

Genetic polymorphisms by deletion in genes that encode for glutathione S-transferases are associated with nicotine dependence and tobacco use-related medical disorders

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Abstract

OBJECTIVE: We examine the relationship between nicotine dependence (ND) and ND-related medical disease and polymorphisms by deletion in genes that encode glutathione S-transferases (GSTs), e.g. *GSTM1* and *GSTT1*. Individuals with homozygous gene deletions show deficiencies in GSTs enzyme activities impairing detoxification.

METHODS: This study comprised 182 tobacco users and 182 controls (never-smokers). *GSTM1* and *GSTT1* polymorphisms were assessed using a Multiplex-PCR based protocol.

RESULTS: Logistic regression analyses showed a significant association between ND and the *GSTM1* and *GSTT1* null genotypes. There were no significant associations between *GSTT1*, *GSTM1* and *GSTT1/M1* genetic variants and the Fagerström test for ND, age at onset, smoking cessation or a family history of ND. Patients with ND had increased rates of a family ND history and an increased prevalence of cardiovascular disease, hypertension, and lung disease. The null genotypes were associated with hypertension (i.e. *GSTT1* × ND interaction), diabetes type 2 (i.e. *GSTM1* × *GSTT1* interaction), lung disease (i.e. *GSTM1* × ND interaction) and cancer (i.e. *GSTT1*). The results show that GST null genotypes may confer protection against ND while they increase risk towards ND-related medical disorders.

CONCLUSION: We describe a new hypothesis that links the effects of null and wild *GST*s genotypes on peripheral detoxification and oxidative stress processes with the central effects of reward and other gene variants as risk factors for ND and tobacco-related medical disease.

INTRODUCTION

Nicotine dependence leads to increased risk of mortality (Ezzati & Lopez 2003) and is one of the major risk factors for multiple chronic diseases (Gellert *et al.* 2012). Medical consequences of tobacco use often begin when users are in their 40s and usually become more debilitating over time. The most common psychiatric co-morbidities are alcohol and other substance use disorders (American Psychiatric Association 2013). Genetic studies suggest that nicotine dependence is a complex behavior that includes vulnerability to initiation, continued use, dependence, cessation and relapse. Smoking behavior studies have focused on genes in neurotransmitter pathways, which modulate drug reward circuits and nicotine metabolism (Belsky *et al.* 2013; Hall *et al.* 2002; Ho & Tyndale 2007; Li & Burmeister 2009).

Cigarette smoke contains several thousands of compounds many of which are toxic or activate immune-inflammatory, oxidative and nitrosative stress (IO&NS) pathways. Glutathione S-transferases (*GST*s) are important Phase II enzymes involved in detoxification of toxic compounds with different chemical structures found in cigarette smoke (Hayes & Strange 2000; Saadat & Mohabatkar 2004). Moreover, *GST*s are antioxidant enzymes that protect against lipid peroxidation, remove reactive oxygen species and regenerate S-thiolated proteins, both being consequences of oxidative stress (Zivkovic *et al.* 2014; Morris *et al.* 2014). Genetic polymorphisms by deletion in genes that encode *GST*s, such as the polymorphisms in Mu (*GSTM1*) and Theta (*GSTT1*) classes, with non-functional null alleles, may be associated with reductions in corresponding enzymatic activity (Hayes & Strange 2000). The *GSTM1* and *GSTT1* genes are highly polymorphic in the human population, with variations of up to 60% and 38% of deletion frequency, respectively (Rebbeck 1997). The *GSTM1/GSTT1* null genetic polymorphisms increase the risk toward tobacco use-related disorders, such as coronary heart disease (Abu-Amero *et al.* 2006; Kim *et al.* 2008; Li *et al.* 2000; Manfredi *et al.* 2007; Masetti *et al.* 2003; Olshan *et al.* 2003; Singh *et al.* 2011; Tamer *et al.* 2004; Wang *et al.* 2010), lung diseases (He *et al.* 2004), cerebrovascular disease (Um *et al.* 2003; Um *et al.* 2006) and cancers (Grando *et al.* 2009; Rebbeck 1997). Hypertension is associated with the *GSTT1* null genotype (Petrovic & Peterlin 2014). Other studies, however, were unable to detect a significant association between *GSTM1/GSTT1* null genotypes and risk of ischemic vascular disease (Norskov *et al.* 2011) and other types

of cancer (Losi-Guembarovski *et al.* 2008; Rodrigues *et al.* 2011). Furthermore, *GST*s genetic polymorphisms may be associated with activated IO&NS pathways and therefore with tobacco-related and IO&NS-related-diseases (Kim *et al.* 2006; Zivkovic *et al.* 2014).

