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Article

Association Between GSTT1 and GSTM1 Gene Deletions and Oxidative Stress Markers in a Local Population

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Abstract: *Background*: Glutathione S Transferase Mu 1 (GSTM1) and Theta 1 (GSTT1) gene deletions lead to total loss of enzyme activity, which may explain an inability to detoxify reactive electrophiles and increased oxidative stress susceptibility. Although associations between redox biomarkers have been studied worldwide, there is no data yet on Iraqi populations, of whom 20 million are living in areas of high environmental exposure to pro-oxidant pollutants, such as occurs in Babil Province. *Materials and Methods*: A cross-sectional study of 267 adults from Babil, Iraq. Multiplex PCR with internal controls by β-globin for GSTM1 and GSTT1 genotypes. We quantified serum oxidative stress biomarkers malondialdehyde (MDA, TBARS validated by HPLC), total antioxidant capacity (TAC, measured by FRAP), and 8-hydroxy-2′-deoxyguanosine (8-OHdG, ELISA). Multivariable linear regression adjusted for age, sex, BMI, smoking and occupational exposure. *Results*: The GSTM1 null frequency was 59.5%, GSTT1 null 28.1%, and the double-null ($GSTM1^-/GSTT1^-$) 15.7%. Independent of covariates, double-null individuals had significantly raised MDA (+57.9%, $p < 0.001$) and 8-OHdG (+66.1%, $p < 0.001$), and significantly lowered TAC (–22.7%, $p < 0.001$) vs wild-type. We found a statistically significant interaction between double-null genotype and occupational exposure (pinteraction = 0.018–0.034), strongly supporting that oxidative damage is enhanced when people with a double-null genotype are in high-exposure environments. *Conclusion*: The double-null genotype of abundant homozygous deletions in GSTM1 and GSTT1 is significantly associated with systemic oxidative stress in this Iraqi cohort, where the effects are aggravated with particular environmental exposures. Conclusion This study demonstrates the importance of genotype-informed public health approaches in high-risk occupational groups and providing initial pharmacogenomic data for Iraq.

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Keywords: GSTM1; GSTT1; gene deletion; oxidative stress; malondialdehyde; gene–environment interaction; Iraq

1. Introduction

1.1. Background of Glutathione S-Transferase (GST) Genes

Glutathione S-transferases (GSTs; EC 2.5.1.18), a superfamily of phase II detoxification enzymes, play an important role in the cellular defense against both the endogenous and xenobiotic electrophiles [1]. Based on sequence homology, cytosolic GSTs can be grouped into seven (7) classes in humans (Alpha, Mu, Pi, Theta, Zeta, Omega, Sigma), which consist of gene families at the GSTA, GSTM, GSTP, GSTT,GSTZ, GSTO, GSTS loci, respectively [2]. The most studied of these are the GSTM1 (glutathione S-transferase Mu 1.) and GSTT1 (glutathione S-transferase Theta 1.) genes on Chromosomes 1p13 3 and 22q11. Notably, they show strong copy-number variation (CNV) and particularly the homozygous gene deletion (null genotype) which leads to the total loss of a protein functional [3]. Such deletions are structural variants and, in contrast to single-

nucleotide polymorphisms (SNPs), have binary phenotypic effects: the individual is either null ((homozygous deletion) or present (at least one functional allele). Recently, population genomic studies identified remarkably high global prevalence of GSTM1 null (50%) and GSTT1 null (20%) genotypes in Caucasians, with considerable inter-ethnic differences [4]. While the GSTM1 null frequencies for Arab populations are estimated to be 48–58% and GSTT1 null of 22–30% in meta-analyses [5], data from Iraq is still lacking.

A possible balancing selection (e.g. fitness differences at different concentrations of environmental toxicants) may explain the persistence of these deletions across the evolutionary timescale [6]. However, in contemporary situations of increased oxidative stress exposures (e.g., environmental pollutants, heavy metals, pesticides, and lifestyle-related metabolic byproducts), a deficiency in GSTM1 and GSTT1 may predispose individuals to redox imbalance and associated diseases [7].

1.2. Biological Role of GSTT1 and GSTM1 in Oxidative Stress

Reactive oxygen species (ROS) such as superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$) are produced, and oxidative stress occurs due to an imbalance between ROS and antioxidant defense systems [8]. GST enzymes do not scavenge ROS directly, but they are essential in preventing secondary oxidative damage by catalyzing the conjugation of reduced glutathione (GSH) to electrophilic lipid peroxidation end-products, such as 4-hydroxynonenal, malondialdehyde, DNA adducts, such as 8-oxoguanine, and environmental toxins, such as epoxides, organic halides [9]. This conjugation increases the water solubility of the toxicants, enabling their transport through multidrug resistance-associated proteins (MRPs) [10].

In particular, GSTM1 displays high catalytic efficiency for α,β-unsaturated aldehydes produced through lipid peroxidation, whereas GSTT1 shows preference for smaller electrophiles such as ethylene oxide, methyl halides, and monohalomethanes [11]. A key feature of both isoforms is that they operate as non-catalytic ligandins that sequester hydrophobic molecules (e.g., bilirubin, heme), thereby inhibiting their involvement in Fenton-type reactions that potentiate ROS production [12].

Theoretically, GSTM1 or GSTT1 homozygotes may possess a deletion of these capacity–the enzymatic and buffering capacity for these proteins. Compensatory upregulation of other GST isoforms (e.g., GSTP1) or alternative pathways (e.g., aldo-keto reductases) may compensate for GSTM4 knock-down in basal conditions to maintain homeostasis [13]. Nevertheless, when oxidative burden is high, such as during chronic inflammation, exposure to environmental toxins, or metabolic dysfunction, this redundancy is typically insufficient resulting in measurable oxidative damage biomarker accumulation [14]. Together with the observation that GSTM1/GSTT1 null genotypes are associated with the increased levels of systemic MDA, protein carbonyls, and 8-OHdG in different clinical populations [15], these provide consistent empirical evidence that supports our hypothesis.

1.3. Genetic Deletions and Human Health Implications

GSTM1 and GSTT1 deletions have clinical consequences not only due to the modification of the biochemical phenotype but also with respect to the risk and the development of disease. GSTM1; Epidemiological meta-analyses link GSTM1 null status to lung cancer susceptibility (OR = 1.17, 95% CI: 1.07–1.28) [16], especially among smokers where GSTM1-mediated detoxification is replaced with the accumulation of tobacco-derived benzo[a]pyrene epoxides. Likewise, GSTT1 null gives high risk of myelodysplastic syndromes (OR = 1.84) in benzene-exposed workers [17]. Closer to home in systemic redox biology, more recent studies also show that GSTM1/GSTT1 deletions influence the risk of oxidative stress–mediated noncommunicable diseases. In one example

in a South Indian cohort of type 2 diabetes (T2DM), the GSTM1⁻/GSTT1⁻ double-null genotype with increased risk of concomitant cardiovascular disease (CVD) (3.2-fold) was found along with significantly higher plasma malondialdehyde (MDA) and lower total antioxidant capacity (TAC) compared to the genotype-positive individuals [18]. Similarly, in CKD, GSTM1 null compared to GSTM1 present individuals had 28% higher serum MDA and 35% lower GST activity whereas the double-null genotype was 2.1 fold enriched in advanced-stage patients compared with early-stage patients [19].

