

## Comparative analysis of GSTM1/GSTT1 null alleles and Ile105Val GSTP1 variant in patients with Nasal Polyposis and hyposmia in a Romanian population group

### Analiza comparativă a polimorfismelor GSTM1/GSTT1 și Ile105Val GSTP1 la pacienții cu polipoză nazală și hiposmie într-un grup populațional din România

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#### Abstract

**Background.** Polymorphisms for genes encoding glutathione S-transferase (GSTM1/GSTT1/GSTP1) might be one of the factors that can influence the variability in susceptibility for hyposmia in normal and ENT pathology associated individuals. The role of GST family enzyme might be important in exposure to xenobiotic induced damage of nasal mucosa. **Objectives.** To evaluate of distribution of GST variants (GSTM1/GSTT1 null alleles and Ile105Val GSTP1 polymorphism) among patients with hyposmia and normal individuals by using a case-control study. **Subjects** The study included 75 cases of hyposmic patients (evaluated with "Sniffin' Sticks" olfaction Test), recruited from the Otorhinolaryngology Department of Emergency County Hospital, Cluj-Napoca and 124 healthy unrelated controls. **Methods.** GSTM1 and GSTT1 variants genotyping was accomplished using a Multiplex PCR method, followed by agarose gel electrophoresis. GSTP1 Ile105Val gene variant was genotyped using PCR-RLFP technique. **Results.** Comparative analysis for Ile105Val variant of GSTP1 gene revealed no statistical differences among patients and controls ( $\chi^2 = 3.012$ ,  $p = 0.087$ ,  $OR = 1.514$ ,  $CI = 0.491$  to  $1.572$ ). Molecular analysis did not reveal an increased frequency for GSTT1 and GSTM1 null alleles in the patients group compared to controls (GSTT1 - 95%  $CI = 0.332$  to  $1.261$ ,  $p = 0.192$ ,  $OR = 0.641$ ,  $\chi^2 = 2.120$ , GSTM1 - 95%  $CI = 0.171$  to  $0.592$ ,  $\chi^2 = 2.017$ ,  $OR = 0.321$ ,  $p = 0.062$ ). Significant statistical differences were found when combined GSTM1 and GSTT1 null genotypes (double-null genotypes) were compared between patients and controls ( $p=0.0015$ ,  $OR=4.0351$ ;  $CI=1.706-9.543$ ) and when comparing allergic NP patients with non-allergic NP patients ( $p=0.027$ ,  $OR=3.455$ ,  $CI=1.147-10.406$ ). **Conclusions.** The presence of both GSTM1/GSTT1 null genotypes (double null genotypes) is considered to be a risk factor for NP and hyposmia development in allergic individuals. The results of our study show no correlation between Ile105Val polymorphism of GSTP1 gene and nasal polyposis associated hyposmia in this Romanian group population.

**Keywords:** GST, Polymorphism, Nasal polyposis, Hyposmia

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## Rezumat

**Introducere.** Polimorfisme genelor care codifică glutatión S-transferazele (GSTM1/GSTT1/GSTP1) ar putea fi unul dintre factorii care pot influența variabilitatea susceptibilității pentru polipoză nazală și hiposmie la indivizii sănătoși sau pacienții cu afecțiuni ORL. Rolul enzimelor din familia GST ar putea fi important în expunerea la leziunile induse de xenobiotice la nivelul mucoasei nazale. **Obiective.** Evaluarea distribuției variantelor polimorfice GST (alele nule GSTM1/GSTT1 și polimorfismului Ile105Val GSTP1) în rândul pacienților cu polipoză nazală și hiposmie într-un studiu de tip caz-control. **Subiecți.** Studiul include 75 de cazuri de pacienți cu polipoză nazală și hiposmie (evaluați cu Testul de olfacție "Sniffin'Sticks"), recrutați din cadrul Departamentului de Otorinolaringologie a Spitalului Județean de Urgență, Cluj-Napoca și 124 controale fără hiposmie și patologii ORL asociate. **Metode.** Genotiparea GSTM1 și GSTT1 a fost realizată folosind metoda Multiplex PCR, urmată de electroforeză în gel de agaroză. Varianta Ile105Val a genei GSTP1 a fost genotipată cu ajutorul tehnicii PCR-RLFP. **Rezultate.** Analiza comparativă pentru varianta Ile105Val a genei GSTP1 nu a relevat diferențe statistice semnificative între pacienți și controale ( $\chi^2 = 3.012$ ,  $p = 0.087$ ,  $OR = 1.514$ ,  $CI = 0.491 - 1.572$ ). Analiza moleculară nu a evidențiat o frecvență crescută pentru alele nule GSTT1 și GSTM1 în grupul de pacienți, comparativ cu controalele. GSTT1, (95%  $CI = 0.332 - 1.261$ ,  $p = 0.192$ ,  $OR = 0.641$ ,  $\chi^2 = 2.120$ ), GSTM1, (95%  $CI = 0.171 - 0.592$ ,  $\chi^2 = 2.017$ ,  $OR = 0.321$ ,  $p = 0.062$ ), însă au fost identificate diferențe semnificative între purtătorii ambelor alele nule (heterozigoți dublu-nuli) și riscul de a dezvolta polipoză nazală comparativ cu lotul de control ( $p = 0.0015$ ,  $OR = 4.0351$ ;  $CI = 1.706 - 9.543$ ). De asemenea s-a evidențiat un risc crescut pentru polipoză nazală și hiposmie la pacienții alergici comparativ cu cei fără alergii ( $p = 0.027$ ,  $OR = 3.455$ ,  $CI = 1.147 - 10.406$ ). **Concluzii.** Prezența concomitentă a genotipurilor nule GSTM1/GSTT1 (dublu - nule) este considerată un factor de risc pentru dezvoltarea NP și hiposmiei la persoanele alergice. Rezultatele studiului nostru nu identifică nici o corelație între polimorfismul Ile105Val al genei GSTP1, polipoza nazală și hiposmie.