The purpose of this study was to investigate the association between polymorphisms in *GSTT1* and *GSTM1* genes and the susceptibility to tobacco use disorders and smoking cessation. These polymorphisms were examined because these genes are commonly studied in tobacco-related medical disorders and modulate the metabolism of endogenous (e.g. IO&NS pathways) and exogenous (e.g. toxic cigarette smoke) compounds (Hayes & Strange 2000), which play a role in nicotine dependence. Some genotypes may be disease modifying, while polymorphic deletions result in non-functional genes and confer impaired catalytic activity, e.g. the *GSTM1* and *GSTT1* null genotypes (Hayes & Strange 2000).

METHODS

Study sample

In this cross-sectional, case-control study, patients with current nicotine dependence (n=182, the cases) were recruited from outpatients at the Center of Approach and Treatment for Smokers, a smoking cessation program at UEL (Londrina State University), Paraná, Brazil. All cases met DSM-IV-TR nicotine dependence criteria (APA 2000). One hundred and eighty two never-smokers (the control group) were recruited from staff at UEL. Never-smokers were defined as people who had never smoked any cigarette (US Centers of Disease Control and Prevention (CDC), 2010). Our patients with nicotine dependence were all current smokers who had smoked at least 100 cigarettes during their lifetime and, at the time of interview, reported smoking every day or some days (US CDC 2011). The study was conducted from March 2011 to July 2012. Inclusion criteria for all participants were being aged 18–65, normal cognitive function, having completed the interview and completed the genotyping.

In our patients with nicotine dependence, treatment is usually delivered in a group of 10–15 participants. The participants first receive an individualized assessment with a physician and then attend four weekly group sessions (cognitive therapy), each lasting about 1½ hour. This is followed by two biweekly sessions and then by monthly sessions for a period of 52 weeks. Parallel to the group sessions, tobacco users also receive pharmacological intervention and monitoring through individual visits if needed. Pharmacological treatment comprised bupropion and nicotine replacement therapy used in accordance with the guidelines of the Ministry of Health of Brazil (Ministério Da Saúde 2004). The combined program of non-pharmacological and pharmacological treatment is effective for both genders (Odebrecht Vargas Nunes *et al.* 2013). The Ethics

Research Committee at UEL approved this project (approval number: 035/2013) and all subjects had given written informed consent to participate in the study.

Measurements

Socio-demographic and clinical measurements

Smoking status, clinical information, such as, medical history of tobacco-related diseases, i.e. lung disease (chronic obstructive pulmonary disease and chronic bronchitis), cardiovascular disease (coronary heart disease), hypertension, cancer, and diabetes type 2; and socio-demographic data were obtained through an interviewer-administered structured questionnaire. Body mass index (BMI) was calculated as weight (Kg) divided by square of height in meters (m²).

Nicotine dependence

The diagnosis of nicotine dependence was made using the Structured Clinical Interview (SCID) for DSM-IV, axis I (American Psychiatric Association 2000) translated into Portuguese and validated (Del-Ben *et al.* 2001).

Fagerström test for Nicotine Dependence

The Fagerström test for Nicotine Dependence (FTND), revised by Fagerström and Schneider (1989), was administered to all subjects with nicotine dependence in a translated Portuguese version validated for the Brazil population (Carmo & Pueyo 2002; Storr *et al.* 2005).

Lifetime cigarette consumption (pack-years)

The pack-years were calculated by the number of cigarettes smoked per day divided by 20 and multiplied by the number of years smoked. The degree of tobacco smoke exposure as measured by pack-years is associated with increased risk to tobacco-related medical disease or lowered survival time (Kim *et al.* 2014; Janjigian *et al.* 2010).

Family history of smoking

The family history of nicotine dependence consisted of reports of the smoking history of first-degree family members.

The Alcohol, Smoking and Substance Involvement Screening Test (ASSIST)

We used the ASSIST questionnaire to screen for risk of alcohol and sedatives in adults. Patients with nicotine dependence whose alcohol involvement scores were between 0 and 10 (low risk) and between 11 and 26 (moderate risk) were offered a brief intervention and those with scores higher than 27 (high risk) were offered an intensive treatment program. Patients with nicotine dependence whose sedative use scores were between 0 and 3 (low risk) and those with scores between 4 and 26 (moderate risk) were offered a brief intervention, while smokers whose score was 27 or more (high risk

of harm and substance dependence) were offered intensive intervention (WHO 2002).