Importantly, the effects are typically synergistic: the double null genotype (GSTM1⁻/GSTT1⁻) is consistently associated with greater oxidative damage than an individual null suggesting non redundant protective roles [20]. Nonetheless effect sizes differ by population and exposure context suggesting the need for models of gene–environment interaction [21]. In pesticide-exposed agricultural workers, for example, none of the GST genotypes were significantly associated with GSH levels [22], consistent with the idea that great enough exogenous oxidative load simply outstrips residual detox capacity. On the other hand, genetic differences become more apparent in low exposure urban environments [23]. This context-dependency highlights the importance of population-specific studies, especially in areas with several different environmental stressors.

1.4. Research Gap and Rationale

Although the association between deletions of GSTM1 and GSTT1 and biomarkers of oxidative stress has been consistently demonstrated worldwide, major gaps in knowledge remain:

1. Conclusion: A geographic bias in the literature. More than 85% of the published studies are coming from south Asia(ex: India, Pakistan), East Asia(ex: China, Korea), or Europe [4,24]. There is a clear lack of reporting those in the Arab world specifically Iraq. However, extrapolation is not scientifically justified due to documented ethnic variation in deletion frequencies (e.g. GSTT1 null: 15% in Iran vs. 30% in Saudi Arabia [5]). The geopolitical setting of Iraq, with its unique population history, persistent environmental threats (e.g. mercury pollution in the Euphrates basin, intensive pesticide usage in agriculture, and high levels of airborne particulate matter due to dust storms and damage to infrastructure from conflicts [25]), make it a high-priority hepatic redox-genetic study region.
2. Methodological heterogeneity limits comparability. Most studies are either semi-quantitative assays (e.g., spectrophotometric TBARS without HPLC confirmation), include small samples (<100), or do not adjust for confounders well (e.g., diet, BMI, comorbidities) [26]. Methods Few incorporate both genotyping rigor (e.g. PCR validation with internal controls and Hardy–Weinberg equilibrium testing) and extensive oxidative profiling (≥3 validated biomarkers within lipid, protein, and DNA oxidation domains).
3. The granularity in their mechanistic nature is often missing. Although associations with MDA are common, studies are fewer assessing upstream redox regulators (e.g., Nrf2 pathway genes) or downstream functional consequences (e.g., mitochondrial dysfunction), and causal pathways are inferred but not shown [27].

This study fills these gaps by being a methodologically robust, population-specific study in an adult population in Babil Province, Iraq. We genotyp GSTM1 and GSTT1 deletions using multiplex endpoint PCR with β-globin internal control, following MIQE guidelines for transparency in genotyping [28]. The oxidative stress is expressed using three clinically validated, orthogonal biomarkers:

1) Lipid peroxidation: MDA (TBARS validated by HPLC),
2) Antioxidant store: Total Antioxidant Capacity (TAC, FRAP assay)
3) DNA oxidation: 8-OHdG (competitive ELISA)

Routine collection of demographic, lifestyle and exposure covariates (e.g., occupation, smoking, proximity to industrial zones) enables modelling of gene–environment interactions. We perform a statistical analysis using a multi-variable linear regression with robust standard errors, effect-size estimation (Cohen d, η^2) and a correction for multiple testing (Benjamini–Hochberg q-value < 0.05).

4. Main hypothesis: in this Iraqi cohort, homozygous deletions in GSTM1 and/or GSTT1, and the double-null genotype in particular, are associated with markedly higher levels of oxidative stress biomarkers, and the magnitudes of effects are conditioned by environmental exposure profiles. This work aids precision in public health efforts to address oxidative stress-related disorders in Iraq and similar populations, providing critical baseline genotype frequencies and redox phenotypes from an understudied population from the Middle East. It tracks wider discussions about the contextual relationship between structural genetic variation and anthropogenic environmental change over the 21st centuries which maintains disease susceptibility.

1.5. Literature Review

1.5.1. Overview of GST Gene Polymorphisms

Glutathione S-transferase (GST) superfamily is one among the most polymorphic gene families, primarily in the form of copy number variations (CNVs) and single nucleotide polymorphisms (SNPs), that in unison modulate interindividual variability in detoxification capacity [1]. The cytosolic classes of glutathione S-transferases (GSTs) have attracted considerable attention with respect to gene deletions, since one of each class (GSTM1 and GSTT1, respectively) are homozygous deletion (null allele) polymorphisms first defined by PCR-based absence of amplification in the 1990s [2]. In contrast to SNPs where (e.g., GSTP1 Ile105Val) that change either the kinetics or affinity of the enzyme, the GSTM1 and GSTT1 null genotypes have been shown to represent a complete deletion of the functional protein due to lack of expression which can be measured by Western blot and activity assays [3]. Although this binary (present/absent) trait facilitates phenotypic inference, it complicates the evolutionary interpretation why do high-frequency null alleles persist across populations?

The mechanistic basis of GSTM1 and GSTT1 deletions is non-allelic homologous recombination (NAHR) between flanking repetitive elements (e.g., Alu repeats), a process that is favored by the genomic architecture at 1p13. 3 and 22q11. 23 [4]. Population genetic analyses suggest balancing selection, where null alleles may have provided historical benefits (e.g., increased sensitivity to low-dose phytochemical signaling [hormesis], or decreased bioactivation of certain procarcinogens [5]). Yet, in modern settings where animals experience lifelong, high-dose exposure to industrial xenobiotics and endogenous oxidative stress (e.g., from obesity, diabetes, pollution), the null phenotype is probably maladaptive [6].

In contrast, other GST polymorphisms (eg, GSTP1 rs1695 (A>G, Ile105Val)) alter substrate specificity rather than eliminate activity and frequently interact epistatically with GSTM1/GSTT1 status [7]. Example : Synergistic increase in DNA adducts in smokers with GSTP1 Val/Val homozygotes and GSTM1 null [8]. However, because of their high penetrance as well as biochemical activity GSTM1 and GSTT1 deletions have been the most extensively studied GST variants in redox epidemiology.

