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Original article

Hyperhomocysteinemia in pediatric β-thalassemia: links to vitamin cofactor deficiencies and oxidative stress

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Background: Homocysteine metabolism is crucial to maintaining vascular and metabolic homeostasis, yet its dysregulation in pediatric β-thalassemia major (β-TM) remains poorly understood.

Purpose: This study investigated the prevalence and determinants of hyperhomocysteinemia in pediatric β-TM with a focus on vitamin B9 (folate), B12, and B6 deficiencies, oxidative stress marker levels, and the impact of splenectomy.

Methods: A cross-sectional study was conducted of 92 pediatric β-TM patients. Levels of plasma homocysteine, vitamins B9, B12, and B6, and oxidative stress marker (protein carbonyls, thiols, nitrotyrosine, and nitric oxide metabolites) levels were measured. The patients were grouped based on their splenectomy status. The *MTHFR* C677T polymorphism was genotyped in a subset of patients (n=39). The statistical analyses included *t* tests, analysis of variance, Pearson's correlation, and multivariate regression.

Results: Overall, 93% of patients had hyperhomocysteinemia (≥15 μM), with the values of 50% exceeding 30 μM. Homocysteine levels were negatively correlated with folate levels (r=-0.22, P=0.03) and weakly correlated with B_{12} levels (r=-0.18, P=0.08). Vitamin B_6 levels were not significantly associated with homocysteine levels. Post-splenectomy, patients had significantly higher homocysteine levels (43.3 μM vs. 32.3 μM, P=0.002) but lower nitrotyrosine levels (P=0.035), suggesting reduced nitrative stress. The *MTHFR* C677T genotype did not significantly influence homocysteine levels in our cohort.

Conclusion: Hyperhomocysteinemia is prevalent in pediatric β-TM, driven primarily by severe folate and B12 deficiencies. Splenectomy exacerbates hyperhomocysteinemia but reduces nitrative stress, indicating complex metabolic shifts postsplenectomy. These findings highlight the need for routine homocysteine monitoring and targeted vitamin supplementation to mitigate the potential vascular risks of pediatric thalassemia.

Key words: Homocysteine, Thalassemia, Splenectomy, Vitamin B, Folate, Oxidative stress

Key message

Question: What are the biochemical and clinical correlates of hyperhomocysteinemia in pediatric β-thalassemia, and how does it relate to vitamin status, oxidative stress, and splenectomy?

Finding: Most pediatric β-thalassemia patients exhibited severe hyperhomocysteinemia, which was strongly associated with folate and B_{12} deficiencies and influenced oxidative stress patterns, particularly in splenectomized individuals.

Meaning: These findings suggest that routine monitoring and correction of B-vitamin deficiencies may mitigate hyperhomocysteinemia-related risks in pediatric thalassemia.

Introduction

Thalassemia is an inherited hemoglobin disorder characterized by ineffective erythropoiesis, chronic hemolysis, and the need for regular blood transfusions in severe cases.[1,2] Beyond anemia, transfusion-dependent β-thalassemia patients often suffer iron overload and heightened oxidative stress—excess ferritin from transfusions drives peroxidative tissue damage.[3,4] This pro-oxidant milieu can disrupt normal metabolism, including the pathways of homocysteine clearance.[5,6] Homocysteine is a sulfur-containing amino acid formed from methionine; its

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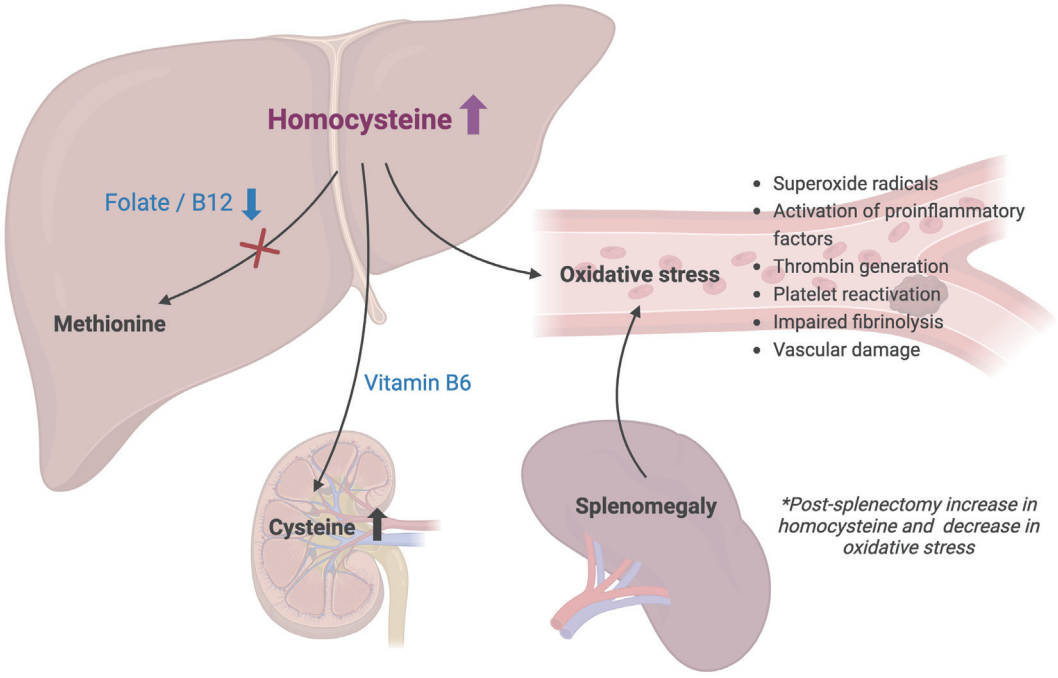