**Cuvinte cheie:** GST, polimorfism, polipoză nazală, hiposmie

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## Introduction

Glutathione S-transferases (GSTs) are biological compounds with functions involved in phase II detoxification processes, inflammation, cell proliferation, oxidative stress response, carcinogenesis and tumor progression and drug resistance (1-3). GSTs isoenzymes are divided in four major classes:  $\alpha$ ,  $\theta$ ,  $\mu$  and  $\pi$  (4) and were demonstrated to have an important role in inflammatory diseases and cancer (5-7).

The lack of GSTs activity could be the result of inherited non-functional null alleles for these genes (8-10). The studies showed that approximately 50% of the Caucasian individuals could be homozygous for GSTM1 null alleles, 15-30% are homozygous for GSTT1 null alleles and 15-20% are positive for Ile105Val GSTP1 polymorphism (11-13).

It has been showed that the olfactory

epithelium exposed to the xenobiotic compounds such as odorants, airborne and environmental polluting toxic chemicals (cigarette smoke), presents a high concentration of biotransformation enzymes, therefore GSTs could play an important role in detoxification processes in this tissue (14). According recent studies, GSTs has an increased activity in rat olfactory epithelium, 5-7 times higher than in other airway tissues, suggesting an important role of these enzymes in chemoreception and sensory cell protection against chemical compounds (14).

## Aims

The aim of our study was to assess the possible relation between null genotypes for the GSTM1/GSTT1 and Ile105Val GSTP1 polymorphism in normosmic subjects and hyposmic nasal polyposis patients.

## Patients and methods

The study was designed as a case control study and comprised a group of randomly selected 75 patients with nasal polyposis (NP) all with hyposmia and 125 normosmic controls were recruited from cases admitted and investigated in the Ear, Nose and Throat (ENT) Department of the Emergency County Hospital Cluj-Napoca. All cases and controls received extensive anamnesis, general physical examination, routine biochemical blood count analysis and a complete ENT evaluation. Nasal polyposis was confirmed by anterior rhinoscopy, nasal endoscopy and sinus computerized tomography (CT). For a detailed analysis of the studied polymorphisms among patients, the study group was split in two subgroups: “de novo” polyposis and recurrent nasal polyposis associated with hyposmia. Subjective hyposmia was evaluated in a bilateral mode using the “Sniffin’ Sticks” test package (Burghardt, Wedel, Germany) (15) in all patients with nasal polyposis. The “Sniffin’ Sticks” test battery included specific tests for odor threshold, odor discrimination and odor identification with Romanian cross-cultural adaptation (16, 17).

After the initial evaluation, allergy assessment was performed in all patients using specific serologic and skin prick tests, and patients with asthma received special evaluation in the Pneumology Department.

For the specific genetic investigation a sample of 2 ml of venous blood was collected on EDTA from all patients and controls. DNA extraction and purification was performed using a commercial DNA Purification Kit (Wizard® Genomic DNA Purification Kit, Promega, MA, USA).

