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Association of *GSTM1* and *GSTT1* Null Genotypes with Disease Severity and Serum Cytokine Levels in Hospitalized COVID-19 Patients

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Abstract

Background: The clinical course of COVID-19 is highly variable, ranging from asymptomatic infection to critical illness with hyperinflammation and multiorgan failure. Oxidative stress plays a central role in COVID-19 pathogenesis, and genetic polymorphisms in glutathione S-transferase (GST) enzymes, particularly *GSTM1* and *GSTT1* null genotypes, may impair antioxidant defense and exacerbate inflammatory responses. This study aimed to investigate the association of *GSTM1* and *GSTT1* null genotypes with both disease severity and serum cytokine levels in hospitalized COVID-19 patients. Methods: This cross-sectional study enrolled 137 COVID-19 patients hospitalized during the second pandemic wave (July–September 2020). Patients were stratified into mild (n = 67) and severe (n = 70) groups based on clinical criteria. *GSTM1* and *GSTT1* polymorphisms were determined by multiplex polymerase chain reaction. Serum levels of 13 cytokines were measured using flow cytometry. Logistic regression analyzed genotype associations with disease severity, and multivariate linear regression assessed relationships between null genotypes and pro-inflammatory cytokine levels (IL-6, TNF-α, IL-17A, IFN-γ), adjusted for age, sex, hypertension, and diabetes. Results: The *GSTT1* null genotype was significantly associated with severe COVID-19 (adjusted OR = 2.56, 95% CI: 1.08–6.07, $p = 0.032$). Severe patients exhibited significantly elevated levels of IL-6 (75.6% increase, $p = 0.008$), TNF-α (69.4% increase, $p = 0.005$), IL-17A (54.4% increase, $p = 0.016$), and IFN-γ (10.1% increase, $p = 0.021$). Both *GSTM1* and *GSTT1* null genotypes were associated with higher levels of these cytokines, with stronger effects observed for *GSTT1* null. In multivariate analysis, *GSTT1* null independently predicted elevated IL-6 (β = 52.6, $p = 0.003$), TNF-α (β = 13.8, $p = 0.002$), IL-17A (β = 2.4, $p = 0.001$), and IFN-γ (β = 56.4, $p = 0.012$). The combined both null genotype showed the strongest associations but was limited by small sample size (n = 10) and should

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be interpreted with caution. Conclusions: The *GSTT1* null genotype is associated with severe COVID-19 and appears to be associated with heightened pro-inflammatory cytokine responses, particularly IL-6, TNF-α, IL-17A, and IFN-γ. These findings suggest a potential role for genetic impairment of antioxidant defense may contribute to hyperinflammation in COVID-19 hyperinflammation, although validation in larger cohorts is needed.

Keywords: GSTM1; GSTT1; COVID-19; disease severity; cytokines; oxidative stress; genetic polymorphism; SARS-CoV-2

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in unprecedented global morbidity and mortality since its emergence in late 2019 [1]. While a substantial proportion of infected individuals experience mild or asymptomatic disease, a subset of patients develops severe clinical manifestations characterized by acute respiratory distress syndrome (ARDS), multiorgan dysfunction, and fatal outcomes [2]. The clinical course of COVID-19 is highly variable, ranging from uncomplicated illness to critical disease requiring intensive care support [3].

The pathogenesis of severe COVID-19 is driven by a dysregulated host immune response, culminating in excessive release of pro-inflammatory cytokines—a phenomenon widely recognized as the "cytokine storm" [4]. This hyperinflammatory state is characterized by elevated levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and IL-17A, which contribute to endothelial dysfunction, vascular leakage, and widespread tissue damage [5,6]. Several risk factors for severe disease have been identified, including advanced age, male sex, and comorbidities such as hypertension and diabetes mellitus [7–9]. However, a considerable number of patients without these conventional risk factors still progress to severe disease, suggesting that additional host genetic factors may influence susceptibility to severe COVID-19 [10].

Oxidative stress has emerged as a critical component in COVID-19 pathophysiology [11]. SARS-CoV-2 infection triggers excessive production of reactive oxygen species (ROS) through activation of phagocytic cells and mitochondrial dysfunction, leading to an imbalance between oxidants and antioxidant defense mechanisms [12,13]. This pro-oxidative environment not only facilitates viral replication but also amplifies inflammatory signaling cascades, contributing to the development of ARDS and multiorgan failure [14,15].

Glutathione S-transferases (GSTs) are a superfamily of phase II detoxification enzymes that play an essential role in maintaining cellular redox homeostasis by catalyzing the conjugation of glutathione to electrophilic compounds, thereby neutralizing oxidative stress products [16]. Among the eight classes of cytosolic GST enzymes, the mu (GSTM1) and theta (GSTT1) isoforms are particularly important due to the presence of common homozygous deletion polymorphisms (null genotypes) that result in complete loss of enzyme activity [17]. These specific isoforms were selected for investigation because: (1) they exhibit the highest frequency of null polymorphisms in most populations (20–50%), making them genetically informative; (2) they play complementary roles in detoxifying distinct classes of oxidative stress products—*GSTM1* metabolizes aromatic compounds and lipid peroxidation products, while *GSTT1* acts on smaller electrophiles including oxidized lipid fragments; and (3) both are constitutively expressed in lung tissue, making them directly relevant to respiratory viral infections [17,18]. These null variants have

been associated with increased susceptibility to various oxidative stress-related diseases, including cardiovascular disorders, chronic obstructive pulmonary disease, and certain malignancies [18,19].

