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Genetic polymorphisms in metabolizing enzymes modify the association between Smoking and Inflammatory Bowel Diseases

Ashwin N Ananthakrishnan, MD, MPH[1,2], **Deanna D Nguyen, MD**[1,2], **Jenny Sauk, MD**[1,2], **Vijay Yajnik, MD, PhD**[1,2], and **Ramnik J Xavier, MD, PhD**[1,2,3,4]

[1]Division of Gastroenterology, Massachusetts General Hospital, Boston, MA

[2]Harvard Medical School, Boston, MA

[3]Center for Computational and Integrative Biology, MGH, Boston, MA

[4]Broad Institute, Cambridge, MA

Abstract

Introduction—Cigarette smoking is a well established environmental risk factor for Crohn's disease (CD) and ulcerative colitis (UC). The exact mechanism of its effect remains unexplained. Genetic polymorphisms in metabolizing enzymes may influence susceptibility to the effect of smoking and shed light on its mechanism of action.

Methods—We utilized a prospective cohort of patients with CD, UC, and healthy controls. Smoking status was defined as current, former, or never smoking. Patients were genotyped for polymorphisms in CYP2A6, glutathione transferase enzymes (GSTP1 and GSTM1), NAD(P)H quinone oxidoreductase (NQO), and Heme Oxygenase 1 using a Sequenom platform. Multivariate logistic regression models with CD or UC as the outcome, stratified by genotype were developed and interaction p-values calculated.

Results—Our study included 634 patients with CD, 401 with UC, and 337 healthy controls. Ever smokers had an increased risk of CD (OR 3.88, 95% CI 2.35 – 6.39) compared to non-smokers among patients with AG/AA genotypes at CYP2A6. However, ever smoking was not associated with CD among patients with the AA genotype ($p_{interaction}$ 0.001). Former smoking was associated with an increased risk for UC only in the presence of GG/AG genotypes for GSTP1, but not in those with the AA genotype ($P_{interaction}$ 0.012). Polymorphisms at the NQO and HMOX loci did not demonstrate a statistically significant interaction with smoking and risk of CD or UC.

Correspondence: Ashwin N Ananthakrishnan, MD, MPH, Massachusetts General Hospital Crohn's and Colitis Center, 165 Cambridge Street, 9th Floor, Boston, MA 02114, aananthakrishnan@partners.org, Tel 617-724-9953, Fax 617-726-3080.

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D Nguyen, data collection, critical revision of the manuscript for important intellectual content.
J Sauk, data collection, critical revision of the manuscript for important intellectual content
V Yajnik, data collection, critical revision of the manuscript for important intellectual content
R Xavier, data collection, critical revision of the manuscript for important intellectual content

Conclusion—Genetic polymorphisms in metabolizing enzymes may influence the association between smoking and CD and UC. Further studies of gene-environment interaction in IBD are warranted.

Keywords

ulcerative colitis; crohn's disease; smoking; genetics

Introduction

The key mechanism underlying the development of Crohn's disease (CD) and ulcerative colitis (UC) is a dysregulated immune response to intestinal microflora in a genetically susceptible host[1-3]. The external environment is an important contributing factor but remains poorly studied. Smoking is the most consistently described environmental risk factor for inflammatory bowel diseases (IBD); however, demonstrates a complex relationship with IBD[4-7]. Since the inverse association between UC and smoking was first described in 1982[8], subsequent studies confirmed the protective effect of current smoking on the development of UC[5, 9]. Contrary to its effect on UC, smoking increases the risk of CD by two-fold[7, 9], and in those with established disease, current smoking is associated with increased risk of disease flares, greater need for steroids, and a higher rate of post-operative disease recurrence[4, 10-14]. There is significant heterogeneity in the susceptibility of IBD patients to the effect of cigarette smoke with influences by ethnicity and gender[15].

