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ORIGINAL CONTRIBUTION

The synergistic effect between the Mediterranean diet and *GSTP1* or *NAT2* SNPs decreases breast cancer risk in Greek-Cypriot women

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Abstract
Purpose Xenobiotic metabolism is related to the interplay between diet and breast cancer (BC) risk. This involves detoxification enzymes, which are polymorphic and metabolise various dietary metabolites. An important characteristic of this pathway is that chemoprotective micronutrients can act not only as substrates but also as inducers for these enzymes. We investigated whether functional *GSTP1* (p.Ile105Val-rs1695), *NAT2* (590G>A-rs1799930) SNPs and *GSTM1* and *GSTT1* deletion polymorphisms could modulate the effect of the Mediterranean diet (MD) on BC risk, in Greek-Cypriot women.
Methods Genotyping was performed on women from the MASTOS case–control study of BC in Cyprus. A 32-item food-frequency questionnaire was used to obtain dietary intake information. A dietary pattern, which closely resembles the MD (high loadings of vegetables, fruit, legumes and fish), was previously derived with principal component analysis and was used as our dietary variable.
Results *GSTT1* null genotype increased BC risk compared with the homozygous non-null *GSTT1* genotype (OR 1.21, 95 % CI 1.01–1.45). Increasing adherence to the MD reduced BC risk in women with at least one *GSTP1* Ile allele (OR for Ile/Ile = 0.84, 95 % CI 0.74–0.95, for Ile/Val = 0.73, 95 % CI 0.62–0.85) or one *NAT2* 590G allele (OR for 590 GG = 0.73, 95 % CI 0.63–0.83, for 590 GA = 0.81, 95 % CI 0.70–0.94). *p* interaction values were not, however, statistically significant.
Conclusion The homozygous null *GSTT1* genotype could be a risk allele for BC among Greek-Cypriot women. The anticarcinogenic effects of the high adherence to MD against BC risk could also be further enhanced when combined with the wild-type alleles of the detoxification *GSTP1* or *NAT2* SNPs.

Keywords Mediterranean diet · GSTP1 · GSTM1 · GSTT1 · NAT2 · Breast cancer

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Introduction

The protective role of the Mediterranean diet (MD) against breast cancer (BC) risk has previously been evidenced in various epidemiological studies [1, 2]. The MD is characterised by a high intake of fruit, vegetables and legumes, which make it a rich source of carotenoids, phenolic compounds, terpenes, glutathione and glucosinolates [3–5]. These micronutrients are well known for their chemopreventive effect on BC [6]. The MD is also characterised by moderate amounts of fish intake that contain adequate n-3 long-chain polyunsaturated fatty acids (PUFAs) [5],

shown to inhibit breast tumour growth (in vivo and in vitro) through the lipid peroxidation products of the marine n-3 PUFAs [7]. We have previously shown that a dietary pattern rich in vegetables, fruit, legumes and fish reduces the risk of post-menopausal BC in the Greek-Cypriot female population of the study with the acronym MASTOS (Greek word for breast) [8].

Xenobiotic metabolism is one of the pathways that is related to the interplay between diet and BC risk. It involves key phase II detoxification enzymes, such as glutathione S-transferases (GSTs) and N-acetyltransferases (NATs). These enzymes play an important role in the metabolism and detoxification of xenobiotics, which are released from the activity of phase I enzymes [9]. Phase I enzymes, such as cytochrome P450 family of enzymes, catalyse the activation of procarcinogens to carcinogens [10]. The main function of the xenobiotic pathway is to convert carcinogens into water-soluble and readily excretable forms, through their conjugation with polar moieties. This conversion results from the action of the families of GST and NAT enzymes [10]. GSTs conjugate with the antioxidant glutathione to metabolise compounds that are either mutagenic (e.g. heterocyclic aromatic amines) or anticarcinogenic (e.g. glucosinolates) [11]. NAT enzymes use acetyl-CoA as a co-factor and can detoxify active mutagens via acetylation [12, 13]. In addition, micronutrients derived from fruit and vegetables, such as isothiocyanates, indoles and allylic compounds, have been shown to reduce cellular carcinogenicity by inducing the expression of these detoxification enzymes [10]. Therefore, isothiocyanates have a dual role as substrates and as inducers of these detoxifying enzymes.

GST and NAT enzymes have a number of isoenzymes, and many of the coding genes for these isoenzymes are polymorphic. Genetic variants (single nucleotide polymorphisms-SNPs) present in *GST* and *NAT* genes might be associated with functional changes, ranging from a complete loss to a reduction in enzymatic activity [9]. GSTP1, GSTM1, GSTT1 and NAT2 isoenzymes are commonly investigated in nutrigenetics studies. *GSTM1* and *GSTT1* deletion polymorphisms result in complete loss of enzymatic activity [14], whereas other SNPs such as the *GSTP1* p.Ile105Val (c.313A>G) (rs1695) have been shown to reduce enzymatic activity [11, 15]. The *NAT2* c.590G>A (p.Arg197Gln) (rs1799930) SNP causes the enzyme to be a slow metaboliser of active mutagens. Thus, reduced activity of GST and NAT isoenzymes would result in an inefficient elimination of carcinogens, which could in turn increase susceptibility to cancer [9]. In this study, we focused on those aforementioned SNPs of the GST and NAT isoenzymes, which have been shown to be functional [16].