The study was approved by the Ethics Committee of the „Iuliu Hatieganu” University of Medicine and Pharmacy of Cluj Napoca, and all participants were provided a written informed consent.

### *GSTM1 and GSTT1 genotyping*

GSTM1 and GSTT1 allele genotyping was carried out by using a multiplex PCR protocol

(18). Briefly, the amplification was made in a total volume of 25 µl reaction mixture containing 12.5 µl 2xPCR Master Mix (Thermo Fischer Scientific Inc., MA, USA), 10 pmoles of each forward and reverse specific primers and free nucleases water to 25 µl. For GSTM1 and GSTT1 null allele identification we used 3 pairs of primers (Eurogentec, Belgium) and therefore we obtained 3 different amplified fragments, one of 215 bp (for GSTM1), one of 480 bp (for GSTT1) and a 268 bp fragment (for the amplification of a β Globin gene control fragment). Thermocycling conditions consisted of denaturation at 94°C for 5 minutes and then 35 repetitive cycles of initial denaturation at 94°C of 1 minute each, primers annealing for 1 minute at 58°C, elongation at 72°C for 1 minute and then a 10 minute final polymerization at 72°C. (Mastercycler Gradient®, Eppendorf, Germany).

The resulted amplification products were then analyzed by agarose 2% gel electrophoresis (MetaPhor® Agarose, Cambrex Bio Science Inc.). The absence of the amplification specific products demonstrated the presence of the null genotypes. This protocol easily identifies the GSTT1 and GSTM1 homozygous null genotypes but cannot distinguish between homozygous and heterozygous positive state for GSTT1 and GSTM1 genes.

### *GSTP1 genotyping*

The GSTP1 Ile105Val gene variant was analyzed using a PCR-RFLP method as described (18). DNA was amplified in a mixture containing the same reagents as previously described for GSTM1 and GSTT1 amplification. Amplification was carried out in the following conditions: 5 minute of an initial denaturation at 95°C and 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds than a final polymerization for 5 minute at 72°C.

The amplification products were digested overnight with 5 units of BsmAI enzyme (Thermo Fischer Scientific Inc., MA, USA) and the resulting DNA fragments then separated on a 3% agarose gel (MetaPhor® Agarose, Cambrex Bio

Table 1. Association between GST genotypes, NP and hyposmia risk in patients and controls

	NP/hyposmia (n=75)	Control (n=124)	NP/asthma (n=23)	NP/non-asthma (n=52)	NP/allergy (n=26)	NP/non -allergy (n=49)	NP/de novo (n=38)	NP/recurrent (n=37)
<b>GSTM1</b>	$\chi^2 = 2.017, p = 0.062$ OR = 0.321 CI = 0.171 to 0.592		OR=0.688, CI=0.211-0.218		OR=0.981, CI=0.332-2.861		OR=1.36, CI=0.48-3.18	
Present	55 (73%)	58 (47%)	18 (78%)	37(71%)	19 (73%)	36 (73%)	29 (76%)	26 (70%)
Null	20 (27%)	66 (53%)	5 (22%)	15 (29%)	7 (27%)	13 (27%)	9 (24%)	11 (30%)
<b>GSTT1</b>	$\chi^2 = 2.120 p = 0.192,$ OR = 0.641 CI = 0.332 to 1.261		OR=0.461, CI=0.161-1.342		OR=3.421, CI=0.100-1.151		OR=0.84, CI=0.30-2.31	
Present	21 (28%)	25 (20%)	9 (39%)	12 (23%)	4 (15%)	17 (35%)	10 (26%)	11 (30%)
Null	54 (72%)	99 (80%)	14 (61%)	40 (77%)	22 (85%)	32 (65%)	28 (74%)	26 (70%)
<b>GSTP1</b>	$\chi^2 = 3.012, p = 0.087,$ OR = 1.51, CI = 0.491 to 1.572		OR=1.462, CI=0.541-3.952		OR=0.731, CI=0.280-1.937		OR=0.480, CI=0.18-1.22	
Ile/Ile	42 (56%)	66 (53%)	10 (43%)	31 (60%)	12 (46%)	28 (57%)	18 (47%)	23 (62%)
Ile/Val	28 (37%)	51 (41%)	10 (43%)	19 (36%)	11 (42%)	19 (39%)	18 (47%)	11 (30%)
Val/Val	5 (7%)	7 (6%)	3 (14%)	2 (4%)	3 (12%)	2 (4%)	2 (6%)	3 (8%)