The mechanism how smoking impacts IBD remains unclear as does the reason for its protective effect in UC but deleterious impact on CD[6]. One component of cigarette smoke that could contribute to its effect is nicotine; however, clinical trials of nicotine in UC have yielded equivocal results[16, 17]. More recent studies suggest that the effect of smoking may instead be mediated through oxidative stress[18]. With either (or both) of these mechanisms playing a role, it is conceivable that genetic polymorphisms affecting the activity of one or more enzymes involved in these biologic processes may influence the association between smoking and IBD. The glutathione transferase gene superfamily (GST) encodes for a set of enzymes that are involved in the scavenging of oxidative free radicals through conjugation with glutathione, and have been described to modulate the association between heavy cigarette smoking and rheumatoid arthritis (RA)[19]. A second enzyme involved in oxidative free radical scavenging is Heme Oxygenase 1 (HMOX1), also influencing the association between smoking and chronic inflammation[19]. In contrast, the cytochrome P450 enzyme system, in particular CYP2A6, is involved in the metabolism of nicotine and cotinine; variants in this enzyme system are associated with smoking behavior in genome wide association studies[20-25]. Gene-environment (GxE) interactions between smoking and such enzyme variants have not been examined previously in the context of IBD. Yet, given the incomplete contribution of genetic risk alleles alone in explaining the heritability of CD or UC, GxE effects may further enhance our understanding of the pathogenesis of these complex diseases.

Thus, we performed the study to (1) define the association between polymorphisms in the GST, HMOX, Nicotinamide adenine dinucleotide phosphate (NAD(P)H)quinone

oxidoreductase (NQO)[26], CYP2A6 and CD and UC in a large cohort; and (2) analyze if the strength of association between smoking and CD or UC is differential based on underlying genotype of these enzyme systems.

Methods

Study Population

The primary data source for our study was a prospective registry of patients with IBD at the Massachusetts General Hospital (PRISM). Details of this registry have been described in previous publications from our center. In brief, the PRISM registry is a prospective cohort of patients receiving care for their CD or UC at the MGH Crohn's and colitis center in 2005. After obtaining informed consent, a trained study coordinator interviewed each patient to obtain demographics, disease characteristics including date of diagnosis, IBD type, disease location and phenotype as well as treatments utilized during the course of disease including need for surgical resections. Our case population consisted of all patients in our registry with a diagnosis of CD or UC. The diagnosis was made based on accepted criteria including clinical history of at least 3 months duration of diarrhea, abdominal pain, bleeding, or weight loss, and confirmatory endoscopic, histologic or radiologic findings of CD or UC[27-29]. Healthy controls were recruited through advertisements as well as from patients attending the gastroenterology practices at MGH for other diagnoses or for colorectal cancer screening. All cases and controls were of Caucasian ethnicity.

Our main predictor variable of interest was smoking status at the time of diagnosis, which was obtained both by patient report and review of the medical records. Concordance between smoking status obtained on patient report and medical record review was high, disagreements were infrequent and were resolved through consensus. Patients were classified as current smokers with any (≥ 1/day) ongoing use of cigarettes or cigars at the time of diagnosis, while those with use in the past but not at the time of diagnosis were labeled former smokers. Smoking status in healthy controls was classified at the time of recruitment. Patients with unknown smoking status were excluded from the analysis. Detailed quantitative information about amount or duration of cigarette use was not obtained in our cohort. We obtained information on age at diagnosis of IBD, gender, presence of IBD in one or more first degree relatives, disease location and behavior in CD and disease extent in UC characterized using the Montreal classification[30].

Genotyping

Genomic DNA was isolated from blood samples of consented patients. Oligonucleotides were synthesized and quality control performed. Genotyping was performed using a Sequenom genotyping platform (Sequenom, Inc. San Diego, CA). Five specific SNPs involved in the processing and metabolism of nicotine or related to clearance of free radicals generated during smoking were selected for genotyping. For nicotine metabolism, we selected a SNP at the CYP2A6 locus (rs3733829), which is located 40kb upstream from the 3′ end of the CYP2A6 that is involved in the metabolic inactivation of nicotine to cotinine[25]. The specific glutathione transferase enzymes assessed include GSTM1 and GSTP1 (rs1695); however, all patients in our cohort were wild type for GSTM1 and so analysis was restricted

to the GSTP1 enzyme[19]. We additionally assessed for polymorphisms in NAD(P)H quinone oxidoreductase (NQO) (rs1800566) that catalyzes reduction of quinone and prevents generation of free radical and reactive oxygen species, and protects cells against oxidative stress. Finally, we examined the presence of polymorphisms in HMOX1 (rs2071746) that also protects against oxidative stress and has been demonstrated to interact with heavy smoking in conferring risk of rheumatoid arthritis[19]. All SNPs passed our threshold of Hardy-Weinberg p-value < 0.001 and a call rate > 99%. Individuals with < 80% genotyping success rate were excluded. Supplementary Table 1 presents the allele distribution for the selected SNPs among the cases and controls.