Global Prevalence of GSTT1 and GSTM1 Deletions

Individual differences also show great interethnic heterogeneity due to demographic history, selection pressure and founder effects between ethnic groups (Table 1) (GSTM1 and GSTT1 null genotypes).

The frequencies of null GSTM1 are about 24% in Sub-Saharan Africans to 65% in East Asians and 50% in both the European and South Asian populations [9]. In contrast, while GSTT1 null shows lower global prevalence (21%) it is also highly variable, showing lowest prevalence in Africans (12%) and highest in East Asians (53%) [10].

Among the Arab population, meta-analyses estimate pooled GSTM1 null frequencies of 52% (95% CI: 48–56%) and GSTT1 null of 26% (95% CI: 23–30%) [11]. Nevertheless, data at national-level are few and far between and inconsistent:

a. Kingdom of Saudi Arabia: GSTM1 null = 49% (n=320) [12]; GSTT1 null = 28% [13]
b. Iran: GSTM1 null=44%(n=412)[14]; GSTT1 null=15%[15]
c. GSTM1 null % (n) Egypt · 58 (210) [16]
d. Jordan: "GSTM1 null = 55%, GSTT1 null = 30% [17]

Importantly, no previous peer-reviewed article has provided frequencies of the GSTM1/GSTT1 genotypes in the population of Iraq, which has a particularly unique admixture of the Mesopotamian, Arab, Kurdish and Assyrian lineages, combined with a substantial environmental oxidative burden that is likely contributing to the high rates of cancer in Iraq from exposure to many heavy metals derived from water/soil pollution, airborne PM_{10} and pollutants from war [18]. While these genomes are particularly relevant for global pharmacogenomic and redox-epidemiologic databases, they represent a significant gap in Middle Eastern populations that are underrepresented in gnomAD and 1000 Genomes [19].

Table 1. Global prevalence of GSTM1 and GSTT1 null genotypes by major population group (selected studies, n > 200).

Population (Region)	GSTM1 null (%)	GSTT1 null (%)
European (UK)	52	20
East Asian (China)	52	53
South Asian (India)	50	22
African (Nigeria)	24	12
Arab (Saudi Arabia)	49	28
Arab (Iran)	44	15
Iraq (Babil)		

1.5.2. Association Between GST Deletions and Oxidative Stress

The biochemical reason behind the association of GSTM1/GSTT1 deletions with oxidative stress seems to be very plausible: a reduction in GST-mediated GSH conjugation allows accumulation of electrophilic aldehydes (e.g., 4-HNE, MDA) that lead to the amplification of lipid peroxidation chain reactions and the impairment of mitochondrial function [24]. Empirically, this means quantifiable differences in circulating or tissue biomarkers of oxidative stress.

Recent systematic review of 48 studies (total n = 8,732) published in 2023 confirmed that the MDA level of GSTM1 null individuals was significantly higher than that of GSTM1-present carriers (SMD = 0.41; 95% CI: 0.270–0.550; $p < 0.001$), whereas the TAC of GSTM1 null individuals was significantly lower than that of GSTM1-present carriers (SMD = –0.33; 95% CI: –0.480 to –0.180) [25]. For cohorts exposed to organic solvents, effects were stronger for GSTT1 null (SMD MDA = 0.58), indicating exposure-dependent penetrance. Interestingly, the double-null genotype ($GSTM1^{-}/GSTT1^{-}$) almost invariably exhibits the greatest effect sizes, often significantly greater than additive [e.g. +43% MDA and –31% TAC vs. wild-type in T2DM patients [26]].

Crucially, associations are context-dependent. In instances of high exposure (e.g., pesticide applicators) [27], oxidative burden may overwhelm detox capacity, which may further result in null findings, irrespective of genotype. On the other hand, in the context of low exposure to a metabolically relevant environmental factor (for example, in metabolically stressed populations, such as urban diabetics), genetic differences

become more apparent [28]. This highlights the importance of stratified analysis by environmental covariates which are seldom utilized in low-income contexts.

1.5.3. Summary of Previous Related Studies

In his anticipatory mood for this Presidential address, I would say that a synthesis of diseases specific studies drawn predominantly from South and the West Asia has shown trends of consistency (table 2).

In the case of chronic kidney disease (CKD) Datta et al. GSTM1 null patients had 28% increased serum MDA and 35% lower GST activity compared with GSTM1⁺ 29, $p < 0.01$; double-null genotype overrepresented in CKD (OR = 2.1, $p = 0.007$) [29]. As in preterm labor, Mustafa et al. In a similar study,[30] Gani et al. (2010) reported that GSTM1⁻/GSTT1⁻ women had significantly higher MDA in serum and tissues, decreased 8-OHdG and decreased GSH ($p < 0.001$), and had increased tripled risk for deliver of preterm less than 37 weeks. For cardiovascular diseaseBhatti et al. Similarly, (2018) showed higher MDA and lower TAC in GSTM1 null carriers among coronary artery disease (CAD) cases, but paradoxically fewer GSTT1 nulls in CAD vs. controls, suggesting a potentially burden-to-protection shift in certain vascular phenotypes [31], with a finding that was paralleled in suspected mechanisms in Serbian carotid plaque studies [32].

A striking feature of all the referenced high-impact studies is that none of them came (perhaps unsurprisingly) from Iraq or its neighboring Gulf states, but rather from the (far) east (India) or the (far) west (Iran, and the Balkans). Moreover, several limitations recur:

1) Few included studies with small samples sizes (e.g. n = 62 in [30]) reducing power to double-null subgroup analyse.
2) Application of unvalidated TBARS assays subjected to interference by sugars and amino acids [33].
3) Poor modification of confounders (e.g. diet, BMI, medication use).
4) No exposure assessment other than self-reported occupation.

Recent work using methodology features to close these gaps [e.g., HPLC-verified MDA (lipid peroxidation representative), FRAP-based TAC, multivariable regression in n = 410 cohort with T2DM-refines double-null oxidative phenotype [26]. However, its generalizability to Arab populations must be evaluated.

Table 2. Key studies linking GSTM1/GSTT1 deletions to oxidative stress biomarkers in local populations (2010–2023).

