 doi:10.20944/preprints201909.0033.v1

Major depression in children with β-thalassemia major is strongly associated with the number of blood transfusions, iron overload and increased levels of interleukin-1β.

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 doi:10.20944/preprints201909.0033.v1

Abstract

Beta-thalassemia major (β-TM) patients are treated with repeated blood transfusions, which may cause iron overload (IO), which in turn may induce immune aberrations. Patients with β-TM have an increased risk of major depressive disorder (MDD). The aims of the present study are to examine whether repeated blood transfusions, IO and immune-inflammatory responses are associated with MDD in children (6-12 years) with β-TM. The Children's Depression Inventory (CDI), iron status (serum iron, ferritin, transferrin, TS%) and serum levels of CCL11, IL-1β, IL-10, and TNF-α were measured in β-TM with (n=54) and without (n=57) MDD and in healthy children (n=55). The results show that MDD in β-TM is associated with a greater number of blood transfusions, increased IO and IL-1β levels. Partial Least Squares path analysis shows that 68.8% of the variance in the CDI score is explained by the number of blood transfusions, IO, and increased levels of IL-1β and TNF-α. The latter two cytokines partly mediate the effects of IO on the CDI score, while the effects of blood transfusions on the CDI score are partly mediated by IO and the path from IO to immune activation. IO is also associated with increased IL-10 and lower CCL11 levels but these alterations are not significantly associated with MDD. In conclusion, blood transfusions may be causally related to MDD in β-TM children and their effects are in part mediated by increased IO and the consequent immune-inflammatory response. The results suggest that not only IO and its consequences including inflammation and ferroptosis, but also other factors related to the number of transfusions may cause MDD including psychosocial stressors. Current treatment modalities with folic acid and vitamin C are insufficient to attenuate IO and immune-inflammatory responses and to prevent MDD is children with β-TM undergoing blood transfusions.

 doi:10.20944/preprints201909.0033.v1

Keywords: depression, cytokines, neuro-immune, inflammation, oxidative stress, antioxidants.

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Introduction

Beta-thalassemia major (β-TM) is a genetic disorder with mutations in the β-globin gene on chromosome 11 resulting in a lack or reduction of the beta-globin chain causing globin chain imbalances [1]. Excess of free α-globin chains in maturing red blood cells may lead to their destruction with consequent anemia [2, 3]. When the thalassemia symptoms are severe and the patients become blood transfusion-dependent the thalassemia is named "thalassemia major" implicating that other organs are or will be affected [4, 5]. β-TM patients have an increased risk for systemic infections, suggesting that a basic defect in the host defense is present [6]. Infections and heart failure are the first two causes of death and hepatic disease is the third most common cause of death in TM [7, 8]. Immune defects in β-TM comprise the innate and the adaptive immune systems including aberrations in cell-mediated immunity, neutrophil chemotaxis, antibody response and decreased activity of T- and B-lymphocytes [6, 9, 10].

Patients with β-TM require lifelong blood transfusions to maintain levels of hemoglobin as normal as possible and to suppress ineffective erythropoiesis [11]. Chronic red blood cell (RBC) transfusions may cause transfusional haemosiderosis, a type of iron overload (IO), which may have clinical consequences by affecting liver, heart, and endocrine organs [12, 13]. IO toxicity has been implicated as main cause of the immune aberrations in β-TM, including defective phagocytic and chemotaxis activity of macrophages and neutrophils, decreased natural killer cell activity, alterations in T and B lymphocytes subsets and functions, impaired lymphocytes proliferative responses to mitogens and antigens, increased immunoglobulin production as well as alterations in cytokines responses [14-17]. Most importantly, increased iron stores are associated with inflammatory responses and increased vulnerability to both bacterial and viral infections [18].

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Intracellular iron accumulation may induce M1 macrophage polarization [19] and increased inflammatory gene expression [20].

Iron chelation therapy, which is used in β-TM to reduce IO, is widely used to prevent the consequences of IO on heart, liver, bones, and immune system [21] but is usually associated with many adverse effects including infections [9, 22]. Some clinicians propose that treatment with vitamin C and folic acid in TM patients may improve erythropoiesis and the secondary deficiencies in these vitamins [23, 24]. The iron status in β-TM patients undergoing blood transfusions can be monitored by noninvasive methods including measurements of serum iron (Fe), which is distributed within the body via plasma transferrin (Tf), a transport protein that mediates iron exchange between tissues, total iron-binding capacity (TIBC), Tf levels, Tf saturation percentage (TS%), unsaturated iron-binding capacity (UIBC) and ferritin levels [25]. Ferritin is the primary storage compound for the body's iron while serum ferritin concentrations offer a reliable index of iron stores and inflammation as well [26-28].