Graphical abstract. Simplified homocysteine metabolism in thalassemia. Schematic diagram visualizing how vitamin deficiencies in folate/B_{12} (common in thalassemia) lead to impaired homocysteine-to-methionine recycling, while vitamin B_6 is needed to convert homocysteine to cysteine (for glutathione, an antioxidant). The liver is a major site of homocysteine metabolism – notably, the betaine-dependent remethylation occurs only in the liver and kidney tissues. The kidneys play a role in homocysteine clearance. An elevated homocysteine level in the bloodstream can damage blood vessels (endothelium), contributing to thrombosis. In the context of β-thalassemia, inadequate liver/kidney cofactor availability (folate, B_{12}, B_6) leads to a systemic homocysteine level elevation, which in turn may harm the vascular organs. Our findings suggest that patients had lower nitrotyrosine levels postsplenectomy, indicating reduced peroxynitrite stress. This implies that splenectomy may alleviate certain aspects of oxidative stress, potentially reducing oxidative tissue damage.

metabolism requires adequate folate (vitamin B_9), vitamin B_{12}, and vitamin B_6 as cofactors. Folate and B_{12} are particularly critical for the remethylation of homocysteine to methionine, while B6 is needed for the transsulfuration of homocysteine to cysteine. Deficiencies in these vitamins lead to the accumulation of homocysteine.[7,8] Indeed, previous studies have noted that thalassemia patients often have reduced folate levels, and homocysteine serves as a sensitive biochemical marker of folate or B_{12} deficiency.[3]

Elevated homocysteine (hyperhomocysteinemia) is of clinical concern because it can impair endothelial function and promote thrombosis through multiple mechanisms.[9] Biochemically, hyperhomocysteinemia induces oxidative stress (via auto-oxidation generating reactive oxygen species) and endothelial injury, triggers proinflammatory pathways, and perturbs nitric oxide (NO) bioavailability. It also promotes a prothrombotic state by upregulating tissue factors, enhancing platelet reactivity, and inhibiting anticoagulant mechanisms.[9] In β-thalassemia, such effects are particularly relevant as patients (especially those who have undergone splenectomy) already face an elevated risk of thromboembolic events.[10] Notably, splenectomy is associated with a 4-fold increase in thrombosis risk in transfusion-dependent thalassemia, potentially exacerbated by hyperhomocysteinemia and oxidative endothelial damage.[10]

The interplay between homocysteine and oxidative stress in thalassemia appears to be complex. On one hand, thalassemia-related oxidative stress may alter homocysteine metabolism: one report suggests that increased oxidative stress can stimulate cystathionine β-synthase (CBS) activity, shunting homocysteine to cysteine and thus lowering plasma homocysteine levels in β-thalassemia.[4] This could partly explain why some well-transfused thalassemia patients paradoxically exhibit homocysteine levels in the normal or even low range despite folate deficiency. On the other hand, if vitamin deficiencies are profound, they may overwhelm such compensatory mechanisms, leading to significant homocysteine elevation—a scenario that could further fuel oxidative damage. Hyperhomocysteinemia itself has been linked to increased lipid peroxidation (e.g., high malondialdehyde) and reduced antioxidant capacity in β-thalassemia.[11] Moreover, the common methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism can modulate this balance: the homozygous 677TT genotype impairs folate

metabolism and is associated with higher homocysteine, especially under low-folate conditions.[11] Patients with β-thalassemia who carry the 677TT variant have been shown to be at increased risk of hyperhomocysteinemia and related oxidative vascular complications.[11]

In pediatric thalassemia, the role of homocysteine and its biochemical consequences remain underexplored. There is a relative paucity of literature on homocysteine levels in thalassemic children and their interplay with vitamin status and oxidative markers. Most studies to date have focused on adult or mixed-age cohorts, or have only examined limited facets (for instance, vitamins without oxidative stress markers, or vice versa). The present work addresses this gap by investigating homocysteine metabolism in a pediatric thalassemia population, with a special focus on biochemical mechanisms and clinical implications. We conducted a comprehensive analysis of plasma homocysteine levels in relation to B-vitamin status, genetic polymorphisms, and oxidative stress indicators (including NO and protein oxidation products) in children with β-thalassemia. In particular, we evaluated how splenectomy status and homocysteine-related genotypes influence these biochemical profiles. Through this, we aim to elucidate the significance of homocysteine in pediatric thalassemia and assess the originality of our findings in the context of existing literature.

Methods

1. Study design and participants

This study analyzed data from a cross-sectional cohort of N=92 pediatric patients with β-thalassemia (ages 1.8–15 years). All patients were diagnosed with thalassemia major or a severe thalassemia syndrome requiring regular transfusions, and many received chelation therapy for iron overload. Clinical data included age, sex, and whether the child had undergone a splenectomy. Patients were categorized as either presplenectomy (those with intact spleen, n=45) or postsplenectomy (those who had surgical spleen removal, n=47) at the time of evaluation. Splenectomy had typically been performed to alleviate hypersplenism or reduce transfusion requirements, and the postsplenectomy group was on average older (mean, ~10.5 years vs. ~5.5 years in presplenectomy), reflecting the clinical timing of this intervention. Inclusion criteria encompassed confirmed thalassemia diagnosis and pediatric age; patients with other chronic illnesses or on medications affecting folate/homocysteine metabolism (e.g., anticonvulsants, high-dose vitamins) were excluded to minimize confounders.

Informed consent was obtained from guardians, and the study was conducted with institutional ethical approval (Azerbaijan Medical University's IRB [No. 27102021.01]).

2. Biochemical measurements

Fasting blood samples were collected from all participants prior to their routine transfusion (to avoid transfusion-related transient changes). Plasma or serum was analyzed for total homocysteine and a panel of related biochemical markers. Homocysteine concentration (μmol/L) was measured using a chemiluminescent immunoassay on an automated analyzer, with an internal reference range of 5–15 μmol/L. Vitamin B_9 (folate) and B_{12} levels were quantified by immunoassay (chemiluminescence), and vitamin B_6 (pyridoxal-5'-phosphate) by high-performance liquid chromatography—results for vitamins are reported in ng/mL for folate (normal, 5–20 ng/mL), pg/mL for B_{12} (normal, 279–996 pg/mL), and μg/L for B_{12} (normal, ~5–30 μg/L, assay-adjusted). Markers of oxidative stress and NO metabolism were also assessed: protein carbonyl content was measured by DNPH (dinitrophenylhydrazine) assay as an index of protein oxidative damage; total free thiol groups in plasma (μmol/L) were measured by Ellman's reagent to reflect antioxidant thiol reserves; NO levels were determined by measuring stable oxidation products nitrate/nitrite (NO_x, μM) via the Griess reaction, providing an estimate of systemic NO production. 3-Nitrotyrosine (nmol/L) was measured by an enzyme-linked immunosorbent assay as a marker of protein tyrosine nitration, indicative of peroxynitrite-mediated oxidative stress. In addition, routine hemolysis and liver function markers were recorded: conjugated (direct) bilirubin and unconjugated (indirect) bilirubin (μmol/L), along with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (U/L), to gauge hemolytic burden and hepatic stress (e.g., from iron overload). All assays were performed in duplicate for accuracy, and quality controls were within acceptable ranges.