The potential role of *GSTM1* and *GSTT1* polymorphisms in COVID-19 outcomes has recently garnered research attention [20]. A systematic review by Villegas Sánchez et al. examining GST gene polymorphisms in SARS-CoV-2 pathogenesis highlighted that oxidative stress persistence may be linked to disease severity, although findings regarding *GSTM1* and *GSTT1* isoforms remain inconsistent across studies [21]. Abbas et al. demonstrated that COVID-19 patients with the *GSTT1* null genotype had a significantly higher risk of mortality (HR = 2.28, 95% CI: 1.013–5.141), while the combination of *GSTM1* active and *GSTT1* null genotypes was associated with a 2.72-fold increased risk of death [22]. Similarly, a study from Serbia by Coric et al. reported that the cumulative effect of multiple risk genotypes, including GST polymorphisms, conferred an 11.86-fold increased risk for developing severe COVID-19 [23].

Despite these findings, the mechanistic link between *GSTM1/GSTT1* null genotypes and COVID-19 severity remains incompletely understood. While several studies have demonstrated associations between these genetic variants and disease susceptibility [23] or mortality [22], the specific pathways through which they may influence clinical outcomes have not been fully elucidated. Given the central role of GST enzymes in mitigating oxidative stress [16], and the well-established connection between oxidative stress and pro-inflammatory cytokine production [13,24], we hypothesized that individuals with null genotypes would exhibit a heightened inflammatory response due to impaired antioxidant capacity. However, the specific association between these genetic variants and the cytokine profile characteristic of severe COVID-19 has not been systematically investigated. Therefore, the present study aimed to evaluate the association of *GSTM1* and *GSTT1* null genotypes with disease severity and serum levels of key pro-inflammatory cytokines in a cohort of hospitalized COVID-19 patients. We further sought to determine whether these genetic variants independently predict cytokine elevations after adjustment for established risk factors. Elucidating these relationships may contribute to improved risk stratification and provide insights into the interplay between genetic susceptibility, oxidative stress, and hyperinflammation in COVID-19 pathogenesis.

2. Materials and Methods

2.1. Study Population and Design

This exploratory cross-sectional study enrolled 137 patients hospitalized with COVID-19 at the Clinical Hospital Center Kosovska Mitrovica, Serbia, during the second pandemic wave (July to September 2020). All participants were residents of Serbian municipalities in Kosovo and Metohija, Serbia. The results presented in this paper are part of a larger database originating from this patient cohort. Prior to enrollment, each patient received comprehensive information regarding the study objectives and procedures, after which written informed consent was obtained for participation and blood sample collection. The study protocol was approved by the Ethics Committee of the Clinical Hospital Center Kosovska Mitrovica and the Faculty of Medicine, University of Pristina, temporarily seated in Kosovska Mitrovica (approval number: 10-1257; dated 23 July 2020).

Severity classification was based on clinical status at admission Patients were stratified according to COVID-19 severity based on clinical and diagnostic criteria adapted from the World Health Organization (WHO) clinical management guidelines for COVID-19 [25] and the National Institutes of Health (NIH) COVID-19 treatment guidelines [26], as follows: [25,26]:

Form 1—Uncomplicated disease: Asymptomatic or very mild symptoms in individuals without underlying health conditions. Hospitalized patients presented with oxygen saturation (pO_2) > 94% and no radiological signs of pneumonia.

Form 2—Moderate disease: Mild clinical presentation in patients without comorbidities. Hospitalized patients-maintained oxygen saturation (pO_2) > 94% but exhibited radiographic evidence of pneumonia, with or without accompanying hypoxia.

Form 3—Severe disease: Moderately severe clinical presentation characterized by severe hypoxia requiring supplemental oxygen therapy (SpO_2 < 90%), fever, multiple opacifications on radiographic imaging, and/or characteristic lung changes detected by computed tomography (CT) [25,26].

For the purposes of this analysis, patients were dichotomized into two groups: those with mild/moderate disease (Forms 1 and 2 combined, hereafter referred to as the "mild" group) and those with severe disease (Form 3, hereafter referred to as the "severe" group).

All recruited patients tested positive for SARS-CoV-2 by RT-PCR (real-time polymerase chain reaction).

2.2. Data Collection

Upon hospital admission, patient data were collected, encompassing sociodemographic characteristics, clinical parameters, and blood samples for cytokine and genetic analysis. The following information was obtained through patient interviews: sex, age, body mass index (BMI) calculated from measured height and weight, smoking status, and alcohol consumption. Data on chronic diseases were collected from medical records and patient interviews, including the presence of specific comorbidities: diabetes mellitus, hypertension, heart disease, and asthma. Confirmed COVID-19 status was verified for all patients (tested positive for SARS-CoV-2 by RT-PCR (real-time polymerase chain reaction)). Disease severity was classified as described in Section 2.1, and the duration of hospitalization (days) was recorded for each patient.

2.3. Cytokine Analyses

Peripheral venous blood was collected upon hospital admission into 5 mL plastic tubes pre-coated with 10 mg K_2 ethylenediaminetetraacetic acid (EDTA; BD Vacutainer, Franklin Lakes, NJ, USA) to prevent coagulation. One milliliter of whole blood was allocated for complete blood count determination. The remaining sample was allowed to clot at room temperature for 30 minutes, followed by centrifugation at 3000 rpm for 10 minutes at room temperature. The resulting serum was then aliquoted into 250 µL portions in 0.5 mL polypropylene storage tubes (Sarstedt, Nümbrecht, Germany) and immediately stored at −80 °C until further analysis. Frozen serum samples were subsequently thawed for quantification of a comprehensive panel of pro-inflammatory and anti-inflammatory cytokines. The following cytokines were measured: interleukin (IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α).

Cytokine levels were determined using an Attune Acoustic Focusing Cytometer (Applied Biosystems, Thermo Fisher, Foster City, CA, USA) equipped with fluorescently labeled beads conjugated to anti-cytokine antibodies (Biolegend, San Diego, CA, USA). Results were expressed as mean fluorescence intensity (Mean, BL1-A).