\*Statistic significant for  $p < 0.05$

**Table 2. Nasal Polyposis associated hyposmia risk evaluation for GSTP1 gene, Ile105Val polymorphism  
Fisher Test (Dominant model - Ile/Val+Val/Val vs. Ile/Ile)**

Genotype	$\chi^2$	p	Odd ratios	95% CI	
				Inferior limit	Superior limit
GSTP1 Ile <sup>105</sup> Val	0.032	0.523	1.645	1.000	2.876

**Fisher Test (Recessive model - Val/Val vs. Ile/Val + Ile/Ile)**

Genotype	$\chi^2$	P	Odd ratios	95% CI	
				Inferior limit	Superior limit
GSTP1 Ile <sup>105</sup> Val	0.028	0.723	0.784	0.512	1.651

**Table 3. Association between GSTM1/GSTT1 double - null genotypes, NP and hyposmia risk  
in patients and controls**

Study group	p	Odd ratios	95% CI	
			Inferior limit	Superior limit
Patients/control	0.015*	4.035	1.706	9.543
De novo/recurrent NP	0.635	0.778	0.277	2.190
Asthma/no asthma NP	0.406	0.632	0.213	1.867
Allergic/non allergic NP	0.027*	3.455	1.147	10.406

\*Statistic significant for  $p < 0.05$ .

Science Inc.) .The homozygous Ile/Ile genotype is corresponding to undigested 176 bp fragment. The complete digestion of the amplified product results in two fragments of 91 and 85 bp and defines the presence of the homozygous Val/Val genotype, while the presence of all three fragments (176, 91 and 85 bp), highlights the presence of the Ile/Val heterozygous genotype.

#### Statistical analysis

For statistical analysis we used the SPSS 18.0 for Windows software (SPSS, Inc, Chicago, IL., USA).

Odds ratio (OR) assessment with 95% confidence limits were calculated by logistic regression for proper statistical analysis. GSTM1 and GSTT1 genotypes were classified as homozygous deletion (null alleles) or not deleted. P value was also calculated for a more accurate risk evaluation. Ile105Val variant of GSTP1 gene statistical assessment was also evaluated by logistic regression and quantified by odds ratio (OR) with 95% confidence limits and fol-

lowed by comparative analysis according to dominant and recessive models.

#### Results

Statistical analysis did not reveal an increased frequency for GSTM1 and GSTT1 null genotypes in the study group compared to controls. (GSTM1  $\chi^2 = 2.017$ ,  $p = 0.062$ , OR = 0.321 CI = 0.171 to 0.592, GSTT1  $\chi^2 = 2.120$   $p = 0.192$ , OR = 0.641 CI = 0.332 to 1.261). Also, comparative analysis for Ile105Val variant of GSTP1 gene revealed no statistical differences between patients and controls ( $\chi^2 = 3.012$ ,  $p = 0.087$ , OR = 1.514, CI = 0.491 to 1.572) (Table 1). Molecular analysis of GSTP1 Ile105Val using the Fisher test by autosomal recessive model (variant homozygous genotype vs. heterozygous and common homozygous genotype) and autosomal dominant model (variant homozygous genotype and heterozygous vs. common homozygous genotype) reveals no statistically significant differences

between patients group compared to the control group ( $\chi^2=0.028$ , OR= 0.784, CI= 0.512-1.651,  $p=0.723$ ;  $\chi^2=0.032$ , OR=1.645, CI=1-2.876,  $p=0.523$ ) (Table 2).

No statistical differences were found between de novo nasal polyposis hyposmic patients and recurrent nasal polyposis hyposmic patients for GSTT1/GSTM1 null genotypes (GSTM1 OR=1.36,  $p=0.554$ , CI=0.48-3.18, GSTT1 OR=0.84,  $p=0.742$ , CI=0.30-2.31 and Ile105Val GSTP1, OR=0.480,  $p=0.124$ , CI=0.18-1.22).

There were no statistical significant differences between asthma diagnosed patients when compared to non-asthmatic patients regarding the GSTT1/GSTM1 null genotypes (GSTM1, OR=0.680,  $p=0.522$ , CI=0.215-2.182, GSTT1, OR=0.466,  $p=0.157$ , CI=0.162-1.343) and Ile105Val GSTP1 with OR=1.466,  $p=0.448$ , CI=0.544-3.943).

The same non-significant differences were highlighted allergic and non-allergic individuals (GSTM1, OR=0.980,  $p=0.970$ , CI=0.334-2.868; GSTT1, OR=0.342,  $p=0.084$ , CI=0.101-1.155 and Ile105Val GSTP1 with OR=0.738,  $p=0.537$ , CI=0.282-1.932).