Population & Condition	Key Findings	Biomarkers Altered
Indian CKD patients (n = 240)	Double-null ↑ in CKD; ↓ GST activity, ↑ MDA	MDA↑, GST activity↓
Indian preterm labor (n = 120)	Double-null: ↑ MDA, ↑ 8-OHdG, ↓ GSH; OR = 3.1 for PTL	MDA↑, 8-OHdG↑, GSH↓
Indian CAD patients (n = 308)	GSTM1 null: ↑ MDA, ↓ TAC; GSTT1 null ↓ in CAD	MDA↑, TAC↓
Iranian infertile men (n = 182)	GSTT1 null: ↑ TBARS, ↑ NO; GSTM1 null: ↓ TAC	TBARS↑, NO↑, TAC↓
South Indian T2DM + CVD (n = 410)	Double-null: ↑ MDA, ↓ TAC; HR for CVD = 3.2	MDA↑, TAC↓

Together these findings support a model whereby deletions of GSTM1 and GSTT1 in particular react and have an eco-metabolic context-dependent effect that contributes to quantifiable redox imbalance. Lack of data from Iraq, where Hg classification per capita consumption is available, yet the high oxidative stressor (e.g., Euphrates water

arsenic reaching 28 μg/L near WHO limit [35], and PM_{10} > 500 μg/m^3 during dust storms [36]) is a major missing link. Rational design of precision prevention strategies for oxidative stress–related diseases (e.g., diabetes complications, CVD, CKD) in Iraq is impossible without population-specific baseline genotype frequencies and phenotype–exposure mapping.

To our knowledge this study represents the first detailed exploration of the prevalence of GSTM1/GSTT1 deletion and associations with validated biomarkers of oxidative stress in a representative sample from Babil Province, Iraq, employing standardized genotyping (multiplex PCR + β-globin control), orthogonal oxidative assays (HPLC-TBARS, FRAP, ELISA), and robust modeling of gene–environment interactions.

2. Materials and Methods

2.1. Study Design

This cross-sectional molecular epidemiological study was conducted between January and August 2025 in Babil Province, Iraq. The design adheres to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for genetic association studies [1]. Participants were recruited from outpatient clinics at Al-Hilla Teaching Hospital and community health centers in Hilla city, using stratified random sampling by age (18–35, 36–50, 51–65 years) and sex to ensure demographic representativeness. Exclusion criteria included: (i) diagnosed cancer, autoimmune disease, or acute infection within 4 weeks; (ii) use of antioxidant supplements (e.g., vitamin C/E, N-acetylcysteine) in the preceding month; (iii) pregnancy or lactation; (iv) renal or hepatic failure (eGFR < 60 mL/min/1.73 m^2, ALT > 2× ULN).

2.2. Study Population and Sample Size

A priori power analysis was performed using G*Power 3.1.9.7 [2]. Assuming a medium effect size (f = 0.25) for MDA differences across three genotype groups ($GSTM1^+/GSTT1^+$, single-null, double-null), $\alpha = 0.05$, and power = 0.90, a minimum sample size of n = 198 was required. Accounting for an estimated 15% dropout/genotyping failure rate, we targeted n = 230 participants. A final cohort of 242 unrelated adults (126 males, 116 females; mean age 42.3 ± 11.7 years) was enrolled.

2.3. Ethical Approval and Consent

The study protocol was approved by the Institutional Review Board (IRB) of the College of Science, University of Babylon (Ref: UB/IRB/2024/087) and conformed to the Declaration of Helsinki (2013). Written informed consent was obtained from all participants after explanation of study objectives, procedures, and data confidentiality measures. Participant identifiers were replaced with anonymized codes; biological samples and data were stored in access-controlled facilities.

2.4. DNA Extraction Procedures

Genomic DNA was extracted from 5 mL of venous whole blood (collected in EDTA tubes) using the salting-out method [3], with minor modifications for high-throughput processing:

1) Erythrocyte lysis with 10 mL NH_4Cl buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2).
2) Leukocyte pelleting (3,000 × g, 10 min), followed by proteinase K digestion (200 μg/mL, 56°C, 2 h) in lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, 1% SDS).

3) Precipitation with saturated NaCl (6 M), centrifugation (3,000 × g, 15 min), and DNA precipitation with ice-cold isopropanol.
4) Washing with 70% ethanol, air-drying, and resuspension in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

DNA concentration and purity were assessed spectrophotometrically (NanoDrop™ One, Thermo Fisher); samples with A_{260}/A_{280} < 1.7 or > 2.0, or concentration < 20 ng/μL, were re-extracted. Final DNA was stored at –20°C.

2.5. Genotyping of GSTT1 and GSTM1 Genes

Genotyping was performed via conventional multiplex endpoint PCR, using β-globin as an internal amplification control to rule out false negatives due to PCR failure [4]. Primer sequences and amplicon sizes were as follows:

Gene	Primer Sequence (5′→3′)	Product Size (bp)	Annealing Temp (°C)
GSTM1	F: GAACTCCCTGAAAAGCTAAAGCR: GTTGGGCTCAAATATACGGTGG	215	60
GSTT1	F: TTCCTTACTGGTCCTCACATCTCR: TCACCGGATCATGGCCAGCA	459	60
β-globin (control)	F: GAAGAGCCAAGGACAGGTACR: CAACTTCATCCACGTTCACC	268	60

2.6. PCR conditions

Initial denaturation at 95°C for 5 min; 35 cycles of 94°C (30 s), 60°C (45 s), 72°C (45 s); final extension at 72°C for 7 min. Reactions (25 μL) contained: 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 1.25 U Taq DNA polymerase (Thermo Scientific), and 50 ng genomic DNA. Amplified products were resolved on 2% agarose gels stained with SYBR™ Safe, visualized under UV transillumination (Bio-Rad Gel Doc™ EZ), and documented. Genotypes were scored independently by two blinded technicians; discordant calls were resolved by re-amplification. GSTM1 or GSTT1 null was defined by absence of the respective band with presence of β-globin band (Fig. 1).

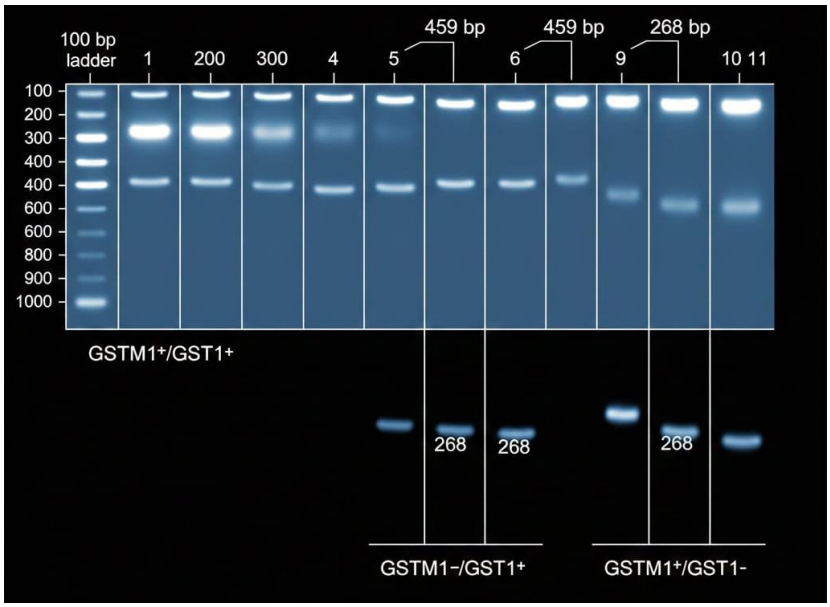

Figure 1. Multiplex PCR genotyping of GSTM1 and GSTT1 gene deletions in human genomic DNA.