3. Genetic analysis

To investigate the role of homocysteine metabolism polymorphisms, genotypic screening was performed for the common *MTHFR* C677T variant on a subset of patients (n=39) where DNA was available. Genomic DNA was extracted from peripheral blood leukocytes, and the *MTHFR* 677C>T polymorphism was genotyped by DNA sequencing, amplifying the relevant gene region through polymerase chain reaction. The genotypes are classified as wild-type (CC), heterozygous (CT), or homozygous mutant (TT). This study specifically focused on the *MTHFR* C677T polymorphism due to its known effect on homocysteine levels, while other genetic variants (e.g., *MTHFR* A1298C, CBS mutations) were not routinely tested in this cohort.

4. Statistical analyses

All data were analyzed using IBM SPSS Statistics ver. 22.0 (IBM Co., USA) and Python SciPy. Continuous variables were checked for normality and summarized as mean±standard deviation. Comparisons between the presplenectomy and postsplenectomy groups were made using independent-sample *t* tests (or the nonparametric Mann-Whitney *U* test if distributions were skewed) for biochemical parameters. Categorical comparisons (e.g., sex distribution, proportion of vitamin deficiencies) utilized chi-square tests. One-way analysis of variance (ANOVA) was employed to evaluate differences in homocysteine levels across *MTHFR* genotype categories (CC vs. CT vs. TT); given the small TT sample, a nonparametric Kruskal-Wallis test was also considered for robustness. Pearson correlation analysis was performed to assess linear relationships between homocysteine and other continuous variables (vitamin levels, oxidative markers, and age). In particular, correlations of homocysteine with folate, B_{12}, and B_6 were examined to elucidate which vitamin deficiencies most strongly influence homocysteine in this cohort. We also explored correlations among oxidative stress markers (e.g., between thiols and nitrotyrosine) to see if these formed distinct clusters. A multivariable linear regression was planned to jointly assess predictors of homocysteine (with potential inputs like folate, B_{12}, splenectomy status, and genotype), though given the sample size, we primarily report univariate analyses. All statistical tests were two-tailed with a significance threshold of $P<0.05$.

To enhance the interpretation of results, subgroup analyses were carried out. We stratified certain analyses by age group (e.g., <10 years vs. ≥10 years) to account for the older age of postsplenectomy patients and the possibility of age-related differences in vitamin status or homocysteine. We also examined the postsplenectomy subgroup for any correlation between time-since-splenectomy and homocysteine or oxidative markers (as a proxy for adaptation after splenectomy). We referenced liver enzymes (ALT/AST) and bilirubin as indirect reflections of iron-related organ stress and hemolysis, respectively, and correlated these with homocysteine to probe any link between iron overload and homocysteine metabolism.

Results

1. Patient characteristics and vitamin status

The cohort consisted of 92 children (mean age, 8.1±5.0 years, 62% boys) with β-thalassemia. All were transfusion-dependent, receiving packed red cell transfusions roughly every 3–4 weeks, and many had evidence of iron overload (reflected by elevated liver enzymes and skin hyperpigmentation clinically). As expected, the postsplenectomy group was significantly older (mean, ~10.5 vs. 5.5 years, $P<0.001$) since splenectomy is typically performed in later childhood. Nutritional and biochemical assessment revealed widespread B-vitamin deficiencies. Mean serum folate (B_{12}) in the cohort was 3.16±2.73 ng/mL, and mean B_{12} was 159.8±235 pg/mL—both below pediatric reference ranges (folate normal, 5–20 ng/mL; B_{12} normal, ~300–1,000 pg/mL) (Table 1). Over 70% of children had folate levels indicative of deficiency (<5 ng/mL), and about half had low B_{12} (<200 pg/mL). Vitamin B_6 levels averaged 26.3±17

Table 1. Key biochemical parameters of pediatric thalassemia patients (overall and by subgroup)

Parameter	All patients (N=92)	Presplenectomy (n=45)	Postsplenectomy (n=47)	*P* value
Age (yr)	8.1±5.0	5.5±3.2	10.5±4.0	**<0.001**
Homocysteine (μmol/L)	37.2±17.2	32.3±16.7	43.3±16.0	**0.002**
Folate (vit B_9) (ng/mL)	3.16±2.73	3.41±2.79	2.92±2.68	0.39
Vitamin B_{12} (pg/mL)	159.8±235.4	171.1±167.0	147.8±281.6	0.63
Vitamin B_6 (μg/L)	26.3±17.2	27.6±17.8	24.7±16.7	0.42
Protein carbonyls (nmol/mg)	321.8±135.0	344.1±148.1	301.9±119.9	0.27
Total thiols (μmol/L)	179.2±83.0	201.5±71.7	158.3±88.4	0.08
Nitric oxide (μM)	45.7±10.8	46.8±10.1	44.6±11.5	0.53
3-Nitrotyrosine (nmol/L)	18.6±9.6	21.4±9.8	15.9±9.1	0.035
Unconjugated bilirubin (μmol/L)	23.3±2.9	22.4±3.0	24.0±2.6	**0.014**
Conjugated bilirubin (μmol/L)	18.7±2.9	18.0±3.2	19.4±2.5	**0.032**
ALT (U/L)	41.4±4.9	39.2±4.7	43.5±5.0	**0.016**
AST (U/L)	41.5±4.3	39.1±5.0	43.9±3.0	0.066

Values are presented as mean±standard deviation.
Reference ranges: homocysteine, 5–15 μmol/L; folate, 5–20 ng/mL; B_{12}, 279–996 pg/mL.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Pre- vs. postsplenectomy comparisons were made using *t* tests.
Boldface indicates a statistically significant difference with $P<0.05$.

