# Analysis Association between Mitochondrial Genome Instability and Xenobiotic Metabolizing Genes in Human Breast Cancer

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The aim of this study was to determine the existence of association between the genetic polymorphisms of metabolizing genes *GSTM-1*, *GSTT-1*, and *NAT-2*, and the presence of mitochondrial genome instability (mtGI) in breast cancer cases. Ninety-four pairs of tumoral/nontumoral breast cancer samples were analyzed. Our samples showed 40.42% of mtGI by analysis of two D-loop region markers, a (CA)n mtMS starting at the 514-bp position, and four informative *MnlI* sites between the 16,108-16,420-bp. *GSTM-1* null genotype has shown a significant association with mtGI presence ($\chi^2$ = 7.62; *P* = 0.006) in breast cancer cases; moreover, these genotypes also are related to an increased risk for mtDNA damage (odds ratio (OR) = 3.71 (1.41–9.88); 95% Cornfield confidence interval (CI)). These results suggest that the absence of *GSTM-1* enzymatic activity favors chemical actions in damaging the mtDNA. Analysis of *GSTT-1* and *NAT-2* polymorphisms showed no association with mtGI ($\chi^2$ = 0.03; *P* = 0.87 and $\chi^2$ = 2.76; *P* = 0.09, respectively). The analysis of invasive breast cancer cases showed mtGI in 74.36% of ILC cases (29 of 39 samples), and in only 18.75% (9 out of 48) IDC cases; this result suggests a possible relation between mtDNA mutations and variations in molecular pathways of tumor development.
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## INTRODUCTION

Worldwide, breast cancer is the fifth most common cause of cancer death and the first cause among women (1). Approximately 40% of breast cancer cases show nuclear genome instability (NGI), due to the malfunction of one or more genes forming the cohort of mismatch repair genes (MMR) (2,3), while more than 60% show mtGI, a second form of instability associated with tumors and characterized by the presence of point mutations or length tract instabilities in mtDNA (4,5).

The finding of mutations and deletions in mtDNA of tumor cells has focused the attention on these cytoplasmic organelles as concurrent factors in the start or progress of tumors (6). However, little is known about the mtGI, their presence and frequency variation among tumors, the mechanisms involved in their origin, and their role in human cancers.

The human mtDNA is a 16,569-bp, circular and double-stranded molecule. All the mt genome encoded genes are organized continuously with noncoding intervening sequences except for a small segment called displacement-loop (D-loop), which comprises some of the replication and transcription origins of the mt-genome (7).

Trying to clarify the origin of mtGI and their role in human cancers, particularly in breast cancer, our group previously showed that causes of the appearance of NGI are not involved in mtGI (8). This result was confirmed later by other work (9,10,11), allowing us to conclude that NGI and mtGI are independent events with different mechanisms of origin.

Moreover, in the last year, there was significant progress regarding the function of the metabolizing enzymes and their role in the elimination of a very wide class of DNA damage compounds (1). The xenobiotic metabolizing enzymes (XME) are a family of enzymes coded by the xenobiotic metabolizing genes (XMG). There are more than twelve different families of enzymes that have been described for almost all genetic polymorphisms that modified their activity efficiency, and that are responsible for wide inter-individual variation in drug metabolism (12).

The human mammary gland is exposed to agents capable of inflicting DNA damage and thereby initiating tumor induction (13,14,15). Most of these potential human mammary carcinogens require enzyme-catalyzed steps to effect

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biotransformation to DNA-reactive metabolites. Although hepatic enzymes are likely to play a role in the metabolism of potential breast carcinogens, it is important to consider that enzymes expressed locally in the target site also have relevant influence in modulating DNA-reactive species levels (1).

Glutathione S-Transferase isoforms, μ (*GSTM-1*) and τ (*GSTT-1*), and *N-Acetyl Transferase 2* (*NAT-2*) are enzymes which demonstrate expression in mammary tissues (1). For both the GST enzymes, the deficiency is due to a deletion of the entire genomic coding region, giving rise to the allele known as "null." The deletion in a homozygous state determines the polymorphism called "null genotype," with the consequent absence of total enzymatic activity. Regarding the *NAT-2* gene, at least 19 variant alleles have been detected in the human population (16), but the *NAT-2*5*, **6*, and **7* alleles account for virtually all of the slow acetylator alleles in the Caucasian population, providing evidence for a high concordance between genotype and phenotype (17).