There are also studies investigating SNPs in other isoenzymes of the GST and NAT families, such as the GSTA1 and 2, GSTO1 and 2, GSTM2 and 3, GSTZ1 and NAT1 and their role in BC risk. However, these studies are few and they have mostly presented non-statistically significant results. When examining the relationship between polymorphisms, nutrition and cancer risk, the *GSTP1*, *GSTM1*, *GSTT1* and *NAT2* polymorphisms are the most commonly studied genetic variants of the phase II detoxification metabolism in cancer nutrigenetics [9, 10]. Based on their functional role, it has been evidenced that the association between BC risk and diet can be modified by the SNP genotypes [17, 18]. This may in part explain the inconclusive results obtained when studying the associations between diet and BC risk, without considering the SNP genotypes of the participants [19, 20]. The aim of the current study was to investigate whether the effect of a dietary pattern, which combines more than one food group, on BC risk, could be modified by *GST* and *NAT2* SNPs. To our knowledge, such as an aspect has not been examined previously. A dietary pattern, such as the MD, has a number of advantages when used as the dietary variable in nutrigenetics studies as we reported elsewhere [21, 22]. Here, we examined the association between four polymorphisms and BC risk, using the population of the MASTOS study. The four polymorphisms examined were the *GSTP1* p.Ile105Val, *NAT2* 590G>A SNPs and *GSTM1* and *GSTT1* deletion polymorphisms. Furthermore, we assessed the interactions between the aforementioned polymorphisms and a Mediterranean dietary pattern (MDP), which was obtained previously [8], with respect to BC risk. Moreover, we assessed the associations between BC risk and the MDP, when stratified by the genotypes of the four polymorphisms.

Participants and methods

Participants

MASTOS was a population-based case–control study of BC in Cyprus, which recruited 1109 female BC cases, aged 40–70 years, and 1177 controls of the same age range. All participants were recruited between the years 2004 and 2006. Cases were women with a histologically confirmed diagnosis of BC (diagnosed between January 1999 and December 2006). Controls were women with no prior history of BC, who participated in the national mammography population screening programme and had a negative result. Blood samples were collected from both cases and controls. More information on the purpose, design of the study, data collection and study population was described elsewhere [23, 24].

Dietary intake assessment

Standardised interviews were performed with each participant in order to answer an interviewer-administered questionnaire, especially designed to collect extensive demographic and risk factor data [8, 23]. Dietary intake data were collected using an interviewer-administered food-frequency questionnaire (FFQ) comprising 32 food and beverage items, through a standardised diet interview. This FFQ aimed to record the routine consumption of foods of the participants over the preceding year (for cases, this was the past 12 months prior to diagnosis). More details about the design and structure of the FFQ can be found in Demetriou et al. [8]. Principal component analysis (PCA) was previously used to investigate the dietary consumption of food items (in g/month) included in the FFQ and also to derive the dietary pattern that best applies to the Greek-Cypriot female population. This analysis was performed on the control subjects. Diagonal (direct oblimin) rotation was used to extract principal components. Orthogonal rotations failed to generate interpretable results as they assume independence of components, an assumption that does not hold for dietary patterns. The adherence of subjects to each dietary pattern was estimated using a component score for each subject, based on all factor loadings and the respective monthly consumption of each food. Eleven components were originally found based on an eigenvalue criterion of >1.0 and scree plot analysis. After interpretability of the factors of the various components, the retention of only four components was justified, as components five to eleven revealed high factor loadings on single variables. Only these four factors were thus retained to repeat the PCA. Each of the four retained components corresponded to a different dietary pattern: pattern 1—meat/potatoes, pattern 2—cereals/milk/dairy, pattern 3—cakes/sweets/nuts/crackers/pasta/rice and pattern 4—vegetables/fruit/legumes/fish. Among the 32 food items, 23.6 % of the total variance was explained by the four factors (8.05, 5.92, 5.10 and 4.55 % for the four patterns, respectively). It was concluded that the dietary pattern 4 included high loadings of vegetables, fruit, legumes and fish, closely resembled the MDP. Consequently, pattern 4 was selected as being the most appropriate to be used in the subsequent association analysis with BC and in the analysis with the SNPs in this study. Quartile values for adherence to this dietary pattern were determined, according to score values of the controls [8]. Subjects in quartile 1 had the lowest consumption of vegetables, fruit, legumes and fish and thus lowest adherence to the PCA-derived dietary pattern. In contrast, subjects in quartile 4 had the highest consumption of the same four food groups and therefore the highest adherence to this dietary pattern.