Patients with β-TM show increased vulnerability to develop emotional and behavioral problems and this is further increased in children [29, 30-32]. Many β-TM patients (up to 50%) have some type of mental disorder including depression and anxiety, which are additionally negatively associated with lowered quality of life [30, 32, 33]. Other studies showed high rates (30.8%-49.0%) of depressed mood or major depressive disorder (MDD) in those patients [31, 34, 35]. Moreover, MDD in β-TM is associated with increased suicide attempts, increased social isolation, low school performance, limited life opportunities, maladaptive surviving strategies, and lower physical abilities [34, 36].

The interplay between the immune and nervous systems plays a pivotal role in the pathophysiology of MDD [37]. This mental disorder is characterized by activation of different

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immune cell phenotypes, including M1 macrophages with elevated levels of interleukin (IL)-1β and tumor-necrosis factor (TNF)-α, T helper (Th)1, Th2 (with increased levels of CCL11 or eotaxin) and T regulatory (Treg) (with increased production of IL-10) cells [38]. As such, MDD is characterized by increased production of pro-inflammatory and neurotoxic products (IL-1β, TNF-α, CCL11) as well as immune-regulatory (IL-10) cytokines, which downregulate the primary immune-inflammatory response and have protective properties [38]. The aggregate of all immune-regulatory components that exert negative feedback on the immune response is named the "compensatory immune-regulatory system" (CIRS) [38]. MDD is also accompanied by iron-related aberrations including the "anemia of inflammation" with lowered levels of iron and Tf, aberrations in the erythron, increased ferritin [39] and maybe ferroptosis [40]. Ferroptosis is a programmed cell death characterized by the accumulation of iron, lipid hydroperoxides and their metabolites in the cytosol, and peroxidation of polyunsaturated fatty acids in the plasma membrane [40-42]. Nevertheless, there are no data whether MDD in β-TM is associated with blood transfusions, iron overload or immune-inflammatory responses.

Hence, the present study was conducted to examine the iron-related and immune-inflammatory biomarkers of children with β-TM with and without MDD versus normal controls. The specific hypothesis is that MDD in children with β-TM is associated with an increased number of blood transfusions leading to increased IO which may cause inflammatory responses and, therefore, MDD.

Subjects and Methods

Participants

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One hundred and eleven Iraqi β-TM male and female children (aged 6-12 years) participated in the present study. The β-TM patients were recruited at the Thalassemia Unit at Al-Zahra'a Teaching Hospital, Najaf, Iraq. The diagnosis of β-TM was made by pediatricians according to the 2019 ICD-10-CM Diagnosis Code D56.1. The diagnosis was based on the clinical symptoms (including severe anemia, hepato-splenomegaly, and abnormal bone growth), hematological parameters (including hemoglobin <7g/dl; microcytic hypochromic RBCs with anisopoikilocytosis and increased reticulocyte percentage), and Hb HPLC (VARIANTTM β-Thalassemia Short Program).

Fifty-five apparently healthy children (28 male and 27 female) were selected as control group and their age ranges were comparable to that of the patients. None of these subjects was anemic or had an obvious systemic or inflammatory disease. We excluded patients or controls with splenectomy, systemic diseases such as diabetes mellitus, hypertension, renal failure or patients with overt inflammation defined as serum C-reactive protein (CRP) levels > 6mg/l. In addition, the exclusion of subjects with serum CRP > 6mg/l indicates that increased levels of ferritin are probably associated with IO rather than with an acute phase response [43].

All patients were treated with blood transfusions as a part of their treatment regimen. All patients had regular blood transfusion units of packed RBCs at two to four week intervals as needed in order to maintain the hemoglobin levels above 9 g/dL. Moreover, patients were treated with iron-chelating therapy with deferoxamine mesylate USP (Desferal®) infusions at a dose range between 25-50 mg/kg/day over 8 hours/day depending on the ferritin levels (3-5 times/week). Thalassemia patients were given vitamin C to facilitate the binding of Fe to Desferal through increased Fe release from the reticuloendothelial system. Folic acid was given to reduce ineffective

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erythropoiesis. The patients were administered One-alpha capsules when the patients had calcium metabolism disorder due to reduced 1-α hydroxylation.

Symptoms of depression were measured using the Children's Depression Inventory (CDI), a self-rating screening tool with 27 items rated on a 3-point scale [44]. For each item, the children are asked to select the response that best describes their feelings in the preceding two weeks. The total CDI score was used as an index of severity of depression and children were considered depressed when the total CDI score was ≥19. The CDI score is validated in children and is one of the most commonly used screening methods for childhood depression [45]. β-TM patients were classified into two groups according to the presence or absence of MDD. The β-TM with MDD subgroup comprised 54 patients (29 male, and 25female) while the β-TM without MDD subgroup comprised 57 patients (24 male, and 33 female).

Written informed consent was obtained from the patients' first-degree relatives (mother or father). The study was approved by the IRB of University of Kufa number 487/2018.