In this report, we first analyze pairs of breast nontumoral/tumoral tissues for the presence of mtGI, studying two markers of the D-loop region which have a low spontaneous rate of mutation in germline cells, a (CA)n mt MS (microsatellite) starting at the 514-bp position, and four informative *Mnl*I sites located between the 16,108-bp and 16,420-bp position (8). Second, we analyze the polymorphism of *GSTM-1*, *GSTT-1*, and *NAT-2* in breast cancer patients with and without mtGI, trying to determine whether this instability is associated with a specific allele for the XMG. Furthermore, we analyzed the possible correlation between the presence of mtGI and the histopathological types of breast cancer samples.

## MATERIALS AND METHODS

### Samples

Ninety-four breast cancer samples and their adjacent nontumoral tissues were obtained from female patients at a Clinical Center from La Plata City (Buenos Aires, Argentina), who underwent surgical resection between 1997 and 2004, according to the Institutional Review Board approved protocol. We have selected only those patients who did not undergo pretreatments (radio- or chemotherapy) before surgery. A representative tissue section was stained with haematoxylin and eosin and semiquantitatively assessed for tumor area percentage over the total sample area by one of the authors (M Laguens); cancer regions selected for DNA extraction showed >90% abnormal cells. Histological typing was based on standard criteria (18). All the donors gave informed consent for the study. The DNA extraction was performed using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA).

### mtDNA Analysis

The (CA)*n* MS, starting at 514 bp and showing five haploid alleles differing in size by the number of dinucleotide repeats (7), was amplified with the primers and polymerase chain reaction (PCR) conditions reported by Szibor *et al*. (19). Microsatellite allele identification was performed by electrophoresis in 15% neutral polyacrylamide and Sybr-Green fluorescence.

Restriction fragment length polymorphisms (RFLP), for *Mnl*I sites [CCTC↓$(N)_7$] between nontumoral (Nt) and tumoral (T) tissue, were detected in an mtDNA fragment of 312 bp extending from the 16,108 bp position to the 16,420 bp position (7). PCR conditions and primers were as reported previously in Richard *et al*. (8). According to the reference sequence of Anderson *et al*. (7), the 312 bp fragment comprises five *Mnl*I sites producing fragments of 118, 89, 39, 35, 28, and 3 bp. With the exception of the 3 bp fragment, which could not be identified due to its small size, all of the other fragments and their corresponding RFLP patterns were detected by electrophoresis in 10% neutral polyacrylamide visualized by Sybr-Green fluorescence staining.

Those pairs of tumoral and nontumoral samples showing mt markers heteroplasmy and/or changes between both tissues were tested 2 to 4 times in independent studies performed by two researchers to confirm the consistency of mt marker identifications and to exclude possible sample cross-contamination.

### Analysis of Xenobiotic Metabolizing Genes

*GSTM-1* genotype analysis was carried out by a modification of the duplex PCR procedure described by Hou *et al*. (20), replacing the internal PCR-positive control reaction (*NAT-2*), by a segment of the syntenic gene *GSTM-2* (211pb, accession number STS-RH65389). Primers for *GSTM-2* were: Fw: 5′- TgA AgT CCT TCA ggT TTg gg –3′; Rw: 5′- CAg CCA Tgg TTT CTT CTT ggg –3′, and the annealing temperature was 58°C. This method allows us to identify individuals with one or two copies of the *GSTM-1* allele (showed both the *GSTM-1* and the control band), from individuals with a homozygous deletion (null genotype only amplifies the control gene). *GSTT-1* genotype was determined using the same duplex PCR conditions and primers reported by Baranova *et al*. (21).

Identification of *NAT-2* slow acetylators was performed by PCR method and specific restriction endonucleases analysis as reported previously in Hou *et al*. (20). The *NAT-2* mutant alleles were identified by the substitution nucleotides, and loss of a restriction site, in C481T (*Kpn*I), G590A (*Taq*I), and G857A (*BamH*I), allele *5, *6, and *7, respectively. The wild-type allele (*NAT-2*4*) was defined by the absence of such substitutions. Correlation phenotype/genotype, the presence of at least one wild-type allele, confers an intermediate acetylator phenotype (*NAT2*I*), whereas two mutant alleles are required for the slow acetylator phenotype (*NAT2*S*). The wild-type allele in a homozygous state determines the rapid acetylator phenotype (*NAT2*R*) (20,22).

### Statistical Methods

The $\chi^2$ test was used to study the difference in the genotype distributions. Relative risk was estimated with Odds Ratio (OR) and the Cornfield 95% confidence interval (95% CI). $P$ values (two-sided) below 0.05 or Odds Ratio with a 95% CI over or below 1.0 were considered statistically significant.

