Association of GSTT1/GSTM1 and ApoE variants with left ventricular diastolic dysfunction in thalassaemia major patients

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**Abstract**

**Background:** Cardiomyocytes are particularly susceptible to complications from iron loading. The blood transfusions in thalassaemia major create loading of iron that cannot be naturally excreted. Apolipoprotein E and Glutathione S-transferase act as the scavenger of free radicals, which are generated due to excess iron. The variants of Apolipoprotein E (ApoE) and Glutathione S-transferase (GST) may play a role in oxidative damage-induced cardiomyopathy, so we aimed to study the association of genetic variants of these genes on diastolic dysfunction in our patients.

**Materials and methods:** One hundred and five β-thalassaemia patients older than 10 years were enrolled for the study. Two-dimensional and M-mode echocardiography analysis was done in all patients. Genotyping of the genetic variants of aforementioned genes was done using the PCR–RFLP method. Serum Glutathione S-transferase levels were estimated by ELISA.

**Results:** Diastolic dysfunction was observed in 24 (22.8%) patients, whereas left ventricular hypertrophy was present in 37 (35.2%) patients. There was a significant association of GSTM1 null allele with diastolic dysfunction only. Serum GST levels were also positively correlated with e/a and e/e ratio. Positive association of ApoE E2 allele with the diastolic dysfunction was also seen.

**Conclusions:** Patients having Glutathione S-transferase M1 allele and Apolipoprotein E E2 allele are predisposed to oxidative stress-induced cardiac injury.

**Introduction**

Thalassaemia major patients, presenting severe anaemia, require necessary supportive regular red blood cell transfusions that exacerbate iron overload affecting different organs and iron-driven oxidative stress. Compared to other organs, the heart has relatively less well-developed antioxidant defence and iron in excess leads to the formation of oxygen-free radicals which makes cardiomyocytes more susceptible to iron-induced perioxidative damage [1,2].

Diastolic dysfunction is an early sign in heart disease. The ischaemic cascade begins with an imbalance in the supply of oxygen demand and metabolic alterations, identifies diastolic disorders of the left ventricle (LV) as an early phenomenon even before any visible clinical features. Although the physiology of diastolic function is complex, the factors contributing to diastolic disturbances can be attributed to impaired LV relaxation, LV hypertrophy and increased LV asynchrony. Improved insight into the genetic aspects contributing for impaired LV function may help in the management of LV diastolic dysfunction [3,4].

Glutathione S-transferase (GST) plays an important physiological role in the elimination of reactive oxygen species. Glutathione S-transferase genes (GSTT1 and GSTM1) are well-known detoxification agents. Genetically determined variants can cause changes in activity level and/or expression of some GST, and may cause decreased defence capacity against oxidative stress. Glutathione S-transferase T1 (GSTT1) and glutathione S-transferase M1 (GSTM1) are members of θ (theta) and μ (mu) classes, respectively, and have been shown to be polymorphic. The common variant of GSTM1 and GSTT1 genes is the homozygous deletion (null genotype) which leads to reduced enzyme activity and increased risk for various diseases [5,6].

Apolipoprotein E (ApoE) is a well-known lipid transport protein. Its gene has three major alleles including E2, E3 and E4, and has been reported to be associated with increasing risk of cardiovascular disorders which includes atherosclerosis, coronary heart disease and stroke. It acts as the scavenger of free radicals [7,8]. The oxidative damage and ApoE genotype are important factors involved in the pathogenesis of cardiomyopathy in patients with beta-thalassaemia major [8].

Studies regarding the association of GST and ApoE gene variants with left ventricular diastolic dysfunction and left ventricular hypertrophy in Indian thalassaemia patients are lacking. Therefore, the aim of the study is to see the plausible effect of polymorphic variants of GSTT1 and GSTM1.
glutathione S-transferase gene and ApoE with cardiac dysfunction in beta-thalassaemia major patients.

**Material and methods**

**Subjects:** A total of 105 patients of beta-thalassaemia major of above 10 years of age were recruited from the Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, during the study. All patients were on regular blood transfusion and iron chelation therapy. Patients with any congenital or acquired heart disease, concurrent infective disorder, renal failure, diabetes mellitus and patients with a history of any type of cardiac surgery were excluded from the study. Patients on cardioprotective drugs were also excluded from the study. The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all adult patients or if the patient's age was below 18 years consent was taken from their parents.