Statistical Analysis

Continuous variables were expressed as medians with interquartile ranges (IQR) and categorical variables were expressed as proportions and compared using the chi-square test. Smoking status was our primary predictor variable of interest. Multivariate logistic regression models were developed using either a diagnosis of CD or UC as the outcome, adjusting for age, gender, and family history of IBD in first degree relatives. Stratified analysis was performed for each of the genotypes of interest and a formal test of interaction was performed by introducing a cross-product interaction term. This model also adjusted for age, gender, and family history of IBD in addition to smoking status, genotype, and the interaction term. To account for comparison stratifying by 4 specific genetic loci, a p-interaction < 0.0125 (i.e. 0.05/4) was considered to be statistically significant. The study was approved by the Institutional Review Board of Partners Healthcare.

Results

Study cohort

Our study population included 634 patients with CD, 401 with UC, and 337 healthy controls. Table 1 presents the characteristics of the cohort. The median age of diagnosis of patients with CD was younger than for UC, with a slight female predominance for both diseases. Approximately one-fifth of patients in each cohort had a family history of IBD in a first degree relative. One-third of patients with CD had penetrating disease. Pancolitis was the most common disease phenotype in UC. The median interval between diagnosis and recruitment in the IBD cohort was 6 years (interquartile range 3-15 years). Table 2 presents the association between smoking and disease risk. Consistent with the literature, both former and current smoking were associated with an increased risk for CD with odds ratios (OR) of 2.17 (95% confidence interval (CI) 1.50 – 3.13) and 2.19 (95% CI 1.16 – 4.13) respectively. In contrast, only former smoking (OR 2.22, 95% CI 0.98 – 2.13) but not current smoking (OR 1.04, 95% CI 0.49 – 2.18) was associated with an increased risk of UC.

Genetic polymorphisms and interaction with smoking

For CD, since we observed similar risk for former and current smokers, we first stratified patients into dichotomous categories. Ever smokers had a four-fold increase in risk of CD (OR 3.88, 95% CI 2.35 – 6.39) compared to non-smokers among patients with AG/GG genotypes at CYP2A6. However, smoking was not associated with CD among patients with the AA genotype (OR 1.27, 95% CI 0.79 – 2.04) ($p_{interaction}$ 0.001). Stratifying smoking into

three categories of ever, former, or never smokers showed that former smokers with AA genotype at CYP2A6 had no increase in risk of CD (OR 1.16) compared to those with AG or GG genotypes (OR 4.76), while current smoking was associated with CD only in those with AG/GG genotype ($p_{interaction}$ 0.0027) (Table 3). When stratifying further by CYP2A6 genotype, we observed a numerical increase in the strength of association with former and current smoking among wild type, heterozygous and homozygous individuals though the smaller number of individuals homozygous for the G allele precluded statistically meaningful comparisons. The OR for former smoking increased from 1.15 for the AA genotype to 3.93 for the AG genotype and 8.67 for the GG genotype. For current smoking, the OR increased from 1.93 among those with AA genotype, to 1.95 for the AG genotype and 4.64 for the GG genotype. Applying the methods proposed by Ottman et al.[31], our findings favored a multiplicative model of interaction though the wide confidence intervals precluded drawing firm conclusions. Similarly in UC, former smokers had a substantially greater risk of UC with the AG/GG genotype compared to those with the AA genotype, but this did not meet independent statistical significance ($p_{interaction}$ 0.078) (Table 4). We found smoking to be associated with both colonic and small bowel CD. The interaction between smoking and CYP2A6 genotype was similar for both small bowel ($P_{interaction}$ 0.002) and colonic CD ($P_{interaction}$ 0.034). There was no association between smoking status and complicated disease behavior, and no interaction by underlying genotype. The interaction between CYP status and smoking was of similar magnitude in patients diagnosed before the age of 40 years as in those with a later age of diagnosis. Repeating the analysis using non-parametric permutation p-values yielded similar results.