μg/L; while reference standards for pediatric B_6 vary, approximately one-third of patients had suboptimal B_6 status.

2. Homocysteine and correlation with vitamins

Consistent with the vitamin profile, plasma homocysteine was markedly elevated in this pediatric thalassemia cohort. The overall mean homocysteine was 37.2±17.2 μmol/L, well above the upper limit of normal (15 μmol/L). Notably, 86 out of 92 children (93%) had homocysteine levels classified as hyperhomocysteinemia (≥15 μmol/L), and more than half exceeded 30 μmol/L, indicating moderate to severe elevation. Despite these generally high homocysteine concentrations, there was substantial interindividual variation (range, 11.4 to 88.7 μmol/L). There was no evident difference in homocysteine between boys and girls in our cohort (P=0.63), suggesting that the metabolic differences were disease-related rather than sex-related.

We observed an inverse relationship between homocysteine and folate levels (Fig. 1). Pearson analysis showed a statistically significant negative correlation between serum folate and homocysteine (r≈-0.22, P=0.03). In contrast, the correlation between vitamin B_{12} and homocysteine, while negative, did not reach statistical significance in our data (r≈-0.18, P=0.08). Vitamin B_6 levels in our patients did not correlate significantly with homocysteine (r≈-0.17, P>0.1), although a trend of lower B_6 associating with higher homocysteine was present. It is plausible that folate deficiency dominated the effect; additionally, plasma B_6 may not perfectly reflect functional B_6 status (pyridoxal phosphate inside cells).

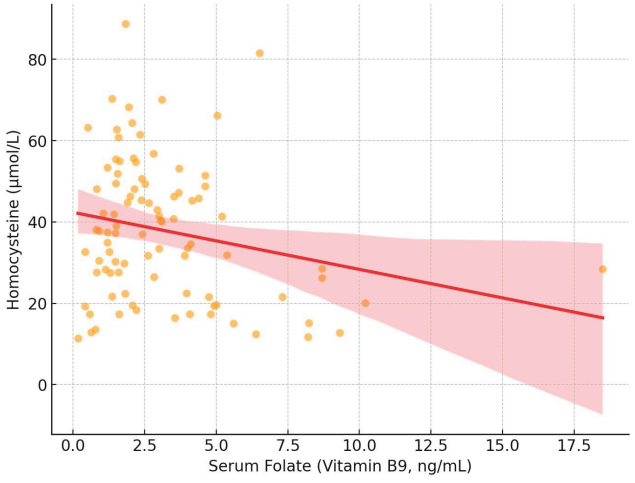

Fig. 1. Correlation between serum folate and homocysteine. Scatter plot depicting the relationship between folate (vitamin B_9, x-axis in ng/mL) and homocysteine (y-axis in μmol/L) for all 92 patients. Each point represents an individual child. A fitted regression line with 95% confidence band is overlaid is shown. The plot demonstrates an inverse correlation: patients with low-folate level tended to cluster at high homocysteine levels. For instance, most children with a folate level <5 ng/mL had a homocysteine level >30 μM. The Pearson correlation coefficient (r≈–0.22) and significance (P=0.03) are indicated on the graph. This visualization reinforces the key finding that a folate deficiency was associated with an elevated homocysteine level in thalassemia. The inclusion of reference lines for normal folate (5 ng/mL) and upper-normal homocysteine (15 μM) levels further emphasizes how many patients fell into the deficient/high quadrant.

3. Homocysteine and oxidative stress markers

To evaluate the biochemical interplay between homocysteine and oxidative stress, we examined markers of redox status. Levels of protein carbonyls were elevated in many patients (overall mean, ~322±135 nmol/mg), indicating significant protein oxidative damage (Fig. 2). Mean 3-nitrotyrosine levels in our cohort were 18.6±9.6 nmol/L, comparable to levels reported in healthy pediatric populations.[12] Total thiol concentrations (which include antioxidant molecules like glutathione and cysteine) were somewhat diminished (mean, ~179±83 μmol/L), though reference pediatric values for total plasma thiols are not well-established. In general, patients with higher homocysteine did not show a linear increase in oxidative markers; in fact, no significant positive correlations were found between homocysteine and nitrotyrosine or carbonyls. If anything, a slight negative correlation was noted (homocysteine vs. nitrotyrosine r≈-0.12, P>0.2, not significant), suggesting that those with the very highest homocysteine tended to have, paradoxically, somewhat lower nitrative stress levels. This counterintuitive trend became clearer upon subgroup analysis (see below "4. Subgroup

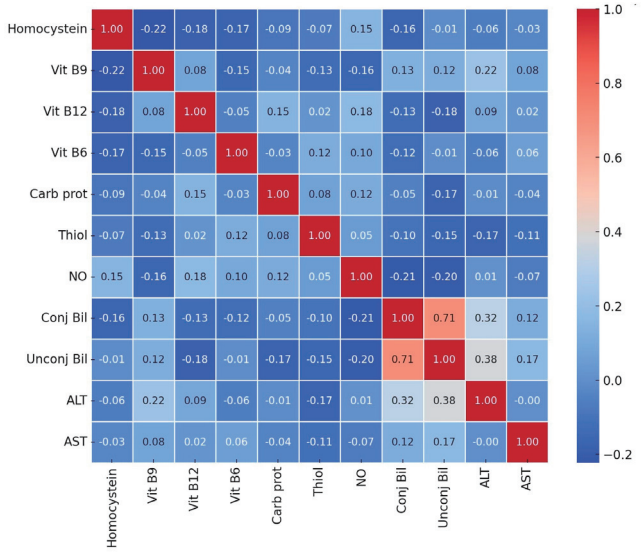

Fig. 2. Heatmap of correlations among key biochemical markers. A color-coded heatmap illustrating the Pearson correlation matrix for select variables including homocysteine, folate, vitamin B_{12}, vitamin B_6, total thiols, protein carbonyls, nitrotyrosine, and nitric oxide (NOx). Each cell in the grid represents the correlation coefficient between the row and column variable, with a color scale from blue (negative correlation) to red (positive correlation). ALT, alanine aminotransferase; AST, aspartate aminotransferase.

analysis – pre- vs. postsplenectomy") and likely relates to splenectomy status.