## RESULTS

### mtDNA Analysis: (CA)*n* mtMSI and *Mnl*I RFLPs in Nt/T Tissue

Over a total of 94 breast tissues analyzed for (CA)*n* MS, we detected eight heteroplasmic samples (8.51%). The analysis of tumor sample counterparts showed allele changes in 24 of 94 cases (25.53%).

The appearance of *Mnl*I mutations was observed in 28 of 94 cases of breast cancer patients (29.79%), comparing the digestion pattern in tumoral tissues with the nontumoral counterpart. Moreover, we observed that five of these samples showed heteroplasmy in the nontumoral tissue (presence of two different digestion patterns).

We considered a case unstable when it had a changed allele in the mtMS, difference in the digestion pattern for *Mnl*I, or both events in parallel; therefore, if we combine MS and *Mnl*I data, the mtGI observed in cancer tissues was 38 of 94 (40.42%).

### Histopathological Types of Breast Cancer and mtGI Event Analysis Association

The possible correlation was evaluated in 94 total cases, consisting of one case of LCIS (Lobular Carcinoma *In Situ*), 39 cases of ILC (Lobular Carcinoma Invasive), six cases of DCIS (Ductal Carcinoma *In Situ*), and 48 cases of IDC (Ductal Carcinoma Invasive).

Forty-one samples from the 94 total cases analyzed showed mtGI, 29 showed ILC (70.73%), whereas only 9 cases showed IDC (21.95%), and the remaining 3 cases showed DCIS (7.32%).

Analyzing particularly invasive case results, we found mtGI in 74.36% of the total ILC cases (29 of 39 samples) and in 18.75% of the total IDC cases (9 of 48 samples). The use of statistical analysis to compare this distribution revealed a significant association between the presence of the mtGI phenomenon and the ILC histophatological type of breast cancer ($\chi^2$ = 24.83; $P$ < 0.001; OR = 12.57 [4.08–40.39]; 95% CI).

**Table 1.** Genotype frequencies of xenobiotic metabolizing genes.

| Gene and genotype | Frequency: Cases,[a] *n* (%) | Frequency: Reported[b] | Reference |
|---|---|---|---|
| *GSTM-1* | 94 | | |
| null/null | 38 (40.4) | 42–60 | (20,29,32,39) |
| +∫+/null | 56 (59.6) | 40–58 | |
| *GSTT-1* | 94 | | |
| null/null | 17 (18) | 16–21.5 | (29,32) |
| +∫+/null | 77 (82) | 78.5–84 | |
| *NAT-2* | 94 | | |
| Rapid | 7 (7.4) 52 (55.3) | 36.8–60.1 | (20,40) |
| Intermediate | 45 (47.9) | | |
| Slow | 42 (44.7) | 39.9–63.2 | |

[a]Frequency found in nontumoral samples with *n* number of case studied for each gene.
[b]Average frequency and standard deviations estimated according to the data published by other groups (bibliographic reference), in normal subjects of similar ethnicity (since it works with the average, the sum of the averages of the two genotypes of a particular gene may be less than 100).

### Analysis of Xenobiotic Metabolizing Genes

None of the obtained frequencies for the xenobiotic metabolizing gene polymorphisms studied showed significant differences with data reported for the same ethnic population (Table 1).

Of 35 mtGI tumors, 21 (60%) were homozygous for the *GSTM-1* null allele. In contrast, 71.2% of the samples carrying the gene in hetero- or homozygosity (42 of 59 cases) showed a stable mt genome (without detection of mutations or instability). Those opposite values of observed percentages indicate that the *GSTM-1* null genotype may be correlated with the presence of mtDNA instability. Evaluating this possibility with statistical methods, we observed that, effectively, there was a positive significant association ($\chi^2$ = 7.62; $P$ = 0.006) for the events mentioned above (Table 2). Moreover, the OR value obtained (OR = 3.71 [1.41–9.88]; 95% CI), confirms the correlation between the homozygous deletion of the gene as a risk factor for mtGI generation.

The analysis for *GSTT-1* gene showed that 32 of 94 samples analyzed showed mtGI, from which only five (15.6%) have the null genotype, whereas the remaining 84.4% (27 of 32) carry one or two copies of the allele. Using a statistical method, we found no significant correlation between mtGI and *GSTT-1* homozygous null ($\chi^2$ = 0.03; $P$ = 0.87), as shown in Table 2.

