Genetic Determinants of Gastric Cancer

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Genetic Determinants of Gastric Cancer

Genetische factoren van maagkanker

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	Prof.dr. L.A.L.M. Kiemeney
	Prof.dr. E.J. Kuipers

For my family

Idealism increases in direct proportion to one's distance from the problem

John Galsworthy
(1867-1933)

CONTENTS

Manuscripts based on the studies described in this thesis

Chapter 2.1

Boccia S, Sayed-Tabatabaei FA, Persiani R, Gianfagna F, Rausei S, Arzani D, La Torre G, D'Ugo D, van Duijn CM, Ricciardi G. Polymorphisms in metabolic genes, their combination and interaction with tobacco smoke and alcohol consumption and risk of gastric cancer: a case-control study in an Italian population. BMC Cancer 2007;7:206.

Chapter 2.2

Boccia S, Gianfagna F, Persiani R, La Greca G, Arzani D, Rausei S, D'Ugo D, Magistrelli P, Villari P, van Duijn CM, Ricciardi G. Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and susceptibility to gastric adenocarcinoma in an Italian population. Biomarkers 2007;12:635-644.

Chapter 2.3

De Feo E, Persiani R, La Greca A, Amore R, Arzani D, Rausei S, D'Ugo D, Magistrelli P, van Duijn CM, Ricciardi G, Boccia S. A case-control study on the effect of *p*53 and *p*73 polymorphisms on gastric cancer risk and progression in an Italian population. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 2009;675:60-65.

Chapter 3.1

Boccia S, De Lauretis A, Gianfagna F, van Duijn CM, Ricciardi G. *CYP2E1Pst*I/*Rsa*I polymorphism and interaction with tobacco, alcohol and *GST*s in gastric cancer susceptibility: a meta-analysis of the literature. Carcinogenesis 2007;28:101-106.

Chapter 3.2

La Torre G, Boccia S, Ricciardi G. Glutathione S-Transferase M1 status and gastric cancer risk: a meta-analysis. Cancer Letters 2005;217:53-60.

Chapter 3.3

Boccia S, La Torre G, Gianfagna F, Mannocci A, Ricciardi G. Glutathione S-Transferase T1 status and gastric cancer risk: a meta-analysis. Mutagenesis 2006;21:115-123.

Chapter 3.4

Boccia S, Hung RJ, Ricciardi G, Gianfagna F, Ebert MPA, Fang JY, Gao CM, Götze T, raziano F, Lacasaña-Navarro M, Lin D, López-Carrillo L, Shen H, Takezaki T, Weng YR, F Zhang FF, van Duijn CM, Boffetta P, Taioli E. Meta- and pooled analyses of the Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and gastric cancer risk: a HuGE-GSEC Review. American Journal or Epidemiology 2008;167:505-516.

General discussion

Gianfagna F, De Feo E, van Duijn CM, Ricciardi G, Boccia S. A systematic review on the published meta-analysis on genetic association studies in gastric cancer research. Current Genomics 2008;9:361-374.

1

Introduction

Gastric cancer is the second leading cause of death from cancer worldwide, with an estimated 700,000 deaths annually, and 876,000 new cases every year (1). The overwhelming majority (around 95%) are adenocarcinoma of the stomach, the remainder comprising non-Hodgkin's lymphomas and stromal tumors. The Laurén classification system classifies gastric adenocarcinoma under two major histopathological variants: a more common intestinal type, and a diffuse type, showing histological characteristics of well- and poorly differentiated adenocarcinoma, respectively. The intestinal type predominates in high-risk regions, which shows a correlation with *Helicobacter pylori* (*H. pylori*) prevalence, is more likely to be sporadic and related to environmental risk factors (2). This gastric cancer type shows large geographic differences in incidence, with relatively low rates in most Western Countries, including the United States and the United Kingdom, and with relatively high rates in Japan, Korea, and China, and part of South America, where the prevalence of *H. pylori* is relatively high. The diffuse gastric cancer type is more uniformly distributed geographically, typically develops from *H. pylori* free individuals, and seems to have a primary genetic aetiology. While intestinal type adenocarcinoma progresses through a relatively well defined series oh histological steps, including atrophic gastritis, intestinal metaplasia and displasia, the diffuse type cancer consists of individually infiltrating neoplastic cells with no glandular structure, and is not associated with definite precancerous lesions (2). Mortality rates for gastric cancer have been decreasing since two decades due to declined incidence and improvement of survival (3), especially for the intestinal histologic type. As for the anatomical sub-site, there are two main types of stomach cancer. Distal gastric cancer involves the lower portion of the stomach, and is the predominant type, while the other type is cancer of the gastric cardia, sometimes grouped with oesophageal junction cancers.

Concerning the environmental risk factors for gastric cancer, there are strong evidences that non-starchy vegetables, allium vegetables and fruit protect from gastric cancer, while salt, salty foods actually increase the risk. There are also suggestive evidence for an increased risk associated with the use of processed and red meat, smoked and grilled food, and cigarette smoking, while for the alcohol the evidences are inconclusive, suggesting a major role especially for the anatomic sub-site of cardia gastric cancer (4,5). Lastly, the lack of food refrigeration, which allows bacteria overgrowth and nitrosamine production played a key role especially in the past (6). As for the hereditary forms of gastric cancer, it is known that a positive family history for gastric cancer confers an increased risk (7). In family studies, first degree relatives of

patients with gastric cancer have two-to-three fold increased risk, which could not be explained by familial clustering of *H. pylori* infection (8). Although most carcinoma of the stomach, both intestinal and diffuse, occurs sporadically without a demonstrated inherited predisposition, a small proportion of gastric cancer arises in clearly inherited predisposition syndromes including hereditary non-polyposis colon cancer syndrome caused by germline mutations in mismatch repair genes (e.g., *MLH1, MSH2, MSH6, PMS1* and *PMS2*); hereditary diffuse gastric carcinoma resulting from germline mutations in the *CHD1* gene (known also as *E*-cadherin gene); Li Fraumeni syndrome caused by mutations in *p*53 gene, and familial adenomatous polyposis due to mutations in the *APC* gene (9).

Most of the research conducted so far, however, concerns the effect of environmental and genetic factors on sporadic gastric cancer. In 1994, the IARC classified the infection with *H. pylori* as a class I human carcinogen (10). This germ causes an initial damage by initiating chronic inflammation of gastric mucosa, which is mediated by an array of pro-and anti-inflammatory cytokines. During the following decades *H. pylori* infection can remain silent or evolve into more-severe diseases, such as atrophic gastritis, peptic ulcer, intestinal metaplasia, dysplasia and cancer. However, even though past or present *H. pylori* infection has been found in up to 70% of patients with sporadic adenocarcinoma of the stomach, only 2% of infected individuals actually develop stomach cancer (11), so that *H. pylori* appears to be neither a necessary nor even a sufficient cause. This has been explained by the complex interplay between other component causative factors for gastric cancer, such as tobacco smoking, low fruit and vegetable intake, high meat and salt intake and alcohol consumption (7). Additionally, more recently the long suspected influence of genetic susceptibility to *H. pylori* damages of gastric mucosa has come to forefront. Since El-Omar et al (12) firstly investigated in 2000 the relationship between the pro-inflammatory *IL*-1 gene cluster polymorphisms and non-cardia gastric cancer, several studies have been published. According to a recent meta-analysis (13), Caucasian individuals carrying the T variant allele of *IL-1B*-511 have an increased risk of developing gastric cancer compared with the homozygous wild types. Another large meta-analysis (14) also showed that Caucasians carrying the *2 polymorphic allele of the receptor antagonist of *IL-1β,* coded by the *IL-1 RN* gene, have a significantly increased risk of gastric cancer, especially in combination with *H. pylori* infection.

Beside the *IL-1* gene cluster, which mainly affects the capacity of the host in handling the *H. pylori* attack and mediating the resulting inflammation, other candidate genes have been investigated in association with the risk of sporadic gastric cancer (9,15). Among the most extensively gene studied are those affecting with the individual ability to detoxify tobacco and alcohol carcinogens that might be relevant for gastric carcinogenesis (16). Most of the published studies, however, considered only few phase I and/or II metabolic gene variants at time, and even less their interaction and combination with tobacco and alcohol exposures (17), so that results concerning the effect of metabolic gene polymorphisms on gastric cancer risk remain inconclusive. Other genes relevant for tumorigenesis were also investigated in relation with gastric cancer. Among them, the methylenetetrahydrofolate reductase (*MTHFR*) gene, which is involved in the DNA synthesis and in gene expression control through DNA methylation, and genes involved in the cell cycle control such as *p*53 and *p*73 tumor suppressor genes. While evidences suggest a key role for *MTHFR* in gastric carcinogenesis (18), results on the potential effect of *p*53 and *p*73 genes are inconclusive, with no one study considering the combination of the two most common gene variants in relation with gastric cancer.

Generally speaking, the majority of the genetic association studies conducted so far in relationship with sporadic gastric cancer, however, are underpowered to detect a robust association, or to explore gene-gene and gene-environment interactions. As such, some authors attempted to quantitatively summarize the results of the literature on certain gene polymorphisms and gastric cancer by using the meta-analytical approach. Currently meta-analysis is the most cited study design in health sciences (19), and it is widely accepted as one of the highest levels of evidence in medicine to evaluate existing data. Initially adopted to summarize the results from clinical trials, meta-analyses were then widely applied to observational studies, including genetic epidemiology studies. The reliability of meta-analysis results, however, depends mainly on a rigorous methodology, on the quality of primary studies included and the availability for individual data collected from primary studie. In this context, the Meta-analysis of Observational Studies in Epidemiology group proposed a checklist for authors of a meta-analysis containing specifications on how to structure the background, search strategy, methods, results, discussion and conclusion (20). More recently, another eminent research group published the PRISMA statement for reporting systematic reviews and meta-analyses, builded on the QUORUM statement (21), that further clarified how to elaborate and report such studies (22).

The objective of the work described in this thesis was dual:

- to investigate the association of candidate genes and gastric cancer risk by selecting genes that, given their function, should have a high probability to be involved in gastric cancer;
- to assess the cumulative evidence on the most extensively studied gene polymorphisms in association with gastric cancer risk through meta-analyses and pooled analysis.

The first aim was conducted at the Institute of Hygiene in collaboration with the Department of Surgery located in the Policlinico 'A. Gemelli' teaching hospital, which enrols since 2002 consecutive cases of primary gastric adenocarcinoma patients with histological confirmation that undergo curative gastrectomy. The study is approved by the local review board and written informed consent is obtained from each subject, and the procedures adopted are in accordance with the Helsinki Declaration. Control subjects are selected from gastric cancer-free patients admitted to the same hospital during the same time period with a broad range of diagnoses.

The candidate genes for the case-control study were selected according to their relevance in different oncogenic process, namely detoxification of carcinogens, synthesis of DNA and cell cycle control. This work is described in the chapter 2 of this thesis. Chapter 2.1 reports the results of the effect of polymorphisms in genes involved in the bioactivation (phase I enzymes) and detoxification (phase II enzymes) of promutagens and carcinogens on gastric cancer risk. Chapter 2.2 describes the potential role on gastric cancer aetiology of two functional polymorphisms in the *MTHFR* gene, which is involved in the metabolism of folate and providing methyl donor for DNA methylation and deoxynucleoside synthesis. Chapter 2.3 studies the association between polymorphisms in *p*53 and *p*73 tumour suppressor genes and gastric cancer. In all the studies the relevant gene-gene and gene-environment interactions were explored.
The second part of this thesis reports the results of three meta-analyses and a pooled analysis summarizing the effect of polymorphisms in a phase I enzyme (Chapter 3.1), phase II enzymes (Chapters 3.2 and 3.3), and in *MTHFR* gene (Chapter 3.4), on the risk of gastric cancer. Chapter 4 reviews the main results of the studies described in this thesis and discusses them in the context of the current knowledge and potential methodological limitations. Finally, some suggestions for future research are provided.

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2

Genetic polymorphisms and risk of gastric cancer in an Italian hospital-based case-control study

2.1

Polymorphisms in metabolic genes, their combination and interaction with tobacco smoke and alcohol consumption and risk of gastric cancer: a case-control study in an Italian population

ABSTRACT

The distribution and the potential gene-gene and gene-environment interaction of selected metabolic genetic polymorphisms was investigated in relation to gastric cancer risk in an Italian population. One hundred and seven cases and 254 hospital controls, matched by age and gender, were genotyped for *CYP1A1, CYP2E1, mEH, GSTM1, GSTT1*, *NAT2* and *SULT1A1* polymorphisms. Haplotype analysis was performed for *EPHX1* exons 3 and 4, as well as *CYP2E1 Rsa*I (**5* alleles) and *CYP2E1 Dra*I (**5A* or **6* alleles). The effect modification by alcohol and cigarette smoking was tested with the heterogeneity test, while the attributable proportion (AP) was used to measure the biological interaction from the gene-gene interaction analysis. Gastric cancer risk was found to be associated with the inheritance of *GSTT1* null genotype (OR= 2.10, 95% CI: 1.27-3.44) and the *SULT1A1* His/His genotype (OR= 2.46, 95% CI: 1.03-5.90). No differences were observed for the haplotype distributions among cases and controls. An increased risk was detected among individuals carrying the *6 variant allele of *CYP2E1* if ever-drinkers (OR= 3.70; 95% CI: 1.45-9.37) with respect to never-drinkers (OR= 0.18; 95% CI: 0.22-1.46) (*p* for heterogeneity = 0.001). Similarly, the effect of *SULT1A1* variant genotype resulted restricted to ever-smokers, with an OR of 2.58 (95% CI: 1.27-5.25) for the carriers of His allele among smokers, and an OR of 0.86 (95% CI: 0.45-1.64) among never-smokers (*p* for heterogeneity = 0.03). Gene-gene interaction analyses demonstrated that individuals with combined *GSTT1* null and *NAT2* slow acetylators had an additional increased risk of gastric cancer, with an OR of 3.00 (95% CI: 1.52-5.93) and an AP of 52%. *GSTT1*, *SULT1A1* and *NAT2* polymorphisms appear to modulate individual's susceptibility to gastric cancer in this Italian population, particularly when more than one unfavourable genotype is present, or when combined with cigarette smoke. All the results need to be confirmed by larger prospective studies.

INTRODUCTION

Gastric cancer is the second most common cause of mortality from cancer, with 647,000 deaths reported worldwide in 2002 (1). In many populations, particularly in high-income countries, in the last decades its incidence has gradually decreased, however it still represents the fifth most common type of cancer in Europe and the fourth internationally (1). The development of gastric cancer appears to be the result of a complex interaction between lifestyle and genetic factors. Among the lifestyle and related risk factors, *Helicobacter pylori* infection, tobacco smoking, a high intake of salt and lack of food refrigeration all seem to play a major role (2). Additionally, gastric cancer shows a familial clustering (3). With regards to genetic factors, several Single Nucleotide Polymorphisms (SNPs) might potentially alter the individual susceptibility to gastric cancer, among them genes coding for metabolic enzymes (4).

A major part of carcinogenic substances require metabolic activation by enzymes to be genotoxic, and inherited variations in carcinogens metabolizing genes may alter enzyme activity and subsequently carcinogens activation or deactivation. Phase I enzymes, including Cytochrome P450 (CYP) and microsomal Epoxide Hydrolase (mEH), activate several compounds to form genotoxic electrophilic intermediates. Activated metabolites are then, in part, detoxified by phase II enzymes, such as glutathione S-transferase (GST), N-acetyltransferase (NAT) and Sulfotransferase (SULT) (5). We recently showed, for the first time, that *SULT1A1* Arg213His polymorphism might affect the risk of gastric cancer (6), while contradictory results concerning several SNPs in metabolic genes have been reported (7-15).

Based on the knowledge that metabolic genes are presumed to modulate an individual's susceptibility to cancer by interacting with carcinogens, and since the inheritance of several unfavourable genotypes is supposed to additionally increase the risk of gastric cancer (8,9,11), this hospital-based case-control study aims to investigate the effect on gastric cancer of selected SNPs of *CYP1A1*, *CYP2E1*, *mEH*, *GSTM1*, *GSTT1*, *NAT2*, *SULT1A1*, and their differential effect according to tobacco smoking and alcohol habits. We also investigated to what extent the inheritance of more than one unfavourable genotype affects the risk of gastric cancer.

METHODS

Study population

The study subjects were selected according to a case-control study design as previously described (16). Briefly, cases were consecutive primary gastric

adenocarcinoma patients, with histological confirmation, who underwent a curative gastrectomy in the "A. Gemelli" teaching hospital, located within the Università Cattolica del Sacro Cuore in Rome. We defined gastric cancer cases as including International Classification of Disease Ninth revision codes 151.0-151.9. Controls were selected from cancer-free patients, with a broad range of diagnoses including around 15% of blood donors, admitted to the same hospital during the identical time period and were frequency matched to cases for age (±5 years) and gender. All subjects were Caucasians born in Italy. According to the Lauren histotype classification (17), the majority (57.8%) of the gastric cancer cases were intestinal. The tumours were located in the antrum (39.3%), in the corpus (14.8%), in the antrum/corpus (28.0%), in the cardia (10.3%), stumps (5.6%) and in the fundum (2.0%). Based on the cytological and architectural atypisms, as well as the histo-pathological reports (18), patients' tumours were classified accordingly: 68.3% scarcely differentiated, 29.2% moderately differentiated, 2.5% well-differentiated, while 53.8% were staged I-II and 46.2% staged III-IV. With a response rate of 95% and 90% respectively for cases and controls, 102 gastric cancer and 254 controls were recruited. A venous blood sample was drawn from each participant, collected into an EDTA-coated tubes from which DNA was isolated from peripheral blood lymphocytes. The study was approved by the local review board and written informed consent was obtained from each subject. The procedures followed were in accordance with the Helsinki Declaration.

Data collection

Cases and controls were interviewed by trained medical doctors using a standard questionnaire to elicit information on demographic variables, tobacco smoking (including cigarette, cigar and pipe) and drinking history, dietary habits and family history of cancer. Questions pertaining to lifestyle focused on the time period ending one year prior to diagnosis. Smoking status was categorized as never and ever-smokers (including both current and former smokers) and alcohol consumption as drinkers/non-drinkers. Fruit and vegetables intake was classified as high if the participant consumed at least two portions of fruit and two portions of vegetables per day. Meals salt addition was referred to the use of adding salt to the entrées during the main meals. Family history (including non-melanoma skin cancer) of cancer referred to parents, siblings and offspring. Data concerning previous *Helicobacter pylori* infection was not available for either cases or controls. The response rate for completing the interview was 99.1% for cases (106/107) and 99.6% (253/254) for controls, with the exception of data relating to a family history of cancer [(unknown in 7.4% (8/107) of cases and 3.5% (9/254) of controls)].

Genotyping

GSTM1 and *GSTT1* null alleles were identified using a multiplex-Polymerase Chain Reaction (PCR)-based method as described by Arand et al. (19). The polymorphic site at nucleotide 638 in exon 7 (Arg213His) of the *SULT1A1* gene was genotyped by PCR-Restriction Fragment Length Polymorphisms (RFLP) analysis as described by Coughtrie et al.(20). Identification of the *mEH* exon 3 (Tyr113His) and exon 4 (His139Arg) polymorphisms was performed using a RFLP-based method (21). *CYP1A1* 3'-flanking region *Msp*I polymorphism (*CYP1A1*2A* allele), *CYP2E1 Rsa*I polymorphism (*CYP2E1*5* alleles) and *CYP2E1 Dra*I *(*5A* or **6* alleles) were also determined by PCR-RFLP analyses (21). Three known slow acetylator alleles, NAT2*5A, *6A and *7A were identified as previously described by Peluso et al (22). Fast acetylator genotypes are the homo-heterozyogous wild-type alleles (*4A), slow acetylator genotypes are those with 2 slow acetylator alleles (23). Quality control for each genotyping was performed in each experiment, and 10% of the total samples were randomly selected and reanalyzed with 100% concordance. The analyst was blinded to the case or control status of the samples.

Statistical analysis

The relationship between gastric cancer and putative risk factors were measured using the adjusted odds ratios (ORs) and their 95% CI derived from logistic regression analysis using STATA software (version 8.2). We considered possible risk factors for gastric cancer as potential confounders if the addition of that variable to the model changed the OR by 10% or greater. Confounding checks were performed in both of the univariate and final multivariate models. If a factor was identified as a confounder of any estimated main effect, it was kept in all models. Based on these criteria, we controlled for age, gender, alcohol consumption and family history of cancer, when appropriate. In the multivariable model, we adjusted for the continuous variables of age and alcohol (g/day).

The genotypes of *GSTM1* and *GSTT1* were dichotomized according to the presence *versus* absence of the null allele, and *NAT2* was dichotomized according to the inferred phenotype (slow *versus* fast). We analyzed exon 3 and exon 4 *mEH* genotypes by "imputed phenotype" as suggested from Smith and Harrison (24). Lastly, we conducted haplotype analysis for *EPHX1* exons 3 and 4, as well as *CYP2E1**5 and **5A* or **6* using Cocaphase software
(http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased).

Hardy-Weinberg Equilibrium (HWE) was tested for separately all of the case and control SNPs. In order to assess if the effect of the studied polymorphisms is modified

by tobacco smoking and alcohol consumption, we performed a stratified logistic regression analysis. An heterogeneity test was then used to test differences among the strata.

Biological interaction between two genes was estimated using departure from additivity of effects as the criterion of interaction, as suggested by Rothman (25). To quantify the amount of interaction, the attributable proportion (AP) due to interaction was calculated as described by Andersson et al (26). The AP due to interaction is the proportion of individuals among those exposed to the two interacting factors that is attributable to the interaction per se and it is equal to 0 in the absence of a biological interaction (25). Finally, in order to test for more than multiplicative effect among two genes, the likelihood ratio test was used, with the homozygous wild-type individuals for both genes as the reference group.

RESULTS

General characteristics of the study population are presented in Table 1. Alcohol consumption and family history of cancer were associated with an increased risk of gastric cancer, with ORs of 2.10 (95% CI: 1.22-3.60) and an OR of 1.93 (95% CI: 1.14-3.26), respectively (Table 1). The genotype frequencies of our control group were in line with those for Caucasians and were in HWE both for cases and controls (p > 0.05) (5, 20). As shown in Table 2, we found a significant difference in the distribution of *GSTT1* null and *SULT1A1* His/His genotype amongst cases and controls: 37.1% *versus* 22.4% (OR= 2.10, 95% CI: 1.27-3.44) and 10.3% *versus* 5.1% (OR= 2.46; 95% CI: 1.03-5.90), respectively. An increased risk was also detected for *NAT2* slow acetylators (OR= 1.38, 95% CI: 0.88-2.19), however not statistically significant. Haplotype analyses indicated that there was no significant linkage disequilibrium between *EPHX1* exons 3 and 4, as well as CYP2E1*5 and *5A or *6, amongst the cases and the controls. Furthermore, the frequency of the estimated haplotypes was the same among the groups (data not shown).

Data were stratified according to the histologic type and similar effects were shown among the two strata (data not shown). From the stratified analysis according to smoking status (Table 3), the significant association for *SULT1A1* observed in the overall analysis seems to be limited to ever smokers, with a p value for heterogeneity among the two strata of 0.03 (Table 3). On the other hand, the increased risk for *GSTT1* null individuals was significant regardless of the smoking status (Table 3).

Table 1. Odds Ratios (95% CI) of gastric cancer according to the collected variables and their frequency distribution among 107 cases and 254 controls

	Cases n (%)	Controls n (%)	OR (95% CI) †
Age (years± SD)	66.4±12.0	64.0±12.8	-
Male gender	56 (52.3)	141 (55.5)	-
Alcohol drinkers			
Never-drinkers°	32 (29.9)	121 (47.6)	1*
Drinkers	73 (70.1)	133 (52.4)	2.10 (1.22-3.60)
Smoking status			
Never	57 (53.3)	146 (57.5)	1*
Ever	50 (46.7)	108 (42.5)	1.10 (0.64-1.90)
Pack-years of smoking			
0	60 (56.0)	147 (57.8)	1*
1-25	21 (19.6)	63 (24.8)	0.97 (0.50-1.92)
> 25	26 (24.4)	44 (17.4)	1.03 (0.50-2.10)
Fruit and vegetables intake			
High‡	19 (17.9)	40 (16.6)	1*
Low	87 (82.1)	212 (83.4)	0.92 (0.45-1.84)
Meals salt addition ^			
No	91 (85.1)	235 (92.9)	1*
Yes	16 (14.9)	18 (7.1)	1.70 (0.78-3.67)
Family history of cancer			
No	61 (61.6)	192 (78.4)	1*
Yes	38 (38.4)	53 (21.6)	1.93 (1.14-3.26)
Family history of gastric cancer			
No	88 (91.7)	237 (94.4)	1*
Yes	8 (8.3)	14 (5.6)	1.88 (0.80-4.44)

* Reference category

† OR adjusted for age, gender, alcohol consumption and family history of cancer

° Never-drinkers defined as those who are teetotaller

‡ High fruit and vegetables consumption is defined as at least 2 portions of fruit and 2 portions of vegetables per day

^ Adding salt to the entrées during the main meals

Table 2. Odds Ratios (95% CI) of gastric cancer for SNPs in metabolic genes and their frequency distribution among 107 cases and 254 controls

		Cases n (%)	Controls n (%)	OR (95% CI) * †
GSTM1 null		59 (56.2)	134 (52.7)	1.13 (0.71-1.79)
GSTT1 null		39 (37.1)	57 (22.4)	2.10 (1.27-3.44)
*CYP1A1*2A*		23 (20.5)	56 (22.0)	0.88 (0.50-1.54)
*CYP2E1*5*		5 (4.7)	20 (7.8)	0.54 (0.20-1.50)
*CYP2E1*5A* or *6		14 (14.5)	27 (10.6)	1.33 (0.67-2.65)
NAT2 Slow ‡		64 (59.8)	131 (51.8)	1. 38 (0.88-2.19)
SULT1A1	Arg/His	39 (36.5)	85 (33.5)	1.35 (0.82-2.21)
	His/His	11 (10.3)	13 (5.1)	2.46 (1.03-5.90)
EPHX1 exon 3	Tyr/His	41 (38.7)	90 (36.0)	1.24 (0.76-2.04)
	His/His	15 (14.1)	28 (11.2)	1.37 (0.67-2.80)
EPHX4 exon 4	His/Arg	32 (30.5)	95 (37.4)	0.77 (0.47-1.27)
	Arg/Arg	6 (5.7)	6 (2.4)	2.28 (0.70-7.20)
Imputed *mEH* phenotypes ^	Rapid	15 (15.8)	59 (23.7)	0.60 (0.30-1.15)
	Slow	24 (25.3)	54 (21.2)	1.00 (0.55-1.78)
	Very slow	8 (8.4)	21 (8.6)	0.82 (0.33-2.00)

* OR adjusted for age and gender

† Reference groups are the homozygous wild genotypes for each gene

‡ Reference group is fast acetylators (homo-heterozygous for the wild-type allele)

^ Reference group is the normal imputed phenotype

As for the effect modification by alcohol habits, drinking subjects carrying the variant allele of *CYP2E*1 (*5A or*6 alleles) had an OR of 3.70 (95% CI: 1.45-9.37) of gastric cancer compared to those drinking without the variant allele, with the result of the heterogeneity test among the strata showing a significant effect modification by alcohol (*p* value = 0.001, Table 3). To reduce the chance of multiple testing, we limited the gene-gene interaction analyses to the three SNPs that exhibited the most prominent association with gastric cancer. It was observed that in all of the combinations individuals carrying two risk genotypes had an additional risk compared to those with only one risk genotype, with an AP greater than 0, however there was no evidence of multiplicative interaction (*p* values > 0.05, Table 4). The observed effect was particularly high amongst individuals with both GSTT1 null and NAT2 slow (OR= 3.00, 95% CI: 1.52-5.93; AP = 52%) (Table 4). Additionally, by stratifying these data according to smoking status (data not shown), ever-smoker individuals with combined *GSTT1* null and *NAT2* slow had an OR of 4.23 (95% CI: 1.49-12.01) compared to ever-

smokers with combined normal variants, while an OR of 2.60 (95% CI: 1.00-6.67) appeared using the same comparators amongst never-smokers (*p* value of heterogeneity among the two estimates = 0.49).

Table 3. Odds Ratios (95% CI) of gastric cancer for SNPs in metabolic genes according to smoking status and alcohol habits

	Never-smokers (57 cases, 146 controls)		**Ever-smokers** (50 cases, 108 controls)		***p* for heterogeneity**
	cases/ controls	OR (95% CI) * †	cases/ controls	OR (95% CI)	
GSTM1 null	33/70	1.55 (0.83-2.90)	26/64	0.70 (0.35-1.39)	0.10
GSTT1 null	21/34	2.09 (1.06-4.11)	18/23	2.17 (1.02-4.59)	0.92
*CYP1A1*2A*	14/33	1.09 (0.53-2.27)	8/23	0.66 (0.27-1.61)	0.40
*CYP2E1*5*	4/11	0.86 (0.26-2.88)	1/9	0.20 (0.02-2.70)	0.24
*CYP2E1*5A* or **6*	10/17	1.59 (0.67-3.79)	5/10	0.99 (0.31-3.13)	0.58
NAT2 Slow ‡	34/77	1.39 (0.74-2.60)	30/54	1.50 (0.75-2.98)	0.87
SULT1A1 His carriers	22/62	0.86 (0.45-1.64)	27/36	2.58 (1.27-5.25)	0.03
EPHX1 exon 3 His carriers	28/62	1.31 (0.70-2.46)	28/56	1.12 (0.57-2.22)	0.69
EPHX4 exon 4 Arg carriers	21/63	0.84 (0.44-1.60)	16/38	0.89 (0.43-1.85)	0.85
	Never-drinkers (32 cases, 121 controls)		**Ever-drinkers** (73 cases, 133 controls)		***p* for heterogeneity**
	cases/ controls	OR (95% CI)	cases/ controls	OR (95% CI)	
GSTM1 null	16/64	0.95 (0.43-2.10)	43/70	1.23 (0.69-2.20)	0.55
GSTT1 null	13/24	3.15 (1.32-7.47)	26/33	1.72 (0.92-3.22)	0.27
*CYP2E1*5*	0/10	-	5/10	0.86 (0.28-2.68)	-
*CYP2E1*5A* or **6*	1/19	0.18 (0.22-1.46)	14/8	3.70 (1.45-9.37)	0.001
SULT1A1 His carriers	16/51	1.42 (0.63-3.17)	33/47	1.56 (0.86-2.82)	0.98

* OR adjusted for age and gender

† Reference groups are the homozygous wild genotypes for each gene

‡ Reference group is fast acetylators (homo-heterozygous for the wild-type allele)

Table 4. Age and gender adjusted Odds Ratios (95% CI) of gastric cancer for selected gene-gene interaction analyses

		GSTT1		***SULT1A1***	
		Present	Null	Arg/Arg	His carriers
NAT2	Fast	1*	1. 38 (0.63-3.01)	1*	1.45 (0. 71-2.95)
	cases/controls	30/93	13/29	23/74	20/48
	Slow	1.07 (0.61-1.88)	3.00 (1.52-5.93)	1.40 (0. 75-2.60)	2.00 (1.03-3.89)
	cases/controls	36/103	26/28	35/81	29/50
		p for interaction† = 0.17		*p* for interaction = 0.97	
		AP‡= 52%		AP= 8%	
SULT1A1	Arg/Arg	1*	1.53 (0.86-2.71)	-	-
	cases/controls	35/122	22/34		
	His carriers	2.30 (1.18-4.45)	2.87 (1.36-6.05)	-	-
	cases/controls	31/75	17/23		
		p for interaction = 0.70			
		AP= 1%			

* Reference category; † By likelihood ratio test; ‡ Attributable Proportion due to biological interaction (see methods)

DISCUSSION

This case-control study of 107 surgical cases of gastric adenocarcinoma and 254 controls born in Italy evaluated the effect on gastric cancer risk of several metabolic gene polymorphisms simultaneously. Results showed a significantly increased risk for *GSTT1* null and for *SULT1A1* homozygotes, and an additional risk for *NAT2* slow acetylator individuals, although not statistically significant. Risks associated with those genes became substantive when two unfavourable genotypes were combined, with evidence of biological interaction between them. From the gene-environment interaction analysis, we showed effect modification of the association between *SULT1A1* and gastric cancer by tobacco smoking, and *CYP2E1 (*5A* or**6* alleles) by

alcohol drinking. In addition, our results confirm previous findings of gastric cancer risk to be increased by alcohol intake and family history for cancer (27,28).

Several limitations should be taken into account in the interpretation of our results. Firstly, based on the prevalence of the analyzed genotypic variants in our population (Table 2), our study was powered to detect an OR of 2.0 for common polymorphisms (with a significance level of 5%), however not for *CYP2E1*5* allele carriers, *CYP2E1*5A* or**6* allele carriers and the homozygotes variants of *SULT1A1*, *EPHX3* and *EPHX4*. The study's sample size limits the ability to explore the combined effects of the genotypes, or gene-environment interactions, which highlights the need to increase the sample size in order to confirm our results. However, when appropriately conducted, large and small studies should give, theoretically, the same results, with just a more precise effect measure estimate from the larger ones (29). Secondly, as in all case-control studies information bias may exist, leading to biased ORs related to the gene-environment interaction results. Thirdly, data on *Helicobacter pylori* infection were not available in our population.

This is the first study conducted on an homogenous ethnic group who evaluated the effect on gastric cancer risk of several metabolic genes SNPs contemporarily, and the effect of their combination with tobacco and alcohol. One of the main source of confounding in the genetic association studies arises from population stratification, since the ethnicity itself may be related to a specific disease and to the allele frequencies as well (30,31). Our study showed a significant association between *GSTT1* null genotype and gastric cancer, which is in keeping with the results of a recent meta-analysis considering only high-quality papers (8). Individuals who have the homozyogous deletion in *GSTT1* have no enzyme activity, and thus are more susceptible to carcinogens such as benzo(α)pyrene-7,8-diol epoxide and smaller reactive hydrocarbons, such as ethylene oxide and diepoxybutane (8). We also reported that individuals carriers of the *SULT1A1* variant allele, who have limited detoxification capability of xeniobiotics through sulfonate conjugation, have an additional risk of gastric cancer if smokers.