Among the oxidative stress parameters themselves, we observed expected interrelationships (Fig. 2). For example, higher protein carbonyl levels tended to coincide with lower thiol levels (inversely, though not a strong correlation), consistent with the consumption of thiol antioxidants during oxidative protein damage. Nitrotyrosine levels showed a positive (but modest) association with protein carbonyls, suggesting that patients experiencing more oxidative protein damage also had more nitrative stress—likely due to overall higher reactive oxygen and nitrogen species in circulation. These patterns validate that our oxidative stress markers are capturing the oxidative burden in patients. The NO (nitrate/nitrite) levels did not differ dramatically across patients (mean, ~45±11 μM), and homocysteine showed no significant correlation with NO metabolite levels.

4. Subgroup analysis – pre- vs. postsplenectomy

A major finding of this study is the difference in homocysteine and related parameters between children with intact spleens and those who had undergone splenectomy. Homocysteine levels were significantly higher in postsplenectomy patients: the postsplenectomy group had a mean homocysteine of 43.3±16.0 μmol/L compared to 32.3±16.7 μmol/L in the presplenectomy group (P=0.002). Fig. 3A illustrates this difference, showing that the distribution of homocysteine in splenectomized children is shifted upward, with very few postsplenectomy patients having homocysteine below 20 μmol/L. This novel observation suggests that splenectomy status (or factors correlated with it, such as age or disease duration) influences homocysteine metabolism. Supporting this, we also found that homocysteine was mildly positively correlated with age (r≈+0.20, P ~0.06), implying that as thalassemic children grow older–and many undergo splenectomy – homocysteine tends to rise.

Nitrotyrosine levels were unexpectedly lower in the postsplenectomy group (mean, 15.9±9.1 nmol/L) compared to the presplenectomy group (21.4±9.8 nmol/L, P=0.035). In other words, children who still had their spleen showed about 35% higher nitrative stress on average than those without spleens (Fig. 3B). Similarly, total thiol levels were lower (worse) in postsplenectomy patients (158±88 μM vs. 202±72 μM in presplenectomy, P=0.08), although this difference was of only borderline significance. Protein carbonyls were slightly (but not significantly) lower in postsplenectomy patients (mean, ~302 nmol/mg vs. 344 nmol/mg, P=0.27). Taken together, these suggest that certain oxidative markers (especially those related to NO/peroxynitrite, like nitrotyrosine) were ameliorated after splenectomy, whereas measures of general oxidative burden did not worsen and, in some cases, trended better.

On the other hand, markers of hemolysis and liver metabolism were higher in the postsplenectomy group: mean unconjugated bilirubin was 24.0±2.6 μmol/L postsplenectomy vs 22.4±3.0 μmol/L presplenectomy (P=0.014), and conjugated bilirubin 19.4±2.5 versus 18.0±3.2 μmol/L (P=0.032). Liver enzyme levels were also elevated postsplenectomy (ALT, 43.5 U/L vs. 39.2 U/L, P=0.016; AST, 43.9 U/L vs. 39.1 U/L, P=0.066).

Folate levels were slightly lower on average in postsplenectomy patients (2.92±2.68 ng/mL) than in presplenec-

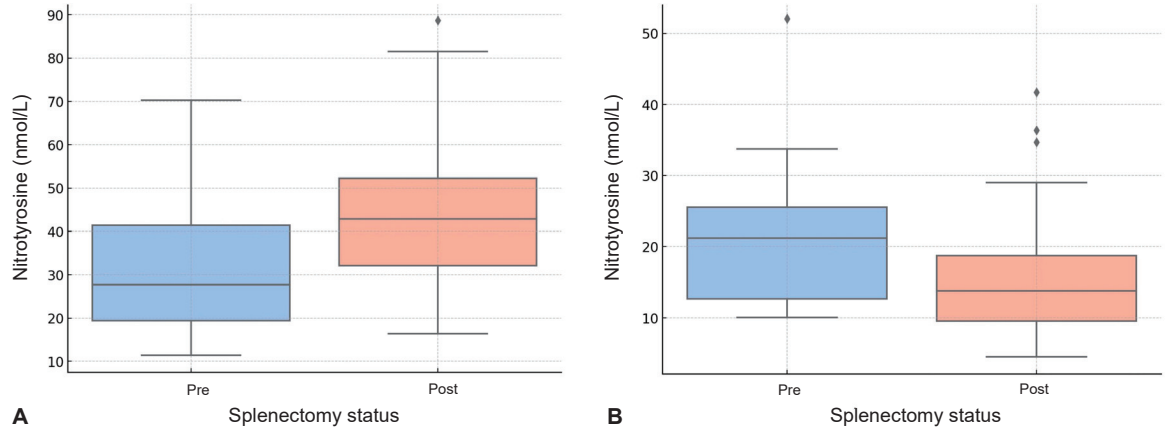

Fig. 3. Group comparisons of homocysteine and nitrotyrosine in pediatric thalassemia. (A) Box plot of plasma homocysteine levels in children presplenectomy (Pre-Spl) versus postsplenectomy (Post-Spl). The Post-Spl group showed a higher median homocysteine level and a distribution shifted toward elevated levels, with a significantly higher mean (43 μM vs. 32 μM, P<0.01). The box indicates the interquartile range with the median line; the whiskers span the 5th–95th percentiles. (B) Box plot of 3-nitrotyrosine levels in the Pre-Spl versus Post-Spl groups. In contrast to the homocysteine level, the nitrotyrosine level was lower in the Post-Spl group (median, ~14 nmol/L vs. ~20 nmol/L in Pre-Spl, P<0.05), suggesting reduced nitrative stress postsplenectomy. These side-by-side plots highlight the divergent effects of splenectomy on homocysteine versus nitrosative stress marker levels.

tomy (3.41±2.79 ng/mL), but this was not significant (P=0.39). Vitamin B_{12} was also somewhat lower postsplenectomy (148±282 pg/mL) vs presplenectomy (171±167 pg/mL), again not significant. Vitamin B_6 showed no significant difference.