Analyzing a total of 94 cases for three *NAT-2* gene polymorphisms (C481T, G590A, and G857A), we found that seven cases were rapid acetylator (7.4%), 45 were intermediate acetylator (47.9%), and 42 were slow acetylators (44.7%) (Table 1). To analyze the possible correlation between the acetylator type for *NAT-2* and the presence of mtGI, we considered the intermediate and rapid acetylator together because of the small number of the latter. The statistical methods, evaluating the *S versus *I/*R acetylators, showed that there is no significant association ($\chi^2$ = 2.76; $P$ = 0.09) (Table 2). We also assessed, separately, the correlation between mtGI and each of the acetylators studied (*R, *I, and *S), and we found no significant association (statistical data not shown).

**Table 2.** Genotype analysis association with mtGI and metabolizing genes.

| | mtGI | | | |
|---|---|---|---|---|
| Gene and Genotype | Absence, *n* (%) | Presence, *n* (%) | Odds ratio (95% CI) | *P* value |
| *GSTM-1* | 59 | 35 | | |
| +[a] | 42 (71.2) | 14 (40) | 1 (reference) | |
| null | 17 (28.8) | 21 (60) | 3.71 (1.41–9.88) | 0.006[b] |
| *GSTT-1* | 62 | 32 | | |
| + | 50 (80.6) | 27 (84.4) | 1 (reference) | |
| null | 12 (19.4) | 5 (15.6) | 0.77 (0.21–2.71) | 0.87[c] |
| *NAT-2* | 62 | 32 | | |
| R/I | 30 (48.4) | 22 (68.8) | 1 (reference) | |
| S | 32 (51.6) | 10 (31.2) | 0.43 (0.16–1.14) | 0.09[d] |

[a]Cases carrying the *GSTM-1* gene in hetero- or homozygosis.
[b]*GSTM-1* null versus "+" genotype, the first had a significant overrepresentation of cases with mtGI.
[c]*GSTT-1* null versus "+" genotype, nonsignificant difference.
[d]*NAT-2* slow versus rapid/intermediate acetylators, nonsignificant difference.

## DISCUSSION

### Mitochondrial Genome Instability

It is believed that mutations in the mitochondrial genome contribute to the process of carcinogenesis. In recent years the existence of alterations in the mtDNA in several human cancers (gastric, colorectal, lung, and breast) has been demonstrated (23).

We found that 38 of 94 cases (40.42%) showed mtGI when both markers from the D-Loop region were analyzed. Our group published one of the first works to evaluate the existence of mtGI in breast cancer (24), finding mutations in the entire mt genome of tumoral cells (5 of 7 cases), but not in nontumoral cells. Subsequently, other works also describe the presence of mutations at the mtDNA when compared with the mammary tumoral sample and paired with adjacent nontumoral tissues from the same patient. Sequencing the entire mt genome, Zhu *et al*. (25) identified one or more somatic mutations in 14 of 15 (93%) breast cancer cases studied; while Tan *et al*. (26) found that 74% (14 of 19 tumors analyzed) displayed at least one somatic mtDNA mutation, and that 81% of the changes were in the D-loop region (22 of 27 different somatic mutations). Richard *et al*. (8), analyzing the D-Loop region in 40 pairs of breast Nt/T tissues, observed a 65% mtGI in cancer tissues; while on the other hand, Alazzouzi *et al*.(9) found 10.8% (4 of 34) of mtGI in ductal breast cancer cases.

The high frequency of somatic mutation reported for the mtDNA in breast cancer cases and the percentage of mtGI found in our series (40.42%) show that this instability is not a sporadic phenomenon, but rather is a detectable feature in a high rate of breast cancer cases studied, as well as for other carcinomas (lung, gastric, and colorectal cancers) (27). Despite this, testicular tumors did not show mtGI (0 of 17 cases) (4), neither did bladder tumors (in a preliminary analysis we found that 0 of 10 cases studied showed this event). Consequently, this data could reflect the existence and/or succession of certain conditions that favor the mtGI generation in some tumors but not in others. One explanation would be the presence of a barrier mechanism acting only in a particular tissue and preventing the induction of mutations locally; for example, specific metabolizing enzyme(s) (1), as we will discuss later for the XMG studied in the present report.

### mtGI and Breast Cancer Histopathological Type

Presently, we have found that 74.36% of ILC samples presented the mtGI phenomenon (29 of 39 samples), while only 18.75% of total IDC samples showed this event (9 of 48 cases). A low percentage of mtGI for IDC (10.8%) was reported previously by Alazzouzi *et al*. (9).

