis type association we used Adjusted Residual Analysis, Crosstabulation between polyposis type and genotype.

RESULTS

The characteristics of the study population are shown in Table 1.

Demographic characteristics of the studied population are presented in Table 1. The 91 cases (44 females and 47 males) ranged in age from 18 years to 77 years, with a mean (standard deviation) age of 51 (15.6) years. The 117 controls (56 females and 61 men) ranged from 18 to 81 years, with a mean (SD) age of 46 (16.1) years.

Comparative analysis between nasal polyposis patients and controls related to the distribution of the variant G alleles respectively GG/GC genotypes did not reach any positive statistical association ($p=0.090$,

OR= 1.630, CI=0.925-2.873, respectively $p=0.552$, OR=0.877, CI=0.5697-1.351).

Polyposis patients and the control group did not show any statistically significant difference of -954G/C polymorphism when using the recessive model (GG+GC vs. CC) association for the Fisher test ($p=0.172$, OR=2.35, CI= 0.67-8.30); still, the dominant model (GC+CC vs. GG) highlights statistical differences between patients and controls ($p=0.019$, OR=1.991, CI=1.08-3.67).

The potential association between the distribution of NOS2 genotypes did not reveal any statistically significant difference between nasal polyposis patients with no olfactory dysfunction and those with present olfactory dysfunction ($p=0.158$, OR=0.362, CI=0.094-1.390).

Table 1
Demographic Characteristics of Subjects and Controls

	N	Age, y	Male, n (%)	Female, n (%)	Smokers n (%)
Controls	117	46	61 (52.13)	56 (47.87)	66 (56.41)
Nasal Polyposis	91	51	47 (51.64)	44 (48.36)	38 (41.75)
Olfactory dysfunction	60	53	19 (31.66)	41 (68.33)	24 (40)
Asthma	27	49	19 (70.37)	8 (29.62)	8 (29.62)
Allergy	38	52	17 (44.73)	21 (55.26)	16 (42.1)

Table 2
Distribution of genotypes and allele frequencies of NOS2 polymorphism -954G/C in Nasal polyposis patients and Controls

954G/C	Polyposis		Olfactory dysfunction		Asthma		Allergies		Controls
	de novo	recurrent	absent	present	absent	present	absent	present	
GG	5	2	3	5	4	3	7	0	6
GC	14	12	9	17	16	10	15	11	26
CC	27	31	19	38	44	14	31	27	85
C	68	74	47	93	104	38	77	65	196
G	24	16	15	27	24	16	29	11	38

Table 3
Risk analysis for NOS2 polymorphism -954G/C in Nasal polyposis patients and Controls (Fisher test, dominant and recessive models)

Genotype Polyposis/Controls (N)	p	Odd ratios	95% IC
<i>Dominant model</i>			
GG			
GC+CC	0.019*	1.991	1.08-3.67
<i>Recessive model</i>			
GG+GC	0.172	2.35	0.67-8.30
CC			

* Significant association for $p<0.050$

We also evaluated the potential association between the distribution of *NOS2* G variant allele distribution and patients with asthma and allergy. The statistical results revealed a relationship between nasal polyposis patients with asthma and non-asthmatic patients ($p=0.119$, $OR=1.825$, $CI=0.875-3.80$). A statistically significant finding was highlighted among allergic and non-allergic patients with nasal polyposis ($p=0.046$, $OR=0.449$, $CI=0.208-0.969$).

DISCUSSIONS

Nasal polyposis is a complex disease with a yet unknown pathogenesis, although there are several different studies that describe associations with various gene variants¹⁷⁻²¹.

In the present study we evaluated the potential implication of -954G/C *NOS2* polymorphism in nasal polyposis pathogenesis and phenotype. We found an association between nasal polyposis and the investigated gene variant and also a statistically significant association between NP and specific phenotypes.