5. *MTHFR* genotype and homocysteine

Of the 39 patients genotyped for the *MTHFR* C677T polymorphism, 18 (46%) were wild-type (CC), 15 (38%) heterozygous (CT), and 6 (15%) homozygous mutant (TT). This frequency of the TT genotype (~15%) is higher than that in many general populations (often ~10%), though our sample is small and possibly enriched for severe cases. We evaluated whether genotype correlated with homocysteine levels. Surprisingly, homocysteine did not differ significantly across *MTHFR* genotypes. Wild-type patients had a mean homocysteine of 39.5 μmol/L, CT heterozygotes 38.0 μmol/L, and TT homozygotes 37.9 μmol/L (ANOVA, P=0.97). In fact, median values were also very similar (~37 μmol/L in each subgroup).

In summary, the key results from this study include: (1) a widespread elevation of homocysteine in pediatric thalassemia, correlated with low-folate levels; (2) a significant increase in homocysteine in postsplenectomy patients, accompanied by distinct changes in oxidative stress markers (notably lower nitrotyrosine) and higher hemolytic markers; (3) confirmation of ongoing oxidative stress in these children (high protein carbonyls, altered thiols), though no direct proportional relationship with homocysteine concentration; and (4) a lack of significant genotype effect on homocysteine, highlighting nutritional status as the dominant influence.

Discussion

In this study, we conducted a detailed biochemical investigation of homocysteine metabolism in pediatric β-thalassemia, revealing important insights into its mechanisms and clinical implications. Our findings demonstrate that hyperhomocysteinemia is prevalent among thalassemic children, likely driven by chronic nutritional deficiencies and high metabolic demand. We also uncovered how splenectomy status and oxidative stress interplay with homocysteine levels, contributing new knowledge to a sparsely studied area.[3]

1. Homocysteine elevation in thalassemia – nutritional and biochemical mechanisms

The pronounced elevation of homocysteine in our cohort underscores a fundamental biochemical imbalance in many thalassemic children: inadequate cofactors for one-carbon metabolism (graphical abstract). Folate (B_9) and cobalamin (B_{12}) are essential to recycling homocysteine into methionine via the methionine synthase pathway.[12-14] In our patients, severe folate deficiency emerged as the key factor associated with homocysteine accumulation. This aligns with general biochemical expectations and is consistent with homocysteine's role as a surrogate marker for folate/B_{12} deficiency.[3] It also complements clinical observations that thalassemia patients often require folate supplementation to support their high-output erythropoiesis. Without sufficient folate, homocysteine cannot be efficiently remethylated, causing it to build up in plasma. Vitamin B_{12} deficiency can produce a similar effect (via the "methyl trap" mechanism), and indeed many of our patients had low B_{12} as well. Interestingly, however, our data suggested folate was the more limiting nutrient: homocysteine correlated significantly with folate levels, whereas the correlation with B_{12} was weaker. This might be a unique aspect of our cohort's diet or supplement regimen – perhaps folate intake was particularly poor. Another consideration is that B_{12} deficiency in early childhood takes time to manifest in rising homocysteine due to liver stores, whereas folate reflects a more immediate dietary status. Regardless, the message is clear that ensuring adequate folate and B_{12} in thalassemia is critical to prevent hyperhomocysteinemia. The role of vitamin B_6, a cofactor for homocysteine's conversion to cysteine, is also notable. Though we did not find a strong direct correlation, suboptimal B_6 could impair the transsulfuration pathway (CBS function), further contributing to high homocysteine.[5,12,14] This pathway is especially relevant under oxidative stress, as transsulfuration helps generate cysteine for glutathione synthesis—a vital antioxidant.[15,16] Our data hint that many thalassemia patients may have insufficient B_6 activity (whether due to diet or increased turnover), which could limit this adaptive response.

The high homocysteine levels observed in our pediatric cohort are in contrast to some reports in adult thalassemia populations. For instance, Ozdem et al.[4] found that adult patients with β-thalassemia major actually had lower homocysteine (mean, ~6.4 μM) compared to healthy controls (~8.7 μM), despite lower folate levels in the patients. They hypothesized that chronic oxidative stress in thalassemia stimulates homocysteine catabolism via the B_6-dependent transsulfuration pathway, thereby keeping homocysteine low. Indeed, oxidative stress can upregulate CBS activity, effectively "shunting" homocysteine into cysteine and mitigating its accumulation.[5,6] Why then do our pediatric patients show the opposite (high homocysteine)? One key difference is the vitamin status: in Ozdem's adult cohort, baseline folate was low but perhaps not critically deficient (mean ~9.1 nmol/L, roughly 4 ng/

mL), and many adult patients receive folate supplements as part of care.[4] In our children, folate levels were frankly deficient (often <3 ng/mL) and likely not supplemented consistently (especially in resource-limited settings). Thus, any CBS upregulation may have been unable to compensate for the massive homocysteine load generated by folate/B_{12} insufficiency. In essence, vitamin deficiency can dominate over oxidative stress adaptation, leading to net hyperhomocysteinemia. This highlights a crucial point of originality and significance: our study shows that without adequate vitamin support, pediatric thalassemia patients are at high risk of severe homocysteine elevation—a finding that calls for greater attention to nutritional management in this population. It addresses a gap where previous studies focused on adults (with better-managed nutrition) might have underestimated homocysteine issues in children.