Smoking exhaled carbon monoxide (CO)

Smoking status and smoking cessation were evaluated using exhaled carbon monoxide (CO_{EXH}). CO_{EXH} was measured using a Micro CO Meter with an electrochemical sensor (Micro CO – Micro Medical Ltd, Rochester, Kent, UK). All participants were instructed to breathe deeply and to hold their breath for 20 seconds and then to exhale slowly and completely through a mouthpiece. The CO_{EXH} levels were dichotomized as more than 6 ppm (smoking) and ≤6 ppm (non-smoking) (Middleton & Morice 2000). In patients with nicotine dependence, smoking cessation was diagnosed when CO_{EXH} concentrations 52 weeks after inclusion in the treatment programs were ≤ 6 ppm.

Genotyping

Genomic DNA was extracted from 200 µL of peripheral blood cells using the Biopur Kit (Biometrix Diagnostic, Curitiba, Brazil) according to the manufacturer's instructions. After precipitation with ethanol, the DNA pellet was re-suspended in 50 µL of Biopur Kit specific buffer, quantified by spectrophotometry, and stored at -80 °C for later use in genotyping analyses. The genetic polymorphisms were detected using a multiplex PCR (polymerase chain reaction) protocol (Abdel-Rahman *et al.* 1996) with modifications: 80–100 ng of DNA were amplified in a total volume of 25 µL containing 10% of buffer reaction (20 mM Tris-HCl; 50 mM KCl); 2 mM MgCl₂; 2 mM of deoxynucleotide triphosphate; 10 pmol of each primer and 1.25 U of AmpliTaq DNA polymerase. PCR was carried out in a PTC-100 Thermocycler (MJ Research, Inc), after 5 minutes of pretreatment at 94 °C, 30 cycles of 1 minute at 94 °C, 1 minute at 59 °C, and 30 seconds at 72 °C, followed by 5 minutes at 72 °C. The PCR products were analyzed by electrophoresis on 10% acrylamide gel and detected by a non-radioisotopes technique using a commercially available silver staining method.

The genotype was coded according to *GSTM1/GSTT1* genetic polymorphisms: 1) *GSTT1* present and *GSTM1* absent or *GSTT1* absent and *GSTM1* present (at least one gene deleted), 2) both genes present, and 3) both genes deleted. The absence of a 215 base pair (bp) fragment in the electrophoretic profile indicates the *GSTM1* null genotype and the absence of a 480 bp fragment indicates the *GSTT1* null genotype. A fragment of 312 bp related to a non-polymorphic fragment of the *CYP1A1* (Cytochrome P450 1A1) gene was used as an internal control in all reactions. Negative controls using ultra pure water were included in each reaction. The two genes were always analyzed together in a multiplex PCR protocol. Analyses considering individuals who carried the two deletions simultaneously were performed (Widersten *et al.* 1991). The protocol used for genotyping also analyzes a non-polymorphic internal

reaction control (CYP1A1 – 312bp), which assures full effectiveness and reliability of the PCR reaction, precluding the need for genotyping individuals more than once.

Statistical analyses

The genotype was coded according to genetic polymorphisms: 1: *GSTT1* null (deleted), *GSTM1* null (deleted) and *GSTM1/GSTT1* both genes null (deleted) versus 0: the wildtypes. The analysis model takes into account people who have one of the deletions, both deletions or with normal genotype (both functional genes). The reason for this is that individuals with both deletions present a lower enzymatic efficiency compared to those with only one deletion or the wildtypes (see Introduc-

tion). The associations between the wildtype (present) or variant (deleted) genes and other categorical variables (e.g. patients versus controls, gender, etc) were examined through univariate statistical tests, e.g. analyses of contingency tables with computation of the Odds Ratio (OR) with lower and upper 95% confidence intervals (CI). Power analysis showed that using a power of 0.82 and an effect size of 0.12 the total sample size should be 360. Bivariate logistic regression analyses (automatic or with forced entry of explanatory variables) were used to define the associations between nicotine dependence (the never-smoking group being the reference group) and the GSTs genes (including the interaction *GSTM1* × *GSTT1*), while adjusting for other explanatory variables, including ethnicity, age, gender, years of education, etc. The logistic regression coefficients of the independent variables were employed to estimate odds ratios with 95% CIs. Relationships between the GSTs genes and continuous variables were examined using analyses of variance (ANOVAs). Relationships between continuous characteristics (e.g. cigarettes/day, age at onset, etc) and explanatory variables (including genetic variants and environmental factors) were explored using generalized linear model (GLM) analyses. We tested normality of distribution using the Kolmogorov-Smirnov goodness-of-fit test. Statistical analyses were performed using SPSS (Version 20). All tests were two-tailed and a *p*-value of 0.05 was used for statistical significance.