Polymorphisms at the GSTP1 locus demonstrated an interaction with smoking and risk of UC. Former smoking was associated with an increased risk for UC only in the presence of AG/GG genotypes, but not in those with the AA genotype ($P_{interaction}$ 0.012) (Table 4). In contrast, current smoking was associated with reduced risk of UC in those with AG/GG genotypes compared to those with the AA genotype though the confidence interval for both estimates overlapped. In contrast, the interaction between smoking and GSTP1 genotype for CD risk did not achieve statistical significance after adjusting for multiple comparisons (Table 3). Polymorphisms at the NQO and HMOX1 loci did not demonstrate a statistically significant interaction with smoking and risk of either CD or UC.

None of the four polymorphisms examined were themselves associated with risk of CD ($p > 0.40$ for all) or UC ($p > 0.25$ for all). We also examined the interaction between smoking and known common risk loci for CD/UC in a subset of our cohort (541 CD, 329 UC, 290 healthy controls). We observed no interaction between smoking and NOD2 (p=0.42), ATG16L1 (p=0.72), IRGM (p=0.31), CARD9 (p=0.56), or IL23R (p=0.59) for CD.

Discussion

The mechanisms wherein environmental factors influence the development of CD or UC remains poorly defined. Despite the consistently described yet complex and poorly understood association between smoking and IBD, there have been few studies examining potential GxE between smoking and polymorphisms in metabolizing enzymes[32]. In a large IBD cohort, we demonstrate that current or former smoking was associated with increased

risk of CD, and former smoking with increased risk of UC in individuals who were hetero- or homozygous for polymorphisms near the CYP2A6 and GSTP1 loci respectively, without a similar effect among those with wild type variants at these loci.

Several different hypotheses have been proposed to understand the effect of cigarette smoking on IBD. One such mechanism is through free radical generation and cellular oxidative stress. Evidence for this being an important mode of action comes from studies demonstrating lack of association between moist snuff or smokeless tobacco and IBD suggesting that non-nicotinic components of cigarette smoke may be mediating its effect[33, 34]. In an elegant study, Bergeron et al. exposed peripheral blood mononuclear cells from IBD patients and healthy controls to lipopolysaccharide (LPS)[18]. CD patients who were smokers demonstrated attenuated production of interleukin-8 in response to LPS stimulation when compared to cells from healthy subjects. In addition, cells from CD patients who were smokers demonstrated reduced antioxidant protection with lower levels of heat shock protein 70 (Hsp70)[18].

The GST genes are a widely expressed supergene family encoding enzymes catalyzing the conjugation of glutathione[35, 36]. GST substrates in cigarette smoke include α- and β-unsaturated carbonyls, polycyclic aromatic hydrocarbons, and reactive oxygen species. Variations in these genes reduce glutathione conjugation and increase susceptibility to oxidative stress mediated through smoking. The GSTP1 polymorphism in our study has been associated with reduced enzymatic stability and activity, decrease in function, and consequently reduced ability to clear oxidative free radicals[37]. Keenan et al. found a two-fold elevated risk of RA in heavy smokers with GSTT1-null polymorphism compared with never or light smokers with normal GSTT1[19]. Heavy smokers with a HMOX1 variant allele also had a two-fold increased risk of RA (RR 1.85, 95% CI 1.29 – 2.65) compared with never or light smokers with wild type HMOX1[19]. In contrast, in our study we observed an interaction between another enzyme in the glutathione family (GSTP1) and smoking in UC and no interaction with HMOX1. Variants in the GST enzymes themselves were not associated with risk of CD or UC in our cohort or in prior studies[38]. A Scandinavian study by Ernest et al. identified an association between smoking and GSTM1*0 genotype for UC further supporting an interaction between GST variants influencing cellular response to oxidative stress and smoking in influencing UC susceptibility[38].