Assays

In the early morning hours (between 8.00 and 10.00 a.m.) fasting venous blood was collected in all participants. Blood was left at room temperature for 15 min for clotting, centrifuged 3000 rpm for 10 min, and then serum was separated and transported into Eppendorf tubes to be stored at -80 °C until analyzed. Serum iron was measured spectrophotometrically by ferrozine method (Linear®, Spain). TIBC was measured by saturation of serum transferrin with iron and the unbound Fe portion is precipitated with magnesium carbonate. The total amount of iron is then determined by the ferrozine method. Serum ferritin levels were measured by using the VIDAS ferritin assay, an enzyme-linked fluorescent immunoassay (ELFA) performed in an automated

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instrument (BioMérieux Co., France). The interassay CV% of ferritin was <5.70% and for iron <2.19%. Unsaturated Iron binding capacity (UIBC) was calculated from the following formula: UIBC= TIBC - serum iron concentration [46]. Transferrin saturation percentage (TS%) was calculated from the following equation: TS% = Iron × 100/TIBC [47]. Transferrin concentrations were estimated from the percentage of transferrin saturation and serum iron using the formula: Tf (g/L) = serum iron (μmol/L) / (3.98 X TS%). The formula is based on the maximal binding of 2 mol Fe^{3+}/mol of transferrin and a molecular weight of 79,570 for transferrin [43].

Commercial ELISA sandwich kits (Elabscience, Inc. CA, USA) were used to measure serum CCL11, IL-1β, IL-10 and TNF-α using an ELISA microplate reader from Biotek (USA) and Biotek software ($KC4^{TM}$). No significant cross-reference with analogues is observed. For samples with highly concentrated biomarkers, we used sample dilution as required. All assyas were run in duplicate. The intra-assay coefficients of variation (CV) (precision within-assay) were < 7.0%. There were no missing values in the data set. Serum CRP was measured using a kit supplied by Spinreact®, Spain. The test is based on the principle of latex agglutination.

Statistical analysis

Analysis of variance (ANOVA) was employed to assess differences in scale variables between diagnostic categories and analysis of contingency tables (χ^2-test) was used to check associations between nominal variables. Associations among variables were computed using Pearson's product-moment and Spearman's rank-order correlation coefficients. We used multivariate general linear model (GLM) analysis to delineate the effects of diagnosis (3 groups: β-TM with and without MDD and controls) while controlling for background variables including age and sex. Protected LSD tests were used to check pairwise comparisons among treatment

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means. Model-generated estimated marginal mean (SE) values were computed after adjusting for covariates. Multiple comparisons and correlations were corrected for false discovery rate (FDR) [48]. Multiple regression analysis was used to delineate the significant biomarkers that are associated with the CDI total score and the results of those analyses were checked for multicollinearity (tolerance and VIF values) and homoscedasticity (White and Breusch-Pagan tests). Binary logistic regression analysis was used to delineate the important explanatory variables that predict MDD (versus no MDD as reference group). All analyses were bootstrapped (2000 bootstrap samples) and the bootstrapped results are reported in case there would be a difference between the classical and bootstrapped approach. In order to normalize the data distribution of the immune and iron biomarkers (tested with the Kolmogorov-Smirnov test) we processed IL-1β in Ln transformation and TNF-α, CCL11, UIBC and ferritin in square root (sqr) transformation. We computed two relevant z unit-weighted composite scores, namely comp1 (indicating M1 macrophage activation) computed as z sqr TNF-α + z Ln IL-1β; and comp2 (indicating M1/CIRS ratio) computed as z (z sqr TNF-α + z Ln IL-1β) – z IL-10. All tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25, 2017.

In order to examine (putative) causal associations between input variables (biomarkers and number of blood transfusions) and the CDI total score (output variable) we used Partial Least Squares (PLS) path modeling using the SmartPLS software [49]. SmartPLS uses structural equation modeling to examine causal paths connecting indicator variables or latent vectors (LV) extracted from a number of indicators [49]. Complete and consistent PLS path analysis was conducted only when the model and latent constructs complied with specific quality criteria, namely the model SRMR < 0.08; all LVs show adequate reliability validity as indicated by

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Cronbach's alpha > 0.7, composite reliability > 0.7, rho_A > 0.8, average variance extracted (AVE) > 0.5; and all outer model factor loadings > 0.6 at p<0.001 [49]. Using complete consistent PLS (5000 bootstrap samples), path coefficients with exact p-values and total effects, total indirect and specific indirect effects are computed.

Results.