**Echocardiography**

Two-dimensional echocardiogram (GE vivid-7 echocardiogram machine) was done by the single author (ST) when patients came for blood transfusion. Left ventricular diastolic dysfunction (LVDD) was determined according to recommendations of the American Society of Echocardiography and the European Association of Cardiovascular Imaging 2016 [9]. Using the M-mode echocardiography, left ventricular end-diastolic dimension (LVEDd), intra-ventricular septal thickness (IVSd) and posterior wall thickness (PWTd) were measured at end-diastole. LV mass was calculated using the formula $\text{LV mass} = 0.8\times(\text{LVEDd} + \text{IVSd} + \text{PWTd})^3 + 0.6$. LV mass index (LVMI) was calculated by dividing the LV mass (g) by the height (in meters)$^2$ to correct the left ventricular mass (LVM) for body size [10]. Left ventricular hypertrophy (LVH) was defined using the age- and gender-specific cut-off values of LVMI.

**Laboratory analysis**

**Sample collection:** 5 ml of peripheral venous blood was taken in EDTA (2 ml) and Plain (3 ml) vacutainer for molecular and biochemical testing, respectively.

**Haematological analysis:** Pre-transfusion haemoglobin (Hb) was measured on an automated cell counter (Sysmex KX-21).

**Biochemical analysis:** Serum Glutathione-S-Transferase (GST) and ferritin levels were measured by enzyme-linked immunosorbent assay (ELISA).

**Molecular analysis:** Genomic DNA was isolated using the standard phenol–chloroform method. Status of ApoE (rs429358, rs7412) was determined by PCR–RFLP as described previously by Hixon et al. [11]. ApoE has three alleles (E2, E3, E4) and six haplotypes (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, E4/E4). The apo E2/E2 was characterized by the presence of the rs429358 T/T and rs7412 T/T, E2/E3 was characterized by the presence of the rs429358 T/T and rs7412 C/T, E2/E4 was characterized by the presence of the rs429358 C/T and rs7412 T/T, E3/E3 was characterized by the presence of the rs429358 T/T and rs7412 C/C, E3/E4 was characterized by the presence of the rs429358 C/T and rs7412 C/C, E4/E4 was characterized by the presence of the rs429358 C/C and rs7412 C/C.

GSTT1 and GSTM1 genotypes were determined by the multiple polymerase chain reaction (PCR) method using primers and reaction profiles, as described by Sclafani et al. [12].

**Statistical analysis:** Data were analysed using the SPSS software (IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY). Fisher exact test or chi-square was used to see the association of different genotypes with gender, diastolic dysfunction and Left ventricular hypertrophy. Kolmogorov–Smirnov test was used to check the normality of data. Data were presented as median and interquartile range (IQR) for non-parametric continuous variables (Age and Ferritin) and as mean ± standard deviation for parametric continuous variable (Haemoglobin and Serum Glutathione S Transferase). Depending upon the normality of data, Independent sample t-test or Mann–Whitney U-test was used to see the difference in continuous variables in different groups. One-way ANOVA test or Kruskal–Wallis test was used to see the significant difference in pre-transfusion Hb, ferritin and serum GST levels with different haplotypes of ApoE. Karl Pearson correlation test was applied to see the correlation of GST levels with pre-transfusion haemoglobin and echocardiography parameters. $P$ value <.05 was considered significant.

**Table 1. Demographic and clinical profile of recruited subjects.**

| Parameter               | Male (N = 66) | Female (N = 39) | Total (N = 105) | $p$-Value |
|------------------------|---------------|-----------------|-----------------|-----------|
| Median age (years)     | 16 (IQR = 12–18.5) | 17 (IQR = 12–20) | 16 (IQR = 12–19.5) | 0.146*   |
| Mean Hb (g/dL)         | 8.8 ± 2.0     | 8.9 ± 2.4       | 8.9 ± 2.1       | 0.865*   |
| Median serum ferritin (ng/mL) | 3000 (IQR = 2760–3902.5) | 3100 (IQR = 2800–3200) | 3000 (IQR = 2773–3885) | 0.571*   |
| Mean serum glutathione-S-transferase (µg/mL) | 49.7 ± 19.0 | 58.2 ± 24.9 | 53.0 ± 21.7 | 0.120*   |
| LVDD                   | 15 (22.7)     | 9 (23.1)        | 24 (22.9)       | 1.000*   |
| LVH                    | 21 (31.3)     | 16 (41.0)       | 37 (35.2)       | 0.400*   |

Notes: IQR: interquartile range. Data are presented as number (percentage) for LVDD and LVH.