The inflammatory and allergic mechanisms are involved in the etiology of nasal polyposis, hence the frequent association of this pathology with atopy and asthma^{22,23}. The gene for *NOS2A*, which is the main NO-producing enzyme in inflammation is found on chromosome 17q11.2-q12 and lies in the CC chemokine cluster region, where some studies reported a linkage with atopy and asthma²⁴⁻²⁶. Previous genetic studies for the pathogenesis of inflammation have shown the association of the genetic variants of NOS with asthma and allergy²⁷⁻³⁰. Two recent studies that have evaluated the relationship between (*CCTTT*)_n polymorphism of *NOS2A* gene in nasal polyposis found that the number of repeats for this genetic variant in the promoter region could be associated with the inflammatory process of nasal polyposis^{31,32}. To our knowledge, there is only one study evaluating 954G/C *NOS2* in relationship to nasal polyposis³³.

One of the findings in our study is that we highlighted an association of -954G/C *NOS2* polymorphism in polyposis patients with allergy. As is already well known, atopy is one of the confounding elements in the etiopathogenesis of nasal polyposis among patients with or without asthma^{34,35}. Increased frequency of -954G/C *NOS2* variant among patients with asthma and allergic polyposis is a proof that polymorphisms of *NOS* gene is implied in the etiopathogenesis of nasal polyposis and associated phenotypes like asthma and atopy.

We also evaluated the potential association between -954G/C *NOS2*, nasal polyposis and olfactory dysfunction. Little is known about the pathogenic mechanisms leading to olfactory dysfunction in nasal polyposis;

recently, it has been shown that few genetic polymorphisms could influence the outcome of the olfactory status in nasal polyposis. In our study, we found no association between -954G/C *NOS2* and the olfactory dysfunction in nasal polyposis.

One of the findings of our study is that -954G/C *NOS2* polymorphism is more frequent in females with nasal polyposis and olfactory dysfunction compared to males. ($p=0.0189$; $OR=2.354$; $CI=1.192-4.650$). There are few studies that have assessed gender differences in patients with olfactory dysfunction in connection with certain genetic polymorphisms. Evaluating different gene variants by gender is quite difficult because it associates a multitude of genes that interact with several sex-specific endocrine and metabolic factors; however, the result of our study would highlight the role of gene variants in evaluating the olfactory dysfunction in patients with nasal polyposis by gender.

CONCLUSIONS

The major finding of our study is that -954G/C polymorphism of *NOS* gene could be considered an independent molecular risk factor associated with an increased risk for the development of nasal polyposis, especially in allergic individuals.

Conflicts of interests: None

Contribution of authors: All authors have equally contributed to this work.

Ethical considerations: All subjects enrolled in the present study signed an informed consent for all ENT maneuvers, molecular genetic analysis and resulting data usage. The current study was approved by the ethics committee of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, according to the Helsinki Declaration.

REFERENCES

1. Hamilos D.L. - Chronic rhinosinusitis: epidemiology and medical management. *J Allergy Clin Immunol.*, 2011;128(4):693-707; quiz 708-709. doi: 10.1016/j.jaci.2011.08.004. Epub 2011 Sep 3.
2. Qidwai T., Jamal F. - Inducible Nitric Oxide Synthase (iNOS) Gene Polymorphism and Disease Prevalence. *Scand J Immunol.*, 2010;72(5):375-387. doi: 10.1111/j.1365-3083.2010.02458.x.
3. Nathan C., Xie Q.W. - Nitric oxide synthases: roles, tolls, and controls. *Cell.*, 1994;78(6):915-918.
4. Vallance P., Collier J. - Biology and clinical relevance of nitric oxide. *BMJ.*, 1994;309(6952):453-457.
5. Konno S., Hizawa N., Yamaguchi E., Jinushi E., Nishimura M. - (*CCTTT*)_n repeat polymorphism in the *NOS2* gene promoter is associated with atopy. *J Allergy Clin Immunol.*, 2001;108(5):810-814.
6. Batra J., Pratap Singh T., Mabalirajan U., Sinha A., Prasad R., Ghosh B. - Association of inducible nitric oxide synthase with asthma severity, total