1. Socio-demographic data

Table 1 shows the socio-demographic and clinical data in the children participating in the current study. There were no significant differences in age, sex ratio, rural/urban ratio between healthy children and β-TM children with and without MDD. The in- vs outpatient ratio did not differ between both β-TM subgroups. β-TM children with MDD had significantly more blood transfusions than those without MDD. The CDI score was significantly different between the three subgroups and increased from controls → β-TM without MDD and → β-TM with MDD. All β-TM children were treated with Desferal while there were no significant differences between both β-TM groups with and without MDD in use of vitamin C and one-alpha drug. All patients with β-TM without MDD were treated with folic acid, while four of the depressed β-TM patients did not receive folic acid.

In the total study group, there were significant associations between the number of blood transfusions and TNF-α (r=0.436, p<0.001, n=164), IL-1β (r=0.490, p<0.001), IL-10 (r=0.301, p<0.001), and the composite scores Comp1 (r=0.546, p<0.001) and Comp2 (r=0.199, p=0.011). There were also significant correlations between the number of transfusions and iron (r=0.487, p<0.001), UIBC (r=-0.392, p<0.001), TS% (r=0.515, p<0.001) and ferritin (r=0.713, p<0.001).

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2. Iron status and β-TM with and without MDD

Table 2 shows the results of multivariate GLM analysis with the 6 iron status variables (Fe, TIBC, UIBC, TS%, Tf and ferritin) as dependent variables and diagnosis (controls versus β-TM without MDD versus β-TM with MDD) as explanatory variables while adjusting for age and sex. We found that there was a significant association between diagnosis and iron status variables with an effect size of 0.569 while there were no significant effects of age and sex. Tests for between-subject effects showed that there were significant associations between diagnosis and iron (effect size: 0.411), UIBC (effect size: 0.251), TS% (effect size: 0.457) and ferritin (effect size: 0.751). The mean ferritin values were very high in both β-TM groups (> 2000 ng/mL) and all patients had serum ferritin values > 1000 ng/mL, except three cases (834, 924 and 884 ng/mL).

Table 3 shows the model-generated estimated mean (SE) values of the iron state variables in the three study groups. Iron was significantly increased in β-TM versus normal controls. TS%, ferritin, and UIBC showed significant differences between the three study groups with TS% and ferritin increasing from controls → β-TM without MDD → β-TM with MDD, while UIBD decreased from controls to β-TM with MDD. Regression #2 in Table 2 shows that there were no significant effects of the number of blood transfusions on the iron status data in subjects with β-TM.

3. Immune variables and β-TM with and without MDD

Table 4 shows the results of multivariate GLM analysis with the 6 immune markers (IL-1β, TNF-α, CCL11, IL-10, and both composite scores) as dependent variables and diagnosis (the same three groups as in Table 2) as explanatory variables while adjusting for age and sex. Table 4 shows that there was a significant association between diagnosis and immune markers with an

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effect size of 0.372 while age and sex had no significant effects. Tests for between-subject effects showed significant associations between diagnosis and TNF-α (effect size: 0.361), IL-1β (effect size: 0.375), CCL11 (effect size: 0.088), IL-10 (effect size: 0.189), Comp1 score (effect size: 0.507) and Comp2 score (effect size: 0.068). Table 3 shows the model-generated estimated marginal means of the immune markers in the three study groups. IL-1β and the Comp1 composite score were significantly different between the three groups and increased from controls → β-TM without MDD → β-TM with MDD. TNF-α and IL-10 were significantly higher in both β-TM groups as compared with controls, while CCL11 was significantly lower in both β-TM groups as compared with controls. The composite score Comp2 was significantly higher in children with β-TM with MDD than in controls, while β-TM children without MDD occupied an intermediate position.

4. Prediction of MDD using iron status and immune biomarkers

Table 5 shows the results of three different binary logistic regression analysis with β-TM with MDD as dependent variable (and β-TM without MDD as the reference group). The first regression analysis in Table 5 shows that MDD was significantly predicted by the number of transfusions with an odds ratio of 2.93 and a Nagelkerke effect size of 0.180. Regression #2 shows the outcome of binary regression analysis with number of transfusions and all 6 iron state variables (and age and sex) as explanatory variables. We found that MDD was best predicted by the number of transfusions and TS% with a Nagelkerke value of 0.233; 77.5% of all β-TM children were correctly classified with a sensitivity of 70.4% and a specificity of 84.2%.

Regression #3 shows the outcome of a regression analysis with number of transfusions and TS% together with the 4 cytokine/chemokine levels and age and sex as explanatory variables. We

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found that number of transfusions, TS% and IL-1β best predicted MDD with a Nagelkerke effect size of 0.286 whereby 77.8% of all β-TM children were correctly classified with a sensitivity of 72.2% and a specificity of 77.2%.