2. Splenectomy and oxidative stress – interpreting the paradox

One of the novel observations from our data is the reduction in nitrotyrosine levels in postsplenectomy children, despite their having more advanced disease (older age, more transfusions). This suggests that the spleen may play a previously underappreciated role in propagating nitrative stress in thalassemia. A plausible explanation lies in the spleen's function: it aggressively sequesters and destroys abnormal red blood cells. In β-thalassemia, the spleen is enlarged and hyperactive, with numerous macrophages ingesting defective erythrocytes.[17,18] These activated macrophages produce high levels of reactive oxygen and nitrogen intermediates as part of the phagocytic process. Nitric oxide (produced by inducible NO synthase in macrophages) can combine with the superoxide from iron-rich red cell debris to form peroxynitrite, which nitrates tyrosine residues on proteins (hence elevated nitrotyrosine).[9] When the spleen is removed, this intense site of reactive species generation is eliminated, which could lead to a drop in certain oxidative markers like nitrotyrosine. Our finding supports this model—postsplenectomy patients had significantly lower nitrotyrosine, indicating less ongoing peroxynitrite stress. Interestingly, their total NOx levels were not higher than presplenectomy (if anything slightly lower), which might reflect the loss of a major NO-producing organ (the spleen's macrophages). This nuanced biochemical change postsplenectomy is a new insight that adds depth to the understanding of splenectomy's impact beyond standard hematologic changes. It suggests that removal of the spleen might alleviate some aspects of oxidative stress (particularly nitrative stress), even as it exacerbates others (like iron deposition in the liver, as seen by ALT increase). Clinically, this could mean that splenectomy might reduce certain oxidative damage to tissues, possibly offsetting some negative effects of iron overload. However, more research is needed to confirm and detail these effects, as our study is one of the first to document changes in nitrotyrosine with splenectomy in thalassemia.

At the same time, not all oxidative indicators improved postsplenectomy. Total thiols were lower (nonsignificantly) in splenectomized patients, implying a trend toward reduced antioxidant reserves. This could be due to the higher iron load and chronic oxidative stress of older patients overwhelming their antioxidant capacity. Protein carbonyls, a marker of cumulative protein oxidation, were high in both groups and only slightly lower after splenectomy. Therefore, while nitrotyrosine (a footprint of reactive nitrogen species) decreases, general oxidative stress remains a concern postsplenectomy. These findings collectively highlight that oxidative stress in thalassemia is multifactorial: some sources (splenic activity) can be removed, but others (iron overload, chronic inflammation) persist or worsen over time. From a therapeutic standpoint, this suggests that antioxidant strategies might need to be tailored—for example, therapies targeting NO/peroxynitrite pathways might be more relevant before splenectomy, whereas broader antioxidants (or iron chelation to reduce Fenton chemistry) remain important throughout.

3. *MTHFR* polymorphism and homocysteine – a subtle effect in a severe setting

The *MTHFR* C677T polymorphism is often linked to elevated homocysteine levels, particularly in individuals with marginal folate status.[11,19] However, in our pediatric cohort, genotype did not significantly impact homocysteine levels, despite a TT genotype prevalence of 15%. This suggests that severe vitamin deficiencies overshadow genetic influences, making environmental factors (e.g., folate and B_{12} depletion) the primary drivers of hyperhomocysteinemia. It is also possible that our sample size was too small to detect minor genotype-related differences, or that in the context of severe deficiency, everyone is at risk, regardless of genetic background.

Notably, the lack of a significant effect of the *MTHFR* C677T polymorphism on homocysteine levels suggests that severe vitamin deficiencies may override genetic predisposition in this pediatric cohort. While previous studies in adults have linked the TT genotype to hyperhomocysteinemia and increased oxidative stress, our findings indicate that nutritional deficiencies dominate homocysteine metabolism in children, regardless of genotype. This highlights the importance of early vitamin supplementation in preventing excessive homocysteine

accumulation.

4. Interplay of homocysteine and oxidative stress

A central theme of this work is the relationship between homocysteine and oxidative stress in thalassemia. Our data illustrate that this relationship is not straightforward. We did not find a direct proportional increase in oxidative damage markers with rising homocysteine levels; in fact, some of the highest oxidative stress readings (nitrotyrosine) were in patients with moderate homocysteine (in the presplenectomy group), whereas postsplenectomy patients had higher homocysteine but somewhat lower nitrotyrosine. This could be interpreted through a mechanistic lens: homocysteine and oxidative stress can influence each other in opposite directions. Oxidative stress can lower homocysteine by activating transsulfuration—essentially a compensatory mechanism to boost antioxidant defenses (producing cysteine and glutathione from homocysteine).[5,20] Conversely, high homocysteine can worsen oxidative stress by generating reactive oxygen species and reducing antioxidant molecules like NO and glutathione.[2,5] In our pediatric patients, we are likely to see both forces at play simultaneously. For example, a child with very high homocysteine due to folate deficiency may start to experience more oxidative stress from that hyperhomocysteinemia, but at the same time, the body may try to catabolize homocysteine via CBS because oxidative stress from iron is present—a tug-of-war of sorts. The net result in a cross-sectional snapshot might be that homocysteine and oxidative markers appear uncorrelated or even inversely correlated in certain subgroups. This complexity is an important insight and speaks to why previous studies have reported seemingly conflicting results regarding homocysteine in thalassemia (some finding low homocysteine due to oxidative stress adaptation.[3,4] others raising concern about high homocysteine causing damage).[11] Our study, by measuring both sides of the equation, suggests that both phenomena are real and might dominate under different conditions. When folate/B_{12} are severely deficient, the "homocysteine-up" phenomenon dominates (leading to hyperhomocysteinemia despite oxidative stress), whereas if vitamins are replete, the "homocysteine-down" mechanism might be more evident (where oxidative stress drives homocysteine into cysteine). This novel conceptual understanding is a significant contribution of our work, as it reconciles prior discrepancies and provides a more nuanced picture of homocysteine biology in thalassemia.

Another finding was that total NOx did not drop with high homocysteine in our data, whereas Abd-Elmawla et al.[11] reported a significant reduction of NO in hyperhomocysteinemic thalassemia patients. One reason could be that many of our patients were very young; endothelial dysfunction from hyperhomocysteinemia might not manifest systemically until later in life. Children's vessels could be more resilient or actively produce NO to compensate. It's also possible that our measure of NO (plasma nitrate/nitrite) is influenced by other factors (dietary nitrites, renal function) and not sensitive enough to homocysteine's effect. Nonetheless, the preservation of NOx in the face of hyperhomocysteinemia might be somewhat reassuring, suggesting that not all protective mechanisms are lost in these children. It might also tie into the splenectomy story—with spleen removal, one loses a sink for NO (macrophages producing peroxynitrite), potentially leaving more NO available in plasma.