- serum immunoglobulin E and blood eosinophil levels. *Thorax*, 2007;62(1):16-22.
7. Leung T.F., Liu E.K., Li C.Y., Chan I.H., Yung E., Lam C.W., Wong G.W. - Lack of association between NOS2 pentanucleotide repeat polymorphism and asthma phenotypes or exhaled nitric oxide concentration. *Pediatr Pulmonol*, 2006;41(7):649-655.
 8. Tatemichi M., Sawa T., Gilibert I., Tazawa H., Katoh T., Ohshima H. - Increased risk of intestinal type of gastric adenocarcinoma in Japanese women associated with long forms of (CCTTT)_n pentanucleotide repeat in the inducible nitric oxide synthase promoter. *Cancer Letters*, 2005;217(2):197-202.
 9. Warpeha K.M., Xu W., Liu L., Charles I.G., Patterson C.C., Ah-Fat F., Harding S., Hart P.M., Chakravarthy U., Hughes A.E. - Genotyping and functional analysis of a polymorphic (CCTTT)_n repeat of NOS2A in diabetic retinopathy. *FASEB J*, 1999;13(13):1825-1832.
 10. Martín M.C., Martínez A., Mendoza J.L., Taxonera C., Díaz-Rubio M., Fernández-Arguero M., de la Concha E.G., Urcelay E. - Influence of the inducible nitric oxide synthase gene (NOS2A) on inflammatory bowel disease susceptibility. *Immunogenetics*, 2007;59(11):833-837. Epub 2007 Oct 23.
 11. Pascual M., Sanz C., Isidoro-García M., Davila I., Moreno E., Laffond E., Lorente F. - (CCTTT)_n polymorphism of NOS2A in nasal polyposis and asthma: a case-control study. *J Investig Allergol Clin Immunol*, 2008;18(4):239-244.
 12. Zintzaras E., Grammatikou M., Kitsios G.D., Doxani C., Zdoukopoulos N., Papandreou C., Patrikidou A. - Polymorphisms of the endothelial nitric oxide synthase gene in breast cancer: a genetic association study and meta-analysis. *J Hum Genet*, 2010;55(11):743-748. doi: 10.1038/jhg.2010.100. Epub 2010 Aug 19.
 13. Zintzaras E., Kitsios G., Stefanidis I. - Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. *Hypertension*, 2006;48(4):700-710. Epub 2006 Aug 28.
 14. Cramer J.P., Mockenhaupt F.P., Ehrhardt S., Burkhardt J., Otchwemah R.N., Dietz E., Gellert S., Bienzle U. - iNOS promoter variants and severe malaria in Ghanaian children. *Trop Med Int Health*, 2004;9(10):1074-1080.
 15. Hummel T., Sekinger B., Wolf S.R., Pauli E., Kobal G. - 'Sniffin' Sticks': olfactory performance assessed by the combined testing of odor identification, odor discrimination and olfactory threshold. *Chem Senses*, 1997;22(1):39-52.
 16. Rajguru R. - Nasal Polyposis: Current Trends. *Indian J Otolaryngol Head Neck Surg*, 2014;66(Suppl 1):16-21. doi:10.1007/s12070-011-0427-z. Epub 2011 Dec 29.
 17. Wang X., Dong Z., Zhu D.D., Guan B. - Expression profile of immune-associated genes in nasal polyps. *Ann Otol Rhinol Laryngol*, 2006;115(6):450-456.
 18. Mfuna Endam L., Cormier C., Bosse Y., Filali-Mouhim A., Desrosiers M. - Association of IL1A, IL1B, and TNF gene polymorphisms with chronic rhinosinusitis with and without nasal polyposis: A replication study. *Arch Otolaryngol Head Neck Surg*, 2010;136(2):187-192. doi: 10.1001/archoto.2009.219.
 19. Rostkowska-Nadolaska B., Kapral M., Fraczek M., Kowalczyk M., Gawron W., Mazurek U. - A microarray study of gene expression profiles in nasal polyps. *Auris Nasus Larynx*, 2011;38(1):58-64. doi: 10.1016/j.anl.2010.05.002. Epub 2010 Jun 15.