To our knowledge, we reported for the first time a strong effect modification by alcohol of the association between *CYP2E1*5A* or **6* alleles and gastric cancer, with an increased risk among ever-drinkers. Two previous studies (32,33) reported no association between *CYP2E1*5A* or *6 allele and gastric cancer, however no one of them stratified data according to alcohol habits. Additionally, one study evaluating the identical association among black South-African males showed an increased risk of oesophageal cancer among drinkers carrying the *CYP2E1*5A* or *6 alleles (34). CYP2E1

is a naturally ethanol-inducible enzyme that is mainly involved in the metabolic activation of *N*-nitrosamines present in tobacco smoke and some dietary compounds, for which a causative role in gastric carcinogenesis has been hypothesised (2), and in the metabolism of fatty acids and several halogenated and aromatic compounds (35). Additionally, CYP2E1 plays a minor role in alcohol metabolism, through the oxidation of ethanol to acetaldehyde and 1-hydroxyacetyl radicals (35). The **5A* or *6 alleles of *CYP2E1* is characterized by some studies in an increased gene expression (36), so that individuals carrying the unfavourable variant might be at higher risk of gastric cancer because of: i) hyper activation of *N*-nitrosamines in more reactive species, especially among drinkers since enzyme activity is induced by alcohol; ii) hyper production of reactive oxygen species and subsequent cell toxicity generated by ethanol metabolism among drinkers. We expected to gain similar results for *CYP2E1 Rsa*I polymorphism, identically associated with increased enzyme activity, however the few subjects in the stratified analysis probably did not show it. Since these results, however, are based on very few subjects (only one case drinker bearing **5A* or *6 alleles) they need to be confirmed by larger studies.

Among the main results of our study, we found that *GSTT1* null genotype individuals contemporarily *NAT2* slow acetylators have a strongly increased risk of gastric cancer, with a more than just the additive effect of the risks associated with each of the two inherited SNPs. N-acetylation is considered a major detoxification step for carcinogenic aromatic arylamines, while *GSTT1* is involved in the detoxification of polycyclic aromatic hydrocarbons, so individuals with one or both depleted phase II enzyme activities might be particularly susceptible to gastric damage from carcinogens, which is supported by the finding of an additional risk for ever-smokers. We used the attributable proportion due to interaction as a measure to quantify the biological interaction between those combined SNPs and showed a strong interaction between them. Assuming that the relationships studied are causal and based on the definition of biological interaction among two component causes (25,37), our results suggest that 52% of gastric cancer cases among *GSTT1* null individuals with combined *NAT2* slow acetylator phenotype are caused through a mechanism in which both risk factors are biological dependent in the same disease process. In other words, since biological interaction among two causes occurs when the effect of one is dependent from the presence of the other, in the absence of either of the two components (*GSTT1* null or *NAT2* slow), than a substantial number of gastric cancer cases would not occur. Given that in our population 25% of cases had a combination of those unfavourable genotypes, this means that a non negligible proportion of gastric cancer cases would have never developed if those enzymatic activities were adequate.

In conclusion, this study suggests that in this Italian population, *GSTT1*, *SULT1A1* and *NAT2* polymorphisms may modulate an individual's susceptibility to gastric cancer, particularly when more than one unfavourable genotype is present and in combination with cigarette smoke. Additionally, we showed that individuals carrying the **5A* or *6 alleles of *CYP2E1* are at increased risk for gastric cancer in drinkers. Clearly, since our study is based on a limited number of cases, it is critical that larger prospective studies possibly based on a single ethnic group confirms our results.

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2.2

Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and susceptibility to gastric adenocarcinoma in an Italian population

ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) plays a central role in the metabolism of folate, which provides methyl donor for DNA methylation and deoxynucleoside synthesis. We performed a case-control study to explore the relationship between two common *MTHFR* polymorphisms (*C677T* and *A1298C*), their combination and interaction with environmental exposures, on gastric adenocarcinoma susceptibility and progression in an Italian population. One hundred and two cases and 254 hospital controls, matched by age and gender, were enrolled. Individuals carrying *MTHFR* 677T allele showed an increased risk of gastric cancer (OR=1.62; 95% CI: 0.98-2.67), particularly among ever smokers (OR= 2.10; 95% CI: 1.07-5.33) and, among 677 TT individuals, those with a low intake of fruit and vegetables (OR= 2.18; 95% CI: 1.05-4.54). The strongest effect, however, was noted for the *MTHFR* 677 TT genotype among the diffuse gastric cancer histotype (OR=2.92; 95% CI: 1.12-7.60). No association was detected for the effect of *MTHFR A1298C* polymorphism. Survival analysis did not show any association between each polymorphism on the overall survival, although when the analysis was restricted to the first year of follow up after the surgical intervention an improved survival was noted among *MTHFR* 677 CC subjects compared to the T allele carriers (*p* value for log-rank test = 0.02). In conclusion, *MTHFR* 677 any T genotype appears to modulate individual's susceptibility to gastric cancer, particularly when combined with cigarette smoke and among those with a low intake of fruit and vegetables. Our results also suggest that aberrant DNA methylation pattern, trough impaired folate metabolism, might play a key role in gastric carcinogenesis. A possible survival effect of *MTHFR C677T* genotype in gastric cancer patients deserves further investigations with larger sample sizes.

INTRODUCTION

Fruit and vegetables intake has been repeatedly reported as protective for cancer occurrence including gastric cancer (1). A recent meta-analysis of prospective studies showed an inverse association between fruit and vegetables intake and gastric cancer incidence, particularly for follow-up periods at least of 10 years (2). The protective effect against cancer might be referable to the combined action of a number of the antioxidants micronutrients, such as β-carotene, vitamin C and E, retinol, and the folate content (3). Folate is a water-soluble B vitamin that plays the fundamental role of providing methyl groups for intracellular methylation reactions and *de novo* deoxynucleoside synthesis (4). Two prominent mechanisms whereby folate deficiency may influence cancer risk have been described (5): low folate levels might induce misincorporation of uracil into DNA, which could lead to chromosomal breaks and mutations; and/or by causing DNA hypomethylation, resulting in altered gene expression (6).

Besides an inadequate folate intake, functional polymorphisms in key enzymes involved in folate metabolic pathway are supposed to modify the risk of cancer. Among them, the methylenetetrahydrofolate reductase (MTHFR) enzyme irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate. Two functional polymorphisms of the *MTHFR*, *C677T* and *A1298C*, have been identified (7), so that heterozygotes (CT) and homozygotes (TT) for the mutant allele of 677 respectively have 65% and 30% of the enzyme activity of individuals with wild-type genotype, while CC homozygotes for *MTHFR* 1298 polymorphism have an enzyme activity around 50-60% of those without the variant allele *(7,* 8). Individuals with the TT genotype for *MTHFR* 677 have significantly lower plasma folate levels than those with the wild-type genotype, while for the 1298 variant the evidences are inconsistent (9). Some nutrients (eg, vitamin B_6 and B_{12}, and methionine) involved in the folate metabolic pathway, as well as alcohol (a folate antagonist) and smoking (which impairs folate stauts) may interact with folate and the *MTHFR* polymorphisms in relation to cancer risk (10,11).

A recently published meta-analysis shows no significant protective effect of dietary folate intake on gastric cancer, while an increased risk associated with the *MTHFR* 677 TT genotype has been detected (5). Few studies, however, explored a possible effect modification of *MTHFR C677T* polymorphism on gastric cancer risk by environmental exposures affecting folate status (12,13,14), and no one ever explored this aspect in relation to *MTHFR A1298C* polymorphism. Furthermore, nothing is known about the influence of those genetic variants on the survival of gastric cancer patients. In the

present study we aimed to investigate the effect of both *MTHFR C677T* and *A1298C* polymorphisms, their combination and the interaction with lifestyle exposures that might affect plasma folate levels, on gastric cancer development and progression in an Italian population.

METHODS

Study population and genotyping

The study subjects were selected according to a case-control study design as previously described (15). Briefly, cases were consecutive primary gastric adenocarcinoma patients, with histological confirmation, who underwent a curative gastrectomy in the "A. Gemelli" teaching hospital, located within the Università Cattolica del Sacro Cuore in Rome. Controls were selected from cancer-free patients, with a broad range of diagnoses, admitted to the same hospital during the identical time period and were frequency matched to cases for age (±5 years) and gender. All subjects were Caucasians born in Italy. According to the Lauren classification, the majority (57.8%) of the gastric cancer cases were intestinal (16). The tumours were located in the antrum (39.3%), in the corpus (14.8%), in the antrum/corpus (28.0%), in the cardia (10.3%), stumps (5.6%) and in the fundum (2.0%). Based on the cytological and architectural atypisms, as well as the histo-pathological reports (17), patients' tumours were classified accordingly: 68.3% scarcely differentiated, 29.2% moderately differentiated, 2.5% well-differentiated, while 53.8% were staged I-II and 46.2% staged III-IV. With a response rate of 95% and 90% respectively for cases and controls, 102 gastric cancer and 254 controls were recruited.

A venous blood sample was drawn from each participant, collected into an EDTA-coated tubes from which DNA was isolated from peripheral blood lymphocytes. Genotyping for *MTHFR C677T* and *A1298C* polymorphisms were performed using a restriction-fragment length polymorphism based method, as already described by Yi et al (18). Quality control for each genotyping was performed in each experiment, and 10% of the total samples were randomly selected and reanalyzed with 100% concordance. The analyst was blinded to the case or control status of the samples. The study was approved by the local review board and written informed consent was obtained from each subject. The procedures followed were in accordance with the Helsinki Declaration.

Data collection

Cases and controls were interviewed by trained medical doctors using a standard questionnaire to elicit information on demographic variables, cigarette smoking and drinking history, dietary habits and family history of cancer. Questions pertaining to lifestyle focused on the time period ending one year prior to diagnosis. Smoking status was categorized as never and ever-smokers (including both current and former smokers). Pack-years were calculated as years smoked multiplied by the current number (or previous number, for those who had quit) of cigarettes smoked per day divided by 20. Fruit and vegetables intake was classified as high if the individual consumed at least two portions of fruit and vegetables per day. Family history of cancer referred to parents, siblings and offspring. Cases were actively followed-up after the day of surgical intervention with a median follow-up time of 19.5 months, and information on all-cause mortality was collected. The proportion of lost to follow-up was 11.7% (12/102).

Statistical analysis

The relationship between gastric cancer and putative risk factors were measured using the adjusted odds ratios (ORs) and their 95% CI derived from logistic regression analysis using STATA software (version 8.2). A variable was defined as confounder if the addition of that variable to the model changed the OR by 10% or greater. If a factor was identified as a confounder of any estimated main effect, it was kept in all models. Based on these criteria, we controlled for age, gender, alcohol consumption and family history of cancer, when appropriate. In the multivariable model, we adjusted for the continuous variables of age and alcohol. Hardy-Weinberg Equilibrium (HWE) was tested for separately all of the case and control polymorphisms.

In order to asses if the effect of the studied polymorphisms is modified by lifestyle exposures that might affect folate status, we performed a logistic regression analysis stratified for alcohol, smoking status and fruit and vegetables intake (the main source of dietary folate). An heterogeneity test was then used to test differences among the strata. In this analysis we used as a reference group those homozygous wild-type individuals who had not been exposed to environmental factors; smoking status was here considered as ever/never cigarette smokers and alcohol consumption as users/non-users.

Finally, the log-rank test was used to evaluate the association between both *MTHFR* polymorphisms and the survival at 1-year, 3-years and the overall survival after gastric surgery intervention. The risk of death was also estimated by Cox's proportional hazards model, when applicable. Hazard ratios (HR) were adjusted for age and gender, with the wild-type genotypes as the reference group.

RESULTS

General characteristics of the study population are presented in Table 1. Alcohol consumption and family history of cancer were associated with an increased risk of gastric cancer, with ORs of 2.10 (95% CI: 1.21-3.67) and 3.74 (95% CI: 1.13-12.45) for moderate and heavy drinkers, respectively, and an OR of 1.80 (95% CI: 1.04-3.06) for individuals with a familial history of cancer (see Table 1). The genotype frequencies of our control group were in line with those for Caucasians (19) and were in HWE both for cases and controls ($p > 0.05$). As shown in Table 2, we found a significant difference in the distribution of *MTHFR* 677T carriers among cases and controls: 71.6% *versus* 61.4%, respectively, with an OR of 1.62 (95% CI: 0.98-2.67). When results were stratified according to tumour histology, the strongest effect was noted among the diffuse type, with an OR of 2.92 (95% CI: 1.12-7.60) for *MTHFR* 677 TT (Table 2).
From the analysis of the combined effect of the *MTHFR C677T* and *A1298C,* no one subject was homozygous for the mutant allele at both sites (data not shown). Among subjects carrying both *MTHFR* 677 TT and 1298 AA, the OR was 2.21 (95% CI: 0.84-5.80), and similarly individuals with both *MTHFR* 677 CT and 1298 AC genotypes had an OR for gastric cancer of 1.95 (95% CI: 0.80-4.81) compared to those with combined 677 CC/1298 AA (data not shown). The heterogeneity test, however, showed that none of these differences was statistically significant, probably due to the very small number of both variant alleles in the analysis.
From our analysis there was no evidence of effect modification of *MTHFR* 677 and 1298 polymorphisms by the lifestyle exposures (Table 3), however ever smokers carrying the *MTHFR* 677 T allele showed a significant increased risk (OR= 2.40; 95% CI: 1.07-5.33) of gastric cancer, while among never smokers that risk appeared not significant (OR= 1.16; 95% CI: 0.60-2.52). Additionally, when *MTHFR* 677 TT genotype was stratified according to the fruit and vegetable intake, an OR of 0.49 (95% CI: 0.04-5.9) and 2.18 (95% CI: 1.05-4.54) resulted among high and low consumers, respectively (data not shown; p value of heterogeneity among the estimates= 0.09).

Table 1. Odds Ratios (95% CI) for gastric cancer according to selected variables and their frequency distribution among 102 gastric cancer cases and 254 controls

	Cases n (%)	Controls n (%)	OR (95% CI) †
Age (years± SD)	66.3±12.1	64.0±13.0	-
Male gender	54 (53.0)	141 (55.5)	-
Alcohol drinkers			
0- 6 g/day	40 (39.6)	150 (59.3)	1*
7- 29 g/day	53 (52.5)	96 (37.9)	2.10 (1.21-3.67)
> 30 g/day	8 (7.9)	7 (2.8)	3.74 (1.13-12.45)
Smoking status			
Never	54 (53.0)	146 (57.5)	1*
Ever	48 (47.1)	108 (42.5)	1.09 (0.63-1.90)
Pack-years of smoking			
0	55 (55.0)	146 (57.7)	1*
1-25	21 (21.0)	62 (24.5)	0.97 (0.50-1.92)
> 25	24 (24.0)	44 (17.8)	1.05 (0.53-2.12)
Fruit and vegetables intake			
High‡	19 (18.8)	40 (15.9)	1*
Low	82 (81.2)	212 (84.1)	0.96 (0.50-1.85)
Meals salt addition			
No	88 (86.3)	235 (92.9)	1*
Yes	14 (13.7)	18 (7.1)	1.52 (0.68-3.41)
Family history of cancer			
No	59 (62.8)	192 (78.4)	1*
Yes	35 (37.2)	53 (21.6)	1.80 (1.04-3.06)

† = OR adjusted by age, gender, alcohol consumption and family history of cancer

* = Reference category

‡= At least two portions of fruit and vegetables per day

Table 2. Distribution of the studied polymorphisms in 102 gastric cancer cases and 254 controls

	Cases n (%)	Controls n (%)	All cases OR (95% CI) †	Intestinal OR (95% CI) †	Diffuse OR (95% CI) †
MTHFR 677					
CC	29 (28.4)	98 (38.6)	1*	1*	1*
CT	51 (50.0)	115 (45.3)	1.53 (0.90-2.62)	1.27 (0.67-2.41)	2.45 (1.09-5.50)
TT	22 (21.6)	41 (16.1)	1.84 (0.95-3.59)	1.27 (0.55-2.92)	2.92 (1.12-7.60)
T carriers	73 (71.6)	156 (61.4)	1.62 (0.98-2.67)	1.27 (0.70-2.32)	2.58 (1.19-5.58)
MTHFR 1298					
AA	50 (49.0)	125 (49.2)	1*	1*	1*
AC	43 (42.2)	107 (42.1)	0.98 (0.60-1.59)	0.47 (0.33-1.63)	0.72 (0.36-1.45)
CC	9 (8.8)	22 (8.7)	0.97 (0.42-2.27)	-	1.50 (0.52-3.91)
C carriers	52 (51.0)	129 (50.8)	0.98 (0.62-1.55)	1.06 (0.60-1.88)	0.84 (0.44-1.60)

† = OR adjusted by age and gender

* = Reference category

The mortality rate in our gastric cancer cases was 1.01/100 person-months (95% CI: 0.70-1.45). Patients carrying at least one *MTHFR* 677 T allele did not show a different median survival time (*p* value for log-rank test = 0.49), with an HR of 1.19 (95% CI: 0.49-2.92) and 1.79 (95% CI: 0.67-4.78) for *MTHFR* CT and TT genotypes, respectively. Namely, 10 out of 51 *MTHFR* CT individuals and 8 out of 22 *MTHFR* TT died in the follow-up period. Similar results were obtained for *MTHFR* 1298T carriers, with an HR of 1.05 (95% CI: 0.50-2.22; *p* value for log-rank test= 0.82) compared with the homozygous wild-type. When the analysis was restricted to 1 year survival, *MTHFR* 677 CC subjects all resulted alive respect to those carrying at least one 677 T allele (*p* value for log-rank test = 0.02) (Figure). However, when the time period was extended to 3-years the effect was not longer detected (*p* value for log-rank test = 0.60). Absence of a survival affect was noted for *MTHFR* 1298C carriers when restricting the analysis to the first year and three years after the surgical intervention (data not shown).

Table 3. Risk of gastric cancer associated with the *MTHFR* C677T and A1298C genotypes according to selected environmental exposures

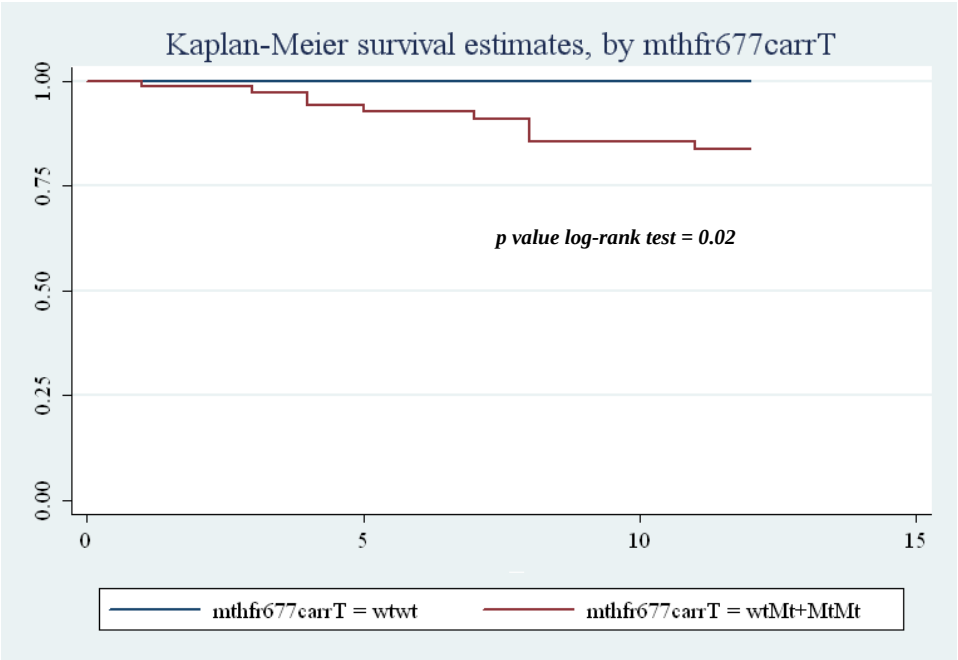
	***MTHFR* 677 T carriers ***			***MTHFR* 1298 C carriers***		
	No. Cases/ No. Controls	**OR (95% CI) †**	***p* value for for heterogeneity across strata**	**No. Cases/ No. Controls**	**OR (95% CI) †**	***p* value for for heterogeneity across strata**
Smoking status						
Never	35/90	1.16 (0.60-2.52)	0.17	27/81	0.78 (0.41-1.48)	0.22
Ever	38/66	2.40 (1.07-5.33)		25/48	1.36 (0.68-2.70)	
Alcohol drinkers						
No	21/71	1.76 (0.71-4.34)	0.67	21/75	1.16 (0.51-2.66)	
Yes	52/85	1.46 (0.78-2.73)		31/54	0.87 (0.49-1.54)	0.61
Fruit and vegetables intake						
High‡	12/21	1.48 (0.48-4.60)	0.94	9/22	0.68 (0.22-2.11)	
Low	61/135	1.68 (0.95-3.00)		43/106	1.06 (0.63-1.77)	0.51

† = OR adjusted by age and gender

* = Reference category is the homozygous wild type genotype

‡= At least two portions of fruit and vegetables per day

Figure. Association between *MTHFR C677T* genotypes and 12 months survival after the gastric surgery intervention



DISCUSSION

This case-control study of 102 surgical cases of gastric adenocarcinoma and 254 controls evaluated the effect on gastric cancer risk of two common *MTHFR* polymorphisms. Results showed an increased risk for *MTHFR* 677T carriers, with a growing trend from individuals carrying only one variant allele to those carrying two (*p* value for trend = 0.05). That risk became higher in patients with the diffuse gastric cancer histological type. No association was detected for the effect of *MTHFR A1298C* polymorphism on gastric cancer risk, also with no evidence of effect modification by the lifestyle exposures. An increased risk was detected among ever-smokers carrying at least one *MTHFR* 677 variant allele and among those *MTHFR* 677 TT with a low intake of fruit and vegetables, despite the results of the heterogeneity test did not support a true effect modification in both instances. Our results also confirm previous findings of gastric cancer risk to be increased by alcohol intake and familial clustering (20,21). Additionally, from the survival analysis a possible effect was noted when considering only the first year follow-up, with all died subjects among those carrying at

least one 677 T allele.

Some limitations of the study need to be considered before interpreting the results. Firstly, based on the prevalence of the analyzed genotypic variants in our control population (see Table 2), our study is powered to detect a minimum OR of 2.0 for the effect of *MTHFR* 677T carriers and *MTHFR* 1298C carriers (with a significance level of 0.05), however the power is lower for the homozygote variants of both genotypes. The study sample size also limits the ability to explore the effect modification of the environmental exposures, or the the combined effects of both genotypes, which highlights the need to increase the sample size in order to confirm our results. However, when appropriately conducted, large and small studies should give, theoretically, the same results, with just a more precise effect measure estimate from the larger ones (22). Secondly, as in all case-control studies information bias may exist, leading to biased ORs related to the lifestyle exposures.

Our study supports the evidence of an increased risk for gastric cancer among individuals carrying the unfavourable variant of *MTHFR* 677, thus confirming the results of the two recently published meta-analyses (5,23). Individuals who are *MTHFR* 677T carriers have reduced enzyme activity and, particularly among those with inadequate folate intake (13), subsequent aberrant genomic DNA methylation. We also observed that the risk for *MTHFR* 677T carriers is higher among those with a diffuse gastric cancer histotype, which is the most deadly form of gastric cancer (24), as already noted by Lacasana-Navarro et al. (25). Recently, aberrant methylation of proto-oncogenes has been explored as both a mechanism and marker of carcinoma progression (26), with some papers reporting a different methylation pattern between the intestinal and diffuse gastric cancer histotype (27,28). Taken together, these results suggest that global aberrant DNA methylation pattern might play a key role in gastric cancer susceptibility and progression, with the MTHFR enzyme playing a central part.

In the present study we observed that the effect of the *MTHFR* 677 variant genotype is particularly strong among ever-smokers, which is in keeping with the results from Gao et al (14). As for the negative effect of smoking on folate status, some authors reported that elevated folate turnover in response to rapid tissue proliferation in aerodigestive tissues among people exposed to tobacco smoke might partially explain this phenomenon, that might be even worsened among individuals carrying the unfavourable *MTHFR* genotype variant (29). We cannot ignore, however that this effect might be confused by alcohol intake or dietary habits (30).

To our knowledge, no one study ever explored whether or not the effect of *MTHFR* 677 TT genotype on gastric cancer is modified by folate levels measured by serological

tests, however two studies reported a strong association between *MTHFR* 677 TT and gastric cancer in populations with folate deficiency (13,31). Also, a prospective study on colorectal cancer risk reported that the protective effect of *MTHFR* 677 TT genotype disappears among those with folate deficiency (32), so we would expect a partial reduction of the negative effect on gastric cancer risk by the *MTHFR* 677 variant among those with adequate folate intake. From our study it appears that the effect of the *MTHFR* 677 variant genotypes on gastric cancer might be modified by fruit and vegetables intake, the main source of dietary folate, particularly among TT individuals. The lack of statistical power, however, limits our result which need to be confirmed by increasing the sample size.

As for the combined inheritance of the two *MTHFR* variants, although all the reported results are not statistically significant for an $\alpha = 0.05$, there is a slight evidence of an increased risk in individuals carrying at least one T allele of *MTHFR* C677T, which is keeping with the results of Miao et al. (31).

Finally, we were not able to detect any effect of the studied polymorphisms on the overall survival after surgical gastrectomy intervention, however when considering the first year of follow-up an increased mortality rate was experienced from *MTHFR* 677T carriers when compared with the CC individuals. Despite the result is based on a very few number of subjects, it suggests that a different pathway of tumour progression might be experienced from gastric cancer patients based on their *MTHFR* 677 polymorphism that eventually affects folate blood levels and DNA methylation status. A recent study on the assessment of folic acid supplementation on colorectal adenomas failed to detect a preventive effect, while an increased risk of colorectal neoplasia was revealed among treated individuals (33). To our knowledge, similar trials have never been conducted on gastritis or gastric cancer individuals, therefore the exact effect on gastric carcinogenesis of folic acid levels, affected by *MTHFR* status, deserves additional investigations in order to integrate all the results into one coherent picture.

In conclusion, this study supports the role of *MTHFR C677T* polymorphism, but not *A1298C*, in gastric carcinogenesis, particularly for the diffuse histotype which is usually associated with a poorer prognosis. In order to gain a clearer picture of the events influencing gastric cancer susceptibility and progression through the folate metabolic pathway, it is critical that larger prospective studies with appropriate collection of data on lifetsyle exposures and folate intake are implemented. This would lay the foundation for evaluating possible benefits from preventive nutritional intervention in at-risk individuals for gastric cancer.

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2.3

A case-control study on the effect of *p*53 and *p*73 polymorphisms on gastric cancer risk and progression

ABSTRACT

p53 protein and its functional homologue p73 share some functions, as modulating cell-cycle control and apoptosis. Based on the functional interaction between p53 and p73 in carcinogenesis, we investigated the combined effect of *p*73 G4C14-to-A4T14 and *p*53 polymorphisms and their interaction with selected environmental factors, on the risk of gastric cancer in hospital-based case-control study conducted in Italy. Their effect on cancer progression has also been investigated. One-hundred fifteen cases and 295 hospital controls were genotyped for *p*73 G4C14-to-A4T14, and *p*53 exon4 (Arg72Pro), intron 3 and 6 polymorphisms. An increased risk of gastric cancer was found to be associated with the inheritance of *p*73 homozygous variant genotype among the gastric cancer intestinal histotype [(odds ratio (OR) = 6.75; 95% confidence interval (95% CI)=1.88–24.24)]. An effect modification of *p*73 variant allele by gender was observed [(OR= 2.82; 95% CI: 1.24–6.40) among females, versus an OR of 0.70 (95% CI: 0.32-1.54) among males; *p* value for heterogeneity among strata estimates = 0.03]. Gene-gene interaction analyses demonstrated that individuals with combined *p*53 exon 4 and intron 6 variant alleles are borderline significantly protected from gastric cancer (OR=0.52; 95% CI: 0.26-1.07; *p* value for interaction=0.005), which was confirmed by the haplotype analysis. Lastly, a poorer survival resulted among gastric cancer cases with an intestinal histotype carriers of the variant allele of *p*53 intron 6 compared to those carrying both wild-type alleles (*p* value for log-rank test = 0.02). This study shows that *p*73 G4C14-to-A4T14 polymorphism might be a risk factor for gastric cancer, as reported from other studies in different tumour sites among Caucasians. Along with the protective effect of *p*53 exon 4-intron 6 allelic variants, already noted for breast and lung cancer, our results require confirmation from larger studies.

INTRODUCTION

The incidence of gastric cancer is decreasing in recent decades in most western countries; nevertheless it remains the second leading cause of death from cancer worldwide (1). Gastric carcinogenesis is a multistep process, in which environmental and genetic factors interact.

The response of gastric mucosa to exogenous damaging injuries is partly regulated by inhibitory and stimulatory factors, which are products of proto-oncogenes and tumor suppressor genes. Improper function of these inhibitory and stimulatory factors is associated with a chronic damage of the gastric mucosa (2). Among these key proteins are: the *p*53 tumor suppressor gene (17p13), which encodes a protein involved in cell cycle regulation and differentiation, DNA repair, and apoptosis (3), and the *p*73 gene (1p36-33), a *p*53 homologue for both the amino acid sequence (4) and DNA binding domain that encodes a protein inhibiting cell growth by inducing apoptosis in a *p*53-like manner (4,5). It has been reported that the *p*73 gene maps on a region often deleted in a variety of human cancers (6). Both *p*53 and *p*73 genes are highly polymorphic, with at least 13 single nucleotide polymorphisms (SNPs) described for *p*53 and 19 for *p*73 (7).

Currently the *p*53 exon4 Arg72Pro polymorphism is the only *p*53 SNP whose role has been extensively studied in relation to gastric cancer. A polymorphism in this codon has been suggested to modulate *p*53-dependent apoptosis and modify sensitivity to chemotherapeutic agents (8). A recent meta-analysis shows a significantly higher frequency of the homozygous variant genotype in gastric cancer cases compared with controls in Asians but not Caucasians (2). Two intronic polymorphisms of *p*53 consisting of a 16-bp duplication in intron 3 and a G→A transition in intron 6 have been investigated in relation to lung, ovarian, breast and colon cancer, with conflicting results (9-12). To our knowledge, no study has investigated the association of these two SNPs with gastric cancer. As for the *p*73 gene, several reports investigated the relationship between the *p*73 G4C14-to-A4T14 polymorphism and cancer (7,13-15). Among these, a study conducted in Asians showed no association with gastric cancer (14). The *p*73 G4C14-to-A4T14 dinucleotide polymorphism consists of two SNPs in complete linkage disequilibrium (13) located at positions 4 (G→A) and 14 (C→T) in the non-coding region of exon 2. These lie just upstream of the initiating AUG of the exon and potentially influence gene expression by forming a stem-loop structure (15). The influence of *p53* intron 3 and 6 polymorphisms on the survival of gastric or other cancer patients has never been investigated. A poor disease-free survival for breast, lung, colorectal and head and neck cancer has been

reported in patients homozygous for the *p*53 exon4 Arg72Pro mutant allele compared with the wild type genotype (8,16-18). One study reports an improved survival of colorectal cancer patients carrying the variant allele of *p*73 G4C14-to-A4T14 (19).

The aim of our hospital-based case-control study was to investigate the effects of the *p*73 G4C14-to-A4T14 polymorphism and three *p*53 polymorphisms in exon 4 and introns 3 and 6, on gastric adenocarcinoma risk and progression in an Italian population. We also explored the impact of different combinations of these polymorphisms and their interaction with environmental factors on the risk of gastric cancer.

METHODS

Study population and genotyping

The study subjects were selected according to a case-control study design as previously described (20,21). Briefly, cases were consecutive primary gastric adenocarcinoma patients, with histological confirmation, who underwent a curative gastrectomy in the "A. Gemelli" teaching hospital during the period from 2002 to 2007. Controls were selected from cancer-free patients, with a broad range of diagnoses, admitted to the same hospital during the same time period. All subjects were Caucasians born in Italy. With a response rate of 95% and 90% respectively for cases and controls, 115 gastric cancer and 295 controls were recruited, the majority (58.2%) of the gastric cancer cases were intestinal, according to Lauren classification (22). The tumours were located in the antrum (40.0%), in the corpus (14.6%), in the antrum/corpus (24.6%), in the cardia (3.6%), stumps (5.4%), in the fundum (1.8%), in the cardia/corpus (7.3%) and the entire stomach (2.7%). Based on the cytological and architectural atypisms, as well as the histo-pathological reports (23), patients' tumours were classified as follows: 69.5% poorly differentiated (G3), 28.1% moderately differentiated (G2), 2.4% well-differentiated (G1). 54.2% of tumors were staged I-II and 45.8% were staged III-IV.

A venous blood sample was drawn from each participant and collected into an EDTA-coated tube from which DNA was isolated from peripheral blood lymphocytes. Genotyping for *p*53 exon 4 codon 72 (rs1042522), *p*53 intron 3 (rs17883323), *p*53 intron 6 (rs1625895) polymorphisms was performed using a polymerase chain reaction (PCR) followed by restriction-fragment length polymorphism, as already described by Wu et al (9). Genotyping of *p*73 exon 2 G4C14-to-A4T14 (rs 2273953/rs 1801173) was performed using PCR as described by Niwa et al (4). We conducted haplotype analysis

for *p*53 intron 3, *p*53 exon 4 codon 72 and *p*53 intron 6 polymorphisms using EH software (24) and cocaphase (25).