Nicotine was initially believed to be an important active ingredient in cigarette smoke mediating its effect. Consequently, it is plausible that variations in enzymes involved in the metabolism of nicotine to cotinine and other active components may influence susceptibility to the effect of cigarette smoke. However, few have examined this association in the context of smoking related diseases, and only one prior study in the context of IBD. Wassenaar et al. demonstrated that genetic polymorphisms at CYP2A6 was associated with nicotine dependence, increased cigarette consumption, and independently influences risk of lung cancer. Other studies have similarly shown a correlation between CYP2A6 variants and serum cotinine levels[24] in response to secondhand smoke, as well as smoking behavior and rates of quitting[20, 21, 39], with many CYP2A6 variants associated with reduced metabolism of nicotine Altarescu et al. found that homozygosity at the CYP2A6*4A locus was more common among UC patients who were never smokers compared to those who were ever

smokers[32]. However, the study included only 69 patients and was limited by lack of a control population for comparison. While our finding of an interaction between CYP2A6 and smoking in influencing CD risk may suggest that nicotine may play a role in the development of CD in response to smoking, another possible explanation exists. Another gene associated with the same locus is *Egl nine homolog 2* (EGLN2) or *hypoxia-inducible factor prolyl hydroxylase* (HIF-Ph1)[20]. This gene is part of the oxygen-sensing pathway in the cells and is widely expressed, modifying transcription of HIF[20]. This further supports the potential role of oxidative stress in mediating the effect of cigarette smoke.

There are a few implications to our findings. First, it highlights the potential role of gene-environment interactions in the pathogenesis of CD and UC, and extends our understanding of the mechanisms behind the effect of the external environment. As none of the polymorphisms in themselves were associated with risk of CD and UC, they likely do not represent pathways involved in disease pathogenesis, but exert their action through modifying the effect of cigarette smoke. Secondly, the lack of interaction between smoking and some of the more common disease risk alleles suggest that the contribution of host genetics to disease risk and heritability may not solely be through disease risk alleles but could additionally be through polymorphisms that interact with the external environment. Similar interactions exist between diet and risk of colorectal cancer[40-42]. Future studies in IBD should expand the examination of GxE to other environmental risk factors. Third, it is difficult to draw conclusive results about whether nicotine is the dominant factor influencing risk of CD or UC or oxidative stress related to cigarette smoking. The potential interactions in our study with GSTP1 and EGLN2, both of which are involved in oxidative stress response, in conjunction with recent laboratory data demonstrating differential effect of cigarette smoking on oxidative stress response in mononuclear cells between CD and UC consistent with epidemiologic associations suggest that the latter may be an important mechanism of effect of smoking. Further studies on gene-environment interactions on other modalities of nicotine delivery that may not be associated with smoking related oxidative stress may also further clarify this hypothesis.

We readily acknowledge several limitations to our study. First, smoking information was assessed as a dichotomous variable; we did not have detailed information on dose or duration or smoking. Future studies should examine if there is a dose-response effect between heavy smoking and genetic variants involved in cellular oxidative stress response or nicotine metabolism. Second, since our cohort is based at a tertiary referral center, there may be over-representation of those with more severe disease. However, the consistency of the magnitude of association between smoking and CD/UC in our cohorts in accordance with that reported in literature suggests that such a bias is unlikely to influence our results. Third, our examination of GxE in this exploratory analysis was restricted to variants that had previously demonstrated strong biologic plausibility in mediating such an effect. Post-hoc power calculation revealed a power of 66-70% to detect an association of similar magnitude given our allele frequencies in CD, and a slightly lower power of 55% in UC. Thus, larger multicenter cohorts with more detailed information on smoking status may be able to identify other significant interactions in an unbiased analysis. Finally, future studies also need to examine whether well described environmental risk factors interact with known CD

or UC risk loci in influencing disease risk though we did not find evidence of this in a subset from our cohort.

In conclusion, we demonstrating an interesting interaction between genetic polymorphisms in CYP2A6 and risk of CD associated with smoking, and between GSTP1 variants and risk of UC in former smokers suggesting a role for smoking related oxidative stress in the pathogenesis of CD and UC. Continued examination of gene-environment interactions may expand our understanding of the pathogenesis of these complex and disabling diseases and explain a portion of the unexplained variance in the risk of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of the study population