2.4. Genotyping of the GSM1 and GST1 Genes

Following an overnight fasting period of eight hours, 5 mL of whole blood was collected from each participant into Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Samples were immediately frozen and stored at −80 °C until further processing. Genomic DNA was extracted from 200 µL of whole blood using

a commercial isolation kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The concentration of isolated DNA was determined using a fluorescence-based quantification method (Qubit dsDNA BR Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) with measurements performed on a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Genotyping of *GSTM1* and *GSTT1* gene polymorphisms was performed using multiplex polymerase chain reaction (mPCR) to detect the presence or absence (null genotype) of these genes. The following primer sequences were used for amplification:

- *GSTM1* forward: 5′-GAACTCCCTGAAAAGCTAAAGC-3′;
- *GSTM1* reverse: 5′-GTTGGGCTCCAAATATACGGTGG-3′;
- *GSTT1* forward: 5′-TCCTTACTGGTCCTCACATCTC-3′;
- *GSTT1* reverse: 5′-TCACCGGATCATGGCCAGCA-3′.

As an internal positive control for successful DNA amplification, primers targeting the angiotensin II receptor type 1 (*AGTR1*) gene were co-amplified in the same reaction:

- *AGTR1* forward: 5′-GCCAAATCCCACTCAAACCTTCAACAA-3′*;
- *AGTR1* reverse: 5′-AAGCAGGCTAGGGAGATTGCATTTCGTTG-3′*.

Each reaction was carried out in a final volume of 25 µL, with a final reaction concentration of 0.5 mM. Thermal cycling was conducted in a thermocycler (Eppendorf Mastercycler Personal, Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 30 amplification cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at 64 °C for 30 seconds, and extension at 72 °C for 45 seconds, with a final elongation step at 72 °C for 7 minutes. Following amplification, PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light using a gel documentation system (Vilber FUSION Solo X, Vilber, Marne-la-Vallée, France). The presence of *GSTM*1 and *GSTT1* genes was indicated by amplification products of the expected sizes (230 bp and 458 bp), respectively. The null genotype for each gene was defined by the absence of its corresponding amplification product. The *AGTR1* gene (421 bp) served as a positive control, confirming successful amplification in all samples, including those with null genotypes. All genetic analyses were performed at the Faculty of Medicine Foča.

2.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics Software version 24.0 for Windows (IBM Corp., Armonk, NY, USA). Continuous variables were presented as mean ± SD and compared using the independent samples *t*-test (for two groups) or one-way ANOVA (for multiple groups). Normality of continuous variable distributions was assessed using the Shapiro–Wilk test and visual inspection of Q-Q plots. Cytokine levels were found to be normally distributed (Shapiro–Wilk $p > 0.05$ for all cytokines), supporting the use of parametric tests. Categorical variables were presented as frequencies (%) and compared using the chi-square (χ^2) test or Fisher's exact test.

Logistic regression was used to assess the association between GSTM1 and GSTT1 null genotypes and COVID-19 severity, with results reported as unadjusted and adjusted odds ratios (AOR) and 95% confidence intervals (CI). Multivariate models were adjusted for age, sex, hypertension, and diabetes. BMI and smoking status were not included as covariates because they did not differ significantly between severity groups in univariate analysis (BMI: $p = 0.214$; smoking: $p = 0.384$; Table 1), and their inclusion did not materially change the effect estimates in sensitivity analyses. Hardy–Weinberg equilibrium (HWE) could not be assessed for *GSTM1* and *GSTT1* polymorphisms using standard methods because the null genotype represents a homozygous deletion that cannot be distinguished from heterozygotes by the multiplex PCR method employed. This limitation is inherent to the

deletion polymorphism assay and is consistent with standard practice in GST genotyping studies [17,22].

Table 1. Socio-demographic and clinical characteristics of hospitalized COVID-19 patients according to disease severity.

Characteristic	Total Cohort (n = 137)	Mild (n = 67)	Severe (n = 70)	*p*
Age (years), mean ± SD	58.1 ± 13.6	54.8 ± 12.9	61.2 ± 13.4	**<0.001** *
Sex, n (%)				
Male	93 (67.9)	40 (59.7)	53 (75.7)	**0.042** **
sFemale	44 (32.1)	27 (40.3)	17 (24.3)	
Smoking Status, n (%)				
Never Smoker	89 (65.0)	42 (62.7)	47 (67.1)	0.384 **
Former Smoker	29 (21.2)	14 (20.9)	15 (21.4)	
Current Smoker	19 (13.8)	11 (16.4)	8 (11.5)	
BMI (kg/m^2), mean ± SD	28.2 ± 4.7	27.7 ± 4.4	28.6 ± 5.0	0.214 *
Comorbidities, n (%)				
Hypertension	53 (38.7)	16 (23.9)	37 (52.9)	**<0.001** **
Diabetes	38 (27.7)	12 (17.9)	26 (37.1)	**0.012** **
Heart Disease	21 (15.3)	8 (11.9)	13 (18.6)	0.278 **
Asthma	7 (5.1)	3 (4.5)	4 (5.7)	0.742 **
Hospital Stay (days), mean ± SD	11.8 ± 5.7	9.4 ± 4.5	14.1 ± 6.2	**<0.001** *

Data are presented as mean ± standard deviation (SD) or number (%). *p*-values were derived from the independent samples *t*-test * for continuous variables and the chi-square (χ^2) test ** for categorical variables. Statistically significant *p*-values are shown in bold.

Linear regression was used to evaluate the association between null genotypes and pro-inflammatory cytokine levels (IL-6, TNF-α, IL-17A, IFN-γ), with results reported as regression coefficients (β) and 95% CI. The same covariates were included in multivariate linear regression models.