 20. Chandra R. - The Role of Atopy in Nasal Polyposis. *J Aller Ther*, 2011;2:e3. doi:10.4172/2155-6121.10000e3.
 21. Muñoz del Castillo F., Jurado-Ramos A., Fernández-Conde B.L., Soler R., Barasona M.J., Cantillo E., Moreno C., Guerra F. - Allergenic profile of nasal polyposis. *J Investig Allergol Clin Immunol*, 2009;19(2):110-116.
 22. Parikh A., Scadding G.K., Gray P., Belvisi M.G., Mitchell J.A. - High levels of nitric oxide synthase activity are associated with nasal polyp tissue from aspirin-sensitive asthmatics. *Acta Otolaryngol*, 2002;122:302-305.
 23. The Collaborative Study on the Genetics of Asthma (CSGA). A genome-wide search for asthma susceptibility loci in ethnically diverse populations. *Nat Genet*, 1997;15(4):389-392.
 24. Dizier M.H., Besse-Schmittler C., Guilloud-Bataille M., et al. - Genome screen for asthma and related phenotypes in the French EGEA study. *Am J Respir Crit Care Med*, 2000;162(5):1812-1818.
 25. Gao P.S., Kawada H., Kasamatsu T., et al. - Variants of NOS1, NOS2, and NOS3 genes in asthmatics. *Biochem Biophys Res Commun*, 2000;267(3):761-763.
 26. Leung T.F., Liu E.K., Tang N.L., Ko F.W., Li C.Y., Lam C.W., Wong G.W. - Nitric oxide synthase polymorphisms and asthma phenotypes in Chinese children. *Clin Exp Allergy*, 2005;35(10):1288-1294.
 27. Payne D.N., Adcock I.M., Wilson N.M., Oates T., Scallan M., Bush A. - Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma, after treatment with oral prednisolone. *Am J Respir Crit Care Med*, 2001;164(8 Pt 1):1376-1381.
 28. Kocyyigit A., Zeyrek D., Keles H., Koçlu A. - Relationship among manganese, arginase, and nitric oxide in childhood asthma. *Biol Trace Elem Res*, 2004;102(1-3):11-18.
 29. Pascual M., Sanz C., Isidoro-García M., Dávila I., Moreno E., Laffond E., Lorente F. - (CCTTT)_n polymorphism of NOS2A in nasal polyposis and asthma: a case-control study. *J Investig Allergol Clin Immunol*, 2008;18(4):239-244.
 30. Benito Pescador D., Isidoro-García M., García-Solaesa V., Pascual de Pedro M., Sanz C., Hernández-Hernández L., Sánchez-López J., Lorente S.F., Picado C., Valero A., Dávila I. - Genetic association study in nasal polyposis. *J Investig Allergol Clin Immunol*, 2012;22(5):331-340.
 31. Wang X., Di Y., Li C. - Allele Distribution Difference of NOS2 Promoter polymorphisms between Patients With Chronic Rhinosinusitis and Non-sinus Disease Controls. Available at: <http://www.ashg.org/genetics/ashg07s/f21246.htm>
 32. Zeitz H.J. - Bronchial asthma, nasal polyps, and aspirin sensitivity: Samter's syndrome. *Clin Chest Med*, 1988;9(4):567-576.
 33. Settignano G.A., Chafee F.H. - Nasal polyps in asthma and rhinitis. A review of 6,037 patients. *J Allergy Clin Immunol*, 1977;59(1):17-21.
 34. Gevaert P., Bachert C., Holtappaels G., Novo C.P., Van der Heyden J., Franssen L., Depraetere S., Walter H., van Cauwenbergher P., Tavernier J. - Enhanced soluble interleukin-5 receptor alpha expression in nasal polyposis. *Allergy*, 2003;58(5):371-379.
 35. Farhadi M., Barati M., Tabatabaie A., Shekarabi M., Noorbakhsh S., Javadinia S. Th1 and Th2 cytokine gene expression in atopic and nonatopic patients with nasal polyposis. *Ear Nose Throat J*. 2015 Jun;94(6):228-35.