Characteristic	Crohn's disease (n = 634) N (%)	Ulcerative colitis (n = 401) N (%)	Healthy controls (n = 337)
Age at diagnosis (median(IQR)) (in years)	24 (18 – 33)	27 (21 – 38)	31 (25 – 51)//
Sex			
Female	359 (54)	223 (52)	206 (61)
Male	307 (46)	205 (48)	131 (39)
Smoking			
Never	407 (61)	264 (62)	211 (62)
Former	186 (28)	130 (30)	43 (13)
Current	53 (8)	18 (4)	13 (4)
Family history of IBD	178 (27)	89 (21)	45 (13)
Disease behavior			
Inflammatory	304 (47)		
Stricturing	143 (22)		
Penetrating	196 (30)		
Perianal	184 (29)		
Extent			
Proctitis		74 (17)	
Left sided colitis		141 (33)	
Pancolitis		213 (50)	

// age at recruitment

Table 2
Smoking Status and Risk of Crohn's Disease and Ulcerative colitis

	Never smoker	Former Smoker	Current smoker
Crohn's disease			
Unadjusted OR (95% CI)	1.0	2.16 (1.51 – 3.11)	2.15 (1.15 – 4.03)
Adjusted OR (95% CI)†	1.0	2.17 (1.50 – 3.13)	2.19 (1.16 – 4.13)
Ulcerative colitis			
Unadjusted OR (95% CI)	1.0	2.31 (1.58 – 3.38)	1.12 (0.54 – 2.35)
Adjusted OR (95% CI) †	1.0	2.22 (0.98 – 2.13)	1.04 (0.49 – 2.18)

OR – odds ratio; IBD – inflammatory bowel disease; CI – confidence interval

† Adjusted for age, gender, family history of IBD in first degree relatives

Table 3

Interaction between genetic polymorphisms in metabolizing enzymes and risk of Crohn's disease associated with cigarette smoking

	Cases/Controls	Never smoker	Former Smoker	Current smoker	P(interaction)
			OR (95% CI)	**OR (95% CI)**	
CYP2A6					
AA	270/138	1.0	1.16 (0.70 – 1.92)	1.93 (0.70 – 5.30)	0.0027
AG / GG	364/199	1.0	4.76 (2.62 – 8.65)	2.33 (1.04 – 5.24)	
GSTP1					
AA	294/157	1.0	1.70 (1.02 – 2.83)	13.12 (1.74-98.98)	0.024
GG / AG	369/189	1.0	3.06 (1.76 – 5.34)	1.29 (0.63–2.64)	
HMOX1					
TT	128/60	1.0	3.84 (1.39–10.61)	7.90 (0.97–64.62)	0.419
AT / AA	511/278	1.0	2.31 (1.52–3.49)	1.78 (0.90 – 3.53)	
NQO					
GG	414/211	1.0	1.64 (1.04 – 2.57)	1.61 (0.74–3.49)	0.08
AA / AG	233/132	1.0	3.92 (2.04 – 7.51)	3.47 (1.14–10.52)	

CYP – cytochrome; GSTP1 – glutathione-S-transferase pi; HMOX – Heme Oxygenase 1; NQO – NAD(P)H oxidoreductase; OR – odds ratio, CI – confidence interval

Table 4

Interaction between genetic polymorphisms in metabolizing enzymes and risk of ulcerative colitis associated with cigarette smoking

	Cases/Controls	Never smoker	Former Smoker	Current smoker	P(interaction)
			OR (95% CI)	OR (95% CI)	
CYP2A6					
AA	168/138	1.0	1.48 (0.86 – 2.56)	1.08 (0.33 – 3.54)	0.078
AG / GG	233/199	1.0	4.15 (2.17 – 7.92)	1.10 (0.42 – 2.90)	
GSTP1					
AA	201/157	1.0	1.44 (0.84 – 2.48)	5.93 (0.73–48.29)	0.012
GG / AG	224/189	1.0	3.45 (1.94 – 6.15)	0.61 (0.25–1.52)	
HMOX					
TT	91/60	1.0	3.25 (1.26 – 8.38)	3.09 (0.34–28.44)	0.454
AT / AA	312/278	1.0	2.14 (1.37 – 3.32)	0.89 (0.39–2.02)	
NQO					
GG	266/211	1.0	1.72 (1.06 – 2.78)	0.60 (0.22–1.63)	0.080
AA / AG	153/132	1.0	3.74 (1.87 – 7.47)	2.29 (0.68–7.67)	

CYP – cytochrome; GSTP1 – glutathione-S-transferase pi; HMOX – Heme Oxygenase 1; NQO – NAD(P)H oxidoreductase; OR – odds ratio, CI – confidence interval