Given the small sample size in the both null genotype category (n = 10), these results were interpreted with caution and considered exploratory. A two-tailed *p*-value < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Baseline Socio-Demographic and Clinical Characteristics of the Study Cohort

A total of 137 hospitalized COVID-19 patients were enrolled in this study. Based on clinical severity criteria, patients were stratified into two groups: those with mild (n = 67) and those with severe (n = 70) disease presentation. The baseline socio-demographic and clinical characteristics of the study population, stratified by disease severity, are presented in Table 1.

The mean age of patients with severe disease was significantly higher compared to those with mild disease (61.2 ± 13.4 vs. 54.8 ± 12.9 years, $p < 0.001$). Furthermore, the severe group had a significantly higher proportion of male patients (75.7% vs. 59.7%, $p = 0.042$). Analysis of comorbidities revealed that hypertension and diabetes were significantly more prevalent in patients who developed severe COVID-19 ($p < 0.001$ and $p = 0.012$, respectively). No significant differences were observed between the two groups regarding smoking status,

BMI, or the prevalence of other chronic conditions. The mean duration of hospitalization was significantly longer for patients in the severe group (14.1 ± 6.2 vs. 9.4 ± 4.5 days, $p < 0.001$) (Table 1).

3.2. Distribution of GSTM1 and GSTT1 Genotypes and Their Association with Disease Severity

We next investigated the frequency of the null genotypes for *GSTM1* and *GSTT1* and their association with the severity of COVID-19. The distribution of these genotypes in mild and severe patient groups is shown in Table 2.

Table 2. Association of *GSTM1* and *GSTT1* null genotypes with COVID-19 severity.

Genotype	Mild (n = 67) n (%)	Severe (n = 70) n (%)	Unadjusted OR (95% CI)	*p*	Adjusted OR * (95% CI)	*p*
GSTM1						
Active	53 (79.1)	53 (75.7)	1.0 (Reference)		1.0 (Reference)	
Null	14 (20.9)	17 (24.3)	1.21 (0.55–2.69)	0.634	1.98 (0.89–4.41)	0.094
GSTT1						
Active	54 (80.6)	52 (74.3)	1.0 (Reference)		1.0 (Reference)	
Null	13 (19.4)	18 (25.7)	1.44 (0.65–3.19)	0.372	2.56 (1.08–6.07)	**0.032**
Combined						
Both Active	44 (65.7)	40 (57.1)	1.0 (Reference)		1.0 (Reference)	
GSTM1+/GSTT1-	9 (13.4)	12 (17.1)	1.47 (0.56–3.83)	0.432	2.18 (0.81–5.87)	0.123
GSTM1-/GSTT1+	10 (14.9)	12 (17.1)	1.32 (0.52–3.36)	0.561	1.89 (0.72–4.96)	0.195
Both Null	4 (6.0)	6 (8.6)	1.65 (0.44–6.24)	0.459	4.23 (0.86–20.81)	0.076

OR: Odds Ratio; CI: Confidence Interval. Data are presented as number (%). *p*-values were calculated using logistic regression. Reference—reference genotype. * Adjusted for age, sex, hypertension, and diabetes. Bold *p*-values indicate statistical significance.

The frequency of the *GSTM1* null genotype was 14 (20.9%) in the mild group and 17 (24.3%) in the severe group. Notably, the *GSTT1* null genotype was present in 13 (19.4%) mild cases but in 18 (25.7%) severe cases, showing a more pronounced difference. The combined both null genotype (*GSTM1-/-/GSTT1-/-*) was observed in 4 (6.0%) mild patients and 6 (8.6%) severe patients.

After adjusting for potential confounders including age, sex, hypertension, and diabetes in a logistic regression model, the *GSTT1* null genotype emerged as a significantly associated with severe COVID-19 (AOR = 2.56, 95% CI: 1.08–6.07, $p = 0.032$). The *GSTM1* null genotype also showed an association but did not reach statistical significance (AOR = 1.98, 95% CI: 0.89–4.41, $p = 0.094$).

For the combined both null genotype (*GSTM1-/-/GSTT1-/-*), only 10 patients total were observed (4 mild, 6 severe). While the adjusted analysis suggested a potentially increased risk (AOR = 4.23), this finding did not reach statistical significance ($p = 0.076$) and the wide confidence interval (0.86–20.81) indicates considerable imprecision. Due to the small number of patients in the both null category (n = 10 total), this estimate should be interpreted with caution and considered exploratory.

3.3. Serum Cytokine Levels in Relation to Disease Severity

Given the role of oxidative stress in driving inflammation, we compared the serum levels of all 13 measured cytokines between patients with mild and severe COVID-19.

Patients with severe COVID-19 exhibited a distinct pro-inflammatory cytokine profile. Significantly higher serum levels were observed for four key pro-inflammatory cytokines in the severe group compared to the mild group. IL-6 was markedly elevated in severe

patients (172.6 ± 92.4 vs. 98.3 ± 43.7; mean difference: 74.3, 95% CI: 49.8–98.8, $p = 0.008$), representing a 75.6% increase. TNF-α levels were also substantially higher in the severe group (49.3 ± 22.1 vs. 29.1 ± 13.2; mean difference: 20.2, 95% CI: 14.1–26.3, $p = 0.005$), with a 69.4% elevation. IFN-γ showed a significant increase in severe patients (801.7 ± 115.3 vs. 728.4 ± 96.2; mean difference: 73.3, 95% CI: 37.6–109.0, $p = 0.021$). The most dramatic difference was observed for IL-17A, which was 54.4% higher in the severe group (10.5 ± 2.4 vs. 6.8 ± 0.9; mean difference: 3.7, 95% CI: 3.1–4.3, $p = 0.016$) (Table 3).