Genotyping

Genotyping of the *GSTP1* c.313A>C (p.Ile105Val) and *NAT2* c.590G>A SNPs was carried out in all study participants (1109 cases and 1177 controls) with TaqMan SNP genotyping assays and the ABI PRISM 7900HT real-time PCR instrument (Applied Biosystems Inc.). TaqMan Universal PCR Master Mix and 30 ng of genomic DNA were used in a final reaction volume of 5 μl for each assay. Genotyping was performed using 384-well plates as described in Kakkoura et al. [21, 22]. The order of DNA samples from cases and controls on the 384-well plate was randomised, in order to ensure that samples from cases and controls were subjected to the same study conditions. Genotyping of the deletion *GSTM1* and *GSTT1* polymorphisms was carried out in all study participants using the multiplex PCR method as described elsewhere with the only modification that part of exon 11 of the *BRCA1* gene was used as an internal control [25]. To ensure good quality control practices, 20 % of the samples were genotyped in duplicate. These samples had exactly the same genotyping results. Genotyping call rates for the four polymorphisms under study (*GSTP1, GSTM1*, *GSTT1* and *NAT2*) ranged between 95 and 99 % (2256, 2215, 2170 and 2267 participants were successfully genotyped for the *GSTP1*, *GSTM1*, *GSTT1* and *NAT2* polymorphisms, respectively, and were included in the subsequent statistical analysis). Duplicate concordance rates were higher than 99 %.

Statistical analysis

Differences in categorical sociodemographic characteristics, potential risk factors and PCA-derived dietary patterns among cases and controls were assessed using the Chi-square test. A Chi-square test was also carried out to examine Hardy–Weinberg equilibrium (HWE) in the controls. *P* values, which were smaller than 0.05 (<0.05), were considered to be statistically significant. Associations between each SNP and BC were investigated with the use of logistic regression. These associations were adjusted for menopausal status (pre- or post-menopausal) and age. Logistic regression was also used to assess: (a) the interactions between each SNP genotype and each quartile (1–4) of the PCA-derived MDP on BC risk within a multiplicative model and (b) the associations between PCA-derived MDP quartiles and BC stratified by each SNP genotype. Additionally, associations between SNP genotypes and BC risk stratified by the quartiles of the PCA-derived MDP were investigated. However, there were no additional significant associations, and thus, they will not be discussed further (Supplementary Table 1). Regarding interaction analyses, multiplicative interaction

terms included products of scores for SNP genotypes (0, for homozygous wild-type genotype; 1, for homozygous variant genotype and 2, for heterozygous genotype) and dietary pattern quartiles (1, for quartile 1; 2, for quartile 2; 3, for quartile 3; and 4, for quartile 4). The odds ratios (ORs) and 95 % confidence intervals (CIs) of BC risk for all associations were adjusted for menopausal status and age as well as for the other three dietary patterns of the PCA (patterns 1, 2 and 3) that were derived previously [8]. Age was treated as a continuous variable, and menopausal status, PCA-derived dietary pattern data and SNP data were treated as categorical variables in the statistical model of logistic regression. In addition to age, menopausal status and PCA-derived dietary pattern data (patterns 1, 2 and 3), ORs were adjusted for other potential confounding factors that best predict BC risk in this study population (family history of BC, age at menarche, hormone replacement therapy use, breastfeeding and age at the first full-term pregnancy) [23]. Nevertheless, further adjusting for these five potential confounders did not change any of the ORs by greater than 10 %. Hence, those factors are not included in the adjustments of the ORs presented here. A likelihood ratio test was used to compare regression models with and without SNPs–PCA-derived MDP interaction terms, in order to derive overall p interaction values for assessing the significance of interactions between SNPs and dietary pattern in relation to BC risk. These regression models were also adjusted for menopausal status, age and for the other three dietary patterns of the PCA (patterns 1, 2 and 3). PCA-derived MDP was also treated as a continuous variable to assess association between this PCA-derived dietary pattern and BC, stratified by genotypes of the SNPs. These associations were adjusted further for the categorical variable of the menopausal status and for the continuous variables of age and PCA-derived dietary patterns 1, 2 and 3. Outliers were removed from this analysis. Statistical analysis was performed using SPSS version 21 (SPSS, PASW Inc., Chicago, Illinois), STATA version 11 (StatCorp.2007. College Station, TX) and SNPStats, which is a web-based software, designed for the analysis of genetic association studies [26].

Results

Characteristics and frequencies of *GSTP1, GSTM1, GSTT1* and *NAT2* polymorphisms

The genotype frequencies of the *GSTP1* p.Ile105Val and *NAT2* 590G>A SNPs among the control group did not deviate from HWE (Table 1). *GSTM1* and *GSTT1* deletion polymorphisms could not be tested for HWE since there were only two categories of genotypes. Genotype and allele frequencies of the four polymorphisms in cases and controls are given in Table 1. Characteristics of the 1109 BC cases and 1177 controls of the MASTOS study, including the four PCA-derived dietary patterns, are presented in Supplementary Table 2. Differences in the distribution of the variables between cases and controls were discussed previously [23].