Quality control for each genotyping was performed in each experiment, and 10% of the total samples were randomly selected and retested with 100% concordance. The analyst was blinded to the case or control status of the samples. The study was approved by the local review board and written informed consent was obtained from each subject. The procedures followed were in accordance with the Helsinki Declaration.

Data collection

Cases and controls were interviewed by trained medical doctors using a structured questionnaire to collect information on demographic data, cigarette smoking, drinking history, dietary habits, physical activity and family history of cancer. Participants were asked to focus on the year prior to diagnosis (for controls the year prior to the interview date) when answering questions regarding lifestyle habits. Smoking status was categorized as never- and ever-smokers (including both current and former smokers). Pack-years were calculated as years smoked multiplied by the current number (or previous number, for those who had quit) of cigarettes smoked per day divided by 20. Fruit and vegetable intake was classified as high if the individual consumed at least two portions of fruit and two portions of vegetables per day. "Meals salt addition" referred to the use of adding salt to the main-meal entrées. Physical activity was classified as high if the individual has physical training at least 2 times/week. Family history of cancer referred to parents, siblings and offspring. Data concerning previous *Helicobacter pylori* infection were not available for either cases or controls. The response rate for completing the interview was 92% for cases and 97% for controls, with the exception of data relating to grilled meat intake (unknown in 12% of cases and 16.6% of controls) and the family history of cancer (unknown in 7.8% of cases and 3% of controls). Cases were actively followed-up after the day of surgical intervention with a median follow-up time of 19 months and information on all-cause mortality was collected. The proportion of lost to follow-up was 11.3% (13/115).

Statistical analysis

The relationship between gastric cancer and putative risk factors was measured using the adjusted odds ratios (ORs) and their 95% CI derived from logistic regression analysis using STATA software (version 8.2). A variable was defined as confounder if the addition of that variable to the model changed the OR by 10% or greater. If a

factor was identified as a confounder of any estimated main effect, it was kept in all models. Based on these criteria, we controlled for age, alcohol consumption, family history of cancer, fruit and vegetable intake and salt use. In the multivariate model, we adjusted for the continuous variables of age and alcohol. Hardy-Weinberg Equilibrium (HWE) was tested separately for all of the case and control SNPs.

In order to examine if the effects of the studied polymorphisms are modified by age, gender, family history of cancer, and some environmental exposures, we performed a logistic regression analysis stratified for age, gender, alcohol, smoking status and family history of cancer. An heterogeneity test was then used to test differences among the strata. In this analysis we used as a reference group those homozygous wild-type individuals who had not been exposed; age was categorized binomially (< 45 and ≥ 45 years old), smoking status was considered as ever/never cigarette smokers, and alcohol consumption as drinkers/non-drinkers (the latter including individuals whose alcohol intake was less than 7 g/day). A gene-gene interaction analysis was performed among the four SNPs, and the likelihood ratio test was used for more than multiplicative effect among each pair of SNPs, with the homozygous wild-type individuals for both genes as the reference group (26).

Additionally, the log-rank test was used to evaluate the effect of the four SNPs on survival at 1-year, 2-years, and the overall survival after gastric surgery intervention. Finally, we stratified survival analyses by cancer histotype (intestinal/diffuse).

RESULTS

General characteristics of the study population are presented in Table 1. Alcohol consumption and meals salt addition were associated with an increased risk of gastric cancer, with ORs of 1.70 (95% CI: 1.02-2.83) and 6.71 (95% CI: 2.19-20.56) for moderate and heavy drinkers, respectively, and an OR of 2.27 (95% CI: 1.05-4.90) for individuals usually adding salt to meals (Table 1). Additionally, family history of cancer was associated with an increased risk of gastric cancer, with an OR of 2.02 (95% CI: 1.18-3.46; Table 1). The genotype frequencies of our control group were in line with those for Caucasians (9) and respected HWE as detailed in Table 2 (p value > 0.05). As shown in Table 2, there were no significant differences in the distribution of the *p*53 polymorphisms among cases and controls, even when cases were stratified by tumour histology.

Table 1. Odds Ratios (95% CI) for gastric cancer according to selected variables and their frequency distribution among 115 gastric cancer cases and 295 controls

	Cases n (%)	Controls n (%)	OR (95% CI) †
Age (years± SD)	66.7±11.7	63.5±13.1	-
Male gender	63 (54.8)	165 (56.0)	-
Alcohol drinkers			
0-6 g/day	49 (42.6)	182 (61.7)	1*
7-29 g/day	53 (46.0)	106 (35.9)	1.70 (1.02-2.83)
≥ 30 g/day	13 (11.4)	7 (2.4)	6.71 (2.19-20.56)
Smoking status			
Never	63 (55.3)	176 (59.7)	1*
Ever	51 (44.7)	119 (40.3)	1.05 (0.63-1.76)
Pack-years of smoking			
0	64 (57.7)	179 (62.0)	1*
1-25	21 (18.9)	66 (22.8)	0.96 (0.50-1.81)
> 25	26 (23.4)	44 (15.2)	1.35 (0.68-2.68)
Fruit and vegetables intake			
High‡	19 (16.8)	40 (14.0)	1*
Low	94 (83.2)	247 (86.0)	0.91 (0.47-1.76)
Meals salt addition			
No	96 (84.2)	266 (93.0)	1*
Yes	18 (15.8)	20 (7.0)	2.27 (1.05-4.90)
Physical activity			
High #	17 (14.9)	30 (10.2)	1*
Low	97 (85.1)	263 (89.8)	0.67 (0.33-1.36)
Family history of cancer			
No	67 (63.2)	232 (81.4)	1*
Yes	39 (36.8)	53 (18.6)	2.02 (1.18-3.46)
Family history of gastric cancer			
No	100 (91.7)	270 (94.7)	1*
Yes	9 (8.3)	15 (5.3)	0.56 (0.20-1.60)

* Reference category

† OR adjusted by age, gender, alcohol consumption, family history of cancer, and meals salt addition

* Reference category

‡ At least two portions of fruit and vegetables per day

At least two times/week

On the other hand, our data showed a significant difference in the distribution of the *p*73 AT/AT genotype among cases and controls: 7.0% versus 3.4%, respectively, with an OR of 4.77 (95% CI: 1.50-15.19) (Table 2).

When results were stratified according to tumour histology, the significant association between *p*73 homozygous variant genotype and gastric cancer was limited to the intestinal type, with an OR of 6.75 (95% CI: 1.88-24.24; Table 2). The estimated *p*53 pairwise haplotype frequencies among the three polymorphisms and their linkage disequilibrium values in cases and controls are shown in Table 3. The pairwise linkage disequilibria were highly significant in both control and patient population (*p* value for χ^2 test < 0.001). The frequency of exon 4 (Pro)/intron 6 A diplotype was lower in cases than in controls (9.8% versus 17.7%), with a decreased gastric cancer risk (OR= 0.56; 95% CI: 0.37-0.97) if compared with the reference exon 4 (Arg)/intron 6 G diplotype.

There were no significant differences in the remaining diplotype frequencies among cases and controls (*p* value > 0.1; Table 3). When the estimation of haplotype frequencies was extended to all three SNPs (Table 4), results show no significant difference in the haplotype frequencies among cancer patients and controls (*p* value > 0.1).

From the analysis there was no evidence of effect modification of the four SNPs by environmental exposures (data not shown), neither by age nor family history of cancer. The only one exception was gender, which was shown to be an effect modifier of the association between *p*73 variant allele carriers and gastric cancer [(OR= 2.82 (95% CI: 1.24-6.40) among females v*ersus* OR= 0.70 (95% CI: 0.32-1.54) in males (*p* value of heterogeneity among the estimates = 0.03)]. From the gene-gene interaction analysis there was no evidence of multiplicative interaction among the four polymorphisms (*p* values > 0.05, Table 5), except for individuals carrying the mutant alleles of both *p*53 exon 4 and *p*53 intron 6 that appeared to be protected from gastric cancer with an OR of 0.52 (95% CI: 0.26-1.07; *p* value for interaction = 0.005), showing a less than multiplicative combined effect. On the other hand an OR of 3.19 (95% CI: 1.01-10.02) was observed for those p53 exon 4 wild-type homozygotes, carrying also one *p*53 intron 6 variant allele at least.

The mortality rate in our gastric cancer cases was 1.01/100 person-months (95% CI: 0.71-1.44). Individuals carrying at least one variant allele for each of the four SNPs did not show a different median survival time (*p* value of log-rank test > 0.05), even when the analysis was restricted to 1 year and 2-years after the surgical intervention (data not shown). However, when the analysis was restricted to the intestinal histotype, an increased overall mortality rate was observed among individuals carrying the *p*53 intron 6 variant allele compared to those with both wild-type alleles (*p* value for log-rank test = 0.02).

Table 2. Distribution of the studied polymorphisms in 115 gastric cancer cases and 295 controls

	Cases n (%)	Controls n (%)	HWE^	All cases OR (95% CI) †	Intestinal (n.61) OR (95% CI) †	Diffuse (n.44) OR (95% CI) †
*p*53 intron 3 (rs1788332)						
WW	80 (70.2)	209 (70.8)		1*	1*	1*
WM	27 (23.7)	76 (25.8)	0.349	0.91 (0.51-1.63)	1.07 (0.53-2.13)	0.69 (0.30-1.59)
MM	7 (6.1)	10 (3.4)		1.65 (0.52-5.22)	1.12 (0.22-5.60)	2.08 (0.55-7.79)
M carriers	34 (29.8)	86 (29.2)		1.02 (0.60-1.76)	1.10 (0.56-2.13)	0.86 (0.41-1.82)
*p*53 exon4 Arg72Pro (rs1042522)						
Arg/Arg	71 (62.3)	169 (57.3)		1*	1*	1*
Arg/Pro	34 (29.8)	102 (34.6)	0.130	0.80 (0.47-1.38)	0.76 (0.39-1.49)	0.80 (0.38-1.67)
Pro/Pro	9 (7.9)	24 (8.1)		0.74 (0.29-1.83)	0.59 (0.18-1.93)	0.89 (0.27-2.92)
Pro carriers	43 (37.7)	126 (42.7)		0.79 (0.47-1.30)	0.72 (0.39-1.36)	0.82 (0.41-1.62)
*p*53 intron 6 (rs1625895)						
GG	82 (71.9)	192 (65.1)		1*	1*	1*
GA	25 (22.0)	87 (29.5)	0.148	0.73 (0.41-1.30)	0.65 (0.32-1.34)	0.80 (0.37-1.72)
AA	7 (6.1)	16 (5.4)		0.96 (0.32-2.83)	0.55 (0.11-2.74)	1.46 (0.42-5.09)
A carriers	32 (28.1)	103 (34.9)		0.75 (0.44-1.27)	0.62 (0.31-1.23)	0.91 (0.45-1.82)
*p*73 exon 2 G4A (rs 1801173/ rs 2273953)						
GC/GC	84 (73.7)	214 (72.5)		1*	1*	1*
GC/AT	22 (19.3)	71 (24.1)	0.183	0.96 (0.53-1.75)	0.84 (0.39-1.81)	1.28 (0.60-2.72)
AT/AT	8 (7.0)	10 (3.4)		4.77 (1.50-15.19)	6.75 (1.88-24.24)	3.33 (0.59-18.78)
AT carriers	30 (26.3)	81 (27.5)		1.25 (0.73-2.16)	1.28 (0.66-2.49)	1.41 (0.68-2.90)

^ *p* value for the χ^2 -test

† = OR adjusted by age and gender

* = Reference category

Table 3. Pairwise haplotype frequency in the gastric cancer patients and controls

	No. alleles	Estimated haplotype frequency				D'	*p* value
		‡ 1 - 1	1 - 2^	2 - 1	2 - 2		
Intron 3-exon 4[†]							
Cases	228	0.709	0.110	0.062	0.117	0.55	<0.001
Controls	588	0.713	0.122	0.031	0.132	0.74	<0.001
Intron 3-intron 6[†]							
Cases	228	0.774	0.046	0.055	0.125	0.67	<0.001
Controls	588	0.763	0.074	0.035	0.128	0.73	<0.001
exon 4-intron 6*							
Cases	228	0.699	0.073	0.129	0.098	0.45	<0.001
Controls	588	0.719	0.026	0.078	0.177	0.83	<0.001

† χ^2 -test: cases versus controls; *p* value >0.1

* *p* value< 0.001

‡ 1= intron 3 (W), exon 4 Arg, intron 6 G

^ 2= intron 3 (M), exon 4 Pro, intron 6 A

Table 4. Estimated p53 haplotype frequencies in the gastric cancer patients and controls

	Alleles	1 - 1 - 1	1 - 1 - 2	1 - 2 - 1	1 - 2 - 2	2 - 1 - 1	2 - 1 - 2	2 - 2 - 1	2 - 2 - 2
Intr 3-ex 4-intr 6									
Cases	228	0.655	0.040	0.119	0.027	0.027	0.029	0.027	0.074
Controls	588	0.693	0.020	0.070	0.053	0.026	0.005	0.008	0.123

χ^2 -test: cases vs controls; *p* value > 0.1

1=intron 3 (W), exon 4 Arg, intron 6 G; 2= intron (M), exon 4 Pro, intron 6 A

DISCUSSION

This case-control study of 115 gastric cancer cases and 295 hospital based controls evaluated the effect of four polymorphisms in the *p*53 and *p*73 genes on the gastric adenocarcinoma risk in an Italian population. To our knowledge, this is the first study showing in a Caucasian population that the *p*73 exon 2 homozygous variant genotype

Table 5. Adjusted † Odds Ratios (95% CI) of gastric cancer for gene-gene interaction analyses

		***p*53 intron3 (rs17883323)**		***p*53 intron6 (rs1625895)**		***p*53 exon4 codon72 (rs1042522)**	
		wt/wt	mt carriers	wt/wt	mt carriers	wt/wt	mt carriers
*p*73 exon 2 G4A	wt/wt	1*	0.81 (0.41 - 1.59)	1*	0.53 (0.26-1.06)	1*	0.60 (0.32-1.14)
	cases/controls	63/152	21/62	63/140	21/74	55/124	29/90
	mt carriers	0.99 (0.50 - 1.94)	1.58 (0.69 - 3.61)	0.74 (0.36-1.50)	0.82 (0.30-2.21)	0.79 (0.38-1.65)	0.68 (0.26-1.79)
	cases/controls	17/56	13/24	19/51	11/29	16/44	14/36
		p for interaction‡ = 0.26		*p* for interaction‡ = 0.48		*p* for interaction‡ = 0.40	
*p*53 exon4 codon72	wt/wt	1*	1.14 (0.37-3.57)	1*	3.19 (1.01-10.02)		
	cases/controls	61/154	10/14	61/157	10/11		
	mt carriers	0.86 (0.41-1.78)	0.72 (0.36-1.41)	1.52(0.73-3.17)	0.52 (0.26-1.07)		
	cases/controls	19/54	24/72	21/34	22/92		
		p for interaction‡= 0.47		*p* for interaction‡ =0.005			
*p*53 intron6	wt/wt	1*	0.63 (0.19-2.07)				
	cases/controls	74/174	8/17				
	mt carriers	0.53 (0.20-1.42)	0.73 (0.38-1.40)				
	cases/controls	6/34	26/69				
		p for interaction‡ = 0.30					

† OR adjusted by age and gender

* Reference category

‡ By likelihood ratio test

increases the risk of gastric cancer 4.77-times compared with individuals with the wild type genotype, and that the risk is particularly increased for the intestinal histotype. Additionally, the gene-environment interaction analysis shows an effect modification by gender on the association of *p*73 and gastric cancer, with an increased risk only among females. Results show the absence of a significant association between *p*53 exon 4 and gastric cancer, which is in line with the results of a recently published meta-analysis (2), while for the first time a borderline significant protective effect was detected for individuals carrying the mutant alleles of *p*53 exon 4 and intron 6. Lastly, our results failed to detect an association between *p*53 introns 3 and 6 and gastric cancer, as previously shown in relation with other tumour sites (27,28), while carriers of the *p*53 intron 6 variant allele had a poorer prognosis for intestinal gastric adenocarcinoma after surgical intervention.

Before interpreting our results, some limitations of the study should be taken into account. Firstly, on the basis of the prevalence of the analyzed polymorphisms in our control population, our study is powered to detect a minimum OR of 1.9 for the effect of mutant allele carriers for all studied polymorphisms (with a significance level of 5%), and an OR of 4.0 for *p*73 homozygous variant genotype. The study's sample size limits the possibility to explore the combined effects of the genotypes, or gene-environment interactions, thus we need to increase the sample size in order to confirm our results. However, when appropriately conducted, large and small studies should give, theoretically, the same results, with just a more precise effect measure estimate in the larger studies (29). Secondly, as in all case-control studies, information bias may exist, leading to biased ORs related to data about the lifestyle and environmental exposures. Thirdly, data on *Helicobacter pylori* infection were unavailable in our population.

Our study reports for the first time a strong association between the *p*73 exon 2 G4C14-to-A4T14 polymorphism and gastric cancer risk in a Caucasian population. When results are stratified according to tumor histology, the effect of the homozygous variant seems to be limited to the intestinal subtype. One study conducted in a Japanese population (14) reported no association between this SNP and digestive tract cancers including gastric cancer, however discrepant results are reported for other tumour sites. In a large hospital-based case control study of a Non-Hispanic White population, Li et al. (30) showed that the *p*73 AT variant allele is associated with an increased risk of head and neck cancer and lung cancer (13). Identical results were reported in a Swedish population of colorectal cancer patients carrying the homozygous variant genotype (19). On the other hand, Ryan et al. (31) showed in a small Irish population that *p*73 AT/AT carriers are significantly protected from oesophageal cancer.

In contrast, several studies conducted in Asians did not provide evidence for an association between the *p*73 G4C14-to-A4T14 polymorphism and the risk of lung (15,32,33), breast (34,35) or oesophageal cancer (14,36), except one study which showed a borderline increased risk for cervical cancer (4). We can argue, as previously suggested (13), that the *p*73 exon 2 G4C14-to-A4T14 polymorphism might play a different role in cancer risk depending on ethnic group and cancer site. In our study gender appears to be an effect modifier of the association between *p*73 AT variant allele and gastric cancer, with females at an increased risk compared to males. Based on previous findings showing that women tend to have a lower capacity for DNA repair with respect to men (31), it is possible that women carrying the *p*73 G4C14-to-A4T14 mutant allele are more sensitive to carcinogens. In order to confirm the potential role of the *p73* G4C14-to-A4T14 polymorphism on gastric cancer risk, both on the intestinal histotype and in females, further studies on its functional effect are needed. Nevertheless, our results are in line with the majority of those from Caucasian studies on different tumour sites. Since this polymorphic site is located in a non coding region it remains to be explained how the variant allele can modulate the protein function. It is possible that the *p73* G4C14-to-A4T14 polymorphism can be functional because it is in linkage disequilibrium with functional alleles at other susceptibility loci (13).

Our study failed to detect any association between the three studied *p*53 SNPs in exon 4, introns 3 and 6, and gastric cancer. This result is confirmed by the haplotype analysis showing no differences in the diplotype distribution among cases and controls. Our gene-gene interaction and haplotype analyses show that individuals carrying both mutant alleles of *p53* exon 4 and intron 6 are protected from gastric cancer risk. These results are in line with those reported for lung and breast cancer (9, 37). In addition a 3.19-fold increased gastric cancer risk was detected among individuals carrying the p53 exon 4 wild-type homozygote genotype and at least one p53 intron 6 variant allele, thus suggesting a positive synergism between the two SNPs only in presence of both variant alleles. Further studies are needed to confirm this result in larger populations and to clarify the mechanism through which the combined effect of these two strongly linked *p53* SNPs influence the protein function allowing for a protective effect against cancer.

The results of the present study show no effect of each of the studied polymorphisms on the overall survival rate after surgical gastrectomy intervention. This issue has never been addressed by other studies on gastric cancer. When the analysis, however, was restricted to the intestinal histotype, an increased mortality rate appears for those carrying the variant allele of *p*53 intron 6 when compared with individuals carrying the wild-type genotype. A similar result has been reported for chronic

lymphocitic leukaemia patients when considering the time of treatment-free survival as the main outcome (38).

In conclusion, our study provides, for the first time, evidence that the *p73* G4C14-to-A4T14 polymorphism is significantly associated with an increased gastric cancer risk in a Caucasian population, and that individuals carrying the mutant allele of both *p*53 exon 4 and *p*53 intron 6 are protected against gastric cancer. Larger prospective studies are needed to confirm our results.

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3

Meta-analyses of genetic polymorphisms and risk of gastric cancer

3.1

CYP2E1PstI/RsaI polymorphism and interaction with tobacco, alcohol and *GSTs* in gastric cancer susceptibility: a meta-analysis of the literature

ABSTRACT

Studies investigating the association between Cytochrome P450 2E1 (*CYP2E1*) 5'-flanking region (*Pst*I/*Rsa*I) polymorphism and gastric cancer risk report conflicting results. The rationale for this meta-analysis was to determine whether c2 variant allele of *CYP2E1* increases gastric cancer risk, especially by interacting with smoking, alcohol and other metabolic gene polymorphisms. Two investigators independently searched the Medline and Embase databases. A qualitative scoring of papers was applied to their evaluation. Authors of the identified papers were contacted to obtain data on the mentioned co-exposures. A measurement of the biological interaction among two putative risk factors was estimated by the attributable proportion (AP) due to interaction. We identified thirteen case-control studies, which included 2066 gastric cancer cases and 2754 controls. Using the random effects model, we found no association between *Pst*I/*Rsa*I genotype and gastric cancer risk [OR= 0.97 (95% CI: 0.79-1.18) for c2 allele carriers and OR= 1.36 (95% CI: 0.82-2.25) for c2 homozygotes compared with homozygotes wild type]. When only high-quality scored studies were considered, a statistically significant increased risk appeared among Asians [OR= 1.50 (95% CI: 1.16-1.94) for c2 carriers and OR= 2.62 (95% CI: 1.23-5.57) for c2 homozygotes]. No interaction was detected between *CYP2E1*-smoking/alcohol (AP= 0), while an AP of 60% appeared for individuals both c2 homozygotes and Glutathione S-Transferase *M1 (GSTM1)* null compared with both homozygotes wild type. This meta-analysis suggests that the *CYP2E1 Pst*I/*Rsa*I polymorphism may be a risk factor for gastric cancer in Asians, and that a synergistic interaction among *GSTM1* and *CYP2E1* may account for a proportion of gastric cancer cases.

INTRODUCTION

Cytochrome P450 2E1 (CYP2E1), a member of the cytochrome P-450 superfamily, is a naturally ethanol-inducible enzyme that is mainly involved in the metabolic activation of low molecular weight compounds such as *N*-nitrosamines, and in alcohol metabolism (1,2). *N*-nitrosamines are formed endogenously in the stomach and are present in various environmental factors including tobacco smoke and some diet compounds (3). Functional *CYP2E1* polymorphisms, whose expression in gastric tissues is well-documented (4), might therefore impact on the susceptibility for gastric cancer, for which *N*-nitrosamines are suspected of having a causative role. Two point mutations in the 5'-flanking region (*Pst*I, *Rsa*I), that are in close linkage disequilibrium, are known to alter the transcriptional activity of the gene (1). These mutations generate the *CYP2E1**1 (c1) allele and the less common *CYP2E1**2 (c2) allele and have been reported to be associated with a greater risk for oral, pharyngeal (5), liver (6) and lung cancers (7, 8). Recent meta-analyses reported, however, the absence of an association between these polymorphisms and oesophageal cancer (9), hepatocellular carcinoma (10) and alcoholic liver disease (11).

Despite the biological plausibility of *CYP2E1* 5'-flanking region polymorphism as a modulator of gastric cancer susceptibility, previously inconsistent results have appeared in the literature (12-23), probably due to the small sample sizes and the lack of investigations of gene-environment or gene-gene interactions. To clarify the effect of *CYP2E1 Pst*I polymorphism on the risk of gastric cancer, we have carried out a meta-analysis of the studies published until 31st April, 2006. Since we would expect to find an interaction between smoking habits, alcohol consumption and other metabolic gene polymorphisms with the *CYP2E1* c2 variant allele, with respect to gastric cancer risk, we performed stratified meta-analyses after collecting the original data from the published papers.

METHODS

Data collection

Papers published before the end of April 2006 were identified through a search of Medline and Embase using the following terms: "Cytochrome p450 2E1/IIE1" or "CYP2E1/IIE1", "gastric" and "cancer" or "carcinoma", without restriction on language. A cited reference search of the retrieved articles was carried out and furthermore publications were also identified by reviewing the bibliographies of the retrieved

articles. Articles reporting on *CYP2E1* genotype identified by *Rsa*I or *Pst*I enzymes in cases of gastric cancer and controls were identified (12-24). If more than one article was published by the same author using the same case series, we selected the study where the most individuals were investigated. In addition, for those papers reporting only the number of *CYP2E1* c2 carriers, the corresponding authors were contacted by email and fax in order to obtain the number of individuals with the homozygous variant genotype.

Since our a priori hypothesis was that the variant allele might affect gastric cancer risk by interacting with smoking habits, alcohol intake and Glutathione S-Transferase (*GST*) M1 and T1 polymorphisms, investigators associated with eligible studies were invited to contribute data to our effort. The corresponding authors of the published papers who reportedly collected data on the mentioned co-exposures without publishing the results in *extenso*, were contacted by e-mail and fax. We invited them to fill in an empty table with the *CYP2E1 Pst*I/*Rsa*I genotype data for cases and controls stratified for smoking status/alcohol consumption/*GSTM1*/*GSTT1* polymorphisms.

After several efforts for data collection, information on the absolute number of homozygous c2 individuals in cases and controls, and on *CYP2E1* genotypes stratified for the mentioned co-exposures, have been received for all the included studies bar one and all bar three, respectively.

Quality assessment and statistical analysis

Each article was blinded with respect to the authors, institutions and journals. The articles were read and scored for quality by two independent researchers using a published quality score system (24), with exception of the articles written in a non-English language (21-23). In brief, papers were rated according to several items on the scale in relation to two areas: the effort of the study to minimize potential bias and the data analysis, with items concerning the first area having twice the weight of those evaluating data analysis. A quality score was then calculated for each paper and reported as a%age of the met applicable criteria from the quality scale. High-quality studies were considered to be those with a value of at least 70% of the total score (19/27). The same two researchers extracted the data from each article using a structured sheet and entered it into a database. The followings items were considered: year and location of the study; ethnicity; characteristics of the case and control groups; number of individuals heterozygous and homozygous for the *CYP2E1* c2 allele in cases and controls; smoking status; alcohol consumption; *GSTM1* and *GSTT1* polymorphisms.

In carrying out the meta-analyses, random effect models were used, taking into account the possibility of heterogeneity between studies, which was tested with the I^2 test and a standard χ^2 test. The ORs of gastric cancer associated with c2 allele carriers and homozygous c2 genotype for the *CYP2E1 Rsa*I/*Pst*I polymorphism were estimated using the homozygous wild-type (c1/c1) as the reference group. In order to detect publication bias, ORs and 95% CI were plotted against standard errors in each study and the Egger test was performed (25). To determine the deviation from Hardy-Weinberg equilibrium among control populations we used a program provided on the web site http://ihg.gsf.de/cgi-bin/hw/hwa2.pl.

Sources of heterogeneity were investigated by subgroup meta-analyses based on the ethnicity (Asian and Caucasian populations) and the quality (high and low) of the studies (26). Subgroup meta-analyses were performed in an attempt to evaluate if heterozygous and homozygous variant genotypes of *CYP2E1 Rsa*I/*Pst*I polymorphism modify the risk of gastric cancer by interacting with smoking, alcohol and *GSTM1* and *GSTT1* polymorphisms. For these purposes, we stratified subjects (both c2 carriers and c2 homozygous) according to smoking status (ever/never smokers); alcohol consumption (users/non-users); *GSTM1* and *GSTT1* polymorphisms (present/null variant).

In order to evaluate the presence of a biological interaction between each couple of the investigated risk factors, additional gene-gene and gene-environment interaction meta-analyses were performed by using the homozygous wild-type individuals not exposed to the environmental factor, or the homozygous wild-type individuals for both genes, as reference groups, as suggested by Botto and Khoury (27). To quantify the amount of biological interaction, the attributable proportion (AP) due to interaction was calculated using departure from additivity of effects as the criterion for interaction, as suggested by Rothman (28). The AP is the proportion of individuals among those exposed to the two interacting factors that is attributable to the interaction per se and it is equal to 0 in the absence of biological interaction (28). When more than additive interaction was evident, we additionally tested for more than multiplicative effect between the two risk factors by using a case-only study design (under the assumption of independence of the two factors in the control population) (29). Statistical analyses were carried out in RevMan program, release 4.2 (30).

RESULTS

Twelve case-control studies (12-23), of which two were written in a non-English language (21,23), were identified, and by adding our recently submitted study (31) we had a total of 2066 gastric cancer cases and 2754 controls (Table). Studies were carried out in Japan, China, Taiwan, Korea, Brazil and Italy. Allele and genotype frequencies in all control groups did not deviate from values predicted by Hardy-Weinberg equilibrium. Quality scores for the individual studies ranged from 56% to 81%, with 6 of the 13 studies being classified as high-quality (12-14,17,22,31), of which three were Asian and three were Caucasian. Our meta-analysis gave an overall OR of 0.97 (95% CI: 0.79-1.18) for gastric cancer risk among c2 allele carriers of *CYP2E1 RsaI/PstI* polymorphism and an overall OR of 1.36 (95% CI: 0.82-2.25) for c2 homozygotes (Figure 1).

Table. Studies of the *CYP2E1- PstI/RsaI* polymorphism and gastric cancer risk

Reference, year	Population	No. cases	No. controls	c2-carriers No. cases/controls
Kato *et al,* 1995 (15)	Japanese	150	203 gastric ulcers	40/41
Wang *et al*, 1998 (21)	Taiwanese	83	83 healthy individuals	39/30
Nishimoto *et al,* 2000 (17)	Japanese	59	133 hospital inpatients	48/48
Nishimoto *et al*, 2000 (17)	Brazilian	189	191 hospital inpatients	6/10
Cai *et al*, 2001 (12)	Chinese	91	94 healthy individuals	36/24
Gao *et al*, 2002 (14)	Chinese	98	196 population	41/38
Tsukino *et al*, 2002 (20)	Japanese	120	158 healthy individuals	41/44
Wu *et al*, 2002 (22)	Taiwanese	356	278 hospital inpatients	40/28
Park *et al*, 2003 (18)	Korean	120	145 cancer free patients	33/35
Ye *et al*, 2003 (23)	Chinese	56	56 healthy individuals	30/54
Colombo *et al*, 2004 (13)	Brazilian	100	150 population	11/11
Suzuki *et al*, 2004 (19)	Japanese	145	177 autopsy subjects	26/37
Nan *et al*, 2005 (16)	Korean	416	630 hospital inpatients	35/36
Boccia *et al* (31) *	Italian	83	254 hospital inpatients	5/8

* Number of cases is smaller than published version of the paper, because cases were added after initial submission and rejection of the manuscript

When stratifying for ethnicity, an OR of 1.01 (95% CI: 0.81-1.25) and 0.72 (95% CI: 0.44-1.18) resulted for c2 carriers, while an OR of 1.44 (95% CI: 0.85-2.42) and 0.42 (95% CI: 0.05-3.85) resulted for c2 homozygotes, among Asians and Caucasians, respectively (Figure 1). When studies were stratified for quality, an overall OR of 1.15 (95% CI: 0.81-1.56) for c2 carriers and an OR of 2.14 (95% CI: 0.96-4.74) for c2

homozygotes emerged for the high-quality scored studies. These results produced a significant OR of 1.50 (95% CI: 1.16-1.94) for c2 carriers and an OR of 2.62 (95% CI: 1.23-5.57) for c2 homozygotes when only high-quality studies among Asians were considered, while the result was not significant among low-quality scored studies (data not shown).

The results of the stratified meta-analyses according to smoking status, alcohol consumption and *GST*s genotypes are shown in Figures 2 and 3. The analysis stratified by smoking status is based on 7 studies (12-14,16,17,20,21), the one by alcohol intake on 6 studies (12-14,16,17,19), the one by *GSTM1* is based on 5 studies (13,16,19,21,22), and the stratified meta-analysis according to *GSTT1* status on 5 studies (13,16,17,22,23). The overall ORs appeared similar among each subgroup, even when further stratified by ethnicity (data not shown). On the other hand, an effect modification due to *GSTM1* genotype for gastric cancer risk appeared for c2 homozygotes individuals compared with c1/c1 homozygotes. In fact, an OR of 4.93 (95% CI: 0.73-33.08) emerged for individuals both c2 homozygotes and *GSTM1* null when compared with c1 homozyogotes, albeit not significant, while an OR of 1.89 (95% CI: 1.00-3.58) appeared for the same comparison among *GSTM1* wild-type individuals (Figure 3).

The computation of the attributable proportion due to interaction showed an absence of biological interaction among each pair of tested risk factors, with an AP=0 for all of the combinations. The only one exception is represented by the interaction between *CYP2E1* c2 homozygotes and *GSTM1* null, for which an OR of 5.36 (95% CI: 1.01-28.47) for gastric cancer risk appeared in comparison to individuals with both wild-type genotype (data not shown). In this case an AP of 60% of gastric cancer cases among the *CYP2E1* c2 homozygotes with *GSTM1* null genotype appeared to be related to the biological interaction among the two unfavourable genotypes, with a more than additive effect on gastric cancer risk. Lastly, case-only meta-analysis showed no evidence of more than multiplicative effect (OR= 1.04; 95% CI: 0.69-1.80) for the association of *CYP2E1* c2 homozygotes and *GSTM1* null on gastric cancer risk (data not shown).