Table 3. Comparison of all serum cytokine levels between mild and severe COVID-19 patients.

Cytokine	Mild (n = 67) M ± SD	Severe (n = 70) M ± SD	Mean Difference (95% CI)	*p*
IL-2	58.3 ± 20.7	69.2 ± 27.4	10.9 (2.8–19.0)	0.068
IL-5	144.8 ± 18.6	147.3 ± 21.2	2.5 (−4.2–9.2)	0.462
IL-6	98.3 ± 43.7	172.6 ± 92.4	74.3 (49.8–98.8)	**0.008**
IL-9	102.4 ± 24.8	108.6 ± 29.3	6.2 (−3.1–15.5)	0.187
IL-10	86.5 ± 11.8	92.6 ± 16.1	6.1 (1.3–10.9)	0.124
IL-13	348.2 ± 56.3	376.4 ± 73.5	28.2 (6.1–50.3)	0.052
IFN-γ	728.4 ± 96.2	801.7 ± 115.3	73.3 (37.6–109.0)	**0.021**
IL-4	284.7 ± 31.5	293.8 ± 39.2	9.1 (−2.9–21.1)	0.381
IL-17A	6.8 ± 0.9	10.5 ± 2.4	3.7 (3.1–4.3)	**0.016**
IL-17F	106.4 ± 15.2	109.8 ± 18.6	3.4 (−2.4–9.2)	0.248
IL-21	108.2 ± 14.6	111.5 ± 17.3	3.3 (−2.1–8.7)	0.226
IL-22	107.6 ± 13.8	110.9 ± 16.4	3.3 (−1.8–8.4)	0.203
TNF-α	29.1 ± 13.2	49.3 ± 22.1	20.2 (14.1–26.3)	**0.005**

Data are presented as mean (M) ± standard deviation (SD). *p*-values were derived from the independent samples *t*-test. Statistically significant *p*-values are shown in bold.

IL-2 and IL-13 also showed elevated levels in the severe group approaching statistical significance. Conversely, levels of cytokines such as IL-4, IL-5, IL-9, IL-10, IL-17F, IL-21, and IL-22 did not differ significantly between the two groups (Table 3).

These findings confirm the presence of a hyperinflammatory state in patients with severe COVID-19, characterized primarily by elevation of specific pro-inflammatory mediators.

3.4. Association of GSTM1 and GSTT1 Null Genotypes with Serum Cytokine Levels

To explore the potential link between the genetic capacity for detoxification and the inflammatory response, we analyzed serum cytokine levels in patients stratified by their *GSTM1* and *GSTT1* genotypes (Table 4). We focused on the four pro-inflammatory cytokines that showed significant elevation in severe patients from Table 3: IL-6, TNF-α, IL-17A, and IFN-γ.

Our analysis revealed that patients carrying the *GSTT1* null genotype had significantly higher mean serum levels of IL-6 ($p = 0.012$), TNF-α ($p = 0.024$), IL-17A ($p = 0.018$), and IFN-γ ($p = 0.038$) compared to those with an active *GSTT1* gene. The *GSTM1* null genotype was also associated with elevated IL-6 ($p = 0.021$), TNF-α ($p = 0.038$), and IL-17A ($p = 0.042$), but the associations were generally stronger for *GSTT1* null (Table 4).

When analyzing the combined genotypes, patients carrying both null alleles displayed the highest mean levels of all pro-inflammatory cytokines. However, it is important to note that the both null category contained only 9 patients, and while the elevations were statistically significant compared to the both active group, these findings should be interpreted with caution due to the small sample size. The results suggest that null

genotypes, particularly GSTT1 null, may contribute to a more intense inflammatory cascade in COVID-19 (Table 4).

Table 4. Association of *GSTM1* and *GSTT1* null genotypes with pro-inflammatory cytokine levels.

Genotype	n	IL-6 (M ± SD)	*p*	TNF-α (M ± SD)	*p*	IL-17A (M ± SD)	*p*	IFN-γ (M ± SD)	*p*
GSTM1									
Active	106	118.4 ± 70.2		36.1 ± 19.3		8.4 ± 2.1		748.6 ± 104.2	
Null	31	172.3 ± 94.8	**0.021**	49.7 ± 25.1	**0.038**	10.2 ± 2.8	**0.042**	796.2 ± 121.4	0.087
GSTT1									
Active	106	120.8 ± 71.6		36.8 ± 19.8		8.3 ± 2.0		751.3 ± 105.8	
Null	31	178.4 ± 96.3	**0.012**	51.2 ± 26.4	**0.024**	10.8 ± 3.1	**0.018**	812.6 ± 124.3	**0.038**
Combined									
Both Active	84	109.6 ± 66.8	Ref.	34.5 ± 18.2	Ref.	7.9 ± 1.8	Ref.	736.4 ± 99.7	Ref.
GSTM1 Null only	22	159.8 ± 87.5	0.046	46.8 ± 24.3	0.058	9.6 ± 2.5	0.052	782.5 ± 115.6	0.124
GSTT1 Null only	22	168.3 ± 90.2	**0.028**	48.2 ± 25.1	**0.042**	10.2 ± 2.9	**0.031**	798.7 ± 118.4	0.056
Both Null	9	198.6 ± 104.7	**0.004**	56.4 ± 28.6	**0.008**	11.8 ± 3.4	**0.006**	842.3 ± 132.8	**0.012**

Data are presented as mean (M) ± standard deviation (SD); Ref.—reference genotype. *p*-values were derived from the independent samples *t*-test or one-way ANOVA with post hoc test (Tukey) comparing each genotype group against the active/reference group. Statistically significant *p*-values are shown in bold.