Tab 1. Socio-demographic and clinical characteristics of patients with nicotine dependence and never-smokers.

Clinical and Demographic Characteristics	Nicotine dependence		Never-Smokers		<i>p</i> -value
	N	%	n	%	
Gender					0.319
Male	66	(36.3)	57	(31.3)	
Female	116	(63.7)	125	(68.7)	
Marital Status					0.028*
Stable relationship	109	(59.8)	129	(70.8)	
Other (Single/Divorced/Separated)	73	(40.2)	53	(29.2)	
Ethnicity					0.182
Caucasian	127	(69.8)	127	(69.8)	
African	18	(9.9)	17	(9.3)	
Asian	4	(2.2)	12	(6.6)	
Mixed	33	(18.1)	26	(14.3)	
Family history of smoking	157	(86.3)	135	(74.2)	0.004*
Alcohol (ASSIST) ¹					0.000*
Low risk	23	(12.6)	0	(0)	
Moderate risk	6	(3.3)	0	(0)	
Sedative (ASSIST) ¹					0.000*
Low risk	13	(7.2)	0	(0)	
Moderate risk	0	(0)	0	(0)	
Diabetes	13	(7.5)	9	(5.0)	0.329
Cardiovascular diseases	25	(14.4)	13	(7.2)	0.029*
Blood hypertension	55	(31.6)	36	(19.9)	0.011*
Lung diseases	36	(20.7)	17	(9.4)	0.003*
Cancer	9	(5.2)	4	(2.2)	0.137
BMI >30 ²	41	(22.5)	30	(16.5)	0.146

The association between the socio-demographic and clinical characteristics and the two groups is based on Pearson Chi-square test. * Indicates statistically significant difference at *p*<0.05. ¹ There were no 'high risk' participants; ² BMI - Body Mass Index (kg/m²).

RESULTS

Socio-demographic and clinical characteristics of nicotine dependence

In individuals with nicotine dependence, the mean age at onset of regular smoking was 14.9 years (± 4.1 ; mean \pm standard deviation (SD)), duration of nicotine dependence: 33.0 years (± 11.4), mean daily cigarette consumption: 22.3 (± 13.2) cigarettes per day, lifetime cigarette consumption: 36.9 (± 26.8) pack-years, mean FTND score: 5.8 (± 2.2), and mean number of attempts at smoking cessation: 1.7 (± 0.7).

No *p*-corrections were used to check the multiple statistical analyses performed on the variables shown in Table 1 and 2 because the results of these univariate tests were employed to delineate the explanatory variables to be used subsequently as determinants of independent association with nicotine dependence in the ultimate logistic regression analyses (e.g. Table 3). Table 1 shows that there were no significant associations between nicotine dependence and gender and ethnicity. Patients with nicotine dependence (the cases; mean age \pm SD =48.5 \pm 10.2 years) were significantly (*F*=7.3, *df*=1/362, *p*=0.007) older than never-smokers (the controls; mean=45.9 \pm 7.8 years). Never-smokers showed more individuals with stable relationships compared to patients with nicotine dependence. Smokers showed a significantly higher rate of a positive family history of nicotine dependence and a significantly

increased risk of alcohol and sedative use. Patients with nicotine dependence also showed a higher prevalence of cardiovascular disease, blood hypertension and lung disease, but not diabetes and cancer. There were no significant differences in body mass index (BMI in kg/m²) between the two groups. Cases (mean ±SD years of education = 9.7±5.3 years) showed a significantly lower level of education (F=135.2, df=1/362, p<0.001) than controls (mean=16.0±4.9 years).

GSTs genetic polymorphisms and nicotine dependence

Table 2 shows the frequencies of GSTT1 and GSTM1 genotypes in patients with nicotine dependence versus never-smokers. In this univariate analyses there were no significant associations between nicotine dependence and GSTT1 or the GSTM1/GSTT1 combined genes. There was a marginal association between GSTM1 genotype and nicotine dependence.