Evidence of heterogeneity appeared when all the studies were pooled, with a I^2 of 51.3% and a χ^2 p value = 0.01 for c2 carriers, and a I^2 of 47.7% and a χ^2 p value = 0.03 for c2 homozygotes (Figure 1). When stratified by ethnicity, the heterogeneity still remained among Asians, but disappeared when only the three high-quality Asian studies were pooled (Figure 1). No evidence of heterogeneity appeared within strata after stratification for smoking status and alcohol consumption (Figure 2), probably due

Fig. 1. Forest plots depicting the Odds ratios (OR) and 95% confidence intervals (CI) from studies examining the association between gastric cancer and *CYP2E1 RsaI/PstI* 5′-flanking gene polymorphisms (c2 variant allele carriers or c2 homozygotes *versus* c1 homozygotes), and stratified meta-analyses according to ethnicity and quality of the studies. The centre of each square represents the OR, the area of the square is the inverse of the variance and the horizontal line indicates the 95% CI. The summary OR is represented by the diamond, where its centre indicates the OR and its ends correspond to the 95% CI. I^2 and p value for χ^2 of heterogeneity are reported for each subgroup analysis. Only one study (19) did not provide information on the number of c2 homozygotes.

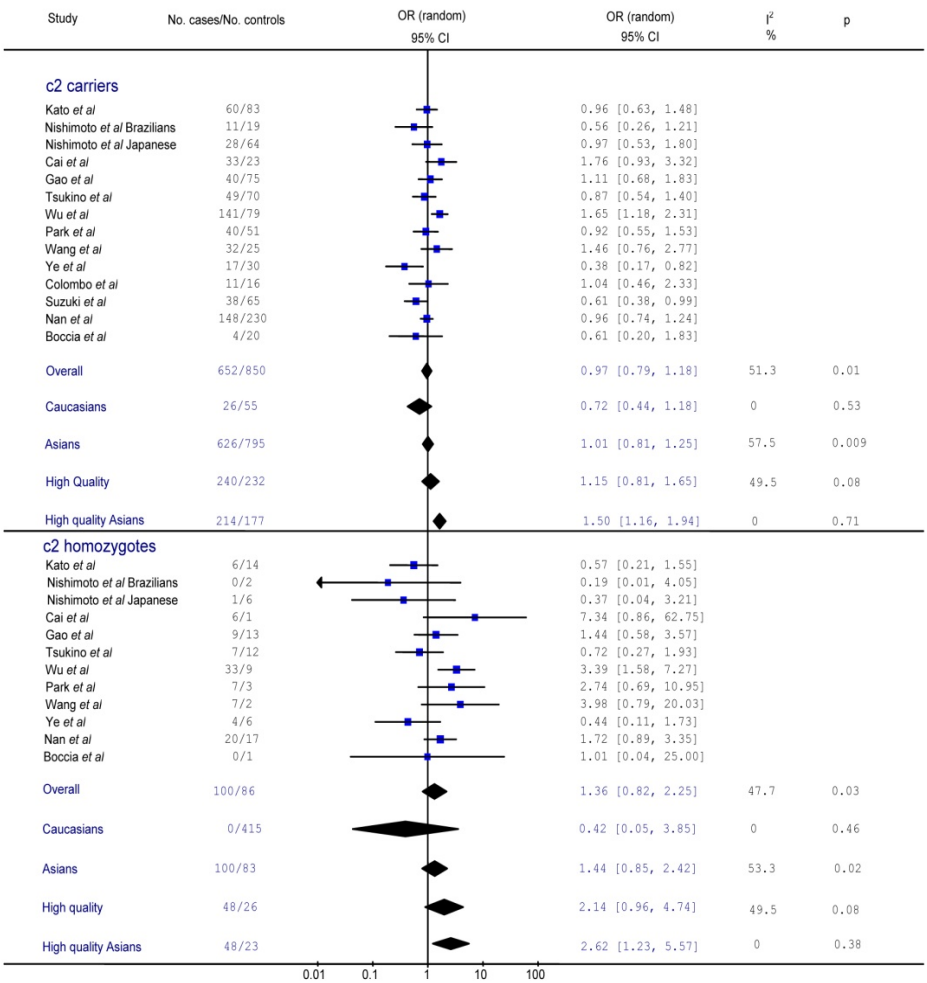


Fig. 2. ORs and 95% CI from the subgroup meta-analyses examining the association between gastric cancer and *CYP2E1 RsaI/PstI* 5′-flanking gene polymorphisms (c2 variant allele carriers or c2 homozygotes *versus* c1 homozygotes), according to smoking status and alcohol consumption (see materials and methods). The centre of each square represents the OR, the area of the square is the inverse of the variance and the horizontal line indicates the 95% CI. The summary OR is represented by the diamond, where its centre indicates the OR and its ends correspond to the 95% CI. I^2 and *p* value for χ^2 of heterogeneity are reported for each subgroup analysis. See results for details of the studies included.

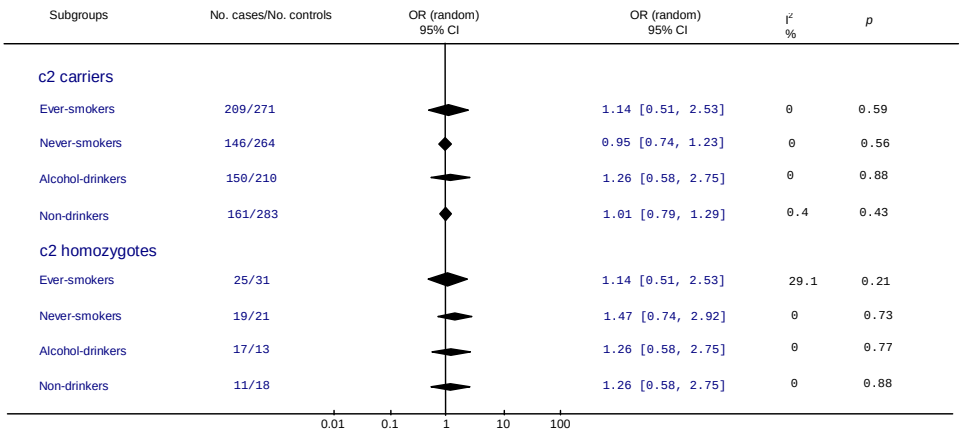
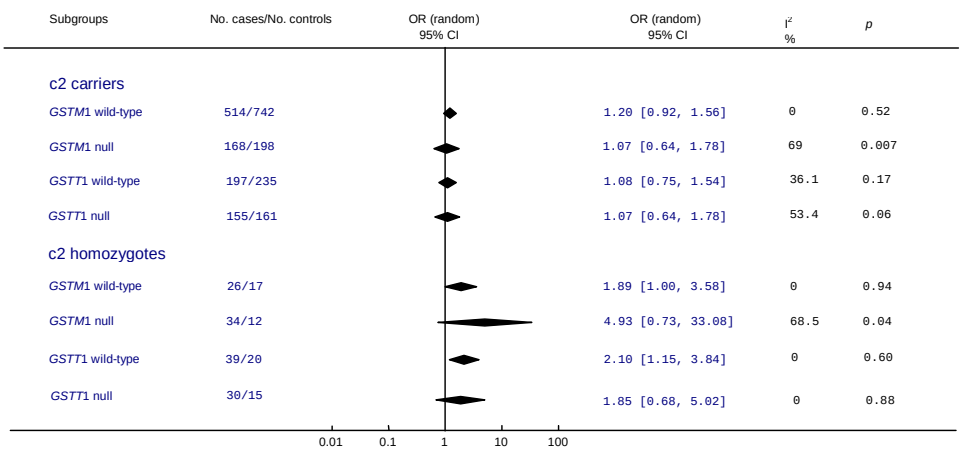


Fig. 3. ORs and 95% CI from the subgroup meta-analyses examining the association between gastric cancer and *CYP2E1 RsaI/PstI* 5′-flanking gene polymorphisms (c2 variant allele carriers or c2 homozygotes *versus* c1 homozygotes), according to *GSTM1* and *GSTT1* gene polymorphisms (see materials and methods). The centre of each square represents the OR, the area of the square is the inverse of the variance and the horizontal line indicates the 95% CI. The summary OR is represented by the diamond, where its centre indicates the OR and its ends correspond to the 95% CI. I^2 and *p* value for χ^2 of heterogeneity are reported for each subgroup analysis. See results for details of the studies included.



to the association between smoking and alcohol with gastric cancer risk *per se*. However, a high heterogeneity emerged when data were stratified by *GST*s genotypes (Figure 3), maybe due to the wide variability in the frequency of this polymorphism among Caucasians and Asians (32).
The funnel plot and the Egger test provided evidence that effect estimates were not related to study size ($p > 0.05$ for both c2 carriers and c2 homozygotes, data not shown).

DISCUSSION

Among the most widely studied metabolic gene polymorphisms as susceptibility factor for gastric cancer, is the *CYP2E1 Rsa*I/*Pst*I polymorphism. The less common c2 variant allele frequency is highly different among Asians and Caucasians, with a prevalence of approximately 25-50% and 5-10%, respectively (32). Since Kato et al. (15) first investigated, in 1995, a possible relationship between *CYP2E1* c2 allele and gastric cancer risk, a further 11 reports mainly conducted in Asian populations have been published examining this hypothesis (12-23), with conflicting results. This led us to undertake the present meta-analysis, which aims to derive an estimate of the gastric cancer risk associated with *CYP2E1 Rsa*I/*Pst*I genotype. The main finding of this meta-analysis of 13 case-control studies involving 4820 subjects is that individual carriers of *CYP2E1* c2 allele or c2 homozygous do not have an increased risk of gastric cancer. Since the result comes from pooling data from different ethnic groups and studies of different quality, we planned a priori to perform subgroup meta-analyses based on ethnicity and study-quality. Considering separately, both Caucasian and Asian populations, the association between *CYP2E1* status and gastric cancer risk did not change, however it did reach a statistically significant level when only high-quality studies among Asians were considered, with a statistically significant 1.50 and 2.62-fold increased risk for gastric cancer among c2 carriers and c2 homozygotes, respectively. The lack of significance for the association in Caucasian population might be explained by substantially lower statistical power to detect an association owing to a lower prevalence of *CYP2E1* c2 carriers (5-10% against 25-50% for Asians).

As expected, there was evidence of between study heterogeneity when all ethnic groups were pooled, which was higher still when only Asian studies were considered. However, Asian reports in the subgroup analysis include a mixture of populations from very distant countries and sometimes very different allele frequencies for the c2 allele. On the other hand, there was no evidence of heterogeneity when only high-quality Asian studies were considered.

Since *CYP2E1* is presumed to confer susceptibility to gastric cancer via an interaction with carcinogens, it is interesting to note that almost all of the studies did not explore the interaction between *CYP2E1* genotype and smoking habits or alcohol consumption. This was probably due to the low statistical power of the individual studies to detect interactions however almost all of the studies collected this data, which was utilised for this meta-analysis. By combining the collected data on *Pst*I/*Rsa*I genotype and smoking habits or alcohol consumption with respect to gastric cancer risk, no statistically significant results emerged from the stratified meta-analyses. Furthermore, the computation of the attributable proportion due to biological interaction was equal to 0 in both cases, thus showing that when the environmental and genetic risk factors are both present, the effect on gastric cancer seems to be no longer than additive of the separate effects. However we cannot ignore that due to the low prevalence of c2 homozygotes in each study, even when data are pooled the statistical power to detect an interaction remains low.

If genetic susceptibility to gastric cancer is, in part, mediated through metabolic gene polymorphisms, it is possible that the combinations of certain genotypes may be more discriminating as risk factors for gastric cancer than a single locus genotype. Among the most investigated metabolic gene polymorphisms, as susceptibility factors, are GSTs enzymes, which are mainly involved in the detoxification of several different xenobiotics (33). Two recent meta-analyses indicated that individuals with both *GSTM1* and *GSTT1* null genotypes are at higher risk for gastric cancer, however no risk emerged when the two unfavourable genotypes were considered separately (24,34). Among the 13 studies included in the present meta-analysis, only one study (19) investigated the interaction between *CYP2E1 Pst*I/*Rsa*I and *GSTM1* polymorphisms, even though nearly half of the studies collected data on *GSTM1* and *GSTT1* status for cases and controls. By pooling the collected data on *CYP2E1 Pst*I/*Rsa*I and *GSTM1/GSTT1* genotypes, a statistically significant 5.36-fold increased risk for gastric cancer appeared for individuals both *CYP2E1* c2 homozygotes and *GSTM1* null, compared with individuals with both homozygous wild genotypes. Based on the definition of biological interaction among two component causes, our result suggests that nearly 60% of gastric cancer cases among individuals both *CYP2E1* c2 homozygotes and *GSTM1* null are caused through a mechanism in which the two risk factors act under biological dependence in the same causal mechanism of disease (28). In other words, in the absence of either of the two risk factors, an important number of gastric cancer cases would not occur.

In interpreting the results the main limitation of the study should be considered. First, only published studies were included in the meta-analysis, therefore

publication bias may have occurred, even though the use of a statistical test did not show it. Second, the subgroup meta-analyses considering interactions between *CYP2E1* genotype and smoking habits/alcohol consumption and gene-gene interactions did not include all of the studies because a small number of authors, three and one respectively, could not share their original data, so selection bias may have occurred and our results should be confirmed with the inclusion of the missing data. Furthermore, when the analysis was restricted to c2 homozygotes individuals, we had a low power to detect an interaction because of the low prevalence of c2 homozygotes in each study, so the results need to be confirmed with a larger sample size. Third, our meta-analysis is based on unadjusted estimates, while a more precise analysis could be performed if individual data were available, which would allow for an adjustment estimate (by age and sex). To be made, however this approach requires the authors of all of the published studies to share their data. Fourth, the quality score of the individual studies included in our meta-analysis was assessed on the basis of efforts to minimize the potential for selection bias, misclassification related to exposure, collection of data on potential confounders and method of statistical analysis. It is known that a validated quality assessment system does not currently exist (35) and it is evident that our quality scale has a subjective component. Despite these limitations, assessment of the quality of individual studies used in our meta-analysis allowed us to see that pooling high-quality scored studies resulted in higher risk estimates than did the pooling of low-quality scored studies. If high-quality scored studies are more likely to yield valid information than low-quality studies, we can conclude that, based on the currently available data, an additional risk of gastric cancer for *CYP2E1* c2 carriers and c2 homozygotes may exist. We argue that the lack of significance for the association in Caucasian population can be most likely explained by the low statistical power to detect an association owing to a lower prevalence of *CYP2E1* c2 carriers and the few number of studies published and included in the present meta-analysis.

Despite all these remarks, some interesting conclusions have emerged. From the results of this quantitative meta-analysis, that combined the data from 4820 people (2066 cases and 2754 controls), it appears that *CYP2E1- PstI/RsaI* polymorphism may be a risk factor for gastric cancer in Asian populations, and particularly for individual homozygotes for the unfavourable gene variant. In addiction, a synergistic interaction among *GSTM1* and *CYP2E1* unfavourable variant genotypes may probably account for a proportion of gastric cancer cases. Since more than half of the included studies were based on a limited number of cases (< 100) it is critical that larger and well-designed multicentric studies based on the same ethnic group confirm our results.

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3.2

Glutathione S-Transferase M1 status and gastric cancer risk: a meta-analysis of the literature

ABSTRACT

Susceptibility to gastric cancer may be in part a attributable to inter-individual variability in metabolic activation or detoxification of carcinogens, and in this context the polymorphic *GSTM1* gene has been extensively studied. Seventeen reports detailing a possible association between *GSTM1* deletion and gastric cancer have been published so far. In order to examine the risk of gastric cancer associated with *GSTM1* null genotype, a meta-analysis of published case-control studies was undertaken using a random effect model. Two studies were excluded because some data were missing in the results. The principal outcome measure was the odds ratio (OR) for the risk of gastric cancer. Pooling all the 15 studies identified, the overall odds ratio of gastric cancer risk associated with *GSTM1* deficiency was 1.24 (95% CI: 1.00–1.54). By pooling 4 studies detailing the possible interaction between *GSTM1* status, smoking habits and gastric cancer risk, an overall estimate of odds ratio of 1.35 (95% CI: 0.98-1.86) for ever smokers with *GSTM1* deficiency compared with *GSTM1* normal genotype has emerged. These results suggest that *GSTM1* status has probably no effect on the risk of gastric cancer per se, but may modulate tobacco-related carcinogenesis of gastric cancer. Greater attention should therefore be paid to the design of future studies: only well designed population-based control studies considering all the possible confounding risk factors and based on a sample size commensurate with the detection of small genotypic risk may allow a more definitive conclusion.

INTRODUCTION

The incidence and mortality of gastric cancer (GC) is decreasing in the world as well as in most European countries, but it still represents the fourth most frequent cancer in the world (1). Given to the low rate of five-year survival of GC, identification and control of risk factors remains the most effective means of prevention (2). An increasing number of epidemiological studies indicate that cigarette smoking (1), H*elicobacter pylori* infection (3) and diet (4) are probably important etiological factors increasing the risk of GC. However, it is currently accepted that the development of GC results from a complex interaction of both environmental and genetic factors (2).

According to a multifactorial model, genetic susceptibility due to specific variant alleles of different genes (polymorphisms) can modify the effect of environmental exposure, probably explaining partly the high variations of GC incidence throughout the world (4). In particular, it is possible that some of the susceptibility to GC is determined by the interindividual difference in the bioactivation of procarcinogens and detoxification of carcinogens due to inherited polymorphisms in low- penetrance metabolic genes (5, 6). Among the latter the most widely studied polymorphism is the glutathione S-transferase M1 (*GSTM1*) null allele, which has been of considerable interest as a GC susceptibility gene (2).

GSTM1 gene is polymorphic, and at least four different alleles exist; the protein is mainly expressed in the liver, brain and stomach (2). Absence of *GSTM1* expression is due to an inherited deletion of the parental allele of *GSTM1* gene and it is found in approximately 50% of Caucasian and Asian populations, and 25% of African population (7). Since GST-μ enzyme is involved in detoxification of various carcinogens, it may be plausible that an increased or decreased activity of this enzyme can be involved in susceptibility to GC (2). Strange et al first reported an association between GST1 deficiency and GC (8). Since this publication in 1991, over 16 studies have appeared in the literature confirming or refusing an association between *GSTM1* deficiency and GC risk (9-24). In order to clarify the effect of *GSTM1* status on the risk of developing GC, we carried out a quantitative meta-analysis of the research published up to the 31st of March, 2004, that have investigated the association between *GSTM1* status and GC.

METHODS

Identification of relevant studies

The studies were searched for using the MEDLINE database of the National Library of Medicine, and the EMBASE database. The key words used for the research were Glutathione S-Transferase M, *GSTM1*, gastric cancer, epidemiology, without restriction on language. The period of research considered included articles published up to 31st of March, 2004. For the meta-analysis, the following inclusion criteria were followed: clear objective in the relation between *GSTM1* status and GC in the introduction/description of the research, and description of *GSTM1* status in cases and controls.

Statistical analysis

Two researchers (GLT and SB) extracted the data from each article using a structured sheet and entered it into a database. The followings items were considered: year and location of the study; ethnicity; characteristics of the control group; number of individuals the number of cases and controls with *GSTM1* deficiency in the compared groups. The heterogeneity was tested by Q statistic. In carrying out the meta-analyses, random effect models were used to take into account the possibility of heterogeneity between studies (25). The summary Odds Ratios (ORs) of gastric cancer associated with *GSTM1* null was estimated using the non null genotype for each genotype as reference group. To check for publication bias, Begg and Egger tests were used.. Statistical analysis was undertaken using the program RevMan, release 4.2 (26). The OR of gastric cancer associated with *GSTM1* deficiency was estimated for each study.

We also computed the power of each selected study, in order to assess the probability of detecting an association between *GSTM1* deficiency and gastric cancer at the 0.05 level of significance, assuming a genotypic risk of 2 and 1.5, using the method described by Schlesselmann (27). Moreover, separate analyses were conducted taking into consideration Asian and Caucasian patients. Lastly, since *GSTM1* genotype is presumed to affect gastric cancer risk also by influencing detoxification of activated tobacco carcinogens, we tried to evaluate this potential modifying effect in patients with *GSTM1* deficiency. An heterogeneity test was then used to test differences among the strata.

RESULTS

Identification of relevant studies

Seventeen studies were identified for the meta-analysis, of which seven were population based case-control and ten hospital based case- control studies. In Table the ORs are reported with their relative 95% CI, including confounding factors that were analysed in each study. Two studies were not included in the meta-analysis as they did not fulfil selection criteria. In fact, results are not expressed as number of *GSTM1* deficiency in cases and controls in Kato S et al. (13), and Conde et al (15) articles. So, the meta-analysis was conducted using the remaining 15 studies (9-12, 14, 16-24). While Strange et al used a phenotypic method for detecting *GSTM1* status (8), all the other studies were based on genotypic methods (PCR) (9-12, 14, 16-24). The ethnicity of cases and controls was detailed in all the 15 studies (Table). In 4 of 15 studies the controls were sex and age-matched individuals from the general population (Table). Smoking histories had been verified from cases and controls in 5 out of 15 studies, while food consumption has been ascertained in only 4 reports (Table 1). In these studies, the relationship between *GSTM1* status and GC risk were analysed in a stratified manner or by logistic regression analysis, taking into account other covariates. In one study, the prevalence of *GTSM1* deficiency was determined in two control groups (8).

Statistical analysis

Figure shows the result of the pooling of data, illustrating a plot of odds ratios and 95% CI for the risk of developing gastric cancer associated with *GSTM1* deficiency in 15 case-control studies. The meta-analysis provides an OR of 1.24 (95% CI: 1.00–1.54; *p* for heterogeneity = 0.002), which testifies the extent to which *GSTM1* deficiency is associated with the risk of gastric cancer. Based on the potential overestimation of the true effect of *GSTM1* deficiency on gastric cancer risk, considering studies using phenotypic method (28), we also conducted a meta-analysis considering only studies using the genotyping methods, giving an overall OR of 1.20 (95% CI: 0.97–1.47; *p* for heterogeneity = 0.005).

Analysis provides different results for Asian and Caucasian patients. In fact, for the former ones (9 studies) we found a significant OR= 1.22 (95% CI: 1.04–1.43), while for the latter (6 studies) the OR was not significant (OR= 1.19; 95% CI = 0.81–1.75, *p* for heterogeneity among the two estimates= 0.80). Taking into account only studies with controls from the general population, an overall OR of 1.25 (95% CI: 0.90-1.73; *p* for heterogeneity = 0.02) was found, whilst an OR of 1.18 (95% CI: 0.91–1.55; *p* for heterogeneity = 0.06, *p* for heterogeneity among the two estimates= 0.91) resulted by considering studies with hospital controls.

Table. *GSTM1* null status and gastric cancer

Investigator, year	Place of study	Analytical method	Cases [a]	% *GSTM1* deficient	Controls	% *GSTM1* deficient	adjusted OR (95% CI)	confounders	Power RR = 1.5	Power RR = 2.0
Strange et al., 1991 (8)	Staffordshire, UK	Starch gel zymogram	19 surgical cases from one hospital; age: NS; sex ratio: NS; Caucasian	73.6	49 cancer- free autopsy English controls died for cardiovascular disease from the same hospital of cases; age 68; sex ratio: NS;453 other control groups 502 (all controls)	40.8 50	4.06 (1.25-6.73)	None	7	19
Harada et al., 1992 (9)	Ibaraki, Japan	PCR	19 cases from a prefecture; age: NS; sex ratio: NS; Asian	73.7	84 healthy donors from a prefecture; age: NS; sex ratio: NS; Asian	47.6	3.08 (0.92-10.9)	None	8	21
Deakin et al., 1996 (10)	Staffordshire, UK	PCR	136 cases from one hospital; age 68; 70.5% male; Caucasian	52.9	577 controls from the same hospital of cases; age 70; 48% male; Caucasian	54.8	0.92 (0.63-1.34)	None	52	100
Katoh et al., 1996 (11)	Kitakyushu, Japan	PCR	139 consecutive cases from three hospitals in the same city; histologically confirmed; age 62.2 (13.2); 70.5% male; Asian	56.8	126 hospital controls from the same city of cases; age 61.9 (16.8); 57.1% male; Asian	43.6	1.32 (1.05-2.08)	Smoking	33	92

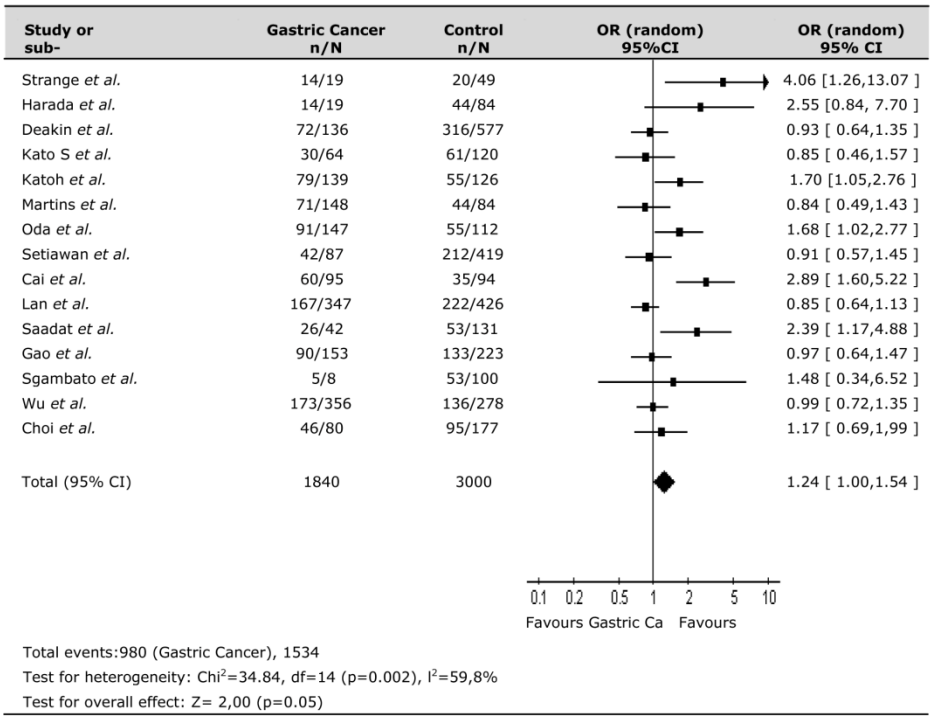
Kato et al., 1996 (12)	Tokyo, Japan	PCR	64 cases from one hospital ; histologically confirmed; ages: 33-84; 57% male ; Asian	45 (intestinal type) 48 (diffuse type)	120 sex and age matched controls with a benign gastric disease; ages: 32-81; 55% male; Asian	51	0.85 (0.47-1.53)	Gender, age, sex, pepsinogen I levels, pepsinogen I/II ratios, *Helicobacter pylori* positivity, polymorphisms of CYP2E1 and L-myc gene	20	56
Martins et al, 1998 (14)	Lisbona, Portugal	PCR	148 incident cases from three hospitals in the same city ; histologically confirmed ; ages: 20-88; 56.7% male; Caucasian	48	84 controls from a Blood Centre; ages: 20-60 ; 77.3% male ; Caucasian	52	0.84 (0.49-1.44)	None	26	72
Oda et al., 1999 (16)	Kanazawa, Japan	PCR	147 surgical cases form two hospitals in the same city; ages: 30-84; 66.6 % male; Asian	61.9	112 age matched cancer- free autopsy control; ages: 20-95; 66.0% male; Asian	49.1	1.68 (1.03-2.75)	None	31	86
Setiawan et al., 2000 (17)	Yangzhong City, China	PCR	91 incident cases; histologically confirmed diagnosis; age: NS; 71.4% male; Asian	48	427 healthy population- based cancer-free individuals; age: NS; 49.7% male; Asian	51	0.6 (0.26-1.38)	Demography, BMI, HP occupation, family history, food consumptions, smoking	36	100

Cai et al., 2001 (18)	Changle County, China	PCR	95 incident cases; histologically confirmed diagnosis; ages: 32-78; 85.3% male; Asian	63.2	94 sex and age matched healthy population- based controls from the same county of the cases; ages: 37-79; 87.2% male; Asian	45.7	2.63 (1.17-5.88)	Age, gender, smoking, alcohol, fish consumption	23	66
Saadat et al., 2001 (19)	Shiraz, Iran	PCR	42 cases; histologically confirmed diagnosis; age: NS; sex ratio: NS; Caucasian	61.9	131 sex and age matched healthy blood donors; age: NS; sex ratio: NS; Caucasian	40.4	2.39 (1.15-4.95)	None	16	45
Lan et al., 2001 (20)	Warsaw, Poland	PCR	347 incident cases from 22 hospitals of one city ; ages: 21-79; 65.8% male; Caucasian	48.1	426 sex and age matched healthy population- based controls from the same city of the cases; ages: 21-79; 64.4% male; Caucasian	52.1	0.92 (0.67-1.26)	Age, gender, smoking, family history, fruit consumption	100	100
Wu et al., 2002 (21)	Taiwan	PCR	356 incident cases from one hospital; histologically confirmed diagnosis; ages: 25-87; 61.2% male; Asian	48.5	278 controls from the same hospital of cases; ages: 22-86; 56.1% male; Asian	48.9	0.98 (0.72-1.34)	None	77	100

Gao et al., 2002 (22)	Huaian City, China	PCR	153 cases (hospital surgical cases and incident cases from a Regional Registry) from the same city; histologically confirmed diagnosis; ages: 40-81; 77.1 % male; Asian	59.6	223 sex and age matched healthy population- based controls from the same city of the cases; ages: 35-81; 66.8% males; Asian	59.6	1.02 (0.67-1.56)	Smoking, alcohol, tea, food consumptions	41	100
Sgambato et al., 2002 (23)	Basilicata, Italy	PCR	8 consecutive cases form one hospital; age: NS; sex ratio: NS; Caucasian	63	100 healthy cases from the same hospital of cases; age: NS; sex ratio: NS; Caucasian	37	1.47 (0.33-6.43)	None	4	11
Choi et al., 2003 (24)	South Korea	PCR	80 surgical cases form one hospital; histologically confirmed diagnosis; age: NS; sex ratio: NS; Asian	57.5	177 healthy cancer-free individuals; age: NS; sex ratio: NS; Asian	53.7	0.86 (0.49-1.51)	None	26	71

[a] Ages of cases and controls: mean (SD) or range given wherever possible

Figure. Forest plot of the published studies considered in the meta-analysis



As far as concerns the interaction between *GSTM1* status and gastric cancer risk in relation to cigarette smoking, we found data available for only 4 out of 15 studies (11,17,18,22), giving an overall estimate of OR= 1.10 (95% CI: 0.76-1.61) for *GSTM1* null versus non null among never smokers, and an OR of 1.35 (95% CI: 0.98-1.86) for ever smokers (*p* for heterogeneity among the two estimates= 0.50). Lastly, making a pooled analysis considering only studies with a power of at least 80% (Table 1), we found an overall estimate of OR=1.05 (95% CI: 0.87-1.28; *p* for heterogeneity=0.12) for a RR=2.0. Results of Begg and Egger tests showed a substantial absence of publication bias (*p* values >0.05).

DISCUSSION

In the last two decade advances in DNA technologies have probably determined an increase in the knowledge of genetic polymorphisms in cancer risk. While rare

alterations of high penetrant susceptibility genes (e.g. tumor suppressor genes) dramatically increase cancer risk for the affected subjects, more common differences in low penetrant susceptibility genes (e.g. drug metabolism enzymes) could be responsible for a relatively small, but rather frequent increase of cancer risk at the population level (6). Given that exposure to carcinogens is one of the most important risk factors for gastric cancer (2, 3), the hypothesis that the modulation of carcinogen metabolism due to inherited polymorphisms in drug metabolism genes, could be a plausible way for explaining interindividual susceptibility.

The most widely studied metabolic gene polymorphism is the *GSTM1* null allele, whose frequency in Caucasian population is approximately 50%. Since Strange et al (8) first drew attention to possible relationship between *GSTM1* deficiency and GC risk, 16 reports have been published examining this hypothesis (9-24), with conflicting results. This prompted us to the present meta-analysis in order to derive an estimate of the risk associated with *GSTM1* status. In our meta-analysis of 15 published case-control studies involving 4797 subjects, *GSTM1* null genotype confers a 1.24 fold statistically significant increased risk of gastric cancer. Our results also suggest that this association might be slightly stronger among Asian than Caucasian population, even if the difference is not statistically significant (p>0.10). Anyway, these OR values obtained support the hypothesis that *GSTM1* deficiency does not influence per se the inter-ethnic variation in GC incidence throughout the world.

Since *GSTM1* is presumed to confer susceptibility to GC by interaction with carcinogens, it is interesting to remark that no data was collected on tobacco or on other environmental carcinogens exposure (e.g., passive smoking) from both cases and controls in most studies. By pooling 4 studies which investigated the possible interactions between *GSTM1* status and smoking habits and the risk of GC, a borderline statistically significant 35% increased risk appeared for smoker individuals with a *GSTM1* null genotype versus *GSTM1* non null individuals. The same effect estimate was far from significant and less pronounced among never smokers, even though the heterogeneity test did not show a true effect modification of *GSTM*1 genotype by smoking status. As this stratified analysis is based on limited sample size (around 200 smokers with a *GSTM1* null genotype) it would be worthy to test on a larger sample.