5. Clinical implications of hyperhomocysteinemia

The consistently high homocysteine levels observed in our cohort raise concerns about potential vascular and systemic complications in pediatric thalassemia. Homocysteine is known to exert toxic effects on the vascular endothelium and promote prothrombotic tendencies.[9] which is particularly concerning in thalassemia patients who already face increased thrombotic risk due to abnormal RBC membranes, platelet activation, and splenectomy.[20-22] Our finding that postsplenectomy patients had the highest homocysteine levels suggests that elevated homocysteine may further amplify thrombotic risk, possibly acting synergistically with hematologic changes after spleen removal.[9,10,23] While prior studies in well-transfused thalassemia patients have questioned homocysteine's role in thrombosis, our cohort exhibited ubiquitous hyperhomocysteinemia, reinforcing the need for monitoring and intervention, particularly in splenectomized individuals.[24] Simple B-vitamin supplementation could help lower homocysteine levels and potentially reduce vascular risk, though outcome trials are needed. Beyond thrombosis, hyperhomocysteinemia may also contribute to neurological and skeletal complications,[25,26] including cognitive impairment, peripheral neuropathy, and osteoporosis, which are already prevalent in thalassemia patients due to iron overload and metabolic imbalances.[27] Although we did not directly assess these clinical outcomes, our findings suggest that homocysteine may serve as a useful biomarker and a modifiable therapeutic target in pediatric thalassemia.

From a clinical perspective, our results reinforce the importance of routine homocysteine monitoring, particularly in splenectomized patients, and the proactive use of folate and B_{12} supplementation. Given homocysteine's established role in endothelial dysfunction and thrombosis, addressing vitamin deficiencies early in thalassemia management may help reduce long-term vascular com-

plications. Future studies should explore longitudinal changes in homocysteine with vitamin therapy and whether targeted interventions can improve clinical outcomes in pediatric thalassemia patients.

6. Comparison with existing literature

Prior to our study, the literature on homocysteine in pediatric thalassemia was limited. The study by Likhitha et al.[3] gave an initial look, finding modest elevations in homocysteine (~11 μM) in Indian thalassemic children and a correlation with B_{12}. Our work expands significantly on that by examining a broader panel of biochemical factors (adding vitamin B_6, oxidative markers, and genetic data) and by focusing on subgroups like splenectomy status. To our knowledge, this is the first study to report how splenectomy might influence homocysteine and oxidative stress markers in thalassemia. It is also one of the first to document such high levels of homocysteine in a pediatric cohort, highlighting a potential unmet need in care.

Our results also complement and contrast with adult studies. The Egyptian study by Abd-Elmawla et al.[11] showed that TT genotype thalassemia patients had worse oxidative profiles; while we did not see genotype effects, we did see that hyperhomocysteinemia (regardless of cause) coexisted with oxidative stress. We thereby reinforce the idea that homocysteine is a contributor to oxidative damage in thalassemia, even if the genotype is not the driver in our cohort. Another adult study from Italy concluded that homocysteine was not a major factor in thrombosis for thalassemia major,[24] which may be true for well-transfused adults with normal homocysteine. Our pediatric data caution that in less-optimized settings, homocysteine can become very high, potentially changing its relevance. Thus, our study adds a pediatric perspective that was missing and suggests that management guidelines consider monitoring homocysteine or at least ensuring vitamin repletion from early in life.

7. Limitations and implications for future research

This study has several limitations, including its cross-sectional design, which captures only a single time-point and does not track longitudinal changes in homocysteine levels postsplenectomy or with age. The modest sample size, particularly for genotyped patients, limits conclusions on genetic influences, and the absence of a healthy control group prevents direct oxidative stress comparisons. Incomplete data on vitamin supplementation may have introduced variability, though pre-transfusion sampling minimized acute fluctuations. Despite these limitations, the study's internal comparisons (e.g., pre- vs. postsplenectomy) remain valid and offer meaningful insights. Future research should explore longitudinal follow-ups to assess whether aggressive vitamin supplementation lowers homocysteine and improves clinical outcomes. Interventional trials comparing high-dose B-vitamin therapy to standard care could clarify its impact on endothelial function, while mechanistic studies should investigate why splenectomy reduces nitrotyrosine, possibly by examining macrophage activity or NO synthase expression. Additionally, exploring other genetic factors (CBS, methionine synthase polymorphisms) in different populations may provide a broader understanding of homocysteine regulation in thalassemia. Clinically, these findings emphasize the importance of proactive vitamin B_9/B_{12} supplementation from diagnosis and suggest that homocysteine monitoring could help identify high-risk patients, particularly postsplenectomy. Whether homocysteine-lowering strategies (e.g., folate/B_{12} or betaine therapy) could mitigate vascular risks in thalassemia warrants further investigation, drawing parallels from homocystinuria and cardiovascular prevention strategies.

In conclusion, our study highlights homocysteine as a crucial biochemical factor in pediatric thalassemia, closely linked to nutritional deficiencies and oxidative stress. We found that elevated homocysteine, particularly in children with low folate and B_{12}, may contribute to endothelial dysfunction and prothrombotic tendencies, compounding vascular risks in this population. Notably, splenectomy was associated with even higher homocysteine levels but lower nitrotyrosine, suggesting a complex metabolic shift where oxidative stress patterns change postsplenectomy. Our findings emphasize that pediatric thalassemia patients may differ from adults in homocysteine behavior, likely due to differences in vitamin management and developmental metabolism. By integrating biochemical, genetic, and oxidative stress analyses, this study fills an important research gap and underscores the clinical need for routine homocysteine monitoring and targeted vitamin supplementation. Given its role as both a nutritional marker and a potential contributor to pathology, homocysteine should be considered in comprehensive thalassemia management. Future research should explore targeted nutritional interventions and long-term outcomes to enhance the quality of life in children with thalassemia.

Footnotes

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