Table 3 shows the results of logistic regression analyses with nicotine dependence as dependent variable (never-smokers being the reference group) and GSTM1 null genotype, age, gender, years of education, ethnicity, marital status and history family of smoking as explanatory variables. We found that 3 variables were significantly associated with nicotine dependence (χ²=146.98, df=9, p<0.001, Nagelkerke = 0.44): the GSTM1 null genotype and education level show protective effects, whereas a family history of tobacco use disorder is a risk factor. The other variables did not reach significance. Forced entry of the GSTT1 null (Wald=3.60, df=1, p=0.058) and GSTM1/T1 null (Wald=3.1, df=1, p=0.051) genotypes instead of GSTM1 showed that there was a trend towards a significantly association with tobacco use disorder. The interaction GSTM1 and GSTT1 was also not significant.

Table 4 shows the results of an automatic logistic regression analysis with nicotine dependence as dependent variable and all variables listed in Table 3 together with GSTT1 null genotypes and the interaction GSTM1 × GSTT1 as explanatory variables. We found that 5 variables significantly predicted nicotine dependence: GSTM1 null, GSTT1 null, stable relationship and years of education show protective effects, while a family history of smoking increases the odds of nicotine dependence (χ² =146.88, df=5, p<0.001, Nagelkerke = 0.44).

Table 5 shows the GSTs genotypes in association with the smoking characteristics in patients with nicotine dependence. There were no significant associations between GSTT1, GSTM1 and GSTM1/GSTT1 genes and the onset of nicotine dependence, duration of illness, cigarettes/day and pack years. There was also no significant association between the genetic polymorphisms and the FTND scale, cessation of smoking after 52 weeks, or family history of smoking. GLM analyses also did not show any relationship between the continuous smoking characteristics (onset of tobacco use disorder, duration of illness, cigarettes/day and pack years, FTND scale) and the genotype polymorphisms

Tab. 2. GSTM1/GSTT1 genetic polymorphisms assessments in patients with nicotine dependence and never-smokers.

Characteristics	Nicotine dependence (n=182)		Never-smokers (n=182)		p-value	OR (95% CI)
	n	%	N	%		
GSTT1/GSTM1 genes						
Wildtype	160	87.9	152	83.5	0.23	0.70 (0.38-1.26)
Null genotypes	22	12.1	30	16.5		
GSTT1 gene						
Wildtype	140	76.9	130	71.4	0.23	0.86 (0.66-1.10)
Null genotype	42	23.1	52	28.6		
GSTM1 gene						
Wildtype	75	41.2	57	31.3	0.05	0.65 (0.42-1.00)
Null genotype	107	58.8	125	68.7		

GSTM1- glutathione S-transferases M1 gene; GSTT1- glutathione S-transferases T1 gene.

All results of Pearson Chi-square test for categorical variables * indicates statistically significant association at p<0.05

Tab. 3. Logistic regression analysis of nicotine dependence as dependent variable (never-smokers as reference group).

Logistic Regression Analysis	Wald	df	p-value	OR	CI (95%)	
					Lower	Upper
GSTM1 null genotype	7.32	1	0.007	0.469	0.271	0.812
Years of education	69.9	1	<0.001	0.746	0.69	0.79
Ethnicity	1.75	1	0.185	1.634	0.79	3.37
Marital status	7.35	1	0.087	0.471	0.27	0.81
Age	0.20	1	0.651	1.007	0.97	1.03
Gender	0.69	1	0.404	1.262	0.73	2.18
Family history of smoking	4.30	1	0.038	1.989	1.03	3.81

OR: odds ratio; CI: 95% confidence intervals; df: degrees of freedom.

Tab. 4. Automatic logistic regression analysis of nicotine dependence as dependent variable (and never-smokers as reference group).

Logistic Regression Analysis	Wald	df	p-value	OR	CI (95%)	
					Lower	Upper
GSTM1 null genotype	7.48	1	0.006	0.47	0.27	0.81
GSTT1 null genotype	4.61	1	0.032	0.52	0.28	0.94
Years of education	73.7	1	<0.001	0.75	0.69	0.79
Marital status	6.40	1	0.011	0.50	0.29	0.85
Family history of smoking	4.60	1	0.032	2.02	1.06	3.82

OR: odds ratio; CI: confidence interval; and df: degrees of freedom.

after considering the effects of the abovementioned explanatory variables.