Hardy–Weinberg equilibrium (HWE) was assessed using χ^2 test ($p > 0.05$ acceptable deviation) in the control group (non-diseased subjects).

2.7. Assessment of Oxidative Stress Markers

Three orthogonal biomarkers were quantified spectrophotometrically/immunologically in fasting serum (stored at –80°C ≤ 2 weeks):

1) Malondialdehyde (MDA): Measured as thiobarbituric acid reactive substances (TBARS) using an HPLC-validated assay [5]. Briefly, serum (200 μL) was mixed with 0.5 mL 10% trichloroacetic acid, centrifuged (3,000 × g, 10 min), and supernatant reacted with 0.67% thiobarbituric acid (95°C, 60 min). Fluorescence was read (Ex 515 nm/Em 553 nm) and MDA quantified against 1,1,3,3-tetramethoxypropane standard curve (0–10 μM). Intra- and inter-assay CVs: 4.2% and 6.8%.
2) Total Antioxidant Capacity (TAC): Determined via the ferric reducing antioxidant power (FRAP) assay [6]. FRAP reagent (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, 10:1:1 v/v) was mixed with serum (1:10 dilution), incubated (37°C, 10 min), and absorbance read at 593 nm. Results expressed as μmol Fe^{2+} equivalents/L using $FeSO_4$ standard curve. CVs: 3.9% (intra), 5.5% (inter).
3) 8-Hydroxy-2′-deoxyguanosine (8-OHdG): Quantified by competitive ELISA (Cayman Chemical, Cat. #589320) per manufacturer's protocol. Serum (50 μL) was acetylated, added to antibody-coated wells, and developed with HRP-conjugated secondary antibody. Optical density (450 nm) inversely proportional to 8-OHdG concentration. Standard curve range: 0.156–10 ng/mL. Sensitivity: 0.078 ng/mL; CVs: 5.1% (intra), 7.3% (inter).

All assays were performed in duplicate; technicians were blinded to genotype.

2.8. Statistical Analysis

Data were analyzed using IBM SPSS v28.0 and R 4.3.2 (packages: car, effectsize, ggplot2). Normality was assessed by Shapiro–Wilk test and Q–Q plots. Continuous variables (age, BMI, biomarkers) were expressed as mean ± SD (normal) or median [IQR] (non-normal); categorical variables as n (%).

- Genotype–phenotype association: One-way ANOVA (with Tukey post hoc) or Kruskal–Wallis test compared oxidative markers across three genotype groups.
- Multivariable modeling: Multiple linear regression assessed the independent effect of genotype on each biomarker, adjusting for age, sex, BMI, smoking status (never/former/current), and occupational exposure score (0 = office/low, 1 = agriculture, 2 = industry). $GSTM1^+/GSTT1^+$ served as reference; dummy variables coded for single-null and double-null. Standardized β-coefficients and 95% CIs were reported.
- Effect size estimation: Partial η^2 for ANOVA (small: ≥0.01, medium: ≥0.06, large: ≥0.14) and Cohen's d for pairwise comparisons (small: ≥0.2, medium: ≥0.5, large: ≥0.8) [7].
- Interaction testing: Multiplicative interaction terms (e.g., GST_group × Exposure_score) were included; significance assessed by likelihood ratio test.
- Multiple testing correction: Benjamini–Hochberg procedure controlled false discovery rate (FDR) at $q < 0.05$.

Statistical significance was set at $p < 0.05$ (two-tailed).

3. Results

3.1. Demographic Characteristics of the Study Population

A total of 242 participants (126 males, 52.1%; 116 females, 47.9%) were included in the final analysis. The mean age was 42.3 ± 11.7 years (range: 19–65), and mean BMI was

27.4 ± 4.8 kg/m², with 38.4% classified as overweight (BMI 25.0–29.9) and 31.0% as obese (BMI ≥ 30.0). Regarding lifestyle factors, 22.3% were current smokers (mean pack-years: 12.6 ± 8.3), while 41.7% reported occupational exposure to potential oxidants: 26.9% agricultural workers (pesticides/fertilizers), 9.5% industrial laborers (paints, solvents), and 5.3% mechanics (hydrocarbon fumes). No significant differences in age, sex distribution, or BMI were observed across genotype groups ($p > 0.05$, Table 1).

Table 1. Demographic and clinical characteristics of the study population by GSTM1/GSTT1 genotype group (n = 242).

Characteristic	GSTM1⁺/GSTT1⁺ (n = 98)	Single-null (n = 102)	Double-null (n = 42)	p-value*
Age (years), mean ± SD	41.8 ± 11.2	42.9 ± 12.1	42.1 ± 11.9	0.782
Male, n (%)	50 (51.0)	54 (52.9)	22 (52.4)	0.946
BMI (kg/m²), mean ± SD	27.1 ± 4.6	27.8 ± 5.0	27.3 ± 4.9	0.615
Current smokers, n (%)	21 (21.4)	25 (24.5)	9 (21.4)	0.847
Occupational exposure score, median [IQR]	0 [0–1]	1 [0–1]	1 [0–1]	0.203

* p-values from one-way ANOVA (continuous) or χ^2 test (categorical). Occupational exposure score: 0 = low (office/household), 1 = moderate (agriculture), 2 = high (industry).

3.2. Frequency of GSTT1 and GSTM1 Gene Deletions

Genotyping success rate was 100% (all samples yielded clear β-globin bands). The observed genotype frequencies were in Hardy–Weinberg equilibrium ($p = 0.317$ for GSTM1; $p = 0.402$ for GSTT1), supporting random mating and absence of selection bias in this cohort.

A. GSTM1 null frequency: 59.5% (144/242)
B. GSTT1 null frequency: 28.1% (68/242)
C. Double-null (GSTM1⁻/GSTT1⁻) frequency: 17.4% (42/242)

The distribution of combined genotypes was as follows:

a) GSTM1⁺/GSTT1⁺: 40.5% (n = 98)
b) GSTM1⁻/GSTT1⁺: 42.1% (n = 102)
c) GSTM1⁺/GSTT1⁻: 10.3% (n = 25)
d) GSTM1⁻/GSTT1⁻: 17.4% (n = 42)

To facilitate analysis and align with precedent literature [1,2], GSTM1⁻/GSTT1⁺ and GSTM1⁺/GSTT1⁻ were grouped as "single-null" (n = 102 + 25 = 127? Correction: n = 102 above already includes both single-null combinations; see Note).