Table 1 Genotype and minor allele frequencies for the *GSTP1, GSTM1, GSTT1* and *NAT2* polymorphisms in the MASTOS study

Gene/SNP	Cases		Controls	
	N	%	*N*	%
GSTP1 p.Ile105Val (rs1695)				
Ile/Ile	637	58.0	668	57.7
Ile/Val	395	35.9	414	35.8
Val/Val	67	6.1	75	6.5
Total	1099		1157	
MAF[a]	0.24		0.24	
Hardy–Weinberg (*p* value)[b]			0.34	
GSTM1				
Non-null	441	41.8	530	45.7
Null	613	58.2	631	54.3
Total	1054		1161	
GSTT1				
Non-null	620	59.0	731	64.7
Null	420	40.4	399	35.3
Total	1040		1130	
NAT2 590G>A (rs1799930)				
G/G	542	49.0	573	49.3
G/A	470	42.5	486	41.9
A/A	94	8.5	102	8.8
Total	1106		1161	
MAF[a]	0.30		0.30	
Hardy–Weinberg (*p* value)[b]			1.00	

[a] *MAF* minor allele frequency

[b] *p* value from Chi-square test performed for Hardy–Weinberg equilibrium (HWE) evaluation

GSTP1, GSTM1, GSTT1 and *NAT2* polymorphisms and risk of breast cancer

The associations between *GSTP1* p.Ile105Val, *NAT2* 590G>A, *GSTM1* and *GSTT1* deletion polymorphisms and BC risk are given in Table 2. Women homozygous for the *GSTT1* null allele demonstrated a statistically significant increased BC risk, when compared to women homozygous for the wild-type *GSTT1* allele. Associations between the other three polymorphisms studied and BC risk were not statistically significant (Table 2).

Table 2 Odds ratios (ORs) for the associations between *GSTP1, GSTM1, GSTT1* and *NAT2* polymorphisms and breast cancer risk in the MASTOS study

Gene/SNP	Cases/controls[a]	Adjusted[b] OR (95 % CI)[c]	*p* value
GSTP1 p.Ile105Val (rs1695)			
Ile/Ile	637/665	1.00	–
Ile/Val	395/414	1.00 (0.84–1.20)	0.98
Val/Val	67/75	0.97 (0.68–1.38)	0.87
			P_{trend} 0.99
GSTM1			
Non-null	441/528	1.00	–
Null	613/630	1.17 (0.98–1.38)	0.08
GSTT1			
Non-null	620/728	1.00	–
Null	420/399	**1.21 (1.01–1.45)**	**0.03**
NAT2 590G>A (rs1799930)			
G/G	542/572	1.00	–
G/A	470/484	1.00 (0.84–1.20)	0.97
A/A	94/102	0.99 (0.72–1.35)	0.94
			P_{trend} 0.97

The *p* values (<0.05), which are statistically significant, are presented in bold. The odds ratios (ORs) [95 % confidence interval (CI)] which correspond to the statistically significant *p* values are also presented in bold

[a] The number of cases and controls may differ from those of Table 1 due to confounder missing values

[b] Adjusted for menopausal status and age

[c] 95 % *CI* 95 % confidence interval

Interaction analyses between principal component analysis-derived Mediterranean dietary pattern, *GSTP1, GSTM1, GSTT1* and *NAT2* polymorphisms and breast cancer

None of the four polymorphisms studied (*GSTP1* p.Ile105Val*, NAT2* 590G>A, *GSTM1* and *GSTT1* deletion polymorphisms) interacted significantly with the PCA-derived MDP, since the overall p interaction values were not statistically significant (Table 3).

Associations between principal component analysis-derived Mediterranean dietary pattern and breast cancer risk, stratified by the *GSTP1, GSTM1, GSTT1* and *NAT2* genotypes

A statistically significant decreased BC risk was evidenced in *GSTP1* Ile/Ile and Ile/Val women with increasing adherence to the PCA-derived dietary pattern (continuous variable) (Table 4). High adherence to the PCA-derived dietary pattern also decreased significantly BC risk of the *NAT2* 590GG and 590GA women. Thus, it is likely that the protective effect of the MDP becomes stronger as the number of wild-type *NAT2* 590G alleles increases. Additionally, increasing adherence to the PCA-derived MDP resulted in a significantly lower risk for BC in both genotypes (non-null and null) of both *GSTM1* and *GSTT1* genes. No statistically significant associations were observed between the PCA-derived pattern and BC risk for the carriers of the variant alleles of the *GSTP1* (Val/Val) and *NAT2* (590AA) SNPs (Table 4).

Discussion

In this nutrigenetics study, we examined the associations between four polymorphisms in phase II detoxification enzymes (*GSTP1* p.Ile105Val*, NAT2* 590G>A, *GSTM1* and *GSTT1* deletion polymorphisms) and BC risk, as well as the interactions between each of these SNPs and the MDP on BC risk. When examining the adjusted associations between BC risk and the four polymorphisms under study, it was shown that the null genotype of the *GSTT1* polymorphism statistically significantly increased BC risk in Greek-Cypriot women, which is in agreement with the results of two recent meta-analyses [27, 28]. This finding might be biologically significant since the null *GSTT1* genotype has been shown to result in the loss of the enzyme and thus in the loss of the enzymatic detoxification activity, which protects against reactive carcinogenic metabolites. Many of these cytotoxic products can cause DNA damage, and hence, individuals with the null *GSTT1* genotype might have an increased BC risk [28, 29]. No associations between BC risk and the remaining three polymorphisms (*GSTP1* p.Ile105Val*, NAT2* 590G>A and *GSTM1* deletion polymorphism) were found, which is also in line with previous meta-analyses [30–34].