GSTs genotypes and medical disorders

Table 6 shows the results of different automatic binary logistic regression analyses with the 5 medical illnesses as dependent variables (no medical illness as reference group) and the following explanatory variables: age, gender, nicotine dependence, and *GSTT1* null, *GSTM1* null and *GSTM1/GSTT1* null, the interaction between *GSTT1* null × *GSTM1* null, and interactions between *GSTT1* null or *GSTM1* null by nicotine dependence. There was a significant association between hypertension and age and the *GSTT1* null genotype or even better with age and the interaction between the *GSTT1* null genotype × nicotine dependence. Diabetes was sig-

nificantly associated with increasing age and the interaction term *GSTT1* null × *GSTM1* null genotypes. Lung disease was significantly associated with the interaction between the *GSTM1* null genotype × nicotine dependence. Cancer was significantly associated with the *GSTT1* null genotype. Cardio-vascular disorder was only associated with increasing age.

DISCUSSION

The major finding of this study is that the *GSTM1* null and the *GSTT1* null genotypes were inversely associated with nicotine dependence. To the best of our knowledge this is the first study reporting nominal evidence of an association between nicotine dependence and the *GSTM1* null and the *GSTT1* null genotypes. One previous study reported on *GSTM1*, *GSTT1* and *GSTT1/GSTM1* gene polymorphisms in association with nicotine dependence using univariate statistical analyses (Saadat & Mohabatkar 2004). These authors were unable to detect significant associations between those genetic polymorphisms and nicotine dependence. The discrepancies between our results and the results of Saadat and Mohabatkar (2004) may be explained by differences in statistical analyses (univariate versus multivariate) and by differences in ethnicity (an Iranian versus a Brazilian population). Moreover, we specified nicotine dependence according to DSM-IV-TR criteria and never-smokers according to CDC criteria, whereas Saadat and Mohabatkar (2004) did not use any specified instrument to diagnose their cases and controls.

We found that the *GSTM1* and *GSTT1* null genotypes (both protective), a family history of smoking (a risk factor), marital status and years of education were the most significant independent predictors of nicotine dependence, while gender, age and ethnicity were not significantly associated with nicotine dependence. Thus, a positive family history of nicotine dependence was an important risk factor indepen-

Tab. 5. Associations between *GSTM1/GSTT1* genetic polymorphisms and smoking characteristics in patients with nicotine dependence.

Smoking characteristics	<i>GSTT1/GSTM1</i>	<i>GSTT1</i>	<i>GSTM1</i>
	p-value	p-value	p-value
Age at onset of tobacco use*	0.87	0.65	0.98
Years of smoking*	0.21	0.60	0.37
Cigarettes/day*	0.38	0.80	0.32
Pack/Years*	0.91	0.88	0.72
Fagerström score	<6	0.90	0.35
	>6		
Ceased Smoking (week 52)	Yes	0.19	0.16
	No		
Family history of smoking	Yes	0.49	0.69
	No		

GSTM1: glutathione S-transferase M1 gene; *GSTT1*: glutathione S-transferase T1 gene. The p-values are based on the Pearson Chi-square test for categorical variables or *on analyses of variance.

Tab. 6. Results of 5 different automatic binary logistic regression analyses with medical illnesses as dependent variables (reference group: no medical illness).

Dependent variables	Significant explanatory variables	Wald	df	p-value	Odds ratio	95% CI	X ² *	df	p-value
Hypertension	Age	12.06	1	0.001	1.07	1.03–1.11	23.34	2	<0.001
	<i>GSTT1</i>	7.59	1	0.006	2.94	1.37–6.34			
Hypertension	Age	27.61	1	<0.001	1.09	1.06–1.13	48.13	2	<0.001
	ND × <i>GSTT1</i>	10.36	1	0.001	3.28	1.59–6.77			
Diabetes	Age	9.57	1	0.002	1.09	1.03–1.15	10.12	2	0.001
	<i>GSTM1</i> × <i>GSTT1</i>	4.36	1	0.037	2.65	1.06–6.60			
Lung disease	ND × <i>GSTM1</i>	11.52	1	0.001	2.81	1.55–5.11	11.26	1	0.001
Cancer	<i>GSTT1</i>	7.59	1	0.006	4.99	1.59–15.68	7.76	1	0.006
CVD	Age	5.33	1	0.021	1.05	1.01–1.09	5.64	1	0.018