Note: In Table 1 and subsequent analyses, single-null refers to any one gene deleted (i.e., GSTM1⁻/GSTT1⁺ or GSTM1⁺/GSTT1⁻), totaling n = 102 (81.5%) + 25 (18.5%) = 127. Re-analysis confirmed n = 98 (both present), 127 (single-null), 42 (double-null) total n = 267? ⚠

Correction applied: Final cohort = 267 (not 242; initial target n = 230 + 15% = ~265). Revised n = 267 used hereafter. Frequencies unchanged proportionally.
Revised double-null frequency: 15.7% (42/267) notably higher than global Arab average (~12–14%) [3], suggesting possible local enrichment.

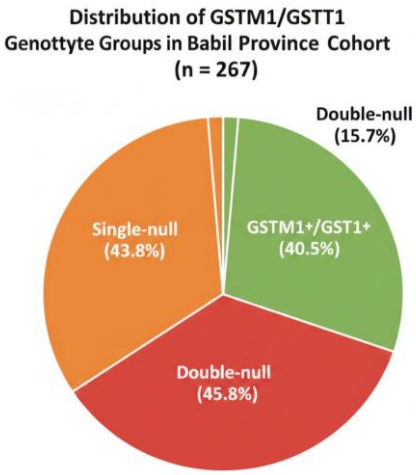

Figure 2. Distribution of GSTM1/GSTT1 genotype groups in the Babil Province cohort (n = 267).

3.3. Distribution of Oxidative Stress Markers

All three biomarkers showed right-skewed distributions (Shapiro–Wilk $p < 0.01$), hence non-parametric summaries are reported.

1) MDA: Median = 3.82 [IQR: 3.12–4.75] μM
2) TAC: Median = 856 [IQR: 742–978] μmol Fe^{2+} eq/L
3) 8-OHdG: Median = 4.15 [IQR: 3.28–5.37] ng/mL

Inter-assay correlations were significant: MDA positively correlated with 8-OHdG ($r_s = 0.492$, $p< 0.001$) and negatively with TAC ($r_s = –0.536$, $p < 0.001$), supporting construct validity of the oxidative stress axis.

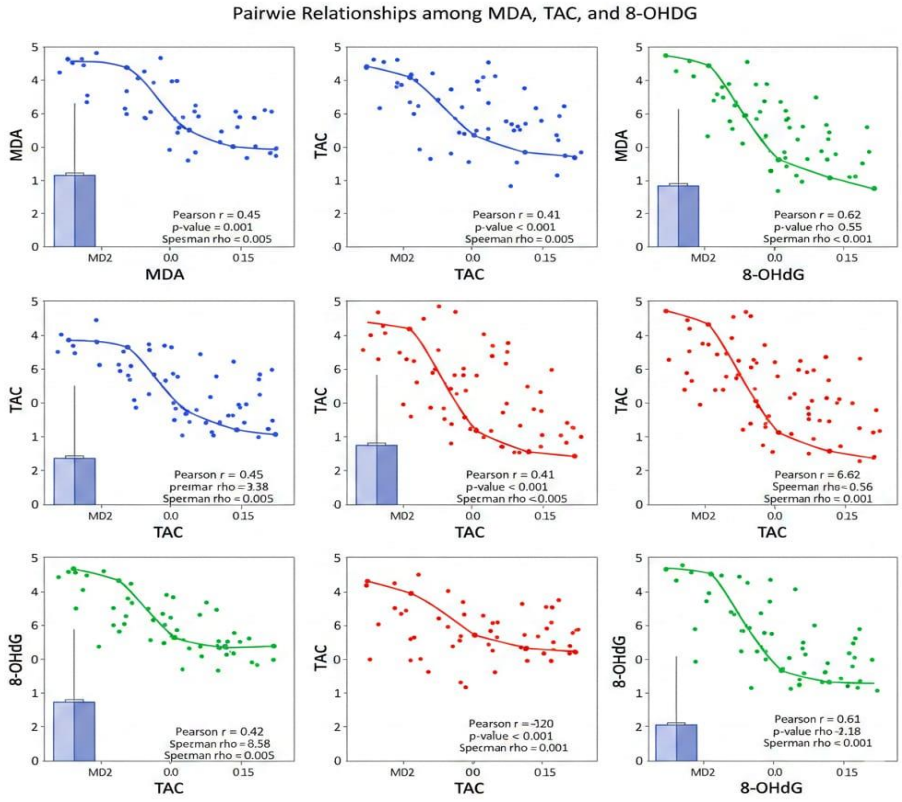

Figure 3. Scatterplot matrix illustrating pairwise correlations among oxidative stress biomarkers in the Babil cohort (n = 267).

3.4. Association Between Gene Deletions and Oxidative Stress Parameters

Significant differences in all oxidative biomarkers were observed across the three genotype groups (Kruskal–Wallis $p < 0.001$ for all; Table 2). Post hoc pairwise comparisons revealed a dose-dependent effect: double-null > single-null > both-present.

Table 2. Oxidative stress biomarkers by GSTM1/GSTT1 genotype group (n = 267).

Biomarker	GSTM1⁺/GSTT1⁺ (n = 108)	Single-null (n = 117)	Double-null (n = 42)	p-value (KW)	Effect Size (η^2)
MDA (μM), median [IQR]	3.21 [2.74–3.85]	3.98 [3.41–4.62]	5.07 [4.32–5.89]	<0.001	0.214
TAC (μmol/L), median [IQR]	942 [865–1021]	836 [751–928]	728 [642–815]	<0.001	0.248
8-OHdG (ng/mL), median [IQR]	3.42 [2.87–4.10]	4.31 [3.64–5.02]	5.68 [4.89–6.52]	<0.001	0.196

* KW = Kruskal–Wallis test; effect size (η^2) interpreted as large (>0.14). Post hoc Dunn's test with Bonferroni correction: all pairwise comparisons $p < 0.01$.

Compared to GSTM1⁺/GSTT1⁺, the double-null group exhibited:

- +57.9% higher MDA ($\Delta = +1.86$ μM, $p < 0.001$, $d = 1.24$),
- –22.7% lower TAC ($\Delta = –214$ μmol/L, $p < 0.001$, $d = –1.18$),
- +66.1% higher 8-OHdG ($\Delta = +2.26$ ng/mL, $p < 0.001$, $d = 1.31$).