The possible correlation between mtGI frequencies and the histopathological features of the samples was evaluated previously by other researchers, both for breast cancer and other types of tumors. In malignant melanomas, the mtMSI incidence increased from 3% (one case) of sporadic malignant melanomas (SSM), to 13% (four cases) of nodular melanoma (NM), and even more in metastatic melanomas (MM) with 20% (nine cases). The mtMSI was determined analyzing insertions and/or deletions at the D-310 homopolymer C tract and the MS (CA)n (nt514-nt523) (28). Although only 4 of 37 (10.8%) ductal type breast cancer cases analyzed by Alazzouzi *et al*. (9) showed mtGI, 3 of the 4 cases corresponded to grade III tumors.

These data suggest that mtGI could be related to variations in molecular pathways of tumor development in breast cancer subtypes. Our results show this as well, with a higher percentage of cases with mtGI in ILC than in IDC breast cancer.

### Analysis Association between mtGI and the XMG Studied

***GSTM-1.*** We found a higher significant association ($\chi^2 = 7.62$; $P = 0.006$), between the null genotype and the presence of mtGI (OR = 3.71 [1.41–9.88]; 95% CI) (Table 2).

The finding of *GSTM-1* null genotype as a susceptibility factor risk for breast cancer has been reported previously (29,30,31). Helzlsouer *et al*. (32) reported an increased breast cancer risk in women with the null genotype (OR = 2.10 [1.22–3.64]; 95% CI). Charrier *et al*. (33) found a positive association in postmenopausal women, while Ambrosone *et al*. (34) found a correlation between *GSTM-1* null and younger postmenopausal women with breast cancer. Moreover, Rebbeck *et al*. (35) examined breast cancer patients with a family history and found no association between *GSTM-1* null allele and tumor risk.

However, we can point out that so far no study has analyzed its possible association with mtGI event, as is done in the present report.

It is known that the *GSTM-1* isoform (mu, μ) is expressed in human breast tissue cells (1,36). Moreover, a study conducted in mouse liver mitochondria demonstrates that its concentration is induced by the presence of toxic metabolites (as in oxidative stress conditions) in the mitochondrial matrix, as well as in the cytosolic compartment (37,38). These enzyme properties, along with the fact that human breast tissues are exposed to a large number of genotoxic compounds (most of which are metabolized by *GSTM-1* enzyme [1], including products such as reactive oxygen species [ROS] [37]), support our correlation result between *GSTM-1* null genotype and an increase in mtGI. Gene deletion in only one allele also causes enzyme activity to decrease, so we could assume that this deletion also could be involved in mtGI generation.

Although in the present report we mentioned that *GSTM-1* null genotype could be a risk factor for mtGI presence, it also is possible that *GSTM-1* "+" acts as a protective factor for mtDNA damage. The *GSTM-1* genotype distribution in cases with mtGI presence is closer to numbers reported previously; whereas *GSTM-1* frequencies in those with mtGI absence are different from the accepted limit percentages in the literature (Tables 1,2). Therefore we should consider the possibility of a protective role of the enzyme encoded by *GSTM-1* gene rather than the null genotype as a risk factor for mitochondrial genome damage.

Figure 1 shows a hypothetical diagram about the processes that occur within the mitochondrial matrix in reference to the damage caused by reactive compounds in the presence or absence of the *GSTM-1* enzyme.