*aIndependent sample t-test was used to calculate the p-value.

*bMann–Whitney U-test was used to calculate the p-value.

'See Fisher’s Exact was used to calculate the p-value.
Results

The study includes 66 males and 39 females with a median age of 16 years (IQR = 12–19.5). The mean pre-transfusion Hb was 8.9 ± 2.1 g/dL. Left ventricular diastolic dysfunction (LVDD) was observed in 24 (22.8%) patients, whereas left ventricular hypertrophy (LVH) was present in 37 (35.2%) patients. The frequency of LVH was higher in females (41.0%) than in males (31.8%), but the difference did not reach the statistically significance (Table 1). Median age was statistically similar in patients with LVDD (median = 17.5 years, IQR = 14.25–21.5) and without LVDD (median = 16 years, IQR = 12–19) (p = 0.182). However, patients with LVH have significantly lower age (median = 14 years, IQR = 10–16 years) as compared to patients without having LVH (median = 17.5 years, IQR = 15.25–20 years) (p < 0.0001).

The frequency of null-GSTT1, null-GSTM1 and double null-GSTT1-GSTM1 in recruited patients were 33.3%, 44.8% and 15.2%, respectively. The distributions were seen between patients with LVDD (median = 17.5 years, IQR = 12–19) and without LVDD (median = 16 Year, IQR = 12–19) (p = 0.182). However, patients with LVH have significantly lower age (median = 14 years, IQR = 10–16 years) as compared to patients without having LVH (median = 17.5 years, IQR = 15.25–20 years) (p < 0.0001).

The frequency of null-GSTT1 genotype was 17.1% in patients with LVDD and 34.3% in a group of patients having LVH. No significant differences in null-GSTT1 distributions were seen between patients with LVDD (p = 0.462, OR = 0.698) and patients without LVDD. Patients having null-GSTM1 genotype have more than 2 times risk for having LVDD as compared to patients having GSTM1 genotype (p < 0.0001, OR = 2.290). It was interesting to see that only one patient with LVDD has double null-GSTT1-GSTM1 genotype. Patients having GSTT1 genotype have significantly higher pre-transfusion haemoglobin levels as compared to patients having null-GSTT1 genotype (Table 3). Also Serum GST levels were significantly lower in patients with null-GSTM1 genotype (46.82 ± 17.5 µg/mL) when compared to patients having GSTT1 genotype (56.3 ± 23.1 µg/mL). Serum GST levels were also found significantly positively correlated with e/e′ (r = 0.320, p = 0.005) and e/a ratio (r = 0.224, p = 0.045), as shown in Table 4.

The ApoE haplotypes E2/E4, E3/E3 and E3/E4 were found in 8 (7.6%), 66 (62.9%) and 31 (29.5%) of patients, respectively. We did not find any patient having E2/E2, E2/E3 and E4/E4 haplotype. There was no association of ApoE haplotypes with gender and age (Tables 2 and 3). Statistically significant association was observed between ApoE haplotypes with diastolic dysfunction only. Patients with E2 haplotype had higher chances of having diastolic dysfunction as compared to patients with E3 and E4 haplotype (p value = 0.007) (Table 2). Although patients with E2/E4 haplotype have lower pre-transfusion Hb when compared to patients with other haplotypes, but the difference was not statistically significant. Serum GST levels were also found elevated in patients having E2/E4 haplotype of ApoE gene.

Discussion

In normal metabolism of the cells, reactive oxygen species (ROS) are generated in small amounts, whereas under conditions of altered cell physiology the production is increased thus they are responsible for many kinds of cell injuries. Owing to the heart’s high need of energy, cardiomyocytes are rich in mitochondria and require more oxygen but, due to low levels of antioxidant enzymes, they are more susceptible to ROS-mediated damage. Iron’s toxicity in the cell arises from its capacity to catalyse the production of ROS that cause lipid peroxidation and organelle damage [13] that leads to cell death followed by impaired systolic and diastolic function. Study in a mouse cardiomyocyte cell line showed that L-type channels mediate the iron uptake [14]. The import of ferrous iron by L-type channels, along with higher ROS production, inhibits calcium influx affecting the cardiac excitation–contraction coupling as it is highly sensitive to changes in cellular redox state [15]. This leads to the impaired diastolic function, characteristic of iron-overload cardiomyopathy in β-thalassaemia [16].