5. Iron status, immune biomarkers, and the CDI

Pearson's correlation analyses performed in the total study group showed significant associations between the CDI score and IL-1β ($r=0.561$, $p<0.001$, all $n=164$), TNF-α ($r=0.531$, $p<0.001$), IL-10 ($r=0.319$, $p<0.001$), CCL11 ($r=-0.185$, $p=0.018$), and the comp1 ($r=0.644$, $p<0.001$) and comp2 ($r=0.264$, $p=0.001$) composite scores. In the restricted study group of subjects with β-TM there were additional associations between the CDI score and IL-1β ($r=0.262$, $p=0.005$, $n=111$) and comp1 score ($r=0.285$, $p=0.002$). Pearson's correlation analyses performed in the total study group also showed significant associations between the CDI score and Fe ($r=0.544$, $p<0.001$, all $n=164$), UIBC ($r=-0.411$, $p<0.001$), TS% ($r=0.563$, $p<0.001$), and transferrin ($r=-0.727$, $p<0.001$), although no significant associations were detected in the restricted study group of β-TM children.

Table 6 shows the outcome of different multiple regression analysis with CDI or cytokines/chemokines as dependent variables. Regression #1 shows the effects of iron variables on the CDI score with ferritin and iron levels explaining 55.9% of the variance in the CDI. Regression #2 shows the effects of the number of transfusions and iron state variables on the CDI with both ferritin and iron coupled with number of transfusions explaining 65.3% of the variance in the CDI score. In regression #3 we examine the effects of number of transfusions and immune biomarkers on the CDI and found that 64.2% of the variance in the CDI was explained by the combined effects of number of transfusions and the composite score C1. In regression #4 we

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examine the combined effects of the significant explanatory variables (as established in regressions #1, #2 and #3) on the severity of depression scale score and found that 68.0% of the variance in CDI score was explained by number of transfusions, the composite score Comp1, ferritin, and iron. Regression #5 shows that in the restricted study sample of children with β-TM, the CDI score was predicted by number of transfusions combined with the Comp1 composite score and in addition use of folic acid (inversely associated), whereas the iron state variables had no significant effect.

Regressions #6 - #10 show the outcome of multiple regression analyses with the immune biomarkers as dependent variables and the iron state variables and number of transfusions (and age and sex) as explanatory variables. We found that 43.9% of the variance in the composite score Comp1 was explained by the regression on ferritin and TS%. Regression #7 shows that 32.4% of the variance in IL-1β was explained by the number of transfusions, ferritin, and TS%. TNF-α, IL-10 (both positively) and CCL11 (inversely) were all three associated with ferritin levels. After removing ferritin from the analysis, it appeared that TS% was the second most important predictor of these cytokines/chemokines.

6. Results of PLS analysis

Figure 1 shows the results of a PLS path analysis with the CDI score as output variable while the number of transfusions, iron load, the composite score Comp1, CCL11, and IL-10 are entered as input variables. Those input variables were entered as single indicators except iron load, which was entered as a latent vector (LV) extracted from ferritin, iron and TS%. The overall model fit was adequate with SRMR=0.035, and the construct reliability validity of the LV was good with Cronbach α= 0.849, composite reliability = 0.843, rho_A = 0.878, and average extracted variance = 0.648 while all outer loadings of the LVs were > 0.677 (all at $p<0.001$). We found that 68.8% of

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the variance in the CDI score was explained by the direct effects of number of transfusions, iron load, and the composite score comp1. There were also direct effects of iron load on IL-10 (positively) and CCL11 (inversely) but these 2 cytokines/chemokines were not associated with the CDI score. Moreover, 52.0% of the variance in the iron load LV was explained by number of transfusions. There were specific indirect effects of number of transfusions on the CDI score mediated by iron load LV ($t=+3.84$, $p<0.001$) and by the path from iron load to the composite score comp1 ($t=2.88$, $p=0.004$). There were also specific indirect effects of number of transfusions on CCL11 ($t=-2.63$, $p=0.009$), IL-10 ($t=6.13$, $p<0.001$) and the composite score comp1 ($t=+8.42$, $p<0.001$), which were all mediated by the iron load LV. Finally, there was also an indirect effect of the iron load LV on the CDI score which was mediated by comp1 ($t=2.88$, $p=0.004$). As such, the CDI score was highly significantly predicted (total effects) by iron load LV ($t=7.29$, $p<0.001$), number of transfusions ($t=19.12$, $p<0.001$) and comp1 ($t=2.94$, $p=0.003$). The latter was highly significantly predicted by number of transfusions (total effects: $t=8.42$, $p<0.001$) and iron load ($t=11.97$, $p<0.001$).

Discussion

The first major finding of this study is that in β-TM there is a significant association between the number of blood transfusion units and MDD and severity of depression. As described in the Introduction, lifelong blood transfusion therapy is the basic treatment for β-TM although multiple transfusions inevitably cause severe IO resulting in progressive organ failure, alloimmunization, and increased risk of infections [50, 51]. The frequency of blood transfusions is a significant factor that may influence psychological disorders such as MDD and anxiety and lowered quality of life in patients with β-TM [52, 53].