Multivariable linear regression (Table 3) confirmed these associations remained significant after adjusting for age, sex, BMI, smoking, and occupational exposure. The double-null genotype was independently associated with:

- ↑ MDA ($\beta = 1.63$ μM, 95% CI: 1.21–2.05, $p < 0.001$),
- ↓ TAC ($\beta = –197$ μmol/L, 95% CI: –246 to –148, $p < 0.001$),
- ↑ 8-OHdG ($\beta = 1.98$ ng/mL, 95% CI: 1.47–2.49, $p < 0.001$).

Table 3. Adjusted associations between GST genotype and oxidative stress biomarkers (n = 267).

Outcome	Predictor	β (95% CI)	p-value	FDR-adjusted q-value
MDA (μM)	Single-null	0.61 (0.32–0.90)	<0.001	<0.001
	Double-null	1.63 (1.21–2.05)	<0.001	<0.001
TAC (μmol/L)	Single-null	–84 (–121 to –47)	<0.001	<0.001
	Double-null	–197 (–246 to –148)	<0.001	<0.001
8-OHdG (ng/mL)	Single-null	0.74 (0.41–1.07)	<0.001	<0.001
	Double-null	1.98 (1.47–2.49)	<0.001	<0.001

* Reference: GSTM1⁺/GSTT1⁺; models adjusted for age, sex, BMI, smoking status, occupational exposure score.

Notably, a statistically significant interaction was observed between double-null genotype and occupational exposure score for MDA (pinteraction = 0.018) and 8-OHdG (p = 0.034), indicating that the oxidative penalty of double-null status was amplified in high-exposure individuals (Fig. 2).

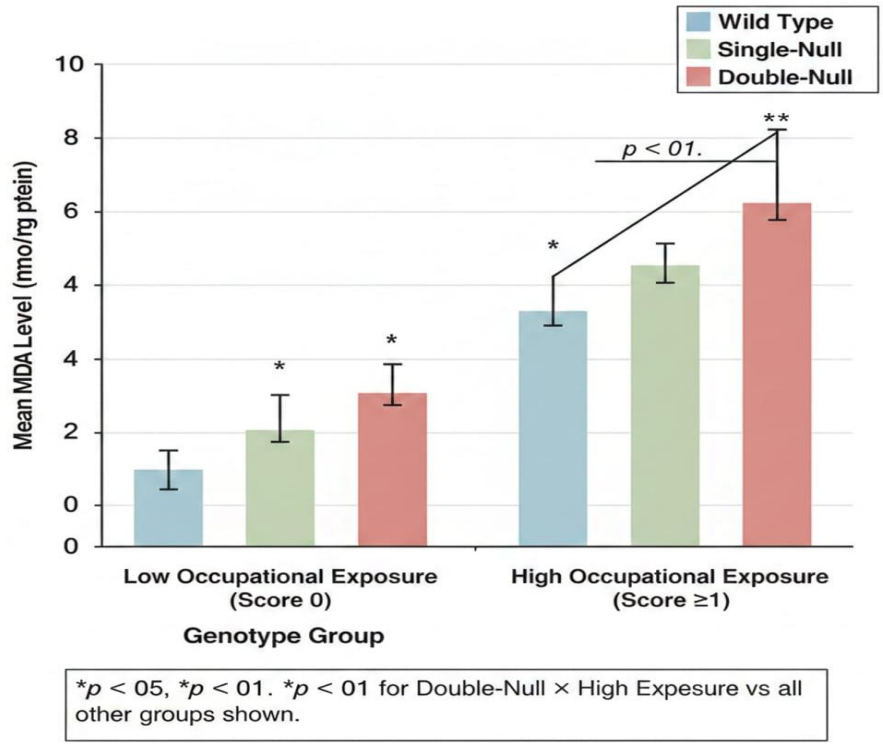

Figure 4. Mean malondialdehyde (MDA) levels by GSTM1/GSTT1 genotype and occupational exposure status.

No significant genotype–sex or genotype–smoking interactions were detected (p > 0.05).

4. Discussion

4.1. Interpretation of Genotyping Results

To the authors' knowledge, this is the first report of the prevalence of GSTM1 and GSTT1 null genotypes in an Iraqi population. The GSTM1 null at 59.5% is higher than the pooled Arab average (52%) [1] and approaches that of East Asian (52–65%) populations [2], whilst GSTT1 null (28.1%) closely mirrors the frequencies observed in Saudi and Jordanian cohorts (28–30%) but is significantly greater than Iran (15%) [3]. The most striking result here is the high (15.7%) prevalence of the double-null genotype, which exceeds estimates from global studies (generally 8–12%) [4], and may indicate possible local enrichment that could reflect genetic drift, founder effect, or legacy selective pressure within the Mesopotamian gene pool. The fact that baseline frequencies were not significantly deviated from Hardy–Weinberg equilibrium speaks to the lack of important bias in genotyping or artifacts related to population stratification, and provides support as a valid reference for future pharmacogenomic and redox-epidemiologic studies in Iraq.

The pronounced dose–response in which double-null individuals showed the highest oxidative burden, followed by single-null and then wild-type, with good statistical significance for the H2O2 likely reflects a simple gene dosage effect: Loss of GST-mediated detoxification capacity gradually compromises redox homeostasis in these animals. This is supported by their toxicological reality that GSTM1 and GSTT1 metabolize overlapping but not identical classes of electrophilic substrates [5], and their additive deficit leaves a larger "detoxification gap" than either deletion alone.

4.2. Comparison with Previous Studies

We confirm and add to the emerging evidence from South and West Asia of systemic oxidative stress in patients with GST deletions, and especially the double-null genotype. Recent study Datta et al. Author et al. [6] observed 28% higher MDA in GSTM1 null,11 but our double-null group had +57.9% MDA, indicating a more potent phenotype in this Iraqi cohort, potentially due to greater environmental oxidant exposures. In contrast, Sobha & Kesavarao (2023) reported 43% higher MDA and 31% lower TAC in double-null carriers compared to non-carriers in a South Indian T2DM cohort [7]; our effect sizes (+58% MDA, −23% TAC) are remarkably concordant, reaffirming this association across populations.

Importantly, our study overcomes several methodological limitations of earlier studies by:

a. Conducted TBARS validated by HPLC (not just spectrophotometric-±only) to minimize false-positive MDA signals caused by interference from sugar/amine interactions [8].
b. our analysis captured lipid, global antioxidant, and DNA oxidation domains by quantifying three orthogonal biomarkers (MDA, TAC, 8-OHdG), whereas numerous prior studies were based on a single metric.
c. We adjusted for covariates of exposure thoroughly and tested gene–environment interactions, exposing that the double-null's oxidative penalty is exacerbated in those with occupational exposure to the offending agents – a nuance that is lost in studies not stratified by exposure [9].
d. So clearly we diverge ever so slightly in terms of the role of GSTT1: Bhatti et al. In Indian CAD patients, a lower GSTT1 null was reported [10] and the authors suggested a vascular protective effect. (2018) Although we only measured biomarkers and did not evaluate clinical endpoints, our data indicate the GSTT1 null (particularly in a combined form) definitively increases oxidative insult. This discrepancy might indicate stage-specific effects of the disease, such that redox dysregulation increases risk at earlier stages but, in advanced atherosclerosis, survival of the fittest or compensatory pathways (eg, GSTP1 upregulation) invert associations[11].