The principal form of possible bias in this study, i.e. the presence of confounding factors which could interfere with the epidemiological meta-analysis, shouldn't have been avoided, given that total estimates reported by each author were adjusted by multivariate analysis only in few studies. A potential- selection bias should be discussed for each individual study considered. Case-control studies have prevalently used population based cancer registries in order to identify the cases. In

this latter type of investigation, only seven of fifteen had used population register to identify controls. Misclassification bias may be considered as negligible in these studies, given that the diagnosis of cancer in the case-control studies has reference to cancer registries which routinely foresee a histological confirmation of cancer cases. Misclassification related to exposure (*GSTM1* status) may be of some interest. By pooling studies that used a genotyping method to ascertain the *GSTM1* status, the overall OR was slightly lower compared with pooling all the studies together, and no longer statistically significant. Therefore misclassification of *GSTM1* status on the basis of phenotype should be taken in great care when pooling data together, even though in our study the different results obtained could be also attributable to the small sample size of the phenotype-based report (8). Moreover, it is well know that using hospital controls could generate some source of bias if not appropriately selected (29), and in this pooled analysis we found that the overall risk of developing gastric cancer in *GSTM1* null patients was underestimated by hospital based case-control studies. In this meta-analysis, only published studies were used. Publication bias is, therefore, an issue even though the statistical tests used did not detect it. Regarding the statistical analysis, this was appropriately guided in most instances, with an adequate indication of 95% CI and associated *p* values.

Despite these limitations, mainly due to a far from perfect in design of some studies, some interesting conclusions emerged. From the results of this quantitative meta-analysis, that pooled together data regarding almost five thousand people (1797 cases and 3000 controls), it appears that *GSTM1* status has probably no effect on the risk of gastric cancer per se, suggesting a potential modulation of tobacco-related carcinogenesis. Future research in this field should take great care in the interaction between healthy risk factor (life-style conditions, such as smoking habits, alcohol and drug consumption) and *GSTM1* status.

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3.3

Glutathione S-Transferase T1 status and gastric cancer risk: a meta-analysis of the literature

ABSTRACT

To clarify the risk of gastric cancer associated with glutathione S-transferase (*GSTT1)* status, a meta-analysis of published studies was performed. Eligible studies included all reports investigating an association between *GSTT1* status and gastric cancer published prior to the 31st of October, 2005. A qualitative scoring of papers was applied to evaluate the quality of the published data. The principal outcome measure was the odds ratio (OR) for the risk of gastric cancer associated with *GSTT1* deletion status using a random effects model. Eighteen case-control studies detailing a possible association between the *GSTT1* null genotype and gastric cancer were selected. Combining data from these studies, totalling 2508 cases and 4634 controls, a non-statistically significant OR for gastric cancer risk associated with *GSTT1* deficiency of emerged (OR= 1.09; 95% CI: 0.97–1.21; I^2 = 0%). When only high-quality scored studies were considered, a statistically significant increased risk appeared (OR= 1.23; 95% CI: 1.04-1.45; I^2 = 0%), as well as considering only Caucasians (OR= 1.23; 95% CI: 1.03-1.56; I^2 = 0%). By pooling data from 7 studies (319 cases and 656 controls) that considered combinations of *GSTT1* and *GSTM1* genotypes, a statistically significant increased risk for gastric cancer (OR= 1.95, 95% CI: 1.42-2.67; I^2 = 0%) was detected for individuals with deletion mutations in both genes compared to wild types. In conclusion, this meta-analysis suggests that the *GSTT1* null genotype may slightly increase the risk of gastric cancer and that interaction between unfavourable GST genotypes may exist. Greater attention should, therefore, be paid to the design of future studies; the investigation of interactions among multiple genotypes and environmental exposures are justified to clarify *GSTT1* null status influence on gastric cancer risk.

INTRODUCTION

Sequence variations in genes coding for phase II enzymes, such as the glutathione S-transferase (GST) family, may potentially alter individual susceptibility to cancer (1). Glutathione S-tranferase are a family of genes with a critical function in protecting against electrophiles and the products of oxidative stress. The GST enzymes are involved in the detoxification of many xenobiotics, including several environmental carcinogens and endogenously derived reactive oxygen species (2). Four major GST families are widely expressed in mammalian tissue: GSTA (α), GSTM (μ), GSTT (θ) and GSTP (π). Certain genes within the *GSTM* and *GSTT* (*GSTM1* and *GSTT1*) subfamilies exhibit homozygous deletion (null genotype) polymorphisms that are considered important modifiers of individual risk for environmentally-induced cancers (1). Individuals who have the homozyogous deletion in one of these genes have no GSTM1 and GSTT1 enzyme activity, and thus are more susceptible to carcinogens such as benzo[α]pyrene-7,8-diol epoxide, the activated form of benzo[α]pyrene, and smaller reactive hydrocarbons, such as ethylene oxide and diepoxybutane (2,3). The prevalence of *GSTM1* and *GSTT1* null genotypes was found to vary among ethnic groups. In human populations, *GSTM1* and *GSTT1* are absent in 10-60% and 13-55% of individuals, respectively (4).

The common expression of GSTα, GSTT1-1 and GSTP1-1 in many cell types along the human gastrointestinal tract suggests an important role in the protection against carcinogens and other xenobiotics (5). The deletion mutations in the *GSTT1* and *GSTM1* genes and their association with gastric cancer have been investigated in a large number of studies. In our recent meta-analysis, which identified 15 studies investigating an association between *GSTM1* null genotype and the risk of gastric cancer, we reported a slight increase in gastric cancer risk associated with *GSTM1* deficiency [odds ratio (OR) of 1.24; 95% confidence interval (CI): 1.00-1.54] (6). A recent review of genetic susceptibility and gastric cancer risk reported that the results of case-control studies detailing associations between the *GSTT1* gene and gastric cancer risk are inconclusive (7). Since Deakin *et al.* (8) first investigated the relationship between *GSTT1* deficiency and gastric cancer in 1996, 17 studies have appeared in the literature, and most of them have refuted an association between *GSTT1* deficiency and gastric cancer risk (3, 9-24). One of the major problems with the published studies is that most of them were based on small numbers of cases and controls. Furthermore, because the *GSTT1* genotype is presumed to affect gastric cancer risk by influencing detoxification of activated environmental carcinogens and by interaction with other unfavourable GST polymorphisms, the potential modifying effect

of *GSTT1* status on the relationship between tobacco smoking, other GST and gastric cancer is of particular interest, even though not often investigated.

To clarify the effect of *GSTT1* status on the risk of developing gastric cancer, we carried out a quantitative meta-analysis of research published through the 31st of October, 2005. In addition, we combined data available from published papers to explore the possible effects of the interactions between the *GSTT1* genotype and smoking habits and between *GSTT1* and *GSTM1* genotypes with respect to gastric cancer risk.

METHODS

Identification of relevant studies

The digital medical databases used for the search were MEDLINE and EMBASE. The key words used for the research were Glutathione S-Transferase T1 or GSTT1, gastric or stomach cancer, without restriction on language. The time period includes research articles published through the 31st of October, 2005.

For the meta-analysis, the following inclusion criteria were considered: presence of a quantitative assessment of the relationship between *GSTT1* status and gastric cancer;

an appropriate description of *GSTT1* status in cases and controls; results expressed as relative risk (RR) or OR; studies with a 95% CI for RR or OR, or with the possibility to calculate these measures if standard deviation (SD) values were present.

We compared the results of our literature search to the review articles found using the previously mentioned databases. Furthermore, when data from one paper was republished by the same author in a larger investigation or written in English, only the most recent article was considered.

Quality assessment and data extraction

Each article was blinded with respect to the authors, institutions and journals. The articles were read and scored for quality by two independent researchers using a system that incorporates elements of the methods developed by Angelillo et al. (25) and Chalmers et al. (26). We also followed suggestions useful to evaluate a molecular epidemiological study from Thakkinistian et al. (27) and Bogardus et al. (28). The criteria employed are shown in the results section below. A quality score was then calculated for each paper as the percentage of applicable criteria that were met in each study. Items concerned with efforts to minimize potential bias (nine points, items A-I, results section) were given twice the weight of those evaluating data analysis (nine

points, items J-R) (25). Lastly, even though reported in the quality score scale, the item related to Hardy-Weinberg equilibrium (item R) was not applicable because it could not have been checked in the included studies due to the analytical method used for *GSTT1* genotyping, which doesn't provide the frequency of heterozygous individuals. Therefore high-quality scored studies were considered as the ones with at least 70% of the total score (19/26), as suggested by Angelillo et al. (25).
The same two researchers extracted the data from each article using a structured sheet and entered into a database. The following data were considered: year and location of study; ethnicity, source, sex ratio and mean age (or range whenever possible) of cases and controls; the number of cases and controls with *GSTT1* deficiency; OR values with their 95% CI and covariates investigated in the study.

Statistical analysis
The OR for gastric cancer associated with *GSTT1* deficiency was estimated for each study. In carrying out the meta-analysis, the random effect model was used, taking into account the possibility of heterogeneity between studies, which was tested with I^2 test and a standard Chi squared test (29). The resulting *P* value of the Chi squared test for heterogeneity is reported in the result section after the result of the I^2 test. The Mantel-Haenszel method (fixed effect model) was also used to assess the effect of the model's assumptions on our conclusions (30,31). Statistical analysis was undertaken using the RevMan program, release 4.2 (32). In order to detect potential publication bias, ORs and 95% CI were plotted against standard errors in each study. We also computed the power of the selected studies, in order to assess the probability of detecting an association between *GSTT1* deficiency and gastric cancer at the 0.05 level of significance, assuming a genotypic risk of 2.0 and 1.5, using the method described by Schlesselmann (33). Based on previous literature findings, we planned to perform several subgroup meta-analyses based on the ethnicity, study design, power of the study and quality score of the papers. Lastly, we performed two additional sensitivity analyses in an attempt to evaluate if the interaction between *GSTT1* and cigarette smoking, as well as between *GSTT1* and *GSTM1* null genotypes, can modify the risk of gastric cancer. The *z* statistic was used to formally assess if any statistically significant difference among the results of each subgroup meta-analyses exists, as reported by Deeks et al. (34). In order to collect the most complete data concerning those interactions, when not extensively published in the results section of a paper, the corresponding author of the individual studies were contacted by e-mail and fax. We thus invited those authors to provide data useful for us in performing the two subgroup meta-analyses.

RESULTS

Identification of relevant studies

Eighteen articles were retrieved by our bibliographic search with three papers written in non-English language (10,17,20). In order to collect data from the last three papers, the corresponding authors were contacted by e-mail and fax and invited to fill in an empty table with the results of their studies. All the authors answered and their data were included in the analysis. Eighteen case-control studies were therefore considered for the present meta-analysis (3,8-24). In Table 1 the ORs are reported with their corresponding 95% CI, including all the data that were extracted from each study. In each report, *GSTT1* status was determined by analysis of the gene via polymerase chain reaction. Ten case-control studies were population based (C-C pb), with 6 of them enrolling sex and age-matched controls, and 8 were hospital based case-control studies (C-C hb), of which only three had sex and age-matched controls (Table 1). History of smoking was verified for cases and controls in 12 of 18 studies, with 4 reporting results of the interaction between *GSTT1* status and gastric cancer risk in relation to cigarette smoking habits (11,12,15,21). Lastly, though 17 of 18 studies collected data on *GSTM1* status, only 5 reported data concerning the combination of those genotypes with respect to gastric cancer risk in a form suitable for a subgroup meta-analysis (3,12,19,21,22). Authors were contacted to obtain data on the interactions between *GSTT1* status and smoking habits, and *GSTT1* status and *GSTM1* status; two authors answered (10,20) and their data were included in the two subgroup meta-analyses.

Quality assessment

Table 2 shows the quality scoring items with the relative%ages of the studies complying with those criteria. The quality scoring procedure was performed for all the studies included in the meta-analysis with the exception of the articles written in a non-English language. The potential for selection bias may be a concern in all of the individual studies considered. Cases and controls were chosen in most of the reports in the appropriate manner: 5 of 15 studies used cancer registries to identify cases and 7 identified randomly selected cases. Most of the studies stated that cancer diagnosis was validated by histology and all the studies specified disease criteria (Table 2).

Table 1. Summary of studies of gastric cancer and *GSTT1* status

Investigator, year	Place of study	Cases [a]	*GSTT1* null %	Controls	*GSTT1* null %	OR (95% CI) [b, c] for null genotype	Covariates	Power (RR>1.5; α=0.05)	Power (RR>2.0; α=0.05)
Deakin *et al,* 1996 (8)	Staffordshire, United Kingdom	114 cases from one hospital; age: 68; 70.5% male; Caucasians	18.4	509 controls from the same hospital of cases; age 70; 48% male; Caucasians	18.5	1.00 (0.59-1.68) [b]	Age, gender, grading, histotype, smoking, *GSTM1* status	52%	100%
Katoh *et al.,* 1996 (9)	Kitakyushu, Japan	139 consecutive cases from three hospitals in the same city; histologically confirmed; age: 62.2 (13.2); 70.5% male; Asians	47.5	126 sex -age matched hospital controls from the same city of cases; age 61.9 (16.8); 57.1% male; Asians	44.4	1.13 (0.70-1.83) [b]	Age, gender, grading, occupation, area of residence, medical history, smoking habits, *GSTM1* status	33%	92%
Wang *et al.*, 1998 (10)	Taiwan	83 cases; age: 59.6 (14.1); 60.2% male; Asians	43.4	83 controls from the same hospital of cases; age 56.7 (14.7); 60.2% male; Asians	54.2	0.65 (0.35-1.19)	Age, gender, *Helicobacter pylori* infection, smoking habits, *CYP2E1* and *GSTM1* status	79%	100%
Setiawan *et al.,*2000 (11)	Yangzhong City, China	81 incident cases; histologically confirmed diagnosis; age: NS; 71.4% male; Asians	54.0	418 healthy population-based cancer-free individuals; age: NS; 49.7% male; Asians	46.0	2.50 (1.01-6.22) [c]	Age, gender, BMI, medical history, education, occupation, family history of cancer, fruit and salt intake, alcohol and green tea consumption, *Helicobacter pylori* infection, smoking habits, *GSTM1* status	36%	100%

Saadat *et al.*, 2001(3)	Shiraz, Iran	42 cases; histologically confirmed diagnosis; age: NS; sex ratio: NS; Caucasians	35.7	131 sex -age matched healthy blood donors; age: NS; sex ratio: NS; Caucasians	31.3	1.22 (0.58-2.57) [b]	Age, gender, *GSTM1* status	16%	45%
Lan *et al.*, 2001 (12)	Warsaw, Poland	293 incident cases from 22 hospitals of one city ; ages: 21-79; 65.8% male; Caucasians	20.4	418 sex and age matched healthy population-based controls from the same city of the cases; ages: 21-79; 64.4% male; Caucasians	15.7	1.48 (0.97-2.25) [c]	Age, gender, education, area of residence, BMI, *Helicobacter pylori* infection, family history, fruit consumption, smoking habits, *IL-1*, *GSTM1*, *GSTM3* and *GSTP1* status	100%	100%
Cai *et al.*, 2001 (13)	Changle County, China	95 incident cases; histologically confirmed diagnosis; ages: 32-78; 85.3% male; Asians	43.2	94 sex-age matched healthy population controls from the same county of the cases; ages: 37-79; 87.2% male; Asians	50	0.76 (0.1-1.4) [b]	Age, gender, education, fish sauce consumption, alcohol, smoking habits, *GSTM1* status	23%	66%
Wu *et al.*, 2002 (14)	Taipei, Taiwan	356 incident cases from one hospital; histologically confirmed diagnosis; ages: 25-87; 61.2% male; Asians	50.8	278 controls from the same hospital of cases; ages: 22-86; 56.1% male; Asians	46.7	1.18 (0.86-1.61) [b]	Age, gender, histotype, anatomy, stage, *GSTM1* and *CYP2E1* status (*Rsa/Pst* and *Dra*)	77%	100%

Gao *et al.*, 2002 (15)	Huaian City, China	153 cases (hospital surgical cases and incident cases from a Regional Registry) from the same city; histologically confirmed diagnosis; ages: 40-81; 77.1% male; Asians	46.4	223 sex-age matched healthy population controls from the same city of the cases; ages: 35-81; 66.8% male; Asians	53.3	0.77 (0.48-1.25) [c]	Age, gender, diet, tea and alcohol consumption, smoking habits, *GSTM1* status	41%	100%
Sgambato *et al.,* 2002 (16)	Basilicata, Italy	8 consecutive cases from one hospital; age: NS; sex ratio: NS; Caucasians	0.0	100 healthy controls from the same hospital of cases; age: NS; sex ratio: NS; Caucasians	18.0	0.26 (0.01-4.75) [b]	Age, gender, occupation, smoking history, *GSTM1* status	4%	11%
Ye *et al.*, 2003 (17)	Wuhan, China	56 cases from two hospitals; histologically confirmed diagnosis; age: 57.6 (22-79); 75% male; Asians	60.7	56 healthy controls from one of the two hospitals of cases; matched for sex, age, smoking, dietary habits and family history of cancer; age: 58.0 (26-86); 69.6% male; Asians	46.4	1.78 (0.84-3.78) [b]	Age, gender, education, occupation, living condition, family history of cancer, dietary habits, drinking, smoking and *CYP2E1* status	18%	42%
Choi *et al.,* 2003 (18)	Iksan, South Korea	80 surgical cases from one hospital; histologically confirmed diagnosis; age: NS; sex ratio: NS; Asians	53.8	177 healthy cancer-free individuals; age: NS; sex ratio: NS; Asians	53.1	0.97 (0.55-1.71) [b]	Stage, grading, histotype, *GSTM1* status	26%	71%

Colombo *et al.,* 2004 (19)	Saõ Jose do Rio Preto and Barretos, Brasil	100 incident cases from two hospitals; histologically confirmed diagnosis; ages: 28-93; 73 % male; 87 Caucasians, 13 Negroids	17.0	150 sex and age matched healthy population-based controls; ages: 20-93; 60 % male; 135 Caucasians, 15 Negroids	18.6	0.89 (0.46-1.73) [b]	Age, gender, family history, occupation, ethnicity, histotype, *Helicobacter pylori* infection, alcohol consumption, smoking habits, *GSTM1* and *CYP2E1* status	29.4%	83.3%
Torres *et al.,* 2004 (20)	Popayàn, Colombia	46 cases from one hospital; histologically confirmed diagnosis; age: 60; 50% male; Caucasians	17.4	96 cancer-free controls from the same hospital of cases; age: 58; 42.7% male; Caucasians	14.6	0.47 (0.09-2.27) [c]	Age, gender, area of residence, family history, occupation, *Helicobacter pylori* infection, diet, alcohol consumption, smoking habits, *GSTM1* and *TNF* status	17.9%	41.4%
Tamer *et al.*, 2004 (21)	Mersin and Kocaeli, Turkey	70 surgical cases from two hospitals; histologically confirmed diagnosis; age: 57.6 (9.8); 67.1% male; Caucasians	30.0	204 healthy cancer-free individuals; ages: 62.0 (7.0); 56.4% male; Caucasians	26.0	1.36 (0.93-2.94) [c]	Age, gender, smoking habits, *GSTM1* and *GSTP1* status	25.2%	74.2%
Palli *et al.,* 2005 (22)	Tuscany, Italy	175 surgical cases from the main hospitals of the area; histologically confirmed diagnosis; age: 68.7 (10.4); 57.1% male; Caucasians	23.4	546 healthy population-based randomly selected controls from the same area of cases; age: 55.5 (7.0); 49.3% male; Caucasians	16.7	1.68 (1.01-2.80)[c]	Age, gender, area of residence, family history of gastric cancer, *Helicobacter pylori* infection, *GSTM1* status	42.7%	100%

Mu *et al.*, 2005 (23)	Taixing City, China	196 cases from Taixing Tumour Registry; age: NS; 66.99% male; Asians	47.4	393 sex-age matched healthy population-based randomly selected controls from the same area of cases; age: NS; 69.16% male; Asians	48.8	1.12 (0.72-2.74) [c]	Age, gender, education, area of residence, income, BMI, very hot food eating habits, family history of stomach disease and gastric cancer, *Helicobacter pylori* infection, alcohol and tea habits, smoking history, *p53*, *GSTP1*, *GSTM1* status	64.6%	100%
Nan *et al.*, 2005 (24)	Cheongju and Daejon, South Korea	421 cases from two hospitals; histologically confirmed diagnosis; age: 60.0 (11.2); 65.6% male; Asians	42.7	632 sex-age matched hospital-based cancer-free individuals from the same hospitals of cases; age: 59.4 (10.7); 65.5% male; Asians	40.2	1.11 (0.86-1.44) [b]	Age, gender, diet, *GSTM1* status	100%	100%

[a] Ages of cases and controls: mean (SD) or range given wherever possible; [b] Crude OR; [c] Adjusted OR

Table 2. Items used in the quality scoring of observational studies in epidemiology

Quality scoring items	% of studies complying[a]
Case-control studies	
A1. Cases either randomly selected or selected to include all cases in a specific population	80
B1. Response rate for identified cases > 75%	40
C1. Controls drawn from the source population of cases	100
D1. Population-based controls	67
E1. Response rate for identified controls > 75%	53
Cohort studies	
A2. Initial response rate > 75%	NA[b]
B2. Comparison of persons who did and did not participate	NA
C2. Follow-up rate > 75%	NA
D2. Comparison of who were and were not lost to follow-up	NA
E2. Exposed/non exposed subjects identified without knowledge of disease status	NA
All studies	
F. Disease validated by histology or other gold standard	69
G. Specific disease/not disease or exposure/not exposure criteria given	100
H. Exposure/disease assessment made blindly with respect to the case-control/exposure status of subjects (analyst unaware of the clinical status/exposure of the sample) [c]	13
I. Reproducibility of laboratory tests mentioned [c]	13
J. Age and sex considered as potential confounders	93
K. Collection of data on other potential confounders/effect modifiers	100
L. Demographic data listed	47
M. Place and time of the study reported	93
N. Power calculations performed	0
O. Precise *p* values or confidence interval given	100
P. Statistic test specified	100
Q. Appropriate statistical analysis	100
R. Hardy-Weinberg equilibrium assessed [c]	NA

[a] If compliance was not specifically indicated in the text, non compliance was assumed

[b] NA: not applicable in the present meta-analysis, see methods and results section

[c] Specific for molecular-epidemiological studies

Furthermore, in all of the studies the controls were drawn from the same population as the cases; 10 reports selected population controls. However, the response rate for cases and controls were frequently less than 75%, which may have led to a selection bias. Misclassification bias should be considered as a concern: only 2 of 15 studies clearly stated that the analyst was unaware of the clinical status of the subjects when

genotyping their samples, so exposure misclassification may exist (Table 2). One other issue is that almost all the studies did not report the reproducibility of the laboratory method used. Age, sex and other potential confounders/effect modifiers were considered in almost of the studies, while demographic data were reported in only 7 reports. Statistical analysis was judged appropriate in all of the studies, and *p* values and/or 95% CI were always listed, while no study provided power calculations. Quality scores for the individual studies ranged from 0.46 to 0.81, and five of fifteen resulted as high-quality scored studies (11, 12, 14, 22, 23).

Statistical analysis

Figure 1 shows the results of the combined data, depicting a plot of ORs (95% CI) for the risk of developing gastric cancer associated with *GSTT1* deficiency in the 18 case-control studies, involving a total of 7142 subjects (2508 cases and 4634 controls). The meta-analysis resulted in a statistically non-significant association between *GSTT1* deficiency and gastric cancer risk (OR= 1.09; 95% CI: 0.97-1.21; I^2 = 0%, p for heterogeneity = 0.48). This analysis is based on combining data from studies based on a number of different ethnic groups. Separate meta-analyses were therefore conducted stratified by ethnicity. For Asians (10 studies) we found an OR= 1.02 (95% CI: 0.89-1.18; I^2 = 12.1%, *p* for heterogeneity = 0.33), while for Caucasians (8 studies) the OR value was 1.27 (95% CI: 1.03-1.56; I^2 = 0%, *p* for heterogeneity = 0.87; *p* value of *z* statistic among the two groups = 0.08). By considering separately C-C hb and C-C pb studies, similar OR values were found [C-C hb: OR of 1.08 (95% CI: 0.92-1.27; I^2 = 0%, *p* for heterogeneity = 0.56); C-C pb: OR of 1.09 (95% CI: 0.92-1.29; I^2 = 16.7%, *p* for heterogeneity = 0.29]. When combining data from the studies with a power of at least 80% for a RR = 2.0 (Table 1), we found an OR of 1.08 (95% CI: 0.95–1.24; I^2 = 17.9%, *p* for heterogeneity = 0.27). In addition, when only high quality papers were considered, a statistically significant increase in risk for gastric cancer appeared (OR= 1.23, 95% CI: 1.04-1.45; I^2 = 0%, *p* for heterogeneity = 0.41), while an OR of 0.99 (95% CI: 0.85-1.15; I^2 = 0%, *p* for heterogeneity = 0.75; *p* value of *z* statistic among the two groups = 0.07) resulted by pooling data from low quality papers.

The funnel plot (Figure 2) shows no evidence of publication bias, with the exception of one outlier (16). As far as the interaction between *GSTT1* status and smoking habits is concerned, in relation to gastric cancer risk, we obtained data from 6 of the 12 studies in a form suitable for a subgroup meta-analysis. After stratification by smoking status, an overall OR of 1.54 (95% CI: 0.95–2.48; I^2 = 59.2%, *p* for heterogeneity = 0.03) for the risk of gastric cancer appears in ever-smokers with *GSTT1* deficiency compared to ever-smokers with a normal *GSTT1* genotype. On the other hand, an overall estimate

Fig. 1. Forest plot of developing gastric cancer associated with *GSTT1* null status and subgroup analyses.

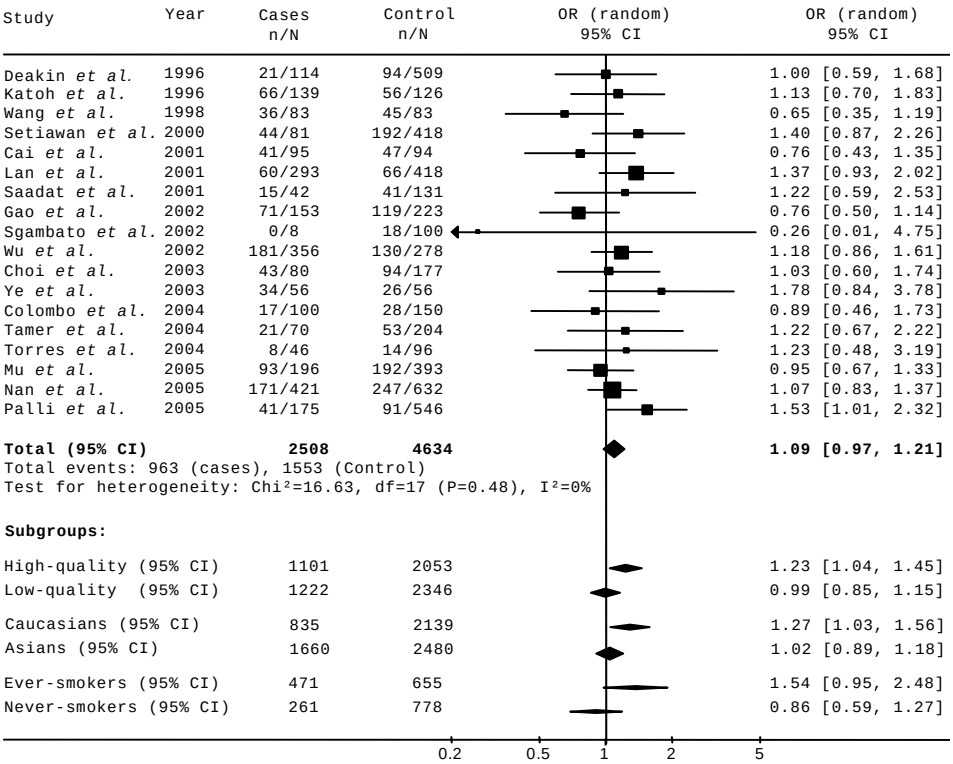
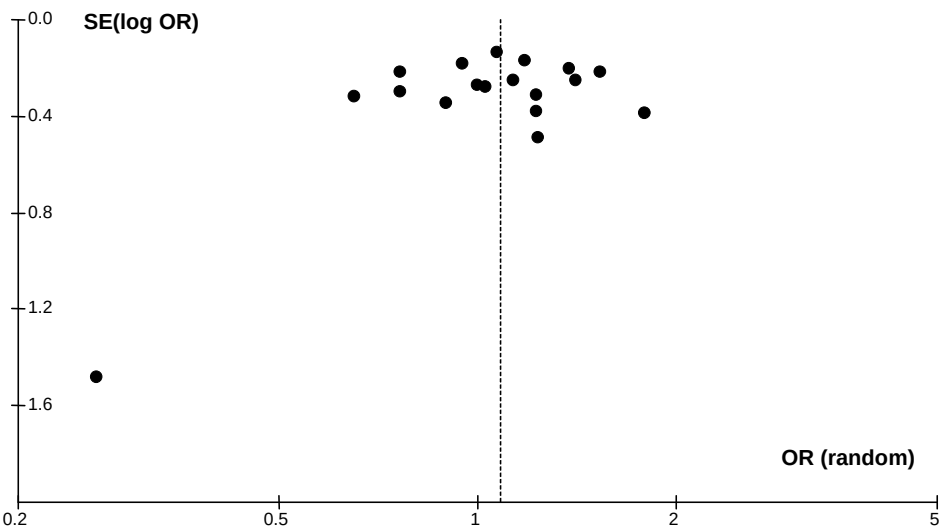


Fig. 2 - Funnel plot of the published studies considered in the meta-analysis.



of OR= 0.86 (95% CI: 0.59-1.27; I^2 = 26.3%, *p* for heterogeneity = 0.24) appears when comparing the effect of *GSTT1* status (null *vs.* normal genotype) in never-smokers with respect to gastric cancer risk. Furthermore, pooling data concerning the combination of *GSTT1* and *GSTM1* genotypes from 7 studies, demonstrated that individuals with combined deletion mutations in those genes have an OR of 1.95 for gastric cancer (95% CI: 1.42-2.67; I^2 = 0%, p for heterogeneity = 0.58) in comparison with individuals with wild type genotypes.

DISCUSSION

Most of the cancer susceptibility genes identified to date are rare and highly penetrant. While the individuals with rare alterations of these genes (e.g. tumor suppressor genes) have a dramatically higher risk of cancer, more common differences in low penetrant susceptibility genes (e.g. drug metabolism enzymes) could be responsible for a relatively small, but observable increase of gastric cancer risk at the population level (35). Although gastric cancer is one of the most common malignancies worldwide, its pathogenesis and the molecular genetic events that contribute to its development are poorly understood (7). One of the most widely studied metabolic polymorphisms examined as a susceptibility factor for gastric cancer is the *GSTT1* null allele; it's frequency in the Caucasian population is approximately 20-30% (4). Since Deakin *et al.* (8) first investigated a possible relationship between *GSTT1* deficiency and gastric cancer risk, a further 17 reports have been published examining this hypothesis (3,9-24), with conflicting results.

This led us to undertake the present meta-analysis, which aims to derive an estimate of the gastric cancer risk associated with *GSTT1* status. The main finding of this meta-analysis of 18 case-control studies involving 7142 subjects is that the *GSTT1* null status seems to be unrelated to gastric cancer risk. Absence of heterogeneity between studies emerged from the statistical analysis, however as Blettner *et al.* pointed out, the tests formally used to assess heterogeneity have low statistical power to detect it (36). Therefore, we decided a priori to perform several subgroup meta-analyses according to ethnicity, study design, quality and power of the studies. Considering separately Caucasian and Asian studies, the association between *GSTT1* null status and gastric cancer risk reaches a slight statistically significant level among Caucasians. In human populations, the frequency of *GSTT1* deficiency is 13-26% and 36-52% in Caucasian and Asian individuals, respectively (4). However, the Caucasian reports in

the subgroup analysis include a mixture of populations from very distant countries, so the result must be interpreted with caution. Unexpectedly, when only high-quality scored studies were considered, a statistically significant increased risk of gastric cancer for *GSTT1* null individuals was detected.

Since *GSTT1* is presumed to confer susceptibility to gastric cancer via an interaction with carcinogens, it is interesting to note that no data was collected from the cases or the controls on tobacco usage, alcohol intake, food consumption, or *Helicobacter pylori* in many of the studies. By combining the data available from 6 studies, which investigated the possible interaction between *GSTT1* status and cigarette smoking with respect to gastric cancer risk, a slightly increased risk appears for ever-smokers with a *GSTT1* null genotype compared to individuals with a *GSTT1* normal genotype. Even though the result is not statistically significant, from the *z* test a statistically significant difference among the two estimates appears, so it would be interesting to pool all the missing data to confirm this result. If genetic susceptibility to gastric cancer is, in part, mediated through polymorphic variation, it is probable that the risk associated with any one locus will be small because an interaction is likely to operate in these circumstances. Hence, combinations of certain genotypes may be more discriminating as risk factors than a single locus genotype. Unfortunately, only a few reports investigated this aspect, even though 17 of 18 selected studies collected data on *GSTM1* status. By pooling the data from 7 available studies investigating a possible interaction between *GSTT1* and *GSTM1* status and gastric cancer risk, a 95% statistically significant increased risk of gastric cancer appeared for individuals with combined deletion mutations in *GSTT1* and *GSTM1* genes in comparison with individuals with both homozygous wild genotypes.

The main limitations of the study have to be considered in interpreting the results. First, only published studies were included in the meta-analysis, therefore a publication bias may have occurred. It is known that positive results usually have a greater probability of being published, and even though unpublished studies are generally of lesser quality than published ones (37), if they are not included an overestimation of the *GSTT1* null effect may appear.

Second, the two subgroup meta-analyses considering interactions between *GSTT1* null genotype and cigarette smoking, as well as between *GSTT1* null and *GSTM1* null genotypes, were performed based on a fraction of all of the possible data to be pooled, so selection bias may have occurred and our results may be over inflated. In this context, it is well known that an important issue in performing a meta-analysis is that literature-based meta-analysis rather than individual data-based meta-analysis could be a potential source of bias. Meta-analyses are in general insufficient to calculate a

pooled estimate since published estimates are based on heterogeneous populations, different study designs and different statistical models. More reliable results can be expected if individual data are available for a pooled analysis, so that confounding factors can be considered, although this approach requires the authors of all of the published studies to share their data.