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Our study showed that blood transfusions were significantly associated with increased IO in β-TM, including higher serum iron, TS%, and ferritin as compared with controls. Our results are in line with previous results reporting a significant elevation in serum iron in β-TM patients as compared with healthy controls [25, 54]. The predominant mechanisms driving the iron loading process includes transfusion therapy and enhanced intestinal absorption as a consequence of the impairments in erythropoiesis [51]. Increased ferritin levels are associated with iron-related damage to tissues including liver and heart [54], which is not only related to increased oxidative stress but also to the direct effects of IO on the tissues.

The second major finding of this study is that a) MDD in patients with β-TM and severity of illness are significantly predicted by IO markers including increased iron, ferritin, and TS% levels; and b) the effect of number of transfusions on severity of illness are partially mediated by those IO biomarkers. Previously, it was detected that higher body iron may be associated with more depressive symptoms in young adult men [55]. Interestingly, MDD in adults is accompanied by the anemia of inflammation with lowered iron stores, lowered Tf levels, increased ferritin levels and aberrations in the erythron including lower number of erythrocytes, and a reduced hematocrit and hemoglobin [39]. Moreover, these alterations in iron status and the erythron were significantly associated with immune-inflammatory biomarkers such as lowered zinc and albumin [39]. Similar findings were reported by Rybka et al. (2013) who reported a decreased hematocrit and hemoglobin in association with increased inflammatory biomarkers including IL-6 in MDD [56]. These findings indicate that the immune-inflammatory response in MDD may be accompanied by increased ferritin, lowered Tf levels (both inflammatory markers) and secondary anemia with a lowered iron status. While those changes in iron status in MDD are secondary to immune

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activation, the current study found that increased iron load may be a cause of MDD in β-TM patients who underwent repeated blood transfusions.

TM patients are dependent on regular blood transfusions which are applicated to attenuate the complications of anaemia and expand bone marrow, although repeated blood transfusions are associated with excessive iron absorption, IO, a chronic hypoxic state, and neurotoxicity due to lifelong chelating therapy while all these factors together may lead to brain dysfunction [57]. Metafratzi et al. (2001) reported that, in patients with β-TM, increased iron deposition may be observed in the putamen, caudate nucleus, and motor and temporal cortex, which are important for cognitive functions including implicit and explicit memory [58]. The damage to the nervous system in those patients may be attributed to many factors acting as cumulative minor injuries over many years including hemolysis and repeated blood transfusions, which lead to a decrease in nitric oxide levels and IO in the brain [59]. The latter, in turn, may lead to oxidative stress aggravating brain tissue damage [60]. Moreover, accumulation of intracellular iron may cause DNA fragmentation, which may ultimately damage lymphocyte functions, leading to immune dysfunction and increased susceptibility to infections in β-TM patients [61].

The third major finding of this study is that IL-1β, TNF-α, and IL-10 (but not CCL11) were higher in β-TM patients as compared with controls, indicating a simultaneous immune-inflammatory response with an activated M1 macrophage profile and activation of the CIRS. Higher levels of both TNF-α and IL-1β were previously observed in the serum of β-TM patients [62, 63]. Our findings extend those of Balouchi et al. [64] who reported that serum levels of pro-inflammatory (e.g. IL-23), as well as immune-regulatory (e.g. transforming growth factor-β) cytokines, were elevated in β-TM patients as compared with controls. These findings are also in agreement with recent findings suggesting a double-faced response of cell-mediated immunity in

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β-TM patients, namely that T cells show a stimulated phenotype while in fact their activity is suppressed [65]. As described in the Introduction, IO is associated with immune-inflammatory responses, M1 macrophage polarization and increased TNF-α expression due to IO and antigenic stimulation induced by chronic transfusion therapy [18-20]. Moreover, chelation therapy may further increase the inflammatory burden by lowering the iron pool thereby augmenting the transcription of IL-1β [66]. Multiple blood transfusions in β-TM patients may explain that the immune system is under constant alloantigen stimulation, despite the suppressed immune responses due to IO [67]. IO also causes neuroinflammation that leads to the upregulation of divalent metal transporter-1 on the surface of astrocytes, microglia and neurones, making them highly sensitive to IO in the presence of high levels of non-transferrin bound iron [40].