Left ventricular systolic function remains normal till late in thalassaemia patients, so echocardiographic left ventricular diastolic evaluation in comparison to systolic evaluation is appropriate for the detection of myocardial dysfunction secondary to iron overload. Similar to our study, Atiq et al. reported that 29% of their studied patients had diastolic dysfunction [17]. Karamanou et al. [18] in their study reported a 34% prevalence of diastolic dysfunction (E/E′>8).

Glutathione S-transferase enzymes work as antioxidants and their activity is determined genetically. Defects in anti-oxidative activity in BTM patients are major contributors to the hypercoagulable state, increased tissue injury, increased lipid peroxidation, and inducing a state of chronic vascular instability that actively contributes to the pathophysiology of thalassaemia complications [19]. In our study, levels of GST were positively correlated with e/a and e/e′ which are markers for diastolic dysfunction [20].

Sclafani et al. [12] reported that the frequency of the GSTM1 null genotype ranges from 23% to 62% in different population around the world which is similar to our study. The present study shows that patients having GSTM1 null genotype are at higher risk of LVDD. Chakarov et al. [21] show that the frequency of GSTT1 null genotypes was significantly higher in β-thalassaemic patients with myocardial siderosis than in
controls. Abo-Shanab et al. [22] studied the 56 thalassaemia patients, including 16 having cardiac complications and 20 healthy controls, and showed that the GSTM1 null genotype frequency has no role in β cardiac complications in thalassaemia patients; however, they have not mentioned the type of cardiac complication and method of assessment of cardiac complication. Wu et al. [23] in their study reported that of the two GSTT1 and GSTM1 variants, only GSTM1 null genotype was found to be associated with a decreased signal intensity ratio on MRI, suggesting the association with cardiac iron deposition. In another study, loss of both alleles was reported to lead decreased value of heart MRI-signal intensity [12]. In the present study, we did not observe any significant difference in ferritin values between GSTM1 and GSTT1 or both null patients which are similar to the study done by Sclafani et al.

### Table 2. Genotypic distribution of glutathione-S-transferase polymorphic variants and ApoE gene in different sub-groups.

| Genotypes          | Gender (male) | Diastolic dysfunction | Left ventricular hypertrophy |
|--------------------|---------------|----------------------|-----------------------------|
| GSTT1              |               |                      |                             |
| Null               | 66 (62.8)     | 24 (22.8)            | 37 (35.2)                   |
| Present            | 25 (71.4)     | 6 (17.1)             | 12 (34.2)                   |
| p-value, OR (95% CI)* | 0.284, 1.768 (0.738–4.238) | 0.462, 0.698 (0.329–1.481) | 1.000, 0.958 (0.541–1.699) |
| GSTM1              |               |                      |                             |
| Null               | 33 (70.2)     | 19 (40.4)            | 13 (27.6)                   |
| Present            | 33 (56.9)     | 5 (8.6)              | 24 (41.3)                   |
| p-value, OR (95% CI)* | 0.223, 1.786 (0.792–4.026) | <0.0001, 2.290 (1.592–3.293) | 0.566, 0.703 (0.427–1.157) |
| GSTT1 and GSTM1 Combined |       |                      |                             |
| Both Null GSTT1-GSTM1 | 12 (75.0)     | 1 (6.2)              | 4 (25.0)                    |
| GSTT1 and/or GSTM1 Present | 54 (60.7)     | 23 (25.8)            | 33 (37.1)                   |
| p-value, OR (95% CI)* | 0.401, 1.944 (0.580–6.513) | 0.031–1.618 | 0.212–1.766 |
| ApoE               |               |                      |                             |
| Haplotype          |               |                      |                             |
| E2/E4              |               |                      |                             |
| Null               | 5 (62.5)      | 5 (62.5)             | 3 (37.5)                    |
| Present            | 39 (59.1)     | 12 (18.2)            | 26 (39.4)                   |
| p-value*b          | 0.529         | 0.019                | 0.422                       |
| Allele             |               |                      |                             |
| E2                 | 5 (62.5)      | 5 (62.5)             | 3 (37.5)                    |
| E3                 | 100 (61.3)    | 31 (19.0)            | 60 (36.8)                   |
| E4                 | 27 (69.2)     | 12 (30.7)            | 11 (28.2)                   |
| p-value*b          | 0.837         | 0.007                | 0.594                       |

Note: Data are presented as number (percentage).
*aFisher’s exact test was used to calculate the p-value.
bChi-square test was used to calculate the p-value.