Third, the quality score of the individual studies included in our meta-analysis was assessed on the basis of efforts to minimize the potential for selection bias, misclassification related to exposure, collection of data on potential confounders and method of statistical analysis. It is known that any quality assessment system has not yet been validated (38) and it is evident that our quality scale has a subjective component. Despite these limitations, assessment of the quality of individual studies used in our meta-analysis allowed us to draw two conclusions: the possibility of a selection bias and even more misclassification related to exposure cannot be ruled out; most importantly, pooling high-quality scored studies resulted in higher risk estimates than did pooling low-quality scored studies. If high-quality scored studies are more likely to yield valid information than low-quality studies, we can conclude that, based on the currently available data, an additional slight risk of gastric cancer for *GSTT1* null individuals may exist.

Despite these remarks, some interesting conclusions have emerged. From the results of this quantitative meta-analysis that combined the data from 7142 people (2508 cases and 4634 controls), it appears that *GSTT1* null status has a very small effect on the risk of gastric cancer *per se*, but it may modulate the tobacco-related carcinogenesis of gastric cancer, and that the combination of unfavourable genotypes may result in an additional risk of gastric cancer. A clearer picture of the interaction between different polymorphisms and environmental factors on gastric cancer risk will be adequately addressed only by large and well-designed epidemiological studies.

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3.4

Meta- and pooled analyses of the Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and gastric cancer risk: a HuGE-GSEC review

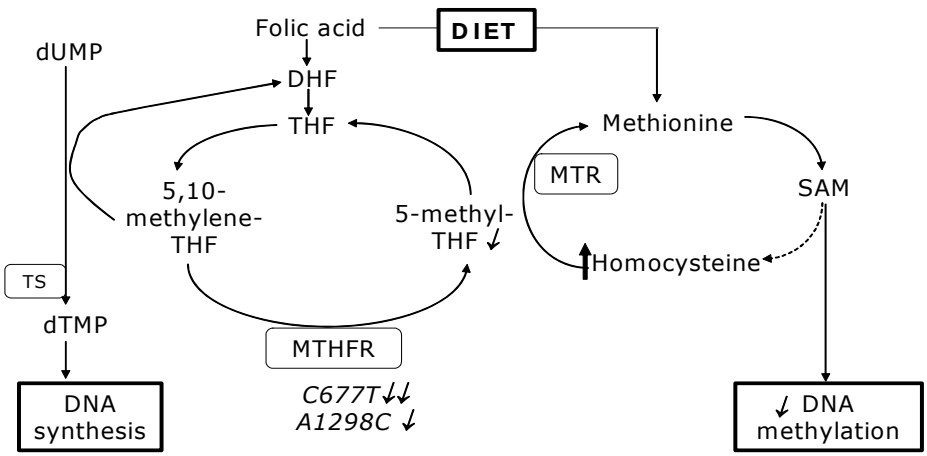
ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the metabolism of folate, whose role in gastric carcinogenesis is controversial. A meta-analysis and individual data pooled analysis of case-control studies that examined the association between *C677T* and *A1298C* polymorphisms (the former being associated with low folate serum levels) and gastric cancer (meta-analyses: 16 studies, 2727 cases and 4640 controls for *C677T*, 7 studies, 1223 cases and 2015 controls for *A1298C*; pooled analyses: 9 studies, 1540 cases and 2577 controls for *C677T*, 5 studies, 1146 cases and 1549 controls for *A1298C*) was performed. An increased risk appeared for *MTHFR* 677 TT in the meta-analysis (OR= 1.52; 95% CI: 1.31, 1.77) and pooled analysis (OR= 1.49; 95% CI: 1.14, 1.95). No association resulted for *MTHFR* 1298 CC (meta-OR= 0.94; 95% CI: 0.65, 1.35; pooled-OR= 0.90 (95% CI: 0.69, 1.34). When results from the pooled analysis of four studies on *C677T* were stratified according to folate levels, results showed an increased risk among individuals with low levels (OR= 2.05; 95% CI: 1.13, 3.72) respect to those with high folate levels (OR= 0.95; 95% CI: 0.54, 1.67). Overall, these findings support the hypothesis that folate play a role in gastric carcinogenesis.

GENE AND FUNCTION

The 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene maps to chromosome 1p36.3 (1). The complementary DNA sequence is 2.2kb long and contains 11 exons (1). The gene product is a 77-kD protein, although a smaller isoform of approximately 70-kD has been observed in some tissues such as liver (2). MTHFR plays a central role in folate metabolism, together with other enzymes, by irreversibly catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate and a cosubstrate for homocysteine methylation to methionine (Figure). In humans, folate plays the fundamental role of providing methyl groups for *de novo* deoxynucleotide synthesis and for intracellular methylation reactions (2,3).

Figure. Folate pathway



dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; DHF: dihydrofolate; MTHFR: methylentrahydrofolate reductase; MTR: methionine synthase; SAM: a-adenosylmethionine; THF: tetrahydrofolate; TS: thymidylate synthase.

MTHFR enzyme function may influence cancer risk in two ways. The substrate of MTHFR enzyme, 5,10-methylenetetrahydrofolate, is involved in the conversion of deoxyuridylate monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP), and low levels of 5,10-methylenetetrahydrofolate would lead to an increased

dUMP/dTMP ratio. In this situation an increased incorporation of uracil into DNA in place of thymine may follow, resulting in an increased chance of point mutations and DNA/chromosome breakage (3). A less active form of MTHFR would lead, all other factors being equal, to an accumulation of 5,10-methylenetetrahydrofolate, thus a lower dUMP/dTMP ratio, and a presumably lower cancer risk (3).
The second way in which an impaired MTHFR activity might influence cancer risk is determined by the level of s-adenosylmethionine (SAM), the common donor of methyl that is necessary for the maintenance of the methylation patterns in DNA. Changes in methylation modify DNA conformation and gene expression. A less active form of MTHFR leads to lower SAM and consequently to hypomethylation; this phenomenon would be expected to increase the risk of some cancers (4) (figure 1). Similarly, low folate intake may modify cancer risk by inducing uracil misincorporation during DNA synthesis, leading to chromosomal damage, DNA strand breaks and impaired DNA repair, and DNA hypomethylation (5).

GENE VARIANTS

Twenty-nine rare mutations of *MTHFR* have been described in homocystinuric patients resulting in very low enzymatic activity (6), while two common polymorphisms are present in healthy individuals with lower enzyme activity: C→T in exon 4 at nucleotide 677, leading to Ala222Val (7); and A→C in exon 7 at nucleotide 1298, leading to Glu429Ala (8,9). These polymorphisms are located 2.1 kb apart, and have been investigated in association with the risk of gastric and other cancers (10). Three additional polymorphisms have been described, *T1059C*, *T1317C*, and *G1793A* (9, 11, 12). While *T1317C* polymorphism is a silent change with no effect on plasma homocysteine and folate concantrations (9), the *T1059C* has been reported to be associated with increased neural tube defects in an Iowa population (11). The *G1793A* allele is least frequent among Ashkenazi Jewish individuals (1.3%) compared to Caucasians (6.9%) (12); it has been reported that individuals with the heterozygous genotype for the variant allele show borderline or deficient values in folate concentration compared to individuals with the wild genotype (13).
Individuals who are homozygous for the *MTHFR* 677 less frequent variant (TT) have 30% of the expected enzyme activity in vitro, compared with those who are homozygous for the common variant (CC), while heterozygous carriers have 65% activity (2). Van der Put et al. (8) evaluated the MTHFR activity according to the combination of *A1298C* and *C677T* genotypes, showing that individuals carrying the 677CC allele and contemporarily the 1298 variant allele (CC) have 60% activity

compared to subjects carrying the 1298AA variant, while in the same population 80% activity was detected for the 1298 heterozygotes (AC). Enzyme activity for individuals who are heterozygous for both *C677T* and *A1298C* appears to be around 50-60% of the activity of those without either variant (9). It has been reported that subjects who are TT homozygous for the *MTHFR* 677 exhibit reduced folate status and higher serum homocysteine levels compared to those who carry at least one 677C allele (14-16). The evidence on the association of the 1298 variant allele with increased folate levels is less consistent (14,15,17,18).
Three recent studies reported that the *MTHFR* TT genotype is related to DNA hypomethylation (19,20), particularly in individuals with reduced plasma folate concentrations (21). Inconsistent results derive from studies on *A1298C* polymorphism, plasma folate and homocysteine levels (15,18,22,23).

POPULATION FREQUENCIES

The T allele frequency of *MTHFR* 677 polymorphism is reported to be of 0.36-0.44 in Europeans, 0.35-0.53 in Asians, 0.33-0.35 in US, and 0.10-0.24 in African Americans (24). The frequency of homozygosity ranges from 1% [95% Confidence Interval (CI): 0.2, 2.0] in US African American populations to more than 20% (95% CI: 14.6, 26.8) in US Latinos; 5% (95% CI: 1.2, 9.6) to 30% (95% CI: 21.4, 38.9) in White populations in Europe and North America; 32.2% (95% CI: 28.3, 36.4) in Mexico; 5.8 (3.5, 9.6) in White Canadians in Alberta to 14.3 (10.9, 17.6) in Canada, Quebec; 0.8 (0.2, 2.1) in West Africa to 0.0 (0.0, 1.2) in Sub-Saharan Africa; 10.7% in Oceania (5.5, 19.7); 11.5% (95% CI: 10.2, 12.7) in Japanese and 16% (95% CI: 8.0, 31.0) in Chinese (24-27). For *A1298C*, the variant allele frequency is reported to be 0.14-0.35 in Europeans and 0.11-0.17 in Asians. The frequency of homozygosity ranges from 10% (95% CI: 9.0, 11.0) in White populations in Europe and North America to 3.5% (95% CI: 0.2, 7.2) in Asians (28).

DISEASE

Gastric cancer is the second most common cause of cancer mortality, with 647,000 deaths reported worldwide in 2002 (29). In many populations, particularly in high-income countries, in the last decades its incidence has gradually decreased, however it still represents the fifth most common type of cancer in Europe and the fourth internationally (30). Infection with *Helicobacter pylori* is the single most common cause of adenocarcinoma of the distal stomach (31), however it is not a necessary neither a

sufficient cause. The development of gastric cancer appears in fact to be the result of a complex interaction between *Helicbacter pylori* infection, lifestyle and genetic factors. Among the lifestyle risk factors tobacco smoking, a high intake of salt and lack of food refrigeration seem to play a major role (32). Lastly, gastric cancer risk shows a familial clustering (33).

With regards to genetic factors, several SNPs might potentially alter the individual susceptibility to gastric cancer (34). Among them are polymorphisms in genes involved in the protection of gastric mucosa against damaging agents and inflammatory response, genes that influence the ability to detoxify carcinogens (metabolic genes) and involved in oxidative damage response and DNA repair, as well as oncogenes (35). Genes involved in folate metabolism have also been considered to play a role in gastric cancer risk (28).

GENE-ENVIRONMENT AND GENE-GENE INTERACTIONS

Some nutrients involved in the folate metabolic pathway (eg, vitamin B_6 and B_{12}, and methionine), alcohol (a folate antagonist) and smoking (which impairs folate level) may interact with plasma folate levels and the *MTHFR* polymorphisms in determining cancer risk (36,37). It has been reported that alcohol perturbs folate metabolism by reducing folate absorption, increasing folate excretion, or by inhibiting methionine synthase (38,39). The inverse association between folate intake and plasma homocysteine levels can be modified by alcohol intake and by the *MTHFR* 677, but not the 1298 polymorphism (40). The inverse effect of smoking on folate status might be confounded by alcohol intake or dietary habits (41,42), even though the association persists after adjusting for dietary folate intake and alcohol (42,43). Additional studies reported that elevated folate turnover in response to rapid tissue proliferation or DNA repair in aerodigestive tissues among people exposed to tobacco smoke might partially explain this phenomenon (44,45).

According to recent reports, alcohol drinkers carrying the *MTHFR* 677 TT genotype had around 5-fold increased risk of gastric cancer compared to drinkers carrying the wild homozygous variant, namely an OR of 5.36 (95% CI: 1.94, 14.83) from Graziano et al (19) and 5.32 (95% CI: 1.66-17.02) from Stolzenberg-Solomon et al. (46) while others did not show such interaction (47, 48). Additionally, Gao et al. reported that smokers carrying the T allele in *MTHFR* 677 had a 7.7-fold increased risk (OR= 7.72; 95% CI: 2.23-26.79) of gastric cancer compared to non-smokers with the CC genotype (49). To our knowledge, no study ever explored if the effect of *MTHFR* 677 TT genotype on gastric cancer is modified by the individual folate intake or by the plasma folate

levels. Finally, the interaction between alcohol, smoking or folate status and *MTHFR A1298C* polymorphism has never been tested in gastric cancer.
The effect of the combination of the two common *MTHFR* polymorphisms on gastric cancer was investigated by Miao et al. and Boccia et al. (50,51), both reporting no interaction between them.

OBJECTIVE

A meta-analysis of prospective studies showed an inverse association between fruit and vegetables intake, the main dietary source of folate, and gastric cancer risk, particularly after 10 years or more of follow-up (52). Discrepant results, however, recently emerged from a large European cohort study, showing no association between fresh fruit intake and gastric cancer, and a slight protective effect of the total vegetable intake only for the intestinal histotype (53). Results from a meta-analysis of prospective and retrospective studies specifically focusing on dietary folate intake and risk of gastric cancer also reported no clear effect of dietary folate intake, with no differences between cohort or case-control studies (54).
On the other hand, two recent meta-analyses show that *MTHFR* 677 TT genotype is associated with an increased risk of gastric cancer, suggesting an important role of folate levels and subsequent impaired chromosomal DNA synthesis and aberrant DNA methylation in gastric carcinogenesis (28,54). Neither meta-analyses, however: included all the published reports available at the time they were published, and specifically included either eight (28) or nine studies (54), compared to 16 studies of the present meta-analysis. In addition, they provided unadjusted overall estimates, and were unable to stratify the results according to potential factors affecting folate status and *MTHFR* polymorphisms, because of the nature of already published data. We accomplished both the last two points by carrying out also a pooled analysis of individual-level data.
With the present meta- and pooled analyses we aim to assess the overall effect of *MTHFR C677T* and *A1298C* polymorphisms on gastric cancer by including all the available published papers, and to contribute in clarifying the interrelations between these polymorphisms with folate, alcohol and smoking in gastric cancer risk.

METHODS

We assessed the association between *MTHFR C677T* and *A1298C* polymorphisms and gastric cancer through meta-analyses of all published papers, and pooled analyses of individual-level data when available.

Meta-analysis

Selection criteria

Identification of the papers was carried out through a search of Medline and Embase up to January 2007 using the following terms: ("methylenetetrahydrofolate reductase" or MTHFR) and (gastric or stomach) and (cancer or carcinoma), without any restriction on language. The research produced 35 articles. A cited reference search of the retrieved articles was carried out, and publications were also identified by reviewing the bibliographies of the retrieved articles. Eligible studies were community-based that reported the frequency of the *MTHFR C677T* and/or *A1298C* polymorphisms as number of gastric cancer individuals and controls according to the three variant genotypes of both polymorphisms. Studies whose allele frequencies in the control population deviated from the Hardy-Weinberg Equilibrium (HWE) at a *p* value equal or less than 0.05 were excluded from the meta-analysis. If more than one article was published from the same case series, we included the paper where the most individuals were reported in the analysis.

Of the 35 articles retrieved, 22 studies resulted eligible for the analysis (19,46-51,55-69). Five reports (55-59) were excluded either because they concerned subjects included in an expanded series (50,60) or because they partially overlapped with another study (49) that was eventually selected because it gave the absolute number of individuals according to the three variant genotypes of *MTHFR* 677 (57-59). Finally, one study was excluded from the meta-analysis for the association between *MTHFR C677T* and gastric cancer (60), and one excluded from the analysis of *A1298C* (46), due to deviations from HWE.

The final number of articles considered for the meta-analysis of the association between *MTHFR C677T* and gastric cancer risk was 16 case-control studies (19,46-51, 61-69), of which three written in Chinese language (49,61,62), comprising a total of 7367 subjects (2727 cases and 4640 controls). A description of the studies is given in table 1. Ten out of 16 were population based; one was a case-control nested in a cohort (46). Among them seven were also included in the meta-analysis of the association between *MTHFR A1298C* and gastric cancer risk (48,50,51,60,61,63,64) for a total of 3238 subjects (1223 cases and 2015 controls).

Statistical analysis

Two researchers (S.B. and F.G.) extracted the data from each article using a structured sheet and entered it into a database. The followings items were considered: year and location of the study; ethnicity; characteristics of the control group; tumour site (cardia/non-cardia gastric cancer); number of individuals heterozygous and homozygous for the *MTHFR* 677 and 1298 variant alleles in the compared groups. The heterogeneity was tested by Q statistic (70). In carrying out the meta-analyses, random effect models were used (71) to take into account the possibility of heterogeneity between studies. The summary Odds Ratios (ORs) of gastric cancer associated with *MTHFR* 677 TT and CT genotypes and *MTHFR* 1298 CC and CA genotypes were estimated using the homozygous wild-type for each genotype as reference group. To determine the deviation from HWE we used the Fisher's exact permutation test with a Monte Carlo technique (72). A visual inspection of Begg's funnel plot and Begg and Egger asymmetry tests (70) was used to investigate for publication bias when appropriate (73).

Since two potential causes of heterogeneity among studies were ethnicity and tumour site, we calculated separate ORs in subgroups of studies performed in different ethnic groups (Asian/Europeans) and in subgroups of studies including cardia and non-cardia gastric cancer cases, when genotype data were tabulated according to the tumour site in the published papers. A heterogeneity test was then performed to test for statistically significant differences among the strata estimates.

Pooled analysis

Data collection

The pooled analysis was performed using the Genetic Susceptibility to Environmental Carcinogens (GSEC) database. The International Collaborative Study on GSEC (http://www.upci.upm.edu/research/ccps/ccontrol/g_intro.html) is a collaborative project that gathers information from both published and unpublished population-based studies on metabolic gene polymorphisms and cancer risk. The design of GSEC study was reported elsewhere (74). Investigators were appositely contacted and asked to provide their data for the pooled analyses. A questionnaire was provided by email to each investigator, collecting information on study design, selection and source of cases and controls, laboratory method used for genotyping, source of DNA used for the genotype analysis, and response rate for cases and controls. We contacted all the authors of the identified published papers including those whose controls subjects were not in HWE for the studied polymorphisms (46,60). Of the 17 eligible data-sets, we were able to obtain data from 10, with one of them (60) later excluded for the pooled

analysis on *MTHFR* 677 and one more (46) on *MTHFR* 1298 since the allele frequency of control population did not respect the HWE. We finally included nine studies for *MTHFR* 677, of which four conducted on Asians, four on Europeans and one on Latinos, totalling for 4117 subjects (1540 cases and 2577 controls) (table 1 for details). As for *MTHFR* 1298, five studies were included, totalling for 2695 subjects (1146 cases and 1549 controls; table 1 for details).

Statistical analysis

To assess the association of *MTHFR* 677 TT and 1298 CC genotypes with gastric cancer, the logistic regression model was used to estimate study specific ORs and 95% CI in each single study. Adjusted ORs were obtained by including age, gender, and smoking status (ever/never) as covariates. In some studies, ORs estimated for individual studies and number of cases and controls did not precisely match those reported in the publications. A pooled OR was estimated by inverse-variance weighting with the random effects model (71), taking into account the possibility of heterogeneity between studies, which was tested with *Q* statistics (70).

We could perform stratified analyses only for *MTHFR* 677 TT, since *MTHFR A1298C* was available only in six studies.

Results were stratified according to ethnicity (Asians/European descendants), alcohol drinking, tobacco smoking and folate status. Since the information of pack-years of smoking was available from only four studies (47,48,50,51), subjects were divided in ever (current and former) and never smokers. For alcohol drinking, individuals were categorized as ever (current and/or former) and never (<1 glass of each alcoholic beverage/month) drinkers. Folate serum levels were obtained from Gotze et al. (66) and gastric mucosa folate levels from Weng et al. (63). Nutrient density of folate (dietary folate intake/total caloric intake: μg/kcal*1,000) was obtained by Zhang et al. (48), while portions intake of fruit and vegetables by Boccia et al (51). For the analysis subjects were categorized in two classes based on the lower quartile of each variable estimated in the control population.

For each stratified analysis a pooled OR was estimated by inverse-variance weighting with the random effects model (71), taking into account the possibility of heterogeneity between studies, which was tested with *Q* statistics. A heterogeneity test was performed to assess for statistically significant differences among the pooled strata estimates.

Statistical analyses were carried out using the STATA software package v.8.2 (Stata Corporation, College Station, Texas).

RESULTS

Meta-analysis of MTHFR C677T

The OR of eleven out of sixteen studies were above the unit, among them four (19,50,65,62) reported a significant positive association between gastric cancer and the *MTHFR* 677 TT genotype (Table 1). The meta-analysis gave an overall OR of 1.52 (95% CI: 1.31, 1.77) and 1.17 (95% CI: 0.99, 1.39) for gastric cancer and *MTHFR* TT (Figure 2) and CT genotypes, respectively. The heterogeneity tests were 0.37 and 0.01 for TT and CT, respectively. The funnel plot (not shown) and the Begg's test provided no evidence of publication bias (p = 0.72) for *MTHFR* 677 TT genotype, while the Egger test provided a p value of 0.007.

When stratifying the data by ethnicity, we observed an OR of 1.34 (95% CI: 0.90, 1.99) and 1.64 (95% CI: 1.36, 1.97) for *MTHFR* 677 TT *versus* CC genotype, from six studies among Europeans and nine studies among Asians, respectively (p value for heterogeneity = 0.38). The analysis by anatomic tumour site showed that both gastric cardia cancer (eleven studies) and non-cardia cancer (six studies) were significantly associated with *MTHFR* 677 TT, with an OR of 1.51 (95% CI: 1.11, 2.05) and 1.57 (95% CI:1.09, 2.24), respectively (p value for heterogeneity = 0.87). There was no evidence of heterogeneity in all the subgroup meta-analyses performed.

Meta-analysis of MTHFR A1298C

All seven included studies reported ORs spread around the null effect (Table 1). From the meta-analysis the association between gastric cancer and *MTHFR* 1298 CC was 0.94 (95% CI: 0.65, 1.35) (Figure 3), while an OR of 1.01 (95% CI: 0.86, 1.18) was found for the association with 1298 AC. There was no evidence of heterogeneity in the overall meta-analysis and in subgroup meta-analyses.

By restricting the analysis of *MTHFR A1298C* to the five studies conducted on Asians (50, 60, 61, 63, 64), an overall OR of 0.81 (95% CI: 0.43, 1.51) emerged. When the analysis was stratified by tumour site, an OR of 0.99 (95% CI: 0.43, 2.28) resulted for cardia cancer, while an OR of 0.81 (95% CI: 0.38, 1.74) was found for non-cardia cancer (p value of heterogeneity = 0.76).

Table 1. Description of the studies included in the meta- and pooled- analyses

First authors (reference no.), year	No. of cases	No. of controls	Country	Source of controls	Gastric tumour site	Crude OR* (95% CI*) *MTHFR** 677 TT vs CC	Adjusted OR^, (95% CI) *MTHFR* 677 TT vs CC	Crude OR (95% CI) *MTHFR* 1298 CC vs AA	Adjusted OR^, (95% CI) MTHFR 1298 CC vs AA
Boccia et al. (51), 2007† #	102 (107) ‡	254	Italy	Hospital	Cardia and non-Cardia	1.81 (0.93, 3.52)	1.95 (1.01, 3.78)	1.02 (0.44, 2.37)	0.44 (0.16, 1.21)
Wang Y et al. (65), 2007	467	540	China	Population	Cardia	1.63 (1.15, 2.33)	NA°		
Gotze et al. (66), 2007†	103 (106)	106	Germany	Population	Cardia and non-Cardia	0.67 (0.28, 1.58)	0.59 (0.21, 1.66)		
Zhang et al. (48), 2007† #	295 (464)	399 (480)	Poland	Population	Cardia and non-Cardia	1.16 (0.69, 1.95)	1.17 (0.70, 1.97)	1.01 (0.60, 1.69)	1.06 (0.62, 1.68)
Weng et al. (63), 2006† #	38	34	China	Hospital	Non-Cardia	0.67 (0.18, 2.54)	0.84 (0.18, 3.98)	0.28 (0.01, 7.30)	††
Zeybek et al. (67), 2006	35	144	Turkey	Hospital	NS§	1.42 (0.45, 4.53)	NA		
Lacasana-Navarro et al. (47), 2006†	201	427	Mexico	Hospital	NS	1.48 (0.95, 2.31)	1.50 (0.96, 2.34)		
Graziano et al. (19), 2006†	162	164	Italy	Population	Cardia and non-Cardia	2.79 (1.48, 5.23)	3.03 (1.60, 5.73)		
Sarbia et al. (69), 2005	332	255	Germany	Population	Cardia and non-Cardia	0.96 (0.57, 1.63)	NA		
Si et al. (61), 2005	122	101	China	Hospital	Cardia and non-Cardia	1.50 (0.61, 3.70)	NA	0.79 (0.22, 2.88)	NA
Kim et al. (64), 2005	133	445	South Korea	Population	NS	1.46 (0.83, 2.57)	NA	0.38 (0.05, 3.11)	NA
Shen et al. (60), 2005 # ‡‡	320	313	China	Population	Cardia and non-Cardia			0.84 (0.33, 2.17)	0.83 (0.32, 2.14)
Wang LD et al. (68), 2005	129	315	China	Population	Cardia	1.78 (1.02, 3.11)	NA		
Mu et al. (62), 2004	194	390	China	Hospital	NS	1.79 (1.06, 3.02)	NA		
Stolzenberg-Solomon et al. (46), 2003†ç	90	398 (405)	China	Population	Cardia	1.14 (0.60, 2.18)	1.06 (0.55, 2.05)		

Miao et al. (50), 2002† #	217	468	China	Population	Cardia	2.02 (1.28, 3.19)	2.03 (1.33, 3.36)	1.30 (0.31, 1.59)	1.32 (0.31, 5.71)
Gao et al. (49), 2002†	107 (155)	200 (223)	China	Population	NS	1.81 (0.89, 3.66)	1.27 (0.68, 2.38)		

* OR, Odds Ratio; CI, Confidence Interval; *MTHFR,* methylenetetrahydrofolate reductase

^ OR adjusted for age, gender and smoking status (ever/never)

† Studies included in the pooled analysis of *MTHFR C677T*

Studies included in the pooled analysis of *MTHFR A1298C*

‡ (number of individuals included in the pooled analysis, when different from the published)

§ NS, Not Specified

° NA, Not Applicable because not included in the pooled analysis

†† Study included only in the pooled analysis of *MTHFR* 1298 CA versus AA (materials and methods for details)

‡‡ Study excluded from the meta- and pooled analysis of *MTHFR C677T* because not in Hardy Weinberg Equilibrium (materials and methods for details)

ç Study excluded from the meta- and pooled analysis of *MTHFR A1298C* because not in Hardy Weinberg Equilibrium (materials and methods for details)

Fig. 2. Forest plot of the Odds Ratios and 95% confidence intervals of studies on the association between gastric cancer and *MTHFR C677T* polymorphism (TT *versus* CC). On the left, the first author of the study is followed by the publication year in parentheses. The size of the black box corresponding to each study is proportional to the sample size; the horizontal line shows the corresponding 95% confidence interval of the odds ratio. The combined estimate is based on a random-effects model shown by the diamond. The solid vertical line represents the null result.

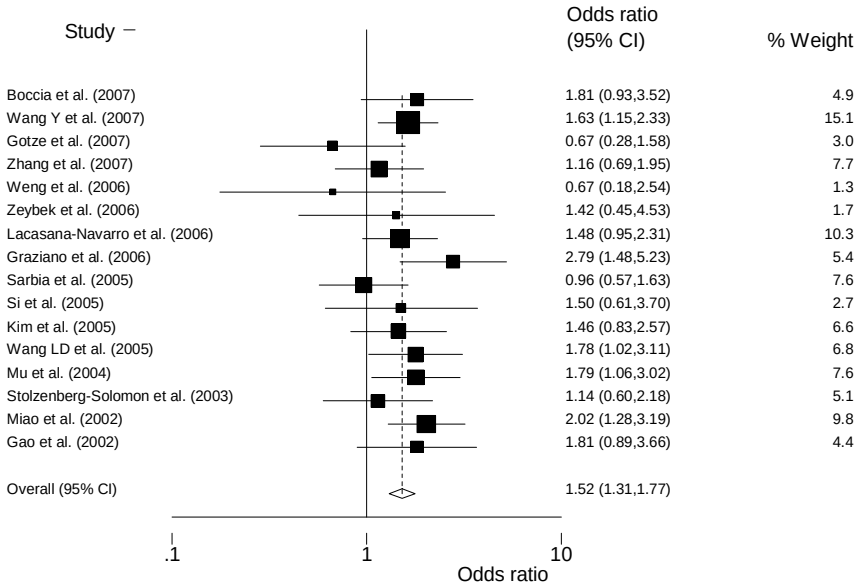
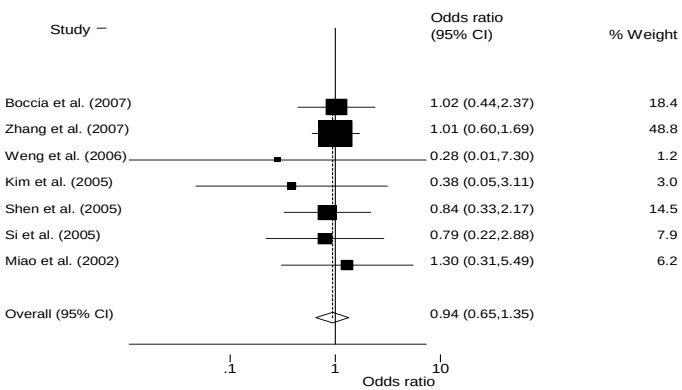


Fig. 3. Forest plot of the Odds Ratios and 95% confidence intervals of studies of the association between gastric cancer and *MTHFR A1298C* polymorphism (CC *versus* AA). On the left, the first author of the study is followed by the publication year in parentheses. The size of the black box corresponding to each study is proportional to the sample size; the horizontal line shows the corresponding 95% confidence interval of the odds ratio. The combined estimate is based on a random-effects model shown by the diamond. The solid vertical line represents the null result.



Pooled analyses

The study specific adjusted ORs for *MTHFR* 677 TT are reported in Table 1. Out of the nine studies included in the pooled analysis, ORs were above the unit in six, among them three (19,50,51) reported a significant positive association between gastric cancer and *MTHFR* 677 TT genotype (Table 1). Results from the pooled analysis are shown in Table 2. The overall OR adjusted for age, gender and smoking status was 1.49 (95% CI: 1.14, 1.95; *p* value for heterogeneity= 0.06) for *MTHFR* 677 TT, while an OR of 1.21 (95% CI: 0.90, 1.62; *p* value for heterogeneity= 0.03) was detected for *MTHFR* 677 CT. Publication bias was not tested because of the low statistical power of the tests when the number of studies is ≤10 (73,75,76).

The pooled OR for *MTHFR* 677 TT among Asians was 1.54 (95% CI: 1.09, 2.15; *p* value for heterogeneity= 0.34), among Europeans was 1.52 (95% CI: 0.84, 2.76; *p* value for heterogeneity= 0.03). The *p* value for heterogeneity test among Asians and Caucasians was 0.86 (Table 2).

When stratifying for smoking habits, an OR of 2.04 (95% CI: 1.27, 3.26) for *MTHFR* 677 TT appeared among ever-smokers, while an OR of 1.36 (95% CI: 1.03, 1.80) appeared among never-smokers, with a *p* value for heterogeneity test among them of 0.14 (Table 2). The stratified analysis according to alcohol intake included 6 studies; similar risk estimates were found for ever-drinkers versus never-drinkers (*p* value for heterogeneity test = 0.49; Table 2). The stratified analysis according to estimated folate status showed an OR for gastric cancer of 2.05 (95% CI: 1.13, 2.72) for *MTHFR* 677 TT individuals with low folates, while an OR of 0.95 (95% CI: 0.54, 1.67) in those with high folates (*p* value for the heterogeneity among the two estimates of 0.06) (Table 2).

The study specific adjusted odds ratios for *MTHFR* 1298 CC are reported in Table 1. It was not possible to compute the adjusted OR for the homozygous variant genotype in one study because of the small amount of subjects (60). The overall OR adjusted for age, gender and smoking status was 0.90 (95% CI: 0.69, 1.34; *p* value for heterogeneity= 0.50, 4 studies) for *MTHFR* 1298 CC, while an OR of 1.01 (95% CI: 0.83, 1.22; *p* value for heterogeneity= 0.50, 5 studies) was found for *MTHFR* 1298 AC.