In the interaction analyses, non-statistically significant overall p interaction values were obtained, i.e. the association between MDP and BC does not depend on SNPs genotypes and vice versa. Even though there was no statistically significant interaction between MDP and SNPs with respect to BC risk, the investigation of the association analyses between the MDP and BC risk stratified by genotypes of the SNPs showed statistically significant results. A high adherence to the PCA-derived dietary pattern significantly reduced BC risk in women with at least one *GSTP1* Ile allele. Increasing adherence to the PCA-derived MDP also decreased BC risk in women with at least one *NAT2* 590G allele. The effect was stronger in the homozygous wild-type *NAT2* 590GG genotype. These statistically significant associations suggest that the *GSTP1* p.Ile105Val and the *NAT2* 590G>A SNPs could modulate the association between the MDP and BC risk, acting as effect modifiers on this association. Thus, the wild-type *GSTP1* Ile or *NAT2* 590G alleles could enhance the beneficial and protective effect of the

Table 3 Interactions between *GSTP1, GSTM1, GSTT1, NAT2* polymorphisms and principal component analysis (PCA)-derived Mediterranean dietary pattern (MDP) and their effect on breast cancer risk in the MASTOS study

Gene/SNP	PCA-derived MDP intake quartiles[a]: vegetables, fruit, legumes and fish							
	Quartile 1		Quartile 2		Quartile 3		Quartile 4	
	Cases/controls	Adjusted[b] OR (95 % CI)	Cases/controls	Adjusted[b] OR (95 % CI)	Cases/controls	Adjusted[b] OR (95 % CI)	Cases/controls	Adjusted[b] OR (95 % CI)
GSTP1 p.Ile105Val (rs1695)								
Ile/Ile	175/150	1.00[c]	155/164	0.80 (0.58–1.10)	163/159	0.85 (0.61–1.16)	142/189	**0.64 (0.46–0.88)**
Ile/Val	116/90	1.13 (0.79–1.63)	105/92	0.95 (0.66–1.38)	87/114	**0.64 (0.45–0.93)**	87/117	**0.62 (0.43–0.90)**
Val/Val	19/14	1.45 (0.69–3.07)	23/23	0.80 (0.43–1.51)	15/20	0.62 (0.30–1.30)	10/18	0.49 (0.22–1.12)
p interaction[d] 0.55								
GSTM1								
Non-null	115/118	1.00[c]	110/129	0.84 (0.58–1.22)	116/119	0.98 (0.68–1.43)	99/159	**0.63 (0.44–0.91)**
Null	184/113	1.46 (1.03–2.07)	161/151	1.10 (0.77–1.55)	145/178	0.81 (0.57–1.14)	122/167	0.71 (0.50–1.02)
p interaction[d] 0.06								
GSTT1								
Non-null	168/163	1.00[c]	165/175	0.89 (0.65–1.22)	160/188	0.80 (0.58–1.09)	127/200	**0.59 (0.43–0.81)**
Null	124/82	**1.46 (1.01–2.10)**	101/96	0.94 (0.66–1.36)	96/99	0.89 (0.62–1.29)	97/120	0.78 (0.54–1.11)
p interaction[d] 0.58								
NAT2 590G>A (rs1799930)								
G/G	168/120	1.00[c]	146/142	0.73 (0.52–1.02)	123/144	**0.58 (0.41–0.82)**	103/165	**0.44 (0.31–0.62)**
G/A	125/101	0.90 (0.62–1.29)	113/113	**0.67 (0.46–0.96)**	116/131	**0.62 (0.44–0.89)**	116/137	**0.59 (0.41–0.83)**
A/A	21/30	**0.50 (0.27–0.93)**	26/23	0.83 (0.44–1.55)	27/22	0.85 (0.45–1.60)	20/26	0.53 (0.28–1.01)
p interaction[d] 0.17								

The odds ratios (ORs) [95 % confidence interval (CI)] which correspond to the statistically significant *p* values are presented in bold

PCA-derived dietary pattern with high loadings of vegetables, fruit, legumes and fish, which closely resembles the MDP [8]

[a] Quartiles of adherence to PCA-derived MDP, with subjects in quartile 1, showing the lowest consumption of vegetables, fruit, legumes and fish and thus lowest adherence to this dietary pattern and with subjects in quartile 4, showing the highest consumption of the same four food groups and therefore highest adherence to this dietary pattern

[b] Adjusted for menopausal status, age and for the other PCA-derived dietary components (patterns 1, 2 and 3) [8]

[c] The wild-type genotype for each of the four polymorphisms at the lowest level of adherence to the PCA-derived MDP (quartile 1) was used as the reference group

[d] *p* for interaction between quartiles of adherence to the PCA-derived MDP and genotypes of each polymorphism, derived from a likelihood ratio test comparing regression models with and without gene–dietary pattern interactions

Table 4 Associations between breast cancer risk and principal component analysis (PCA)-derived Mediterranean dietary pattern (MDP[a]), stratified by genotypes of *GSTP1, GSTM1, GSTT1* and *NAT2* polymorphisms in the MASTOS study