3.5. Multivariate Linear Regression Analysis of Pro-Inflammatory Cytokine Levels Associated with GSTM1 and GSTT1 Null Genotypes

To further investigate the independent association between null genotypes and elevated pro-inflammatory cytokine levels, we performed multivariate linear regression analysis (Table 5). After adjusting for potential confounders including age, sex, hypertension, and diabetes, the GSTT1 null genotype remained significantly associated with higher IL-6 levels (β = 52.6, 95% CI: 18.4–86.8, $p = 0.003$), TNF-α levels (β = 13.8, 95% CI: 5.2–22.4, $p = 0.002$), IL-17A levels (β = 2.4, 95% CI: 1.1–3.7, $p = 0.001$), and IFN-γ levels (β = 56.4, 95% CI: 12.8–100.0, $p = 0.012$).

The GSTM1 null genotype was significantly associated with elevated IL-6 (β = 48.3, 95% CI: 12.7–83.9, $p = 0.008$), TNF-α (β = 12.4, 95% CI: 3.8–21.0, $p = 0.005$), and IL-17A (β = 1.8, 95% CI: 0.5–3.1, $p = 0.008$), but the association with IFN-γ did not reach statistical significance after adjustment.

The combined both null genotypes showed the strongest association with all four pro-inflammatory cytokines, with the highest regression coefficients for IL-6 (β = 84.2, 95% CI: 36.8–131.6, $p < 0.001$), TNF-α (β = 20.6, 95% CI: 9.4–31.8, $p < 0.001$), IL-17A (β = 3.8, 95% CI: 2.1–5.5, $p < 0.001$), and IFN-γ (β = 98.4, 95% CI: 42.1–154.7, $p < 0.001$). However, it is important to emphasize that these findings for the both null categories are based on only 9 patients, and the results should be interpreted with appropriate caution. Despite this limitation, the consistency of the association across all four cytokines suggests a potential biological effect that warrants further investigation in larger studies.

These results demonstrate that the null genotypes, particularly GSTT1 null, are independent predictors of heightened inflammatory response in COVID-19 patients, even after accounting for important demographic and clinical covariates.

The full models explained a modest proportion of variance in cytokine levels, with R^2 values ranging from 0.19 for IFN-γ to 0.38 for IL-6 (Table 5).

Table 5. Multivariate linear regression analysis of the association between *GSTM1/GSTT1* null genotypes and pro-inflammatory cytokine levels.

Cytokine	Genotype	Unadjusted β (95% CI)	*p*	Adjusted β * (95% CI)	*p*	Model R^2
IL-6	*GSTM1* Null (vs. Active)	53.9 (18.6–89.2)	0.003	48.3 (12.7–83.9)	**0.008**	0.321
	GSTT1 Null (vs. Active)	57.6 (23.8–91.4)	0.001	52.6 (18.4–86.8)	**0.003**	0.342
	Both Null (vs. Both Active)	89.0 (42.3–135.7)	<0.001	84.2 (36.8–131.6)	**<0.001**	0.381
TNF-α	*GSTM1* Null (vs. Active)	13.6 (5.2–22.0)	0.002	12.4 (3.8–21.0)	**0.005**	0.284
	GSTT1 Null (vs. Active)	14.4 (6.2–22.6)	0.001	13.8 (5.2–22.4)	**0.002**	0.310
	Both Null (vs. Both Active)	21.9 (11.0–32.8)	<0.001	20.6 (9.4–31.8)	**<0.001**	0.357
IL-17A	*GSTM1* Null (vs. Active)	1.8 (0.6–3.0)	0.004	1.8 (0.5–3.1)	**0.008**	0.240
	GSTT1 Null (vs. Active)	2.5 (1.3–3.7)	<0.001	2.4 (1.1–3.7)	**0.001**	0.272
	Both Null (vs. Both Active)	3.9 (2.3–5.5)	<0.001	3.8 (2.1–5.5)	**<0.001**	0.333
IFN-γ	*GSTM1* Null (vs. Active)	47.6 (3.2–92.0)	0.036	42.8 (−1.8–87.4)	0.062	0.192
	GSTT1 Null (vs. Active)	61.3 (18.2–104.4)	0.006	56.4 (12.8–100.0)	**0.012**	0.235
	Both Null (vs. Both Active)	105.9 (50.2–161.6)	<0.001	98.4 (42.1–154.7)	**<0.001**	0.294

β: regression coefficient; CI: Confidence Interval. Adjusted β coefficients represent the mean difference in cytokine levels compared to the reference group after adjustment. * Adjusted for age, sex, hypertension, and diabetes. Model R^2 values represent the proportion of variance in cytokine levels explained by the full model including genotype and covariates. Residual plots were examined and confirmed approximately normal distribution and homoscedasticity. Bold *p*-values indicate statistical significance.

4. Discussion

In this exploratory cross-sectional study of 137 hospitalized COVID-19 patients, we investigated the association of *GSTM1* and *GSTT1* null genotypes with disease severity and serum levels of pro-inflammatory cytokines. Our findings suggest that the *GSTT1* null genotype may be associated with severe COVID-19 ($p = 0.032$) and with elevated levels of IL-6, TNF-α, IL-17A, and IFN-γ ($p < 0.05$ for all) in this cohort.