Table 2. Odds Ratio and 95% confidence intervals from the pooled analysis for the association between *MTHFR C677T* and gastric cancer

	No. of studies	No. of cases	No. of controls	No. *MTHFR**677TT cases	No. *MTHFR* 677TT controls	OR* †	95% CI*	*p* value for heterogeneity within strata	*p* value for heterogeneity across strata
All studies	9	1540	2577	309	502	1.49	1.14, 1.95	0.06	
Asians	4	507	1147	138	278	1.54	1.09, 2.15	0.34	0.86
Europeans	4	832	1003	111	120	1.52	0.84, 2.76	0.03	
Never-smokers	8	616	1189	135	274	1.36	1.03, 1.80	0.49	0.14
Ever-smokers	7	834	1225	163	219	2.04	1.27, 3.26	0.02	
Non alcohol drinkers	6	527	995	107	228	1.37	0.97, 1.91	0.84	0.49
Alcohol drinkers	6	782	900	120	150	1.68	1.04, 2.73	0.05	
High folate status‡	4	403	433	31	50	0.95	0.54, 1.67	0.86	0.06
Low folate status	4	242	346	35	42	2.05	1.13, 3.72	0.96	

* OR, Odds Ratio (all ORs from the pooled analysis are adjusted for age, gender and smoking status); CI, Confidence Interval; *MTHFR,* methylenetetrahydrofolate reductase

† The comparison is *MTHFR* 677 TT *versus* CC

‡ High folate status defined a nutrient density of folate (dietary folate intake/total caloric intake: μg/kcal*1,000) > 99 (48); eating at least 2 portions of fruit and vegetables/day for crude dietary folate intake (51); > 5.5 ng/ml for serum folate (66); > 4.0 ng/ml for gastric mucosa folate levels (63). See Materials and Methods-Pooled analysis-Statistical analysis for details.

DISCUSSION

The results from the meta-analysis performed on sixteen studies highlighted a higher risk of developing gastric cancer for subjects carrying the *MTHFR* 677 TT genotype. The results were confirmed by the pooled analysis including nine studies. No association was detected from either the meta-analysis or the pooled analysis between the *MTHFR* 1298 CC genotype and gastric cancer. Our results were consistent with two previously published meta-analyses by Zintzaras E. and Larsson et al. (28,54), showing an increased risk of gastric cancer only for *MTHFR* 677 TT genotype, and an absence of risk for the *MTHFR* 1298 CC. The two previously published meta-analyses, however, included a smaller number of studies and results were based on unadjusted estimates. From our pooled analysis on *MTHFR* 677, an increased risk of gastric cancer was observed among subjects with low folate status compared with those with a high folate status. These results support with our a priori hypothesis of a higher risk of gastric cancer for subjects carrying the variant *MTHFR* 677 homozygous variant who have low folate levels as compared to subjects carrying the same variant but with high folate.

A limitation common to both the meta- and the pooled analysis might be the presence of publication bias. In the meta-analysis of *MTHFR* 677 TT, we did not observe evidence of publication bias from the visual inspection of the Begg's funnel plots and the results of the rank correlation statistical test. Results from the Egger's regression method highlighted some publication bias, however this method is usually more sensitive than the Begg's test, reporting to provide evidences for bias (false positive results) when in fact is not present, especially when the number of studies is low (75, 76). We cannot rule out, however, that the effect of *MTHFR* 677 TT on gastric cancer is overestimated from our meta-analysis because negative results from small studies remained unpublished.

In the pooled analysis we explored a possible effect modification of *MTHFR* 677 TT genotype on gastric cancer by stratifying for tobacco smoking and alcohol drinking, two factors that may affect folate levels. We were unable to observe any effect modification, however we should keep in mind that in both instances the information did not take into account the amount or the duration of alcohol intake and tobacco smoking.

When the results from the pooled analysis were stratified according to folate status (available from 4 studies), a strong association between *MTHFR* 677 TT genotype and gastric cancer was noted among subjects with low folate status compared to subjects with a high folate status. The heterogeneity test showed that results were borderline significantly different, therefore our result needs to be confirmed with a larger

population. This result support our hypothesis, suggesting that concomitant inadequate folate intake and impaired MTHFR activity might be important susceptibility factors for gastric cancer. A limitation is the heterogeneity in which on folate information was collected: gastric mucosa level (63), serum level (66), nutrient density of folate (48), or dietary fruit an vegetables intake (51).

To our knowledge, this is the first pooled analysis assessing the role of two common *MTHFR* polymorphisms on the risk of gastric cancer. In fact, the two previously published meta-analyses did not include individual level data (28,54), therefore the authors were unable to calcualate adjusted estimates and to stratify the results of the meta-analyses according to folate status, alcohol or smoking habits. Because the data sets included information on age, gender and cigarette smoking for all studies, it was possible to adjust for the potential confounding effect of these variables, and to assess consistently the presence of gene-lifestyle interactions for *MTHFR* 677, a factor that makes the pooled-analysis preferable to the meta-analysis (7). The absence of publication bias and statistical heterogeneity among studies strengthens our results.

LABORATORY TESTS

Both *MTHFR C677T* and *A1298C* can be detected by means of polymerase chain reaction (PCR followed by restriction fragment-length polymorphism (RFLP) analysis with *Hinf*I and *Mbo*II for *C677T* and *A1298C*, respectively (7,8). Other methods include direct DNA sequencing or TaqMan assays (48). Most studied did not report the success rate in extracting DNA from samples, the proportion of eligible subjects from whom genotyping failed, while 43.0% (7/16) of them reported the degree of genotyping reproducibility (19,46,48,50,51,61,68). HWE was tested by 87.5% (14/16) of the studies. All the previously mentioned variables are important indicators of the analytical validity of the genotyping methods, also influencing the potential non-differential misclassification of the exposure. In addition, only 31.2% of studies (5/16) clearly reported that the analysts were unaware of the clinical status of the subjects when genotyping the samples, therefore differential exposure misclassification may not be ruled out.

POTENTIAL PUBLIC HEALTH IMPACT

At the moment the potential public health impact of is limited, given the small association between gastric cancer and homozygosis TT for *MTHFR* 677. Additional studies, however, on the possible additional risk of gastric cancer among subjects who are homozygous 677 TT and have low folate levels are urgently needed. If this

preliminary result is confirmed, a proper evaluation of the clinical utility of *MTHFR C677T* testing for identifying gastric cancer susceptibility among populations with folate deficiency, as well as the introduction of specific folate supplementation (*versus* no folate supplementation) would be warranted. Currently, however, population testing for *MTHFR C677T* polymorphism for gastric cancer prevention is not indicated.

CONCLUSION AND RESEARCH PRIORITIES

MTHFR plays a central role in balancing DNA synthesis (which involves 5,10-methylentetrahydrofolate), and DNA methylation (which involves 5,10-methyltetrahydrofolate). Specifically, the 677T allele contributes to DNA hypomethylation, which in turn may lead to altered gene expression; at the same time this polymorphism might exert a protective effect as observed for colorectal cancer (24), by increasing the levels of the MTHFR substrate, essential for DNA synthesis. Therefore, the exact interpretation of MTHFR-cancer association is not straightforward; although the observed increased risk for gastric cancer associated with the *MTHFR* 677 homozygous variant suggests that dietary folate might be protective in gastric carcinogenesis mainly by limiting aberrant DNA methylation in situation of impaired folate status. In general, the study of the association between sequence variants of folate related genes and cancer has the advantage of being less prone to the confounding effect exerted by dietary or lifestyle factors (78). The observed increased risk of gastric cancer among *MTHFR* 677 TT individuals strengthens the hypothesis of a protective effect of folate in gastric carcinogenesis. If this holds true, it would be interesting to explore if the introduction of folate fortification in some common food items (79) in North America since 1998 had actually contributed to the decreasing rates of gastric cancer (80). However, in view of the lag-time for an effect of folic acid and the lengthy induction time for gastric cancer, this issue could probably be addressed only in the next decade.

The observation of a potential role of folate in gastric carcinogenesis is also strengthened by our results of an increased risk of gastric cancer in *MTHFR* 677 homozygous subjects with low folate levels, suggesting that concomitant inadequate folate intake and impaired MTHFR activity might be important susceptibility factors for gastric cancer.

Despite the limitations of this analysis in terms of comparable folate data, which requires confirmation from large prospective studies based on blood folate measurement, the results are in keeping with the model proposed by Friso et al. (21). In circumstances of folate deficiency, a decrease in downstream MTHFR-products

results in a lower global DNA methylation status. Recently, aberrant methylation of proto-oncogenes has been explored as both a mechanism and a marker of carcinoma progression (81), with some studies reporting an altered methylation pattern particularly for diffuse gastric cancer (82). Additionally, it has been recently reported that a significant global DNA hypomethylation occurs in *MTHFR* 677 TT subjects when compared with those with the wild type genotype (19,20), especially when plasma folate level is reduced (21). Taken together, these results suggest that the increased risk for gastric cancer associated with the homozygous *MTHFR* 677 variant might be referable to the subsequent impaired folate levels affecting DNA methylation status. Therefore, the negative association between the homozygous variant *MTHFR* genotype and gastric cancer might be counterbalanced to some extent by an adequate folate intake.

Other genes involved in folate metabolism should be considered for a more comprehensive understanding of the exact role of the folate pathway in gastric cancer susceptibility. Given the controversial evidence from nutritional studies on the effect of fruit and vegetables on gastric cancer, there is a need for large prospective cohort studies based on repeated serological dosage of folate levels and/or detailed and repeated nutritional data which would further clarify the role of folate in gastric carcinogenesis. This would lay the foundation for evaluating the possible benefits of preventive nutritional interventions in individuals at risk for gastric cancer.

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4

General Discussion

BACKGROUND AND KEY OBJECTIVE

Background. Gastric cancer still ranks as the fourth most common cancer and the second most frequent cause of cancer deaths, accounting for 10.4% of cancer worldwide (1). High incidence regions include various countries in Asia and Europe including Japan, China and Russia, where the prevalence of *Helicobater pylori* is relatively high compared to Europe and USA (60-90% versus 30-40% of the population). In contrast, there has been a progressive fall in the incidence of gastric cancer in many Western Countries, particularly for cancers located distal to the cardia and for the intestinal type. The effect has been attributed to food refrigeration, a lower intake of preserved foods and salt, and higher intake of fruit and vegetables.

Gastric carcinogenesis is a multistep and multifactorial process resulting from a complex interaction between environmental and genetic factors. It is currently recognized that multiple genetic abnormalities accumulating during the long process of carcinogenesis are responsible for the onset and progression of cancer, however in gastric carcinoma the molecular mechanism remain poorly understood. Recently, genome-wide array-based comparative genomic hybridization (CGH) has been shown to have a high resolution and high sensitivity in detecting gene-copy aberrations. A study published in 2008 on 38 primary gastric tumor tissues from a population in Helsinki, identified amplification of *ERBB2* gene and gains at 20q13.12-q13.33 (2). Expression analysis showed an overexpression of *MUC1*, *GRB7*, *PPP1R1B* and P*PARBP*, in addition to *ERBB2* among the intestinal type.

Conventional epidemiological research in the last two decades identified *H.pylori* infection, tobacco smoking, low fruit and vegetable intake, salt or salty foods intake, and the lack of food refrigeration as playing a major role in gastric cancer aetiology (3). Individuals infected by *H. pylori* have at least a twofold increase of gastric cancer compared to uninfected subjects. In areas where gastric cancer is highly prevalent the great majority of cases are *H.pylori* related, as 95% of the strains circulating in East Asia are particularly virulent (*cagA* positive) compared to Western clinical isolates (60% are *cagA* +, also showing less virulent properties). (4)

The vast majority of *H. pylori* infected pepole, however, do not develop gastric cancer, suggesting for the potential role of the genetic background and other causative factors. Accumulating evidences report that the individual genetic susceptibility to gastric cancer involves several genes with small effects (5). Genes involved in the protection

of gastric mucosa against damaging agents, in inflammatory response, in detoxification of carcinogens (also known as metabolic genes), in synthesis and repair of DNA, in regulation of gene expression, and in cell adhesion and in cell cycle, have been studied (6). A well known study published in 2000 by El-Omar et al (7) demonstrated that the risk of non-cardia gastric cancer is associated with the gene cluster polymorphisms of *IL*-1 encoding for *IL-1β* promoter (-511 T/T variant) and the receptor antagonist *IL-1 RN* (*IL-1RN**2), especially in combination with *H. pylori CagA* positive and/or *VacA* positive strains (8). These evidences have been confirmed by additional studies, as two recent meta-analyses showed that individuals carrying the T variant allele of *IL-1B*-511 have a significant 25% increased risk of developing intestinal gastric cancer compared with the homozygous wild types, so as subjects carrying the *2 polymorphic allele of the receptor antagonist of *IL-1β* (OR= 1.20, 95% CI: 1.01-1.41), especially in combination with *H. pylori* infection (OR= 4.81, 95% CI: 1.4-17.28) (9, 10). These results, however, were not confirmed among Asians, so as the suspected associations between *IL-1B*-31 *C variant, and +3954 *T, with gastric cancer (10). One possible biological explanation for the ethnic differences in gastric cancer risk associated with the *IL-1β* polymorphisms could be the varying *H. pylori* infection rates between Asian and Caucasians, as the positive associations were more likely detected in low incidence compared to high incidence regions (11).

According to the pie model of disease aetiology (12), we could think of gastric cancer aetiology as determined through several different complete causal mechanisms. Every mechanism is a 'sufficient cause', and it is defined as a set of mininal conditions that inevitably produce disease, involving the action of several component causes. The weight of each component cause inside each causative pie mechanism cannot be distinguished by the weight of the others, as all of them need to be present for the mechanism being complete, however the weight of a single component cause can be larger if it present in many causative pies. According to this concept, even though *H. pylori* infection and its interaction with certain cytokine polymorphisms can be thought as two component causes for gastric cancer that might explain a large proportion of the 70% gastric cancer cases with past or present *H. pylori* infection, other component causes still have to be discovered, as gastric cancer occurs also in *H. pylori* free individuals through different causal mechanisms. The latter component causes might include tobacco smoking, alcohol, low fruit and vegetable intake, high meat and salt intake, and the lack of food refrigeration, together with inherited gene variants affecting their metabolism. These component causes might be involved, however, also

in the disease aetiology of *H. pylori* positive individuals with or without certain cytokine polymorphisms.

The discovery of the genes affecting gastric cancer risk as influencing the individual susceptibility to tobacco and alcohol damages, the effect of a low fruit and vegetable intake, and a high consumption of meat and salt intake may allow for better risk prediction, and the most powerful approach to identify these low risk variants is through association studies. These studies test the frequency of genetic variants in (gastric cancer) cases and controls and are convenient because they do not require high-risk families, as does linkage analysis. The power to detect alleles with moderate effect is much larger for association than linkage studies (13). So far, the vast majority of studies focused on candidate genes, chosen by the investigator because of their potential role in carcinogenesis (3).

In this thesis I followed the classical association approach in which I targeted candidate genes, which, given their function, have a high probability to be involved in gastric cancer. Selected different polymorphic genes involved in the detoxification of carcinogens, in the DNA synthesis, and in the cell-cycle regulation were selected. Additionally, I report the results of three meta-analyses and one pooled analysis on the effect of polymorphisms in selected phase I and II enzymes, and in a gene involved in one-carbon metabolism, and the risk of gastric cancer.

Key objective. The objective of the work described in this thesis was dual:

- to investigate the association of candidate genes and gastric cancer risk by selecting genes that, given their function, should have a high probability to be involved in gastric cancer;
- to assess the cumulative evidence on the most extensively studied gene polymorphisms in association with gastric cancer risk through meta-analyses and pooled analysis.

Study population used in this work. The study subjects were selected according to a case-control study design. Cases were consecutive primary gastric adenocarcinoma patients, with histological confirmation, who underwent a curative gastrectomy in the "A. Gemelli" teaching hospital starting from 2002. Controls were selected from cancer-free patients, with a broad range of diagnoses, admitted to the same hospital during the same time period. All subjects were Caucasians born in Italy. A venous blood sample was drawn from each participant from which DNA was isolated from peripheral

blood lymphocytes. All subject participants filled in a questionnaire for collecting demographic data, lifestyle habits and environmental exposures. The study was approved by the local review board and written informed consent was obtained from each subject. The procedures followed were in accordance with the Helsinki Declaration.

REVIEW AND INTEPRPETATION OF MAIN FINDINGS

The objective of the work described in this thesis was to gain more insight into genetic risk factors underlying gastric cancer aetiology. The research question was addressed by using three approaches. First, within the context of an Italian hospital-based case-control study, we assessed the impact of variation in genes involved in the detoxification of carcinogens, DNA synthesis, and cell cycle regulation, on the risk of gastric cancer. Polymorphisms in Cytochrome P450 1A1 and 2E1, Glutathione S-Transferase T1 and M1, Sulfotransferase 1A1, microsomal Epoxide Hydrolase, N-acetyltransferase 2 (all known as metabolic genes), Methylenetetrahydrofolate reductase, and *p*53 and *p*73 genes were selected.

Then, we performed three meta-analyses and one pooled-analysis on the effect of gene polymorphisms coding for a phase I enzyme (Cytochrome P450 2E1), phase II enzymes (Glutathione S-Transferase M1 and T1), and Methylenetetrahydrofolate reductase gene on the risk of gastric cancer, respectively (one meta-and pooled analysis was on the same gene).

1. Genetic polymorphisms and risk of gastric cancer in an Italian hospital-based case-control study

Metabolic gene polymorphisms.

A major part of carcinogenic substances require metabolic activation by enzymes to be genotoxic, and inherited variations in carcinogens metabolizing genes may alter enzyme activity and subsequently carcinogens activation or deactivation. Based on the knowledge that metabolic genes are presumed to modulate an individual's susceptibility to cancer by interacting with carcinogens, and since the inheritance of several unfavourable genotypes is supposed to additionally increase the risk of gastric cancer (14-16), I investigated the effect on gastric cancer of selected SNPs of genes coding for phase I enzymes [Cytochrome P450 1A1 (*CYP1A1*) and 2E1 (*CYP2E1*)], phase II enzymes [Glutathione S-Transferase T1 (*GSTT1*) and M1 (*GSTT1*),

Sulfotransferase 1A1 (*SULT1A1*), microsomal Epoxide Hydrolase (*mEH*), N-acetyltransferase 2 (*NAT 2*)], and their differential effect according to tobacco smoking and alcohol habits.

The results reported in chapter 2.1 demonstrated an increased risk of gastric cancer among subjects carrying the *GSTT1* null variant genotype, and among those homozygotes for the *SULT1A1* variant allele, especially if smokers. While the first result basically confirms those of the recent literature (14), the second is a new finding as no papers have been published so far at this regard. Individuals carrying the *SULT1A1* variant allele have limited detoxification capability of polycyclic aromatic hydrocarbons and arylamine through sulfonate conjugation, which is confirmed by the additional increased risk among smokers. Additionally, an increased risk of gastric cancer resulted for the carriers of *CYP2E1*5A* or *6 (*Dra*I) variant alleles among drinkers. CYP2E1 s a naturally ethanol-inducible enzyme that is mainly involved in the metabolic activation of *N*-nitrosamines, and it plays a role in alcohol metabolism through the oxidation of ethanol to acetaldehyde and 1-hydroxyacetyl radicals (17). Two previous studies (18, 19) reported no association between *CYP2E1*5A* or *6 allele and gastric cancer, however no one of them stratified data according to alcohol habits. Since the **5A* or *6 alleles of *CYP2E1* are characterized by an increased gene expression (20), I hypothesise that individuals carrying the unfavourable variant are at higher risk of gastric cancer because of: i) hyper activation of *N*-nitrosamines in more reactive species, especially among drinkers since enzyme activity is induced by alcohol; ii) hyper production of reactive oxygen species and subsequent cell toxicity generated by ethanol metabolism among drinkers.

From the gene-gene interaction analyses, we showed that individuals with combined *GSTT1* null and *NAT2* slow acetylator variants had an additional increased risk of gastric cancer, with an attributable proportion due to their interaction of 52%. N-acetylation is considered a major detoxification step for carcinogenic aromatic arylamines, while *GSTT1* is involved in the detoxification of polycyclic aromatic hydrocarbons, so individuals with one or both depleted phase II enzyme activities might be particularly susceptible to gastric damage from carcinogens, which is supported by the finding of an additional risk for ever-smokers carrying the unfavourable combination.

Methylenetetrahydrofolate reductase gene.

Methylenetetrahydrofolate reductase (MTHFR) enzyme irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate, providing methyl donor for DNA methylation and deoxynucleoside synthesis.

Two prominent mechanisms whereby folate deficiency may influence cancer risk have been described (21) low folate levels might induce misincorporation of uracil into DNA, which could lead to chromosomal breaks and mutations; and/or by causing DNA hypomethylation, resulting in altered gene expression (22). Two functional polymorphisms of the *MTHFR*, *C677T* and *A1298C*, have been identified (19), associated with decreased enzyme activity and folate levels *(*23,24). We explored whether *MTHFR C677T* and *A1298C* polymorphisms are related with gastric caner risk and progression, and their differential effect according to histological type and concomitant exposures affecting serum folate levels (smoking status, alcohol, and fruit and vegetables intake). Results showed an increased risk of gastric cancer for *MTHFR* 677T carriers, particularly among ever smokers and, among 677 TT individuals, those with a low intake of fruit and vegetables. The strongest effect, however, was noted for the *MTHFR* 677 TT genotype among the diffuse gastric cancer histotype. Lastly, the survival analysis showed that during first year of follow up after the surgical intervention, *MTHFR* 677 CC subjects have an improved survival compared to those carrying the T variant allele. No association was detected for the effect of *MTHFR A1298C* polymorphism. As *MTHFR* 677 TT genotype is associated with aberrant genomic DNA methylation (25), overall the results suggest that aberrant DNA methylation pattern, trough impaired folate metabolism, might play a key role in gastric carcinogenesis.

Genes involved in the cell cycle regulation.

*p*53 and *p*73 tumor suppressor genes encode for proteins involved in cell cycle regulation and differentiation, DNA repair, and apoptosis (26). Both *p*53 and *p*73 genes are highly polymorphic, with at least 13 SNPs described for *p*53 and 19 for *p*73 (27). We investigated the effect of four selected *p*53 polymorphisms (exon4 Arg72Pro, and intron 3 and 6), and the *p*73 exon 2 G4C14-to-A4T14 dinucleotide polymorphisms, their combination and interaction with environmental exposures, on gastric cancer risk and progression. A recent meta-analysis reported a significantly increased risk og gastric cancer among Asians carrying the homozygous variant of *p*53 exon4 Arg72Pro, but not Caucasians (28). Oppositely, a recent study among Asians (29) reported no association between *p*73 G4C14-to-A4T14 dinucleotide polymorphism and gastric cardia. No one study ever explored the effect of *p*53 intron 3 and 6 polymorphisms and gastric cancer so far.

Results showed that *p*73 G4C14-to-A4T14 homozygous variant genotype increases the risk of gastric cancer 4.77-times compared with individuals with the wild type genotype, and that the risk is particularly strong for the intestinal histotype. The

gene-environment interaction analysis showed an effect modification by gender on the association of *p*73 G4C14-to-A4T14 polymorphism and gastric cancer, with an increased risk among females, known have a lower capacity for DNA repair with respect to men (30). From the gene-gene interaction analysis, a protective effect resulted for individuals carrying the mutant alleles of *p*53 exon 4 and intron 6, with 50% reduced risk compared with those both wild-types, which is in line with results from breast and lung cancer (31,32). Lastly, we showed that carriers of the *p*53 intron 6 variant allele have a poorer prognosis for intestinal gastric adenocarcinoma after surgical intervention, which is in line with a recent report on chronic lymphocitic leukaemia when considering the time of treatment-free survival as the main outcome (33).

2. Meta-analyses of genetic polymorphisms and risk of gastric cancer

In the second approach, the results of three meta-analyses on the effect of gene polymorphisms coding for a phase I enzyme (Cytochrome P450 2E1, *CYP2E1*), phase II enzyme (Glutathione S-Transferase T1, *GSTT1*), and Methylenetetrahydrofolate reductase gene (*MTHFR*) on the risk of gastric cancer are reported. Additionally, we performed a pooled analysis using individual level data through the Genetic Susceptibility to Environmental Carcinogens (GSEC) collaborative group on *MTHFR* polymorphisms and gastric cancer and compared the results with those from the meta-analysis on the same gene.

Meta-analysis of *CYP2E1Pst*I / *Rsa*I polymorphism and gastric cancer.

CYP2E1 is a naturally ethanol-inducible phase I enzyme that is mainly involved in the metabolic activation of *N*-nitrosamines, and it plays a role in alcohol metabolism through the oxidation of ethanol to acetaldehyde and 1-hydroxyacetyl radicals (17). Two point mutations, existing in close linkage disequilibrium in the 5'-flanking region (*Pst*I, *Rsa*I), are known to alter the transcriptional activity of the (34). These mutations generate the *CYP2E1**1 (c1) allele and the less common *CYP2E1**2 (c2) allele and have been reported to be associated with an increased risk for oral, pharyngeal (35), liver (36) and lung cancers (37,38). By pooling together the results of thirteen case-control studies identified through Medline and Embase, that include 2066 cases and 2754 controls, we showed the absence of association between *CYP2E1* c2 variant allele and gastric cancer. By pooling the studies of high-quality among Asians, where the c2 variant is more common, an increased risk appeared for those carrying c2 variant allele. Additionally, by collecting individual level data from the authors of the papers

included in the meta-analysis, a significant 5.36-fold increased risk for gastric cancer was shown for individuals both *CYP2E1* c2 homozygotes and *GSTM1* null (an homozygous deletion causing the absence of enzyme activity), compared with individuals with both homozygous wild genotypes (attributable proportion due to interaction of 60%). Absence of significant associations resulted from *CYP2E1*-alcohol/-smoking interaction analyses. Overall, these results suggest that the c2 variant allele of the phase I enzyme *CYP2E1**2 might affect the risk of gastric cancer, especially by interacting with a key phase II enzyme as *GTSM1*, which is completely deleted among those carrying the null variant.

Meta-analysis of *GSTM1* and *T1* status and gastric cancer.

The GST enzymes are involved in the detoxification of many xenobiotics, including several environmental carcinogens and endogenously derived reactive oxygen species (35). Individuals who have the homozyogous deletion (null genotype) in GSTM1 and GSTT1 enzyme activity are more susceptible to carcinogens such as benzo[α]pyrene-7,8-diol epoxide, the activated form of benzo[α]pyrene, and smaller reactive hydrocarbons, such as ethylene oxide and diepoxybutane (39). By pooling together the results of fifteen case-control studies identified through Medline and Embase, including 1797 cases and 3000 controls, I showed a slight increased risk for gastric cancer among those carrying the *GSTM1* null genotype. By pooling data from 4 studies (478 cases and 870 controls) that considered combinations of *GSTM1* and smoking status, a statistically significant increased risk for gastric cancer was detected for *GSTM1* null individuals if smokers compared to non-smokers *GSTM1* wild types. By pooling together the results of eighteen case-control studies identified through Medline and Embase, including 2508 cases and 4634 controls, we showed the absence of association between *GSTT1* null genotype and gastric cancer. There was a substantial absence of heterogeneity among studies. When I considered, however, only high-quality studies, a significant increased risk of gastric cancer appeared, as well as considering only Caucasians. Additionally, by pooling data from 7 studies (319 cases and 656 controls) that considered combinations of *GSTT1* and *GSTM1* genotypes, a statistically significant increased risk for gastric cancer was detected for individuals with deletion mutations in both genes compared to wild types.

Meta- and pooled analyses of the *MTHFR* C677T and A1298C polymorphisms and gastric cancer.

Results from a meta-analysis of prospective and retrospective studies specifically focusing on dietary folate intake and risk of gastric cancer also reported no clear effect

of dietary folate intake, with no differences between cohort or case-control studies (21). Conflicting results come from studies exploring the association between fruit and vegetables intake, the main dietary source of folate, and gastric cancer. As MTHFR is a key enzyme in folate metabolism, we wished to verify if the variant 677T and 1298C alleles related with reduced enzyme activity and folate serum concentration (especially C677T), are associated with an increased risk of gastric cancer by performing a meta- and pooled analyses of individual level data of the published reports. If folic acid has a role in gastric carcinogenesis, I would expect that *MTHFR* C677T genotype is related to gastric cancer risk to the extent predicted by individuals having a low folate serum concentration. This approach is called 'Mendelian randomization'. From the meta-analysis of 16 studies (2727 cases and 4640 controls), subjects homozygous for the *MTHFR* 677 variant genotype have an increased risk of gastric cancer, as well as from the pooled analysis (9 studies, 1540 cases and 2577 controls). No association was found between *MTHFR* 1298 CC and gastric cancer (7 studies in the meta-analysis, 1223 cases and 2015 controls; 3 studies in the pooled-analysis, 766 cases and 1011 controls). There was no statistical heterogeneity among studies or substantial publication bias in the two meta-analyses. When results from the pooled analysis of four studies on *C677T* were stratified according to estimated folate levels, results showed an additional increased risk among individuals with low levels respect to those with high folate levels. Overall, these findings support the hypothesis that folate plays a role in gastric carcinogenesis.

METHODOLOGICAL CONSIDERATIONS

In this work I aimed to explore the impact of some genetic factors on the risk of gastric cancer through a population based case-control study and meta-and pooled-analyses of the literature. Beside the study design considered, the ability of identifying a factor involved in gastric cancer aetiology depends on the absence of random error (precision of measurement) and systematic error (comparison, information and selection bias), both affecting the validity of a study. In the previous chapter I have discussed the methodological considerations of each study separately. In this section I will give an overall review of the methodological strengths and limitations specifically concerning the studies described in this thesis. For an overview of general methodological considerations in epidemiological research I refer to a standard text (40).

Strengths of the approach used in this thesis.

Case-control study.

The study adopted in this thesis was designed in order to minimize selection bias. Namely, cases were selected as all the incident cases of gastric cancer undergoing a surgical resection at the Department of Surgery, and controls randomly recruited among individuals stemming from the source population for the cases and hospitalized during the same time period for all the diagnosis but gastric cancer. Since the participation rate was similar for cases and controls and above 90%, selection bias should be minimized in this study. As for comparison bias, known also as confounding, I can exclude the potential for confounding by ethnicity, as the present study was performed in an ethnically homogenous sample, namely Italians. This should protect from confounding by ethnicity, also called 'population stratification', which usually results from the mixing of different ethnic groups with different allele frequencies: in this situation confounding arises since the ethnicity itself might be related to a specific disease as well as the allele frequencies (41). Additionally, controls were matched by age and gender in order to control for both confounding factors. Non differential misclassification of the genotype exposure is unlikely in this study, as quality control for each genotyping was performed in each experiment, and 10% of the total samples were randomly selected and reanalyzed with 100% concordance. Lastly, differential misclassification of the disease status is also unlikely as the analyst was blinded to the case or control status of the samples.

Meta-analyses and pooled-analysis.

A primary concern in meta- and pooled-analyses is publication bias. This phenomenon stems from the selective reporting of 'positive' findings, and current statistical tests are not sensitive enough to demonstrate it when the number of studies included is lower than 10 (42). In all the meta-analyses presented in this thesis, however, there was no evidence of publication bias neither from the statistical tests or the visual inspection of the funnel plot. An important strength of the meta-analyses of *GSTT1* and *CYP2E1* was the possibility to explore potential source of heterogeneity by stratifying the meta-analyses according to some covariates, which was possible because individual level data were collected by contacting the authors of the included papers. Lastly, the pooled anlaysis was possible to adjust for the potential confounding effect of several variables individually collected from the collaborating authors, and to assess consistently the presence of gene-lifestyle interactions for *MTHFR* 677, a factor that makes the pooled-analysis preferable to the meta-analysis.

Limitations of the approach used in this thesis.

Case-control study.

In general, a large sample size is the primary way to increase precision in any epidemiological study. Therefore, precision was a concern as gastric cancer cases recruited never exceeded 115 subjects. However, when appropriately conducted, large and small studies should give, theoretically, the same results, with just a more precise effect measure estimate from the larger ones (43). The low power of this study also affected the gene-environment interaction analyses, whose results should be confirmed by replication in a different population. As in any case-control study, differential misclassification of the environmental exposures might exist, thus affecting the results of the gene-environment interaction analyses.

Another limitation of this case-control study was the inability to gain information on *Helicobacter pylori* infection, as all the cases were surgical, thus serological tests for the germ detection were not routinely performed. Also, serum from controls was not routinely collected from the beginning of the study. In order to overcome this issue, we are currently recovering all the gastric biopsies paraffin embedded at the Pathology Department, so to search for *H. pylori* infection at least among the cases by polymerase-chain reaction of the DNA of the extracted tissues. This limitation, however, is not affecting the validity of this study, as all the genes investigated in this thesis are not in linkage with the *IL-1* gene cluster polymorphisms known to influence the host response to *H. pylori* attack and the subsequent gastric mucosa damages. In fact, the candidate genes selected in this thesis are relevant for gastric carcinogenesis as they affect the host capacity in the response to the damages caused by the use of tobacco smoke, alcohol, and a low fruit and vegetables intake, that can act as effect modififers of the genes-gastric cancer relationship. Even though most of the papers published in relation with gastric cancer actually report data on *H. pylori* serology among cases and controls, actually even the larger studies (44) that investigated the effect of the candidate genes considered in this thesis did not test for any effect modification by *H. pylori* infection, as not biologically plausible in view of the current knowledge.

A crucial question in the design of our study is whether we should adjust for *H. pylori* status, which means whether I should think of *H. pylori* as a potential confounder of the relationship between the investigated genes and gastric cancer. I personally do not think that any comparison bias affected our data analysis because, even if future studies highlight that the asset of the selected genes influence the susceptibility to *H. pylori* infection, this would imply that the susceptibility to *H. pylori* infection is an effect of the genotype 'exposure', acting as an intermediate step in the causal pathway

towards gastric cancer. And a key property of a confounding factor is that must not be an effect of the exposure (12), so in any case it would have been inappropriate to adjust for *H. pylori* status as it is beyond a doubt the *H. pylori* is in the causal patway of around 70% of gastric cancer cases.

Meta-analyses and pooled analysis.
Our stratified meta-analyses of *GSTT1* and *MTHFR* according to some covariates did not include all of the studies because a small number of authors could not share their original data, so selection bias may have occurred. Stratified meta-analyses were also based on a few number of subjects, so precision of the estimates is an additional concern. Pooled-analysis was able to show an effect modification of the folate status on the risk of gastric cancer by *MTHFR* C677T genotype. Information on folate status, however, was collected in an heterogeneous way across the studies included (different source of folates, and questionnaires used to collect the information on dietary intake). This situation, however, could lead to non differential misclassification of the folate exposure and bias the effect measures towards the null. Thus, if such bias is present in the stratified pooled analysis by folate status, it would indicate that the underlying true effect modification should be stronger than what observed.