### Table 3. Age, haematological and biochemical parameters in different GST polymorphic variants and ApoE haplotypes.

| Genotypes          | Age (year)* | Hb (g/dL)* | Serum ferritin (ng/mL)* | Serum GST (µg/mL)* |
|--------------------|-------------|------------|------------------------|--------------------|
| GSTT1              |             |            |                        |                    |
| Null               | 16 (12–20)  | 9.5 ± 2.1  | 2940 (2800–3770)       | 46.82 ± 17.5       |
| Present            | 17 (12–19)  | 8.5 ± 2.1  | 3000 (2747–4000)       | 56.3 ± 23.1        |
| p-value            | 0.445       | 0.023      | 0.770                  | 0.040              |
| GSTM1              |             |            |                        |                    |
| Null               | 17 (14–20)  | 8.9 ± 2.1  | 3000 (2682–4000)       | 56.5 ± 23.2        |
| Present            | 16 (12–18.25) | 8.8 ± 2.2 | 3000 (2870–3630)       | 49.2 ± 16.6        |
| p-value            | 0.146       | 0.932      | 0.488                  | 0.422              |
| GSTT1 and GSTM1 Combined |       |            |                        |                    |
| Both Null GSTT1-GSTM1 | 15 (12.5–20.75) | 9.4 ± 2.1 | 2935 (2825–3422)       | 41.6 ± 11.9        |
| GSTT1 and/or GSTM1 Present | 16 (12–19)  | 8.7 ± 2.1  | 3000 (2735–4000)       | 55.0 ± 22.5        |
| p-value            | 0.918       | 0.240      | 0.768                  | 0.014              |
| ApoE               |             |            |                        |                    |
| E2/E4              | 17 (15.5–18) | 7.3 ± 2.6  | 2942 (2887.5–4732.5)   | 90.7 ± 31.1        |
| E3/E3              | 16 (12–18.25) | 8.9 ± 2.1 | 3050 (2707–4000)       | 48.7 ± 17.1        |
| E3/E4              | 16 (12–20)  | 9.3 ± 2.1  | 2970 (2787–3400)       | 49.5 ± 14.4        |
| p-value            | 0.700       | 0.064      | 0.558                  | 0.003              |

*aData are presented as median (interquartile range).
*bData are presented as mean ± standard deviation.
[11]. In the present study, patients having GSTT1 null genotype had higher mean pre-transfusion Hb and lower serum GST levels. Also serum GST levels were negatively correlated with pre-transfusion Hb suggesting that patients with high oxidative stress have low pre-transfusion Hb.

Apoe E2 allele was seen to contribute for diastolic dysfunction in our studied patient group (p-value 0.007). ApoE E2 allele is usually associated with lower levels of low-density cholesterol [24]. There is increasing evidence which suggests us that the oxidative modification of low-density cholesterol actively participates in the sequence of events leading to cardiovascular complications [25]. Recently, Dimou et al. [26] did a meta-analysis and shows E4 allele as a genetic risk factor for LVF in beta-thalassaemia major. Bazrgar et al. [27] show E4 allele as a risk factor for left ventricular systolic dysfunction thalassaemia patients; however, they did not find any association with left ventricular diastolic dysfunction. Left ventricular systolic function remains normal till late in thalassaemia patients and also advanced age is necessary for the effect of E4 on LV function to become manifest. E4 allele is not a necessary factor for developing LVF, but because of its reduced antioxidant and iron-binding activity, it should be considered a predictive genetic factor for cardiac dysfunctions in homozygous β-thalassaemic patients [28]. We also observed that patients having E2/E4 haplotype have almost 2 times more serum GST levels than patients having other haplotype.

There are certain limitations of the study. Firstly, we were not able to measure the myocardial iron overload which influences the oxidative stress in cardiomyocytes and thereby increases the diastolic dysfunction. Secondly, we have not examined the effect of GST and ApoE polymorphism on cardiac dysfunction in healthy individuals of the same age groups. Also we were not able to measure the serum Apolipoprotein E levels in studied subjects.

In conclusion, GSTM1 null genotype and ApoE E2 allele are significantly associated with diastolic dysfunction in thalassaemia patients.

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