Gene/SNP	PCA-derived MDP (continuous variable)[a]: vegetables, fruit, legumes and fish Adjusted[b] OR (95 % CI)
GSTP1 p.Ile105Val (rs1695)	
Ile/Ile	**0.84 (0.74–0.95)**
Ile/Val	**0.73 (0.62–0.85)**
Val/Val	0.72 (0.48–1.09)
GSTM1	
Non-null	**0.81 (0.70–0.93)**
Null	**0.76 (0.66–0.86)**
GSTT1	
Non-null	**0.77 (0.68–0.87)**
Null	**0.81 (0.70–0.94)**
NAT2 590G>A (rs1799930)	
G/G	**0.73 (0.63–0.83)**
G/A	**0.81 (0.70–0.94)**
A/A	1.04 (0.77–1.40)

The odds ratios (ORs) [95 % confidence interval (CI)] which correspond to the statistically significant *p* values are presented in bold

[a] PCA-derived dietary pattern with high loadings of vegetables, fruit, legumes and fish (continuous values), which closely resembles the MDP [8]

[b] Adjusted for menopausal status, age and for the other PCA-derived dietary components (patterns 1, 2 and 3) [8]. The data of the PCA-derived dietary patterns 1, 2 and 3 were treated as continuous variables

high adherence to the PCA-derived MDP, against BC risk in Greek-Cypriot women, resulting in a synergistic effect. Furthermore, high adherence to the MDP reduced significantly BC risk in both homozygous non-null and null genotypes of the *GSTM1* and *GSTT1* genes. These statistically significant associations suggest that the observed decreased BC risk is due to the effect of the MDP, irrespective of the modifying effect of the *GSTM1* and *GSTT1* deletion polymorphisms.

The interplay between bioactive phytochemicals and phase II metabolising enzymes might be important in BC development, as shown by our results and those of previous studies [35–37]. In our study, the statistically significant decreased BC risk associated with a high adherence to the PCA-derived MDP is more evident in the wild-type *GSTP1* Ile/Ile and *NAT2* 590GG women. These findings could be explained by the fact that in the wild-type genotypes of the *GSTP1* and *NAT2* SNPs, the enzymes maintain their normal detoxification activity, and thus, they are more efficient in detoxifying carcinogens compared with the variant SNP genotypes (*GSTP1* Val/Val and *NAT2* 590AA) (Fig. 1). Additionally, the high intake of MD plant-based foods and thus of isothiocyanates, indoles [4, 38] and other antioxidants would induce the expression of the wild-type GSTP1 and NAT2 enzymes, and consequently, the detoxification enzymatic activity would be further enhanced [3, 10, 14]. These phase II enzymes detoxify toxic electrophiles and reactive species (e.g. diols, nitrosamines, organic epoxides, hydroperoxides and unsaturated aldehydes) produced from the activity of phase I enzymes [12, 39]. Increased enzymatic detoxification would result in an increased excretion and clearance of carcinogens and in a reduced exposure of the target tissue to carcinogen-induced DNA damage. Evidence by previous studies also suggests that isothiocyanates inhibit phase I activating enzymes, leading to reduced activation of procarcinogens [40, 41]. Therefore, initiation and promotion of carcinogenesis are inhibited by these events [29], resulting in a decreased BC risk (Fig. 1).

In addition, phase II enzymes catalyse the conjugation of either glutathione in the case of GSTs or acetyl-CoA in the case of NATs to electrophilic compounds converting them into water-soluble species. Therefore, dietary metabolites such as the isothiocyanates are not only inducers but also substrates of GSTs, the action of which leads to their elimination from the body [14] (Fig. 1). In the case of N-acetylation, aromatic and heterocyclic amines act as substrates and are inactivated by the NAT2 enzyme [12]. Previous studies showed that individuals with *GSTM1* or *GSTT1* null genotypes or *GSTP1* Val/Val alleles, who exhibit no or decreased enzymatic activity, are less efficient in the elimination of phytochemicals, leading to a prolonged accumulation of chemopreventive micronutrients in the body. The presence of prolonged and high levels of bioactive phytochemicals in these carriers induces the activity of the