The fourth major finding of this study is that MDD in β-TM is characterized by increased levels of IL-1β and M1 activation as compared with β-TM patients without MDD and that the M1/CIRS ratio was significantly increased in β-TM with MDD versus controls while those with β-TM without MDD occupied an intermediate position. Moreover, the CDI score was significantly associated with both pro-inflammatory cytokines, the M1 macrophage activation index and the M1/CIRS ratio. There is now evidence that MDD is an immune-inflammatory disorder and that increased levels of TNF-α and IL-1β are among the most commonly reported immune aberrations in MDD [38, 68, 69]. Although IL-10 was increased in our β-TM patients, those levels were not associated with MDD. This contrasts with MDD in adults where increased IL-10 levels are frequently observed [38]. As discussed previously, increased levels of TNF-α and IL-1β may participate in the pathophysiology of MDD as both cytokines exert neurotoxic effects, alter the activity of neurons, and may contribute to neuroprogression and neuronal death (70-73). IL-10 secretion plays a key role in protecting cells from IO injury by promoting the secretion of

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intracellular hepcidin to reduce intracellular iron content [74]. Therefore, it could be suggested that the lack of an appropriate IL-10 response in children with β-TM and MDD contributes to a greater impact of the neurotoxic effects of TNF-α and IL-1β. Moreover, the increased levels in TNF-α without a simultaneous response in IL-10 may cause decreased differentiation of erythroid cells leading to exacerbation of ineffective erythropoiesis in β-TM [75]. Activated macrophages may selectively phagocytose apoptotic erythroid precursors, thereby contributing to ineffective erythropoiesis [76]. Although an increase in CCL11 is sometimes reported in MDD patients [77-79], this study found no specific alterations in this cytokine in β-TM with MDD.

Limitations of the study.

Firstly, this is a case-control study and, therefore, the possible causal associations established here should be checked in future prospective studies. Second, it would have been more interesting if we had measured a complete panel of pro-inflammatory and CIRS cytokines as well as oxidative and nitrosative stress biomarkers. Third, our results show that treatment with folic acid may have some protective effects by attenuating the CDI score. Nevertheless, our results show that current treatment modalities with folic acid and vitamin C are insufficient to attenuate IO and immune-inflammatory responses and, therefore, to prevent MDD. Future research should examine the effects of other natural anti-inflammatory and anti-oxidant substances (NAIOSs) including curcumin and N-acetyl-cysteine [80] in preventing the onset of MDD in children with β-TM.

Conclusions

Figure 1 shows the results of a PLS analysis and in fact summarizes the findings of the present study. Thus: a) activated immune-inflammatory pathways together with iron overload and

an increased number of blood transfusions explain 68.8% of the variance in the CDI score (and by inference are associated with MDD); b) immune activation mediates in part the effects of iron overload on the CDI score; c) the effects of blood transfusions on the CDI score are in part mediated by effects of iron overload and the path from iron load to immune activation; and d) blood transfusions have also direct effects on the CDI score suggesting that other factors not related to iron overload or immune activation may be involved, for example, psychosocial stressors. Regular screening for depressive symptoms in patients with β-TM undergoing blood transfusions is indicated to provide appropriate medical and psychological support to improve both emotional and physical health [36]. Clinicians should be aware that IO may be a possible cause of psychiatric illness including MDD and that IO may be treated using chelation therapy [81].

Acknowledgment

We thank the staff of the Thalassemia Unit at Al-Zahra'a Teaching Hospital-Najaf city-Iraq for their help in the collection of samples. We also acknowledge the work of the highly-skilled staff of the Asia Laboratory in measuring the biomarkers.

Funding

There was no specific funding for this specific study.

Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

 doi:10.20944/preprints201909.0033.v1

Author's contributions

All the contributing authors have participated in preparation of the manuscript.

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Figure 1. Results of Partial Least Squares path analysis with the CDI (Children's Depression Inventory) score as dependent variable.

Iron load is entered as a latent vector extracted from ferritin, iron and TS% (transferrin saturation percentage) levels.

IL: interleukin

TNF: tumor necrosis factor

zIL1b+zTNFa: this z unit-weighted composite score was entered as a single indicator and was computed as z transformation of IL-1β + z TNF-α.