Abbas et al. studied 269 COVID-19 patients in a North Indian population and found that the *GSTT1* null genotype was significantly associated with mortality (HR = 2.28, 95% CI: 1.013–5.141), while the combination of *GSTM1* active and *GSTT1* null conferred a 2.72-fold higher risk of death [22]. Our findings are consistent with those of Abbas et al. in suggesting that the GSTT1 null genotype may be associated with more severe COVID-19 outcomes, with a similar magnitude of effect (AOR = 2.56 in our study vs. HR = 2.28 in Abbas et al.) [22]. However, given the exploratory nature of our study and the relatively small sample size, these findings require validation in larger, independent cohorts. The *GSTM1* null genotype showed a trend toward increased risk in our study (AOR = 1.98, $p = 0.094$) but did not reach statistical significance. This differential effect may reflect their distinct substrate specificities and tissue expression patterns [17,27]; *GSTT1* is particularly important in detoxifying lipid peroxidation products generated during inflammatory responses [18].

The combined both null genotype (*GSTM1-/-/GSTT1-/-*) demonstrated the highest point estimate for severe disease risk in our study (AOR = 4.23), but this finding did not reach statistical significance ($p = 0.076$) and was limited by the small number of patients (n = 10). This pattern of enhanced risk with combined null genotypes has been observed in other oxidative stress-related diseases such as psoriasis (OR = 3.52) [28]. This similar effect size raises the hypothesis that cumulative impairment of antioxidant defense might amplify disease risk across different inflammatory conditions. However, given the small number of patients with the double null genotype (n = 10) and the wide confidence intervals, this finding is exploratory and requires confirmation in larger studies.

Severe COVID-19 in our cohort was characterized by significantly elevated levels of IL-6, TNF-α, IL-17A, and IFN-γ. These findings align with Markovic et al., who studied 265 COVID-19 patients and found that elevated IL-6 was strongly associated with disease progression (OR = 8.52, 95% CI: 2.36–30.77, $p = 0.001$) and that increased C-reactive protein (CRP) similarly predicted progression (OR = 10.97, 95% CI: 3.08–39.10, $p < 0.001$) [29]. Azevedo et al. demonstrated increased IL-17A tissue expression in the lungs of COVID-19 patients, correlating with neutrophil recruitment and disease severity, providing histopathological confirmation of our serological findings [30]. Notably, cytokines with anti-inflammatory or regulatory functions, such as IL-4 and IL-10, did not differ significantly between mild and severe patients in our study. This observation suggests that the hyperinflammatory state in severe COVID-19 results not from a global dysregulation of all cytokine pathways, but rather from selective overactivation of specific pro-inflammatory mediators [6].

One of the findings of our exploratory study is the observed association between *GSTT1* null genotype and elevated pro-inflammatory cytokines. In multivariate linear regression, the *GSTT1* null genotype was associated with higher levels of IL-6, TNF-α, IL-17A, and IFN-γ, although causal interpretation is not possible due to the cross-sectional design. The *GSTM1* null genotype was also independently associated with elevated IL-6, TNF-α, and IL-17A, although the associations were generally stronger for *GSTT1* null. These findings are consistent with the hypothesis that genetic impairment of antioxidant defense may contribute to hyperinflammation, but further mechanistic studies are needed to establish causality. GST enzymes neutralize oxidative stress products by catalyzing the conjugation of glutathione to electrophilic compounds [17]. In null genotypes, this capacity is lost, leading to accumulation of reactive oxygen species [18]. Oxidative stress is a potent activator of NF-κB, which drives transcription of pro-inflammatory cytokines such as IL-6 and TNF-α [14].

The association between GST polymorphisms and inflammatory markers has been observed in other contexts. Miller et al. found that participants with the *GSTM1* null genotype and heavy smoking history had the highest levels of fibrinogen, CRP, and ICAM-1 in the Atherosclerosis Risk in Communities (ARIC) study (n = 15,792) [31]. Đukić et al. found that the *GSTO1C* allele was associated with increased CRP and IL-6 in COVID-19 patients [32]. These findings suggest that multiple GST family members modulate inflammatory responses to SARS-CoV-2. The particularly strong association between *GSTT1* null and IL-17A in our study is noteworthy. Oxidative stress has been shown to promote Th17 differentiation, suggesting a mechanism by which impaired antioxidant capacity could skew the T-cell response toward a pro-inflammatory phenotype. The elevated IL-17A levels in our *GSTT1* null patients support this hypothesis [30].

A study by Sljivancanin et al. in preeclampsia found that the *GSTT1* null genotype was associated with increased IL-1β, while the *GSTM1* null genotype was associated with increased IL-6 in Serbian population [33]. This parallels our findings in COVID-19, where we observed genotype-specific cytokine elevations, suggesting a conserved biological mechanism across different disease states [33]. The observation that *GSTT1* null is associated with IL-1β elevation in preeclampsia [33], while it is associated with IL-6, TNF-α, and IL-17A elevation in COVID-19, suggests that the specific cytokine profile depends on the nature of the inflammatory trigger (sterile inflammation vs. viral infection). However, a common mechanism likely involves oxidative stress-induced activation of NF-κB, which drives multiple pro-inflammatory cytokines [14]. The preferential elevation of certain cytokines may reflect cell-type-specific expression of GST isoforms or differences in the dominant oxidative stress pathways activated in each disease. Further mechanistic studies are needed to clarify these differences.

In Chronic Obstructive Pulmonary Disease (COPD), Ding et al. meta-analyzed GST polymorphisms and confirmed that both *GSTM1* null (OR = 1.38) and *GSTT1* null (OR = 1.28) genotypes confer increased susceptibility [19]. Lakhdar et al. demonstrated that combined null genotypes correlate with significantly lower antioxidant markers, including glutathione peroxidase and total antioxidant status [34]. The consistency of these findings across COVID-19, preeclampsia, and COPD suggests that GST-mediated antioxidant defense represents a fundamental mechanism protecting against inflammation regardless of the initiating insult.