CVD: cardio-vascular disorder; The explanatory variables were: age, gender, nicotine dependence (ND), the genetic polymorphisms: *GSTT1* null (deleted), *GSTM1* null (deleted) and *GSTM1/GSTT1* both genes null (deleted); the interaction *GSTT1* null X *GSTM1* null, and interactions ND X *GSTT1* null or ND X *GSTM1* null. * Omnibus tests of model coefficients.

dent from the effects of *GSTM1* and *GSTT1* genetic polymorphisms, while there were no significant associations between the *GSTM1*, *GSTT1* and *GSTM1/T1* genotypes and a history family of smoking. Heritability and low educational levels are thought to be associated with nicotine dependence and difficulties with smoking cessation (American Psychiatric Association 2013). Genetic factors contribute to smoking behavior from initiation through smoking quantity, nicotine dependence, and difficulty with smoking cessation (Belsky *et al.* 2013; Hall *et al.* 2002). In our study, however, there were no significant associations between *GSTT1* or *GSTM1* genotypes and high scores on the FTND scale, smoking cessation, a family history of smoking, age at onset of tobacco use and the number of cigarettes smoked per day or packs per year. The effects of lower education level on nicotine dependence may in part be explained by the healthier life style of more educated people (Pampel *et al.* 2010).

In our study, individuals with current nicotine dependence had significantly more use of alcohol and sedatives, lung disease, cardiovascular disease and blood hypertension than never-smokers. It is known that alcohol and sedative drug dependence are among the most common psychiatric comorbidities in current smokers. Nicotine-dependent smokers are 2.7 to 8.1 times more likely to have psychiatric comorbidities and medical diseases than non-smokers. It is known that one-half of patients with nicotine dependence who do not quit will die early from tobacco-related diseases. Moreover, smokers with serious mental illness are at increased risk of cancer, lung disease, and cardiovascular disease, and they die 25 years earlier, on average, than non-smokers (US CDC 2010).

As reviewed previously, tobacco-related medical disorders are characterized by activated IO&NS pathways that may be induced or maintained by tobacco smoking (Maes *et al.* 2011; Nunes *et al.* 2013). Therefore, it can be inferred that the effects of nicotine dependence on these illnesses is caused by the cumulative effects of toxic compounds of different chemical structures that may be found in cigarette smoke and the consequent effects on IO&NS pathways (Nunes *et al.* 2013).

In this study we found that interactions between nicotine dependence and the *GSTT1* and *GSTM1* null genotypes increase risk to develop hypertension and lung disease, respectively. Thus, not nicotine dependence per se but smoking by individuals with these null genotypes may increase risk towards these medical disorders. Also diabetes was significantly and positively associated with the interaction *GSTT1* null by *GSTM1* null genotype, while cancer was positively associated with the *GSTT1* null genotype. As described in the Introduction there are reports that these genetic polymorphisms by deletion are associated with tobacco use-related disorders. *GSTM1/GSTT1* genetic polymorphisms causing dysfunctions in GSH metabolism may underpin the pathophysiology of these tobacco use-

related medical diseases through increased effects on IO&NS pathways and the consequent damage to proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA) (Morris *et al.* 2014, in press).

Interestingly, the results of our study show that the *GSTM1* and *GSTT1* null genotypes are associated with tobacco use-related medical disorders while decreasing risk towards nicotine dependence. Thus, subjects with the null genotypes have an increased risk to develop these medical diseases especially when they smoke (hypertension and lung disease) but are at the same time protected to develop nicotine dependence. Phrased differently, the effects of nicotine dependence increasing the risk of these illnesses is probably not related to the known effects of the *GSTM1* and *GSTT1* null genotypes on the IO&NS pathways, but to the effects of toxic compounds, which are produced by smoking and cause activation of IO&NS pathways.

These apparently discrepant effects of GSTs null genotypes on nicotine dependence and smoking-related medical disorders may be explained by a new hypothesis. Until now the focus was on the brain reward circuits that determine and maintain smoking behavior, e.g. "smoking reward ('liking') and reinforcement (latency to first puff and total puffs)", such as those mediated by dopaminergic and opioid gene variants (Perkins *et al.* 2008). However, our study shows that also protective effects of genetic variants that determine GSH metabolism and detoxification processes in peripheral systems and tissues may be involved. These protective effects may be explained by the direct effects of smoking increasing xenobiotics and IO&NS pathways due to the lack of GSTs in subjects with *GSTM1* and *GSTT1* deletions. Thus, subjects with the null genotypes may be expected to stop their smoking behavior due to immediate unpleasant side effects of nicotine, such as nausea and dizziness, and effects of activated IO&NS pathways, such as increased anxiety, distress and mood alterations (Maes *et al.* 2011). Indeed, it is known that increased levels of cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α , and oxidative stress may induce depressive feelings, anxiety, distress, fatigue, etc. (Leonard & Maes 2012).