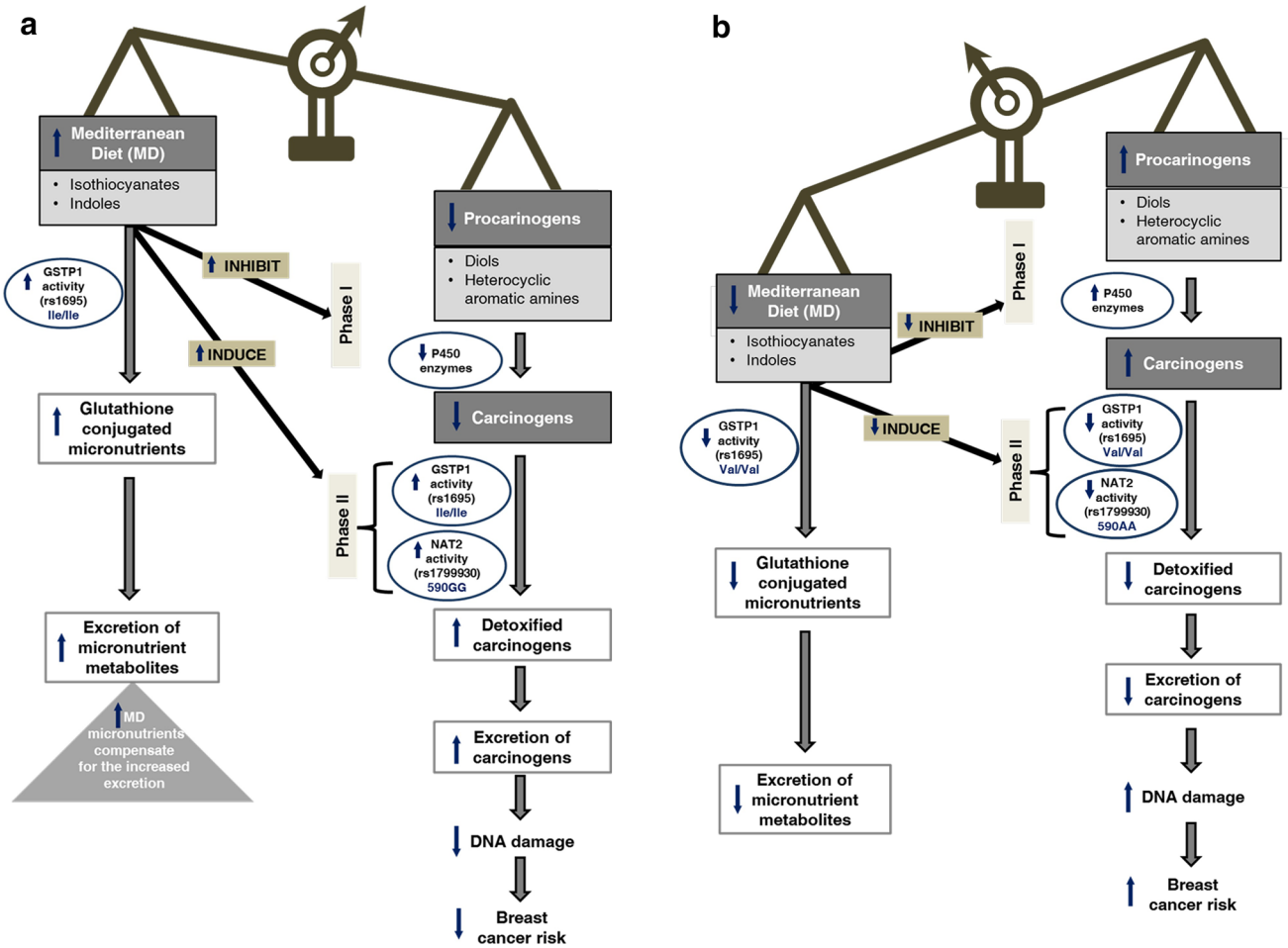

Fig. 1 Schematic diagrams showing a balance in order to explain the relationship between the MD micronutrients, the *GSTP1* p.Ile105Val (rs1695) and *NAT2* 590G>A (rs1799930) SNPs, carcinogens, phase I and phase II enzymes and cancer risk in the xenobiotic metabolism. **a** The synergistic effect between the high intake of the MD micronutrients (high adherence to the plant-based foods of the MD) and the wild-type *GSTP1* Ile/Ile and *NAT2* 590GG SNPs decreases BC risk. **b** The synergistic effect between the low intake of the MD micronutrients and the variant *GSTP1* Val/Val and *NAT2* 590AA SNPs increases BC risk. Microconstituents such as the isothiocyanates and indoles, which are obtained through the MD, can inhibit phase I enzymes that activate procarcinogens to carcinogens and induce phase II enzymes that detoxify carcinogens. MD micronutrients can also act as substrates for GST enzymes (*up arrow* increase, *down arrow* decrease)

detoxification GST enzymes and thus increases protection against cancer [12, 14, 37]. Another study on bladder cancer risk showed that individuals with a *NAT2* slow acetylator genotype might benefit from the high intake of cruciferous vegetables [12]. Nevertheless, in the Greek-Cypriot population we did not observe any statistically significant associations between the MDP and the variant *GSTP1* Val/Val and *NAT2* 590AA genotypes on cancer risk, results that are in agreement with earlier studies [29, 36, 42]. This inconsistency between various studies may be due to different dietary exposures and population differences [14, 43]. Based on our significant findings on the wild-type *GSTP1* Ile/Ile and *NAT2* 590GG SNPs, we hypothesise that the inducing effects of the MD nutrients on the expression of the enzymes might play a more important role in protection against cancer, compared with the eliminating effects of the nutrients themselves, as explained above and supported by others [35, 37, 43–46].