When combined GSTM1 and GSTT1 null genotype (double-null genotype) was compared between patients and controls, significant statistical differences were found ( $p=0.0015$ , OR=4.0351; CI=1.706-9.543). Statistical analysis for combined GSTM1/GSTT1 null genotypes for de novo/recurrent polyposis and respectively asthmatic/non-asthmatic patients revealed no statistical significant differences ( $p=0.635$ , OR=0.778, CI=0.277-2.190;  $p=0.406$ , OR=0.631, CI=0.213-1.867). Although, statistical differences were highlighted when allergic NP patients were compared to non-allergic NP patients ( $p=0.027$ , OR=3.455, CI=1.147-10.406) (Table 3).

## Discussions

GSTs are an important family of enzymes involved in detoxification of several xenobiotics, so this mechanism protects tissues from the harm-

ful effects of oxidative stress, and therefore against chemically induced tissue damage (5, 11, 18). Nasal polyposis and associated hyposmia are considered to be multifactorial disorders (19); multiple factors could be involved in the etiopathogeny of these two related conditions. Although scientific studies demonstrated that individuals with nasal polyposis associate low levels of blood and therefore decreased mucosal tissue antioxidants levels (20), there is no valid data to prove that GSTs are involved in nasal polyposis and hyposmia development. According to statistical analysis our study revealed that there are no significant differences between hyposmic patients with nasal polyposis and controls for GSTM1 and GSTT1 null alleles and Ile105Val GSTP1 polymorphism. Our results are in agreement with another study evaluating the same genetic variants (11). Although comparative analysis did not reveal statistical differences between patient group and controls, GSTM1 null genotypes were more frequent in hyposmic patients with nasal polyposis than in controls. Scientific studies showed that GSTP1 gene polymorphism could influence on the one hand the metabolic activation and also the detoxification of xenobiotics and is therefore, a link between the gene variants and individual susceptibility to different inflammatory disorders (21, 22) but only few studies investigated the relationship between Ile105Val GSTP1 polymorphism, nasal polyposis and hyposmia. The results of our study show no correlation between this genetic variant, nasal polyposis and hyposmia, in agreement with one of the few published researches on this subject (23).

Comparative analysis of GSTM1/GSTT1 null alleles and Ile105Val GSTP1 polymorphism among patients with "de novo" polyposis and recurrent nasal polyposis associated with hyposmia, revealed no statistical differences between these two groups.

Recent studies demonstrated that polymorphism of GST genes are associated with asthma (5, 24, 25) although other studies could not establish an association between these genetic variants and patients with nasal polyposis

and asthma (26, 27). We evaluated the distribution of GSTT1/GSTM1 null alleles and Ile105Val GSTP1 in patients with nasal polyposis and associated allergy or asthma compared to non-allergic patients. There were no statistical significant differences in genotypes distribution between allergic and non-allergic subjects; the same results were highlighted for genetic variants above when compared asthma diagnosed patients to non-asthmatic patients.

An important finding for our study is that patients presenting both GSTM1 and GSTT1 null genotypes (double null genotypes) are more likely predisposed to develop nasal polyposis and hyposmia when compared to controls. Statistical analysis for combined GSTM1/GSTT1 null genotypes for allergic NP patients compared to non-allergic NP patients revealed significant differences; therefore according the results of our study, combined GSTM1/GSTT1 null genotypes could be considered risk factors for NP and hyposmia development in allergic individuals.

Nasal polyposis (NP) is a complex disease with a pathophysiology that is likely to be influenced by multiple environmental and genetic factors (28). Although several studies proved that some genetic polymorphisms (SNPs) are likely to influence the occurrence of NP (29) and hyposmia, no single gene or genetic variant has been shown to be uniquely related to nasal polyposis and hyposmia (30), therefore future studies are needed to identify key genes and control mechanisms involved in NP and hyposmia etiopathogenesis.

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**Conflict of interest** . None to declare.

## Abbreviation list

ENT - ear, nose, throat  
 GST - Glutathione S transferase  
 GSTM1- Glutathione S transferase Mi 1  
 GSTT1 - Glutathione S Transferase T1  
 GSTP1 - Glutathione S transferase Pi 1  
 NP - Nasal Polyposis  
 Ile - Isoleucine  
 Val - Valine  
 PCR - Polymerase Chain Reaction  
 RFLP - Restriction Fragment Length Polymorphism

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