SUGGESTIONS FOR FUTURE RESEARCH

The work in this thesis has provided more insight into the role of several genetic factors in gastric cancer aetiology, and has raised the hypothesis of a potential role of folic acid in gastric cancer prevention. Briefly, results show that gastric cancer risk is increased in an Italian population by the inheritance of the variant alleles of the metabolic genes *SULT1A1* and *CYP2E1* *6, especially among smokers and drinkers, respectively. An additional increased risk is conferred by the inheritance of *GSTT1* null variant, especially if combined with the *NAT2* slow acetylator status. Additionally, I showed that the variant allele of *MTHFR* C677T, associated with inherited low serum folate levels, increases the risk of gastric cancer, especially among those with a low intake of fruit and vegetables. Lastly, I reported that a combination of *p*53 exon 4 and intron 6 variant alleles protects from gastric cancer, thus confirming recent evidences from other tumour sites. Meta-analyses showed that the c2 variant allele of the phase I enzyme *CYP2E1**2 might affect the risk of gastric cancer, especially by interacting with a key phase II enzyme as *GTSM1*, and that the phase II enzyme *GSTT1* confer an increased risk of gastric cancer if combined with *GSTM1* null. Pooled analysis confirmed

the role of folate in gastric carcinogenesis, as individuals with the homozyogous *MTHFR* C677T variant genotype are at increased risk of gastric cancer especially if carrying a low folate status. Our meta-analysis reporting that *MTHFR* C677T is a true risk factor for gastric cancer has been confirmed from a recently published paper that evaluated the false-positive report probability (FPRP) of genetic associations stemming from meta-analyses in the field of cancer research (45). Authors showed that, by assuming a very low prior probability of 0.000001 (as those appropriate for a randomly selected SNP in a genome-wide association study), and a statistical power to detect an OR of 1.5, the FPRP of our reported association [OR= 1.52 (95% CI: 1.31-1.77) between *MTHFR* 677 TT and gastric cancer] was 4.9 x 10^{-8}, thus indicating a noteworthy association.

Beside the eradication of *H. pylori* infection, currently the management of individuals at moderate/high risk for gastric cancer is based on endoscopic screening programme (46). From a public health perspective, however, the results of this thesis raise several questions related to the possibility of preventive nutritional interventions at population level towards individuals at moderate/high risk for gastric cancer. Since 1998 North America introduced the folate fortification of some common food items (47) for the prevention of neural tube defects. Even though it is early to test for a possible decrease in gastric cancer incidence related to the folate fortification, in view of the lag time for the effect of folate, and the lengthy induction time for gastric cancer, it would be worthy to address this issue in the next decade. Given the controversial evidence from nutritional studies on the effect of folate and fruit and vegetables on gastric cancer, however, there is a need for large prospective cohort studies based on repeated serological dosage of folate levels and/or detailed and repeated nutritional data which would further clarify the role of folate in gastric carcinogenesis. Future studies should also consider other genes involved in folate metabolism for a more comprehensive understanding of the exact role of the folate pathway in gastric cancer susceptibility.

This thesis showed for the first time a borderline significant 2-fold reduced risk of gastric cancer for individuals carrying a combination of variant alleles of both *p*53 exon 4 and intron 6. Since the identical result was gained from two studies on lung and breast cancer, it is urgently needed a replication from a large study on gastric cancer, together with functional studies on the effect of such linked SNPs combination on p53 folding and activity.

Despite all these evidences, currrently there is no genetic testing indicated or validated for the prevention of gastric cancer or for its clinical management. As we showed that the odds ratio associated with the most common inherited genetic variants affect the risk of gastric cancer for no more than 50% as in the case of *MTHFR*, it is premature to think about the application of any genetic testing for identify more susceptible individuals, in view of their potential limited predictive value. However, because of the risk for gastric cancer in several hereditary cancer syndromes, genetic testing is currently recommended only in family at high risk (6).

Athough the candidate gene approach that select genes based on their biological plausibility of a role in gastric carcinogenesis currently seems to be the most robust strategy for identifying genetic loci for gastric cancer, it has a potential disadvantage. It only studies the genetic variation in specific candidate gene(s) in relation to the disease of interest. Genetic loci associated with the disease risk but not outside and not in linkage disequilibrium with the gene studied remain therefore undetected. As such, the recent GWA approach is revolutionary as it permits interrogation of the entire human genome at levels of resolution previously unattainable, uncostrained by prior hypotheses regarding genetic associations with disease (48). The Study Group Millenium Project for Cancer in 2008 performed a two-stage GWA and identified the intronic SNP (rs2976392) of *PSCA* gene as a potential gene associated with diffuse gastric cancer (49). Additional GWA studies should confirm this evidence, and new studies are required for understanding the genetic bases of the most common gastric cancer intestinal histotype.

In conclusion, merging the conventional epidemiological research with genome-wide association studies and expression profiling might allow in the next future to identify disease loci that are likely to be involved in the suceptibilty and progression of gastric carcinogenesis not assessed so far in candidate gene studies. To further validate the clinical and biological significance of the loci that will be identified, investigation of the protein level alterations and functional properties should also be warranted.

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Summary

SUMMARY (in English)

Gastric cancer is the fourth most common cancer and the second most frequent cause of cancer deaths, accounting for 10.4% of cancer worldwide. High incidence regions include various countries in Asia and Europe including Japan, China and Russia. In the last decade the incidence of gastric cancer in many Western Countries has declined, mostly due to food refrigeration, a lower intake of preserved foods and salt, and higher consumption of fruit and vegetables.

Gastric carcinogenesis is a multistep and multifactorial process resulting from a complex interaction between environmental and genetic factors. Among the former, *Helicobacter pylori* infection, tobacco smoking, low fruit and vegetable intake, high meat and salt intake, and the lack of food refrigeration play a major role, while for the latter accumulating evidences report that the individual genetic susceptibility to gastric cancer involves several genes with small effects. Polymorphisms in genes involved in the protection of gastric mucosa against damaging agents, in inflammatory response, in detoxification of carcinogens, in synthesis and repair of DNA, in regulation of gene expression, and in cell adhesion and in cell cycle, have been studied in association with gastric cancer. The majority of the population-based studies conducted so far, however, are underpowered to detect a robust association, or to explore gene-gene and gene-environment interactions.

The objective of the work described in this thesis was dual:

- to investigate the association of candidate genes and gastric cancer risk by selecting genes that, given their function, should have a high probability to be involved in gastric cancer;
- to assess the cumulative evidence on the most extensively studied gene polymorphisms in association with gastric cancer risk through meta-analyses and pooled analysis.

After introducing the scientific background and the key objective underlying this work in **Chapter 1**, I summarize in **Chapter 2** studies exploring the association between polymorphic genes involved in the detoxification of carcinogens, in the DNA synthesis, and in the cell-cycle regulation, with gastric cancer risk. The candidate genes were selected, as, given their function, should have a high probability to be involved in gastric cancer.

In the study relating *Cytochrome P450 1A1 (CYP1A1)* and *2E1 (CYP2E1), Glutathione S-Transferase T1 (GSTT1)* and *M1 (GSTM1), Sulfotransferase 1A1 (SULT1A1), microsomal Epoxide Hydrolase (mEH), N-acetyltransferase 2 (NAT2)* polymorphisms, I reported an increased risk of gastric cancer among subjects carrying the *GSTT1* null variant genotype, and among those homozygotes for the *SULT1A1* variant allele, especially if smokers. An increased risk of gastric cancer resulted for the carriers of *CYP2E1*5A* or *6 (*Dra*I) variant alleles among drinkers. I also showed that individuals with combined *GSTT1* null and *NAT2* slow acetylator variants had an additional increased risk of gastric cancer, especially if smokers.

In the study relating *Methylenetetrahydrofolate reductase* (*MTHFR*) gene polymorphisms and gastric cancer, I showed an increased risk for *MTHFR* 677T carriers, particularly among ever smokers and, among 677 TT individuals, those with a low intake of fruit and vegetables. An additional increased risk was noted for the *MTHFR* 677 TT genotype among the diffuse gastric cancer histotype. Survival analysis showed that during first year of follow up after the surgical intervention, *MTHFR* 677 CC subjects have an improved survival compared to those carrying the T variant allele.

In analyses relating three known *p*53 polymorphisms (exon4 Arg72Pro, and intron 3 and 6), and the *p*73 exon 2 G4C14-to-A4T14 dinucleotide polymorphisms, with gastric cancer, I reported that *p*73 G4C14-to-A4T14 homozygous variant genotype is associated with an increased risk for intestinal histotype of gastric cancer. I reported a protective effect against gastric cancer for individuals carrying the combination of mutant alleles of *p*53 exon 4 and intron 6. I also showed that carriers of the *p*53 intron 6 variant allele have a poorer prognosis for intestinal gastric adenocarcinoma after surgical intervention.

In **Chapter 3**, I reported the results of three meta-analyses and one meta- and pooled analysis on the effect of polymorphisms in selected phase I and II enzymes, and in a gene involved in one-carbon metabolism, respectively, and the risk of gastric cancer.

In the meta-analysis of thirteen case-control studies, including 2066 gastric cancer cases and 2754 controls, I showed that the c2 variant allele of the phase I enzyme CYP2E1 might affect the risk of gastric cancer especially by interacting with a key phase II enzyme as *GTSM1*.

In the meta-analysis of fifteen case-control studies, including 1797 cases and 3000 controls, I reported that the phase II enzyme GSTM1 confers a borderline increased risk of gastric cancer, especially among smokers.

In the meta- and pooled analysis of eighteen case-control studies, including 2508 gastric cases and 4634 controls, I reported that the phase II enzyme GSTT1 confer an increased risk of gastric cancer if deleted, especially in combination with the null variant of *GSTM1*.

In the meta-analysis of 16 studies (2727 cases and 4640 controls), subjects homozygous for the *MTHFR* 677 variant genotype have an increased risk of gastric cancer, as well as from the pooled analysis (9 studies, 1540 cases and 2577 controls). When results from the pooled analysis of four studies on *C677T* were stratified according to estimated folate levels, results showed an additional increased risk among individuals with low levels respect to those with high folate levels.

In **Chapter 4**, I reflect on these findings in the context of current knowledge and potential methodological limitations, and give suggestions for future research.

In summary, several genetic polymorphisms of genes involved in the cellular metabolism, DNA synthesis and cell cycle regulation were associated with the risk of gastric cancer. *MTHFR* 677 TT genotype doubled the risk of gastric cancer among subjects carrying a low folate status. The merge of modern genome science with prospective population-based, epidemiological research may provide powerful tools for evaluating possible benefits of public health, preventive nutritional interventions in individuals at risk for gastric cancer.

SAMENVATTING

Maagkanker is de op drie na meest voorkomende kanker en de op een na meest voorkomende oorzaak voor het overlijden aan kanker, en is verantwoordelijk voor 10.4% van alle kankers wereldwijd. Hoge incidentie regio's zijn voornamelijk verschillende landen in Azië en Europa, onder andere Japan, China en Rusland. In de laatste 10 jaar is de incidentie van maagkanker in veel Westerse landen gedaald, voornamelijk door het koud bewaren van voedsel, een lagere opname van gepreserveerd voedsel en zout, en een hogere consumptie van fruit en groenten.

Het ontwikkelen van maagkanker is een proces met meerdere stappen waarbij verschillende factoren een rol spelen, resulterend in een complexe interactie tussen genetische en omgevingsfactoren. Omgevingsfactoren die een rol spelen in dit proces zijn *Helicobacter pylori* infectie, roken, lage fruit en groente consumptie, hoge vlees en zoutconsumptie en het niet koud bewaren van voedsel. Voor genetische factoren is er een groeiend bewijs dat voor het individuele genetische risico voor maagkanker meerdere genen met kleine effecten een rol spelen. Polymorfismes in genen betrokken bij de bescherming van de maagwand tegen schadelijke stoffen, in het immuunsysteem, in de detoxificatie van carcinogene stoffen, in DNA synthese en reparatie, in de regulatie van genexpressie, en in cel adhesie en celcyclus, zijn bestudeerd voor associatie met maagkanker. De meeste van de populatie studies tot

nu toe waren echter niet groot genoeg om een robuuste associatie te vinden, of om naar gen-gen en gen-omgeving interacties te kijken.

Het doel van het werk beschreven in deze thesis was tweezijdig:

- om de associatie tussen kandidaatgenen en maagkanker te bestuderen door genen te selecteren die, gegeven hun functie, een hoge waarschijnlijkheid hebben betrokken te zijn bij maagkanker.
- om het cumulatieve bewijs te bestuderen voor de meest bestudeerde gen polymorfismes in associatie met maagkanker risico door meta-analyses en gepoolde analysis.

Na het introduceren van de wetenschappelijke achtergrond van het belangrijkste doel in dit werk in **Hoofdstuk 1**, vat ik in **Hoofdstuk 2** studies samen die de associatie tussen polymorfismes in genen betrokken in de detoxificatie van carcinogenen, in DNA synthese, en in cel cyclus regulatie, met maagkanker. De kandidaat-genen waren geselecteerd aan de hand van hun functie en moesten een grote kans hebben betrokken te zijn bij maagkanker.

In de studie waar *Cytochrome P450 1A1 (CYP1A1)* en *2E1 (CYP2E1), Glutathione S-Transferase T1 (GSTT1)* en *M1 (GSTM1), Sulfotransferase 1A1 (SULT1A1), microsomal Epoxide Hydrolase (mEH), N-acetyltransferase 2 (NAT2)* polymorfismes, vermeld ik een verhoogd risico op maagkanker onder mensen die de *GSTT1* null variant dragen, en voor mensen die homozygoot zijn voor de *SULT1A1* variant, met name in rokers. Drinkers die dragen waren van de *CYP2E1**5A of *6 (Dra1) variante allelen hadden een verhoogd risico op maagkanker. Ik heb ook aangetoond dat mensen met een combinatie van *GSTT1* null en *NAT2* langzame acetylator variant een extra verhoogd risico voor maagkanker hebben, voornamelijk als ze ook nog eens rookten.

In de studie over *Methylenetetrahydrofolate reductase (MTHFR)* gen polymorfirsmes en maagkanker, toon ik een verhoogd risico voor *MTHFR* 677T dragers, met name onder mensen die ooit gerookt hebben, en voor *MTHFR* 677 TT dragers die een lage consumptie van fruit en groenten hadden. Een extra verhoogd risico is gevonden voor dragers van de *MTHFR* 677 TT onder het diffuse maagkanker histotype. Survival analyse toont dat tijdens het eerste jaar na een chirurgische interventie de *MTHFR* 677 CC mensen een betere overleving hebben vergeleken met mensen met het T variant allel.

In analyses van 3 bekende *p*53 polymorfismes (exon4 Arg72Pro, intron 3 en intron 6), en de *p*73 exon2 G4C14-naar-A4T14 polymorfisme associatie met maagkanker, vermeld ik dat homozygoten voor de *p*73 G4C14-naar-A4T14 een

verhoogd risico hebben op intestinaal histotype van maagkanker. Ik vermeld een beschermend effect voor maagkanker voor individuen die dragers zijn van een combinatie van mutante allelen op *p*53 exon 4 en intron 6. Ik toon ook dat dragers van de *p*53 intron 6 variant allel een slechtere prognose voor intestinale maag adenocarcinoom na chirurgische ingreep hebben.

In **Hoofdstuk 3**, toon ik de resultaten van 3 meta-analyses en 1 gepoolde analyse op het effect van polymorfismes in geselecteerde fase 1 en 2 enzymen, en in een gen betrokken bij 1-koolstof metabolisme, respectievelijk, en het risico voor maagkanker.

In de meta-analyse van 13 case-control studies, met in totaal 2066 patiënten en 2754 controles, toon ik dat de c2 variant allel van de fase 1 enzym *CYP2E1* misschien een effect heeft op de risico voor maagkanker, zeker door interactie met een belangrijk fase 2 enzym zoals *GTSM1*.

In de meta-analyse van 18 case-control studies, met in totaal 2508 patiënten en 4634 controles, toon ik dat de fase 2 enzym *GSTT1* een verhoogd risico geeft voor maagkanker als het gedelete is, zeker in combinatie met de null variant van *GSTM1*.

In de meta-analyse van 16 studies (2727 patiënten en 4640 controles), hadden mensen die homozygoot voor de *MTHFR* 677 variant genotype waren een verhoogd risico voor maagkanker, dit ook in de gepoolde analyse (9 studies, 1540 patiënten en 2577 controles). Wanneer de resultaten van de gepoolde analyse van 4 studies over C677T gestratificeerd werden volgens hun folaat levels toonden de resultaten een extra verhoogd risico voor mensen met lage levels in vergelijking tot mensen met hoge levels.

In **Hoofdstuk 4**, reflecteer ik op deze vondsten in de context van huidige kennis en potentiële methodologische limitaties, en geef ik wat suggesties voor toekomstig onderzoek.

In het kort, verschillende genetische polymorfismes van genen betrokken bij cellulair metabolisme, DNA synthese en cel cyclus regulatie zijn geassocieerd met de risico voor maagkanker. *MTHFR* 677 TT genotype verdubbelde de risico voor maagkanker onder mensen met een laag folaat status. De menging van moderne genomische wetenschap met prospectieve populatie-gebaseerde epidemiologisch onderzoek kan krachtige methoden leveren voor het evalueren van mogelijke voordelen voor volksgezondheid, preventieve nutritionele interventies in mensen met een verhoogd risico op maagkanker.

RIASSUNTO

Il cancro gastrico è la quarta più comune forma di tumore e la seconda più frequente causa di morte per cancro, responsabile del 10.4% dei tumori al mondo. Esistono regionali ad alta prevalenza in alcuni paesi in Asia ed Europa, tra cui Giappone, Cina e Russia. Nell'ultimo decennio l'incidenza del cancro gastrico è diminuita in molti paesi industrializzati, soprattutto per la refrigerazione dei cibi, un minor consumo di cibi inscatolati e di sale, ed un aumento nel consumo di frutta e verdura.

Il processo di cancerogenesi gastrica è multifasica e multifattoriale, risultante da una complessa interazione tra fattori ambientali e genetici. Tra i fattori ambientali più importanti vi è *l'Helicobacter pylori*, il fumo di sigaretta, un basso consumo di frutta e verdura, mentre tra i fattori genetici diversi studi mostrano come la suscettibilità genetica individuale al cancro gastrico coinvolga diversi geni, ciascuno con un effetto piccolo. Diverse classi di geni sono state studiate in associazione con il carcinoma gastrico, tra cui geni coinvolti nella protezione della mucosa gastrica, nella risposta infiammatoria, nella detossificazione di cancerogeni, nella sintesi e riparo del DNA, nella regolazione dell'espressione genica, nella adesione e nel ciclo cellulare. La maggior parte degli studi di associazione condotti sinora, però, sono sottodimensionati per stimare un'associazione robusta, o per esplorare l'interazione gene-gene o gene-ambiente.

L'obiettivo del lavoro descritto in questa tesi è duplice:

- Valutare l'associazione tra geni candidati e rischio di cancro gastrico slezionando geni, che per la loro plausibilità biologica, hanno una elevata probabilità di essere coinvolti nell'eziologia del cancro gastrico;
- riassumere i risultati della letteratura scientifica sui polimorfismi genici più studiati in associazione con il cancro gastrico mediante tre meta-analisi e un'analisi cumulativa della letteratura scientifica.

Dopo aver introdotto il background sulla epidemiologia del cancro gastrico e l'obiettivo precipuo di questo lavoro nel **Capitolo 1**, nel **Capitolo 2** mostro i risultati dello studio caso-controllo relativo all'associazione tra alcune varianti geniche coinvolte nella detossificazione di cancerogeni, nella sintesi del DNA, e nella regolazione del ciclo cellulare, e rischio di cancro gastrico. I geni candidati sono stati selezionati in base alla loro plausibilità biologica in quanto potenzialmente coinvolti nella genesi del cancro gastrico.

I risultati dello studio di associazione tra le varianti dei geni *Citocromo P450 1A1 (CYP1A1)* e *2E1 (CYP2E1), Glutatione S-Transferasi T1 (GSTT1)* e *M1 (GSTM1), Sulfotransferasi 1A1 (SULT1A1), Epossido Idrolasi microsomiale (mEH), N-acetiltransferasi 2 (NAT2)*, mostrano come il rischio di cancro gastrico aumenta negli individui portatori della variante *GSTT1* nullo, e tra coloro che sono omozigoti per l'allele variante di *SULT1A1*, specialmente se fumatori. Un aumento del rischio di cancro gastrico è emerso anche per gli individui portatori della variante genica di *CYP2E1*5A* o *6 (*Dra*I) se bevitori. Ho altresì mostrato che gli individui portatori della combinazione delle varianti *GSTT1* nullo and *NAT2* acetilatore lento hanno un rischio aggiuntivo di cancro gastrico specialmente se fumatori.

Lo studio che ha investigato l'effetto delle varianti geniche di *Metilentetraidrofolatoreduttasi* (*MTHFR*) sul rischio di cancro gastrico mostra che gli individui portatori della variante *MTHFR* 677T hanno un rischio aumentato di cancro gastrico, specialmente se fumatori, e tra gli individui omozigoti *MTHFR* 677 TT, coloro che consumano poca frutta e verdura. Inoltre la variante omozigote MTHFR 677 risulta associata ad un rischio maggiore di cancro dello stomaco di istotipo diffuso. L'analisi di sopravvivenza mostra che durante il primo anno di follow-up dopo l'intervento chirurgico, i soggetti con genotipo *MTHFR* 677 CC hanno una sopravvivenza migliore se confrontata con coloro che sono portatori della variante T.

L'analisi che correla le tre varianti più comuni di *p*53 (Arg72Pro esone 4, e introne 3 e 6), e la variante dinucleotidica G4C14-a-A4T14 di *p*73 esone 2 con il cancro gastrico,

riporta che la variante omozigote di *p*73 G4C14-a-A4T14 è associato ad aumento del rischio di cancro gastrico istotipo intestinale. I risultati mostrano anche un effetto protettivo verso il cancro gastrico per gli individui portatori delle varianti di *p*53 esone 4 e introne 6. Il lavoro mostra anche che i portatori della variante allelica di *p*53 introne 6 hanno una prognosi peggiore per ciò che riguarda l'istotipo intestinale dopo intervento chirurgico.

Nel **Capitolo 3** riporto i risultati di 3 meta-analisi e un'analisi cumulativa relative rispettivamente agli effetti di polimorfismi in alcuni enzimi di fase I e II, e in un gene coinvolto nel metabolismo delle molecole ad un atomo di carbonio, e rischio di cancro gastrico.

La meta-analisi di tredici studi caso-controllo che include 2066 casi di tumore e 2754 controlli, mostra che la variante c2 dell'enzima di fase I CYP2E1 aumenta il rischio di cancro gastrico specialmente interagendo con l'enzima chiave di fase II *GSTM1*.

La meta-analisi di diciotto studi caso-controllo che include 2508 casi e 4634 controlli, mostra che l'enzima di fase II GSTT1 aumenta il rischio di cancro gastrico se deleto, specialmente in combinazione con la variante nulla di *GSTM1*.

La meta-analisi di tredici studi (2727 casi e 4640 controlli) mostra che i soggetti omozigoti per la variante allelica di *MTHFR* 677 hanno un rischio aumentato di cancro gastrico, così come la analisi cumulativa di 9 studi (1540 casi e 2577 controlli). Quando i risultati dell'analisi cumulativa di 4 studi relativi a *C677T* vengono stratificati in base ai valori stimati dei livelli di folati, i risultati mostrano un incremento addizionale del rischio tra individui con bassi livelli di folati rispetto ad alti livelli.

Nel **Capitolo 4** vengono portate avanti alcune considerazioni in merito ai risultati ottenuti alla luce delle conoscenze attuali e di potenziali limitazioni metodologiche, e si propongono alcuni suggerimenti per le ricerche future.

In conclusione, i risultati di questa tesi mostrano che alcuni polimorfismi di geni coinvolti nel metabolismo cellulare, nella sintesi del DNA e nella regolazione del ciclo cellulare sono associati al rischio di cancro gastrico. Inoltre il genotipo *MTHFR* 677 TT sembra raddoppiare il rischio di cancro gastrico tra coloro che hanno livelli di folati bassi. La convergenza delle attuali tecnologie basate sulla conoscenza del genoma con ampi studi di popolazione di tipo prospettico, ci permetteranno di valutare nell'immediato futuro i possibili benefici derivanti da interventi nutrizionali a livello di popolazione in individui ad alto rischio di cancro gastrico.

DANKWOORD

It has been four years since I first started working in the field of epidemiology. At that time, mid-April 2005, I was working on nosocomial infectious disease control by genotyping a plethora of bacterial isolates obtained during routine hospital surveillance. I remember feeling discouraged because I had long since felt that my true calling was in cancer genetics research. I wanted to change, but I wasn't sure where to begin. I realized, however, that I needed a good education in epidemiology and biostatistics to succeed, and I spoke with Walter Ricciardi who suggested that I enroll in a Master of Science program in Epidemiology. It was the beginning of a new found passion for me: researching the genetic epidemiology of cancer.

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SCIENTIFIC PUBLICATIONS

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Contributions to books

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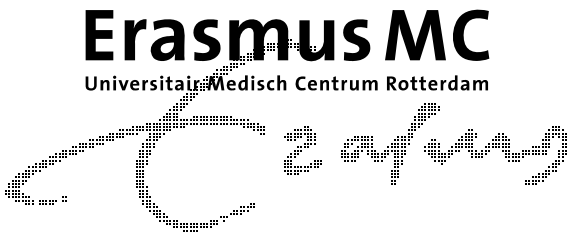
ABOUT THE AUTHOR

Stefania Boccia was born on January 24, 1974 in Naples, Italy. After attending secondary school in Rome, she entered the Faculty of Mathematical, Physical and Natural Sciences of the University 'La Sapienza' in Rome on October 1992. During the first three years she was exempted for merit from University fees. During the last two years of 5-years education in biological sciences, she conducted a research project in environmental hygiene concerning a survey on the health status of landfill workers working in the largest landfill in Europe. This work represented her doctoral thesis that she succeeded in October 1997. From November 1997 to October 2002 she attended the 5-years post-graduate School (Specialization School) in Clinical Pathology at the University 'La Sapienza'. During these years she obtained a fellowship for merit, and contributed to several research projects at the Institute of Hygiene of the University 'La Sapienza' in collaboration with the Institute of Microbiology of the Università Cattolica del Sacro Cuore in Rome. Among the most important research projects were those concerning the molecular basis for drug resistance among *Mycobacterium spp* and *Cryptococcus spp*. On December 2002 she was appointed as Assistant Professor of Hygiene and Preventive Medicine at the Institute of Hygiene (Director. Prof. W. Ricciardi) of the Università Cattolica del Sacro Cuore. She designed and raised funds for two epidemiological research projects at the Institute of Hygiene, namely two hospital-based case-control studies on the genetic and environmental risk factors for gastric cancer, and head and neck cancer. As such, to expand her education in the field of epidemiology, she entered in March 2005 the Master of Science in Epidemiology at the Università Cattolica del Sacro Cuore, from which she graduated in February 2006. In the meanwhile, for increasing her knowledge in genetic epidemiology, she entered in October 2005 the

NIHES Doctor of Science programme in Genetic Epidemiology (program director: Prof. Dr. Cornelia van Duijn), from which she graduated in August 2006. Since then, she funded and coordinates the Genetic Epidemiology and Molecular Biology Unit at the Institute of Hygiene at the Università Cattolica del Sacro Cuore, where she works with Prof. Dr. Walter Ricciardi and in collaboration with Prof. Dr. Cornelia van Duijn at Erasmus MC, Rotterdam. She joined in 2006 the Public Health Genomics European Network (PHGEN) coordinated by Prof. Dr. Angela Brand at the University of Maastricht, The Netherlands, refunded in 2009 as PHGEN II, and since 2007 the International Head and Neck Cancer Epidemiology Consortium (INHANCE) coordinated by Mia Hashibe at the International Agency for Research on Cancer, Lyon, France. Currently she is the Coordinator of the Italian Network for Public Health Genomics and Associate Editor of the Italian Journal of Public Health. In addition of the two aforementioned hospital-based case-control studies on gastric and head and neck cancer, she coordinates two national research projects evaluating the appropriateness of execution of genetic tests for the 1) susceptibility to deep venous thrombosis (funded by the Minister of University, 2008/9), and 2) hereditary breast and colon cancer in some Italian regions (funded by the Abruzzo Regional Health Service, 2009).



PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Stefania Boccia Erasmus MC Department: Epidemiology Research School: NIHES	PhD period: 2005-2009 Promotors: Prof.dr. C.M. van Duijn Prof.dr. W. Ricciardi Supervisor: -

1. PhD training

	Year	Workload (Hours/ECTS)
General academic skills		
- Biomedical English Writing and Communication	2006	4 ECTS
Research skills		
- Master of Science in Epidemiology	2005	80 days
- Doctor of Science in Genetic Epidemiology	2006	60 days
In-depth courses (e.g. Research school, Medical Training)		
- Genetic Epidemiology Research Methods	2005	12 days
- Conceptual Foundation of Epidemiologic Study Design	2006	5 days
- Bayesian Analysis	2006	2.5 days
- Spatial Epidemiology	2006	2.5 days
- Principles of Epidemiologic Data Analysis	2006	5 days
- Advances in Population-based Studies of Complex Genetic Disorders	2006	5 days
- Genetic Linkage Analysis: Model Based Analysis	2006	5 days
Presentations		
- *Meta and pooled-analysis of MTHFR C677T and gastric*	2007	6 hours

cancer, IARC, Lyon, France		
- *Meta-analysis of population-based genetic association studies: potential and limitations from a public health point of view.* Meeting on Consequences from Epigenetics for Nutrition, Environmental and Public Health, Vienna, Austria	2007	6 hours
- *The Italian Task Force of Public Health Genomics*, PHGEN Meeting, Rome, Italy	2007	3 hours
International conferences-oral presentations		
- 13rd EUPHA Annual Conference, Rome, Italy. *Genetic polymorphism of Sulfotransferase 1A1 as susceptibility factor for cancer: a case-control analysis.*	2005	35 hours
- 14th EUPHA Annual Conference, Oslo, Norway. *Head and neck cancer risk in an Italian population is associated with tobacco, alcohol and fruit and vegetables consumption but not with genetic polymorphisms of metabolic genes.*	2006	35 hours
- 15th EUPHA Annual Conference, Lisbon, Portugal. *ALDH2 and head and neck cancer: a meta-analysis implementing a Mendelian Randomization approach.*	2007	35 hours
- 10th ICEM Conference, Florence, Italy. *Public health and genomic epidemiology.*	2009	20 hours
Seminars and workshops		
- Monthly seminars of the post-graduate School of Public Health	2008-on	25 hours
Other		
- Visiting scientist at the IARC, Lyon, France	2007	30 days
2. Teaching activities		
	Year	**Workload (Hours/ECTS)**
Lecturing		
- At the Degree in Medical Laboratory techniques, Università Cattolica del Sacro Cuore, Rome, Italy, she teaches Epidemiology	2005-on	30 hours
- At the Master of Science in Biotechnology, Università Cattolica del Sacro Cuore, Rome, Italy, she teaches	2006-on	30 hours

Molecular Biology and Biotechnology - At the Master of Science in Food and Beverage Quality Control, Università 'La Sapienza', Rome, Italy, she teaches Biomolecular Techniques for pathogen detection and bacteria genotyping - At the Degree in Prevention Techniques in the Environment and at the Workplace, Università Cattolica del Sacro Cuore, Bolzen, Italy, she teaches Cellular Biology - At the Master of Science in Epidemiology, and at the Post-graduate Schools in Biochemistry, Oncology , Psychiatry, Public Health, and Radiology, of the Università Cattolica del Sacro Cuore, Rome, Italy, she teaches the following modules: Measuring the Associations; Study Design (with emphasis on Case-Control Studies); Confounding and Stratification in Data Analysis; Bias (Selection and Information); Public Health Genomics - At the Degree in Nursery, Università Cattolica del Sacro Cuore, Turin, Italy, she teaches Advances in Biomolecular Techniques for the diagnosis and prevention of Oncological diseases	2006-on 2006-on 2007-on 2008-on	20 hours 15 hours 100 hours 15 hours
Supervising practicals and excursions - Supervising practicals on epidemiology	2007/2009	5 hours
Supervising Master's theses - Supervised Alessandra Frustaci: *Meta-analysis of the Brain-Derived Neurotrophic Factor gene (BDNF) Val66Met Polymorphism in Anxiety Disorders and Anxiety-related Personality Traits* - Supervised Rachel Kamangar: *p73 G4C14-to-A4T14 gene polymorphism and interaction with p53 exon 4 Arg72Pro on cancer susceptibility: a meta-analysis of the literature*	2007 2008	20 hours 20 hours
Other - Partner of the European Network of Public Health Genomics (PHGEN I and II) funded by EC	2006-on	20 hours

- Principal Investigator of a Unit inside a national project funded by Italian Minister of University on the study of the epidemiology of Legionellosis in Italy	2006-2007	100 hours
- Partner of the International Head and Neck Cancer Epidemiology (INHANCE), IARC, Lyon	2007-on	40 hours
- Partner of the National Multicenter project *on HTA of genetic tests for venous thromboembolism* (Coordinator: Prof. G. Ricciardi) funded by the Minister of University	2008-on	50 hours
- Scientific Coordinator of the project funded by Abruzzo region on *Predictive genomic tests for hereditary breast and colon cancer: evaluation of the appropriateness of screening individuals at high-risk in selected Italian regions.*	2009	20 hours

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