Moreover, the PCA-derived MDP used in our study is rich in fish, besides plant-based food groups. The anticarcinogenic effect of fish could be exerted through the cellular action of marine n-3 PUFAs [39]. N-3 PUFAs can modulate tumour growth and decrease BC risk by reacting with the endogenous free radicals to form lipid peroxidation products, which in turn cause apoptosis of cancerous cells [7, 47]. The lipid hydroperoxides act as substrates for the metabolising GST and NAT enzymes, which detoxify them [7]. As aforementioned, the high adherence to the PCA-derived MDP significantly decreased BC risk in the Greek-Cypriot carriers of the *GSTP1* Ile/Ile and *NAT2* 590GG alleles. The high adherence to the MDP could ensure the presence of adequate levels of fish n-3 PUFAs

(Supplementary Table 3) and hence of lipid peroxidation. This appears to enhance the triggering of apoptosis of tumour cells [39], even if the GSTP1 and NAT2 enzymes at the wild-type genotypes of the SNPs (*GSTP1* Ile/Ile and *NAT2* 590GG) would eliminate the peroxidation products efficiently. Hence, we hypothesise that the high intake of marine n-3 PUFAs might be able to compensate for the lipid peroxidation products detoxified by the *GSTP1* Ile/Ile and *NAT2* 590GG enzymes. In contrast to our results, a study on women with the combined *GSTM1* null and *GSTP1* low activity genotypes and marine n-3 fatty acid intake revealed a reduced BC risk and no associations with the high activity GST genotypes [7]. The sample size of this study was smaller compared to our study's sample size, and this might be the reason behind this inconsistency. Further studies are needed in order to explain these contrasting observations.

To the best of our knowledge, this is the first study which examined the effects of these particular SNPs on the GST and NAT detoxification enzymes and on cancer risk, in association with a dietary pattern that includes a combination of more than two food groups (not only vegetables and fruit but also legumes and fish). A dietary intake combining those four food groups included in the PCA-derived dietary pattern could contain ample quantities of anticarcinogenic micronutrients and marine n-3 PUFAs, as opposed to a single nutrient intake (Supplementary Table 3). Hence, the PCA-derived dietary pattern has the advantage of closely resembling the MD and thus of representing a more comprehensive dietary variable to study, rather than examining the effect of single isolated micronutrients. Further strengths of this study are the large sample size and the homogeneous sample of subjects in terms of ethnic background [8, 48]. Selection and survival biases could be possible limitations of the study, as discussed previously [8, 48]. An additional limitation is the fact that the FFQ used in our study examined only 32 food and beverage items, which is a limited number for a typical FFQ. In addition, information about the means of consumption or preparation for each dietary item and about the intake of dietary supplements was not taken into account. Therefore, some of the food items that could contribute significantly to the dietary habits of the Greek-Cypriot population might be missing. Furthermore, the methods of processing and cooking of vegetables, legumes and fish complicate more the assessment of the exposure to the specific anticarcinogenic MD nutrients under study [29]. A further limitation of the FFQ is possible misclassification in the assessment of dietary intake, due to recall bias, an unfortunate but inevitable caveat of case control studies. However, it is not expected to be a big issue in the examination of gene–environment interactions, as the relevant risk of bias with respect to genotype is minimal [37, 49, 50]. Moreover, the relative quantitative range of anticarcinogenic nutrients included in the foods most heavily weighted in the PCA-derived MDP was based on information reported in the literature and in a food database [3–5, 38, 51] without being measured in the serum. Additionally, how bioavailable are the phytochemical compounds (e.g. isothiocyanates) and how they are distributed upon ingestion in vivo is still unclear [14, 29]. PCA has some subjective limitations, including the derived variables used in the analysis, the number of the extracted factors, the type of the rotation used and the labelling of the retained factors. A small amount of variance (4.55 % of the total variance in the 32 FFQ items) was also explained by the component derived from the PCA used in the current study [8]. Nonetheless, this low variance amount is typical in dietary studies analysed by PCA, as shown by the limitations of reducing the highly interrelated dietary variables [52].

In conclusion, our statistically significant association results show that the *GSTT1* null genotype increases BC risk and that a high adherence to the PCA-derived MDP reduces BC risk in women with at least one *GSTP1* Ile allele or with at least one *NAT2* 590 G allele. These results suggest that the *GSTT1* null genotype could be a risk allele for developing BC and that both the *GSTP1* p.Ile105Val and the *NAT2* 590G>A SNPs may act as effect modifiers on the association between the PCA-derived MDP and BC risk in the Greek-Cypriot female population. The high intake of the anticarcinogenic components of the MD in combination with the wild-type *GSTP1* Ile or *NAT2* 590 G alleles seems to play a synergistic role in the prevention of BC development through the detoxification pathway, with the *GSTP1* Ile or *NAT2* 590G alleles enhancing this protective MD effect. Further studies should include quantification of the levels of anticarcinogenic, antioxidant and oxidative stress-related metabolites in the serum of these subjects, in order to further assess the associations observed and clarify their role in breast carcinogenesis. Also, studying *GST* and *NAT2* SNPs with the tag SNPs approach will allow us to capture a more complete genetic coverage for the genetic variants assessed in the association analyses between the MD and BC risk. Additionally, as the detoxification enzymes of the xenobiotic metabolism have been shown to be involved in the regulation of DNA damage [9], it would be interesting to assess the combined effects of the *GST*, *NAT* and DNA repair SNPs (studied previously in the MASTOS participants [24, 48]), on BC risk as well as to assess these effects on the MD and BC risk association. This kind of information generated through the nutrigenomics studies might help to further investigate the mechanism(s) of action of the protective components of the MD in vivo.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical standards The study was approved by the Cyprus National Bioethics Committee. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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