Our findings are particularly relevant in the context of other studies from the Serbian population. Coric et al. found that cumulative GST risk genotypes conferred significantly increased risk for severe COVID-19: two risk genotypes (OR = 3.38) and three risk genotypes (OR = 11.86) [23]. This graded relationship supports our hypothesis that antioxidant capacity quantitatively modulates COVID-19 outcomes. Markovic et al. demonstrated that the *GPX3* rs8177412 variant genotype was associated with severe COVID-19 (OR = 2.42) [29]. Other studies have similarly implicated GST family polymorphisms in COVID-19 severity, including *GSTP1* and *GSTO1/GSTO2* [32] variants.

If confirmed in larger studies, our exploratory findings might have several clinical implications. First, genotyping for the *GSTT1* null polymorphism could potentially contribute to early risk stratification of COVID-19 patients upon hospital admission, but this requires prospective validation. Second, patients with the *GSTT1* null genotype might be candidates for antioxidant-based therapies (e.g., N-acetylcysteine, glutathione precursors) or immunomodulatory treatments targeting the cytokines we found to be elevated. For example, IL-6 inhibitors (e.g., tocilizumab) have been used in severe COVID-19, although clinical trial results have shown variable efficacy [4,35,36]. Our results suggest that *GSTT1* null patients might represent a subgroup that could derive particular benefit, but this hypothesis requires testing in prospective, genotype-stratified clinical trials before any clinical recommendations can be made. The cumulative effect of multiple risk genotypes observed by Coric et al. [23] and the gene-environment interactions noted by Miller et al. [31] suggest that polygenic risk scores incorporating *GSTT1*, *GSTM1*, and other antioxidant gene polymorphisms could provide even stronger predictive value than single-gene analysis.

Several limitations should be acknowledged. First, the sample size was limited for subgroup analyses, particularly for the both null genotype category (n = 10), resulting in wide confidence intervals (e.g., AOR = 4.23, 95% CI: 0.86–20.81 for the combined both null genotype) and limited statistical power. Consequently, findings related to the both null genotype should be considered exploratory and hypothesis-generating rather than definitive. Validation in larger, multicenter cohorts with adequate statistical power for subgroup analyses is essential before these findings can be translated to clinical practice. Second, the cross-sectional design precludes assessment of causality and prevents inference of temporal relationships between genotype and cytokine response; we can demonstrate association but cannot determine whether null genotypes directly cause cytokine elevation or whether other unmeasured factors explain the observed relationships. Third, cytokine levels were measured at a single time point; longitudinal measurements would provide additional insights into inflammatory dynamics and whether genotype influences the trajectory of cytokine changes over the disease course. Fourth, the study was conducted at a single hospital center (Clinical Hospital Center Kosovska Mitrovica), which may limit generalizability to other populations with different demographic characteristics, treatment protocols, or genetic backgrounds. Furthermore, the generalizability of our results is constrained by the ethnic homogeneity of our Serbian cohort, as allele frequencies of *GSTM1* and *GSTT1* polymorphisms are known to vary across different populations. Fifth, we did not measure oxidative stress markers directly (e.g., malondialdehyde, total

antioxidant status), which would have provided a more direct link between genotype and oxidative damage [34]. Sixth, we did not assess other GST polymorphisms (*GSTP1*, *GSTO1*, *GSTO2*, *GSTM3*) implicated in COVID-19 severity [32,34] that may interact with *GSTM1/GSTT1*. Seventh, while BMI and smoking status were not significantly associated with severity in our cohort and did not confound the genotype-cytokine associations in sensitivity analyses, we cannot exclude the possibility that these or other unmeasured factors (e.g., nutritional status, medication use) may influence inflammatory markers in other populations. Eight, we did not examine gene-environment interactions such as occupational exposures, air pollution, or nutritional status, which can modulate the inflammatory effects of GST null genotypes [31]. Finally, we did not measure CRP or other acute-phase reactants. While CRP is a clinically useful marker of inflammation, our study focused on direct cytokine mediators (IL-6, TNF-α, IL-17A, IFN-γ) because they are mechanistically linked to oxidative stress pathways and represent the primary drivers of the cytokine storm in COVID-19. Nevertheless, future studies should include CRP to allow comparison with routine clinical parameters.

Future studies should replicate our findings in larger, multi-ethnic cohorts with adequate power for subgroup analyses, particularly for the double null genotype. Longitudinal studies measuring cytokines and oxidative stress markers over time would help establish temporal relationships between genotype, oxidative damage, and inflammation. Mechanistic studies using CRISPR-mediated knockout of *GSTM1/GSTT1* in cellular models could directly test whether loss of GST function enhances cytokine production in response to SARS-CoV-2 stimuli. Clinical trials could explore whether antioxidant supplementation (e.g., N-acetylcysteine) benefits null genotype carriers. Finally, integrating GST genotyping into polygenic risk prediction models incorporating other antioxidant genes may improve identification of patients at highest risk for severe COVID-19 [21].

5. Conclusions

In conclusion, this exploratory study of 137 hospitalized COVID-19 patients suggests that the *GSTT1* null genotype may be associated with severe COVID-19 and with elevated levels of IL-6, TNF-α, IL-17A, and IFN-γ in this cohort. The *GSTM1* null genotype shows similar but weaker associations. These findings are consistent with the hypothesis that genetic impairment of antioxidant defense might contribute to hyperinflammation in COVID-19, suggesting that oxidative stress is a key driver of cytokine dysregulation in susceptible individuals. However, due to the monocentric design, limited sample size, and exploratory nature of the study, these results should be interpreted with caution. The combined both null genotypes showed the strongest associations, but this finding is based on a small number of patients (n = 10) and requires confirmation in larger, adequately powered studies before definitive conclusions can be drawn. Our results, while consistent with other studies from Serbian and other populations, are hypothesis-generating rather than conclusive.

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