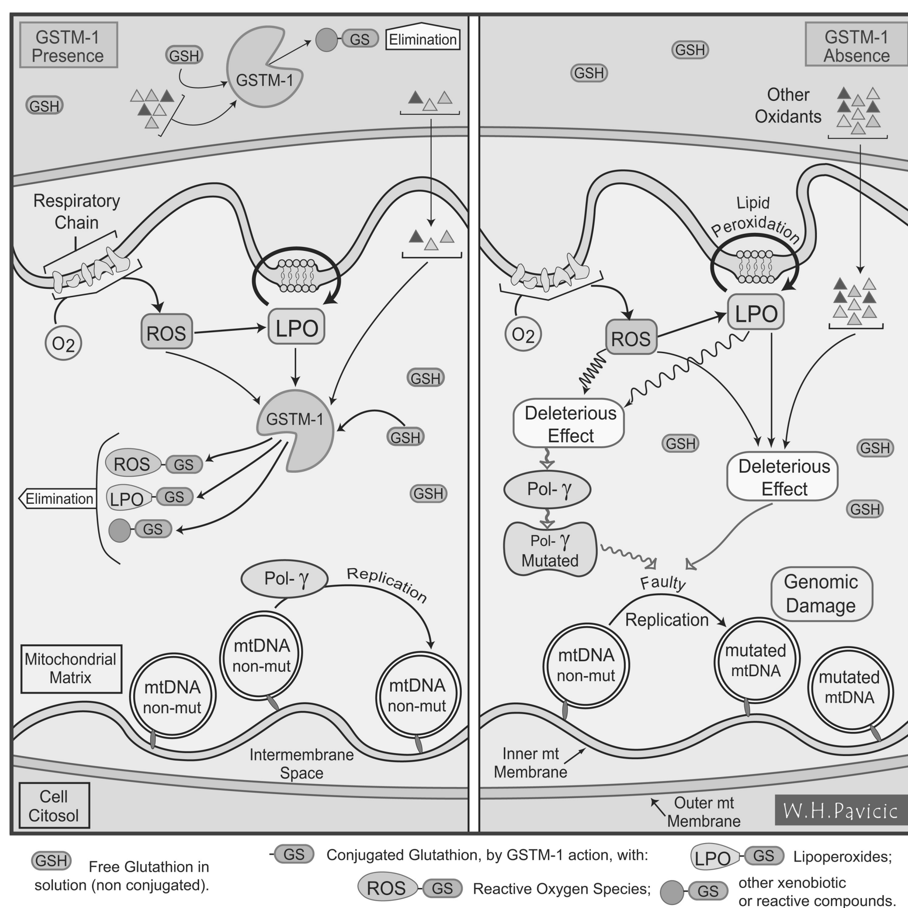


**Figure 1.** Here we diagram the possible role *GSTM-1* enzyme plays inside the mitochondrial matrix as mtDNA protector factor regarding damage caused by reactive species. The biological systems can produce these compounds by different pathways. One of the most important endogenous mechanisms is the respiratory chain, generating great levels of ROS during the $O^2$ reduction. Lipid peroxidation over the membrane lipoprotein, caused by ROS action, is another source of active compounds (HNE: 4-Hydroxynonenal, for example). Another important mechanism involved in ROS generation is the biotransformation of different exogenous compounds or xenobiotics by action of different XME. The ROS action into the cell could produce lesions in different mt compounds, such as lipids; damage different kinds of proteins, Pol-γ for example; damage the mtDNA directly by action of these active species on the bases, or indirectly by DNA replication with a faulty Pol-γ that introduces errors. Altogether, those deleterious processes lead to genotoxic damage, as shown in the right side of the image. The left side shows the possible metabolic pathway of the *GSTM-1* enzyme, promoting the elimination of reactive species and blocking their deleterious effect over the mt component. It is important to note the possible protective action of *GSTM-1* over the mtDNA. The picture shows only a schematic pathway for the *GSTM-1* enzyme; the other mitochondrial matrix events are simplified.

***GSTT-1 and NAT-2.*** Our results have shown that neither the *GSTT-1* null genotype ($P$ = 0.87) nor the different acetylators for *NAT-2* ($P$ = 0.09) have significant association with the presence of mtGI in breast cancer samples (Table 2). A possible explanation is that, although expression of these genes was detected in the mammary tissue, the encoded enzymes do not show enzymatic activity inside the mitochondrial matrix (1). This allows us to conclude that the action of damaging metabolites on the mt genome is not mediated and/or regulated by these enzymes on a local level. So, the presence of mtGI in breast cancer tissues was independent of those XMG.

## CONCLUSION

As we point out above, the action of *GSTM-1* as a barrier defense could prevent the induction and accumulation of mutations at an mtDNA level in breast tissue. Consequently, a locally metabolizing enzyme activity could have an effective and important influence in modulating reactive species levels (Figure 1); this tissue-specific enzymatic activity intervariation could be an explanation for mtGI frequency differences reported for each type of tumor, as mentioned previously in the discussion.

Finally, it would be of great interest to generate a work line to study the role of protection and/or repair for different metabolizing enzymes in relation to the mtDNA damage generation. It also would be of interest to study whether the mtGI/*GSTM-1* null correlation also is present in other tumor types in which the mtGI event was found, or if it is present only in breast cancer tissues. New findings will enable us to have a better understanding about the mechanism of interrelationships which converge in the event of genomic damage, as well as for the carcinogenic process itself.

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## DISCLOSURE

M Laguens predeceased the execution of this disclosure. We declare that the authors have no other competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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