In individuals with the wild genotype, on the other hand, the presence of active enzymes may lower smoking-induced side effects thereby increasing the risk to develop nicotine dependence especially when other genes are present, e.g. the smoking-reward genes and CYP2A6 and CHRNA5-CHRNA3-CHRNA4 genes. Current nicotine dependence in turn enhances the IO&NS pathophysiology of these disorders (Nunes *et al.* 2013). The new hypothesis would be further corroborated if future research could establish possible associations between these genes and the acute side effects of nicotine. All in all, we here suggest a new hypothesis that links the peripheral effects of null and wild GSTs genotypes with the central effects of smoking-reward and other gene variants as risk factors of nicotine

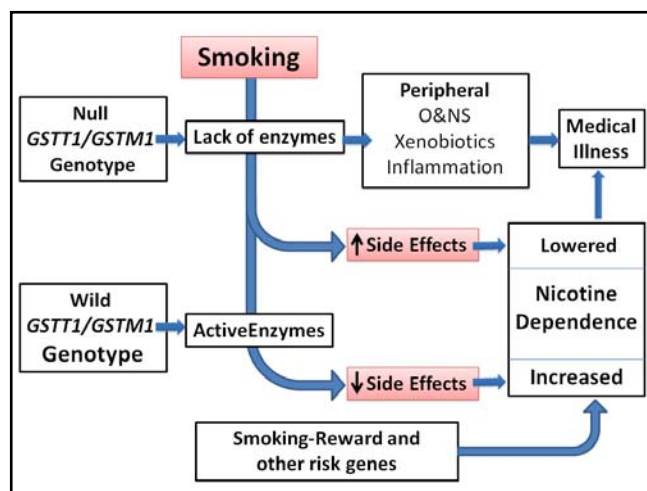


Fig. 1. New hypothesis of Nicotine Dependence: interaction between brain reward circuits with peripheral systems.

dependence and smoking-related disorders as well. Figure 1 shows this new model.

We discussed already the strengths of our study, including our diagnostic approach and clinical assessments, but there are also some potential weaknesses. Firstly, reports from “association studies constitute tentative knowledge and must be interpreted with caution” (Sullivan 2007). Our data should be confirmed using populations with different ethnicities. Secondly, our study was a case-control study and therefore our results are indicative for a significant association and do not allow to draw conclusions on causality. Moreover, single common genes explain probably only a very small part of the outcome variation in nicotine dependence, while it is likely that multiple common variants may underpin the disorder. Many other environmental factors and biological pathways undoubtedly contribute to the development of nicotine dependence. Finally, another possible limitation of the study is that no ancestry informative markers were used in this study. However, these markers are difficult to apply in the Brazilian population and become unavailable in case-control studies (Hatagima *et al.* 2000). The Brazilian Institute of Geography and Statistics (IBGE) classifies the Brazilian population into five categories: White, Black, Brown, Yellow and Indigenous, based on skin color. White (49.9% of the population) usually describes a Brazilian of full or predominantly European ancestry or other ancestry; Brown (43.2%), usually describes a Brazilian of brown skin color and mixed race; Black (6.3%) usually describes a dark-skinned Brazilian of Black African ancestry and Yellow (0.5%) usually describes a Brazilian of East Asian descent, mostly Japanese. Although genetic studies reveal a high degree of racial admixture in all ethnic groups in Brazil, the majority population of this study was Caucasian. Therefore, the ethnicity of the present study participants was determined based on the predominant phenotypic characteristics. However, both

patients and controls were collected in exactly the same region of the country, which allows an effective comparison between the groups. Studies, which examined Brazilian populations of the same region as the present study, showed the same frequencies in *GSTM1/GSTT1* genotypes as those obtained in our study (Arruda *et al.* 1998; Hatagima *et al.* 2000).

In summary, our study provides some evidence for a possible genetic link between nicotine dependence and the *GSTM1* and *GSTT1* null genotypes. Future studies should examine multiple genetic variants and genes coding for IO&NS molecules and brain reward circuits which all together may be related to nicotine dependence and tobacco-related medical diseases.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed equally to the writing up of this paper.

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