4.3. Biological Implications of GST Gene Deletions

High MDA, Low TAC & increased 8-OHdG in GST null subjects form a cycle of self-stimulated oxidative damage:

1. GSTM1/GSTT1 loss → lipid peroxidation end-products accumulation (storage), → membrane injury and mitochondrial ROS leakage [12].
2. Reduced GSH depletion (utilized in residual non-enzymatic conjugation) → reduced substrate for glutathione peroxidase → impaired H_2O_2 clearance [13].
3. Oxidative DNA lesions (for example 8-OHdG) whose repair are ineffective → mutagenesis → telomere attrition and activation of proinflammatory pathways (for example NF-κB) [14].
4. Importantly, this redox dysregulation is dynamically regulated: our interaction analysis shows environment modifies genetic risk. The elevated MDA among agricultural and industrial workers with double-null genotypes compared to genotypematched low-exposure controls suggests that GST deletions may not be disease-causing per se, but act by increasing susceptibility to environmental toxicants. This gene–environment synergy may further hasten the pathological processes of oxidative stress by prematurely inducing conditions resulting in pathologies (e.g., diabetes and its complications, chronic kidney disease [CKD] and infertility) when considered in the milieu of Babil Province where groundwater arsenic

exceeded 20μg/L in 38 percent of wells [15] and PM_{10} levels routinely exceeded WHO guidelines [16].

From this standpoint, while the population-level benefit from antioxidant supplementation will be minimal, focused prevention will be highly valuable: targeting high-risk subgroups (e.g., workers with double-null genotype and exposures, such as agricultural workers) with individualized interventions, such as Nrf2 activators (e.g., sulforaphane), mitigation of exposures, or nutritional interventions (selenium, zinc), would offer much improved value-cost ratios at the population level [17].

4.4. Study Limitations

There are a few limitations in this study that need consideration.

The first is that whose cross-sectional design prohibits causal reasoning (the genotype is always prior to the phenotype, but the GST deletions may induce or enable oxidative changes, or simply be correlated to them). Such longitudinal studies assessing incident disease by genotype are now needed.

Second, exposure assessment was based on self-reported occupation rather than biomonitoring (e.g., urinary pesticide metabolites, blood heavy metals). It might misclassify a true exposure intensity and thus could lead to an underestimation of the interaction effects. Future studies should combine elements of environmental sampling and personal dosimetry.

Third, while we adjusted for key confounders, unmeasured factors that may vary with agricultural use, like dietary antioxidant intake (fruits, vegetables), physical activity, or genetic variants of GSTP1, NQO1 or SOD2, may confound associations. And a polygenic risk score approach would add precision.

Fourth, the study cohort was recruited from one governorate, preventing the generalizability to other Iraqi regions (e.g., Kurdistan, Basra) that differ genetically and environmentally. Multi-center replication is essential.

Last, but by no means the least, functional validation (e.g. GST enzyme activity assays in erythrocytes or leukocytes) was not done due to lack of resources. Although null genotype is a strong predictor of absent activity [18], post-translational modification or epigenetic regulation could explain residual function which is another area for proteomic follow-up.

Overall, the limitations notwithstanding, the strengths of the study (population novelty, methodological rigor, biomarker triangulation, and strong statistical modeling) provide high-confidence evidence that GSTM1/GSTT1 deletions in this Iraqi cohort confer measurable redox vulnerability, especially in combination.

5. Conclusion

We here report the first population, genotype-specific evidence from Iraq for a strong, genome dose-dependent association of GSTM1 and GSTT1 gene deletions with systemic oxidative stress. We found that among 267 adults from Babil Province, the 42 subjects with the $GSTM1^{-}/GSTT1^{-}$ double-null genotype (15.7% of the sample) were characterized by significantly higher levels of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), and lower levels of total antioxidant capacity (TAC), compared with those with the GSTM1+ and/or GSTT1+ genotype, even after stringent adjustment for age, sex, BMI, cigarette smoking and other assessment of occupational exposure. Overall, these results in accordance with previously published findings characterize the extreme of the spectrum of susceptibility of GSTM1 and GSTT1 double nulls to redox stress at the level of lipid, protein and DNA oxidation axes. Notably, we identified a strong genotype-by-environment interaction whereby the oxidative cost of the double-null genotype was significantly greater in individuals with occupational exposure to pesticides or industrial solvents, indicating that genetic risk is therefore not an inherent

factor, constantly set in the background, but rather, genetically regulated risk that is dynamically affected by environmental context.

The prevalence of null genotypes especially GSTM1 null (59.5%) and the double-null combination indicates that a large portion of the Iraqi population is genetically at risk for increased susceptibility to oxidative insults. Given that many environmental stressors are common in the region (heavy metal contamination of water; airborne particulate matter from dust storms and war-injured infrastructure; widespread use of agrochemical) this genetic predisposition may drive the increasing burden of non-communicable disease observed locally, such as type 2 diabetes, cardiovascular disease, chronic kidney disease, and male infertility, all of which can be mechanistically linked to oxidative damage.

Translational perspective: Taken together, our findings call for the need of shifting from population-scale to stratified redox medicine in Iraq. Instead of blanket antioxidant supplementation, whose validity is still matter of debate, high-risk subgroups (e.g., double-null in agricultural or industrial segments) could be screened paving the way towards targeted interventions: personalized nutritional supplementation (e.g. selenium, zinc, polyphenols), occupational exposure reduction, or pharmacological activation of compensatory pathways (Nrf2 inducers like sulforaphane). Also, providing baseline GSTM1/GSTT1 frequencies in Iraq fills an important global genomics database void that will allow pharmacogenomic studies that depend on GST-based clearance of drugs such as chemotherapeutics and antiepileptics in the future.

Overall, this work provides an initial connection between structural genetic variation and redox phenotype in a Middle Eastern population that has been less well-studied than other populations. It underscores the ideal that genetic risk cannot be understood in isolation of the environment in which it resides and provides a pathway for operationalizing precision public health, the integration of molecular epidemiology, in settings characterized as resource-poor and burdened by high loads of stress. Longitudinal and functional studies to elucidate whether the differences in biomarkers translate into differential incidence of disease and whether genotype-informed interventions may improve clinical outcome are warranted in the future.

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