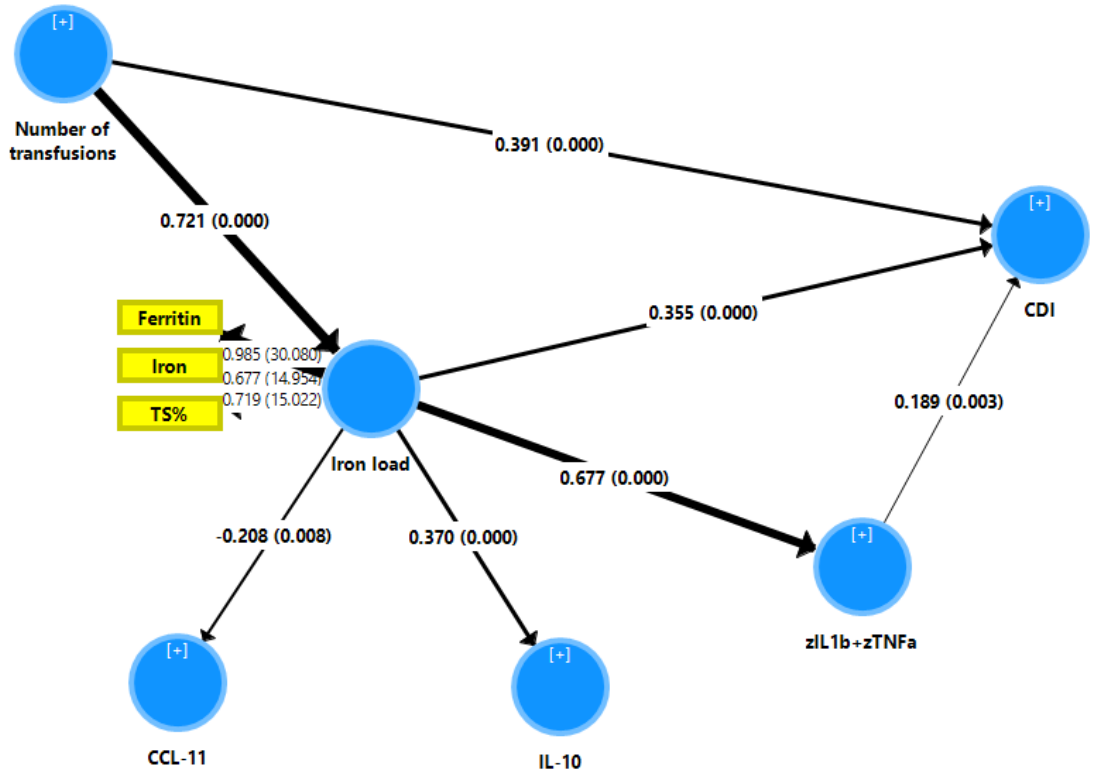

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Table 1. Sociodemographic and clinical data in patients with β-thalassemia major (β-TM) with and without depression (MDD) and healthy controls (HC).

Variables	HC [A]	β-TM-MDD [B]	β-TM+MDD [C]	F / χ^2	df	p
Age (years)	8.9 (1.9)	9.0 (2.4)	8.6 (2.2)	0.58	2 / 161	0.056
Sex (F/M)	27 / 26	33 / 24	25 / 29	1.52	2	0.468
Rural/Urban ratio	10 / 43	17 / 40	17 / 37	2.57	2	0.277
In- / outpatients	-	2 / 55	4 / 50	Ψ= 0.082	-	0.364
Number blood transfusions	-	63.9 (31.1) [C]	91.2 (38.0) [B]	17.24	1 / 109	<0.001
Children's Depression Inventory	3.7 (1.5) [B,C]	11.8 (2.6) [A,C]	21.5 (3.2) [A,B]	640.4	2 / 161	<0.001
Treatment with Desferal	-	57	54	-	-	-
Treatment with vitamin C (N/Y)	-	11 / 46	9 / 45	0.13	-	0.718
Treatment with folic acid (N/Y)	-	0 / 57	4 / 50	Ψ = 0.191	-	0.030
Treatment with 1-Alpha (N/Y)	-	13 / 44	9 / 45	Ψ = 0.077	-	0.417

All results are shown as mean (SD). [A,B,C]: pairwise comparisons between group means

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Table 2. Results of multivariate GLM analysis: associations between diagnosis (3 groups: β-thalassemia major with and without depression and normal controls) and iron (Fe) status variables.

Tests	Dependent variables	Explanatory variables	F	df	p	Partial η^2
#1. Multivariate GLM	Iron, TIBC, UIBC, TS%, Tf, ferritin	Diagnosis	33.91	12 / 308	<0.001	0.569
		Sex	1.17	6 / 154	0.327	0.044
		Age	0.81	6 / 154	0.563	0.031
Test for between-subject effects	Iron	Diagnosis	55.36	2 / 159	<0.001	0.411
	TIBC	Diagnosis	1.72	2 / 159	0.182	0.021
	UIBC	Diagnosis	26.70	2 / 159	<0.001	0.251
	TS%	Diagnosis	66.87	2 / 159	<0.001	0.457
	Tf	Diagnosis	1.88	2 / 159	0.156	0.023
	Ferritin	Diagnosis	239.22	2 / 159	<0.001	0.751
#2. Multivariate GLM	Iron, TIBC, UIBC, Ts%, Tf, Ferritin	#Transfusion	1.06	5 / 103	0.387	0.049

TIBC: Total iron binding capacity

UIBC: Unsaturated iron binding capacity

TS%: Transferrin saturation percentage

Tf: Transferrin

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Table 3. Model-generated estimated marginal means values of iron and immune variables in patient with β-thalassemia major with (β-TM + MDD) and without (β-TM – MDD) depression and healthy controls (HC).

Variables	HC [C]	B-TM - MDD [B]	B-TM + MDD [A]
Fe µM	21.4 (1.5) [B,C]	38.0 (1.4) [A]	41.5 (1.9) [A]
Fe in z score	-0.911 (0.107) [B,C]	0.310 (0.103) [A]	0.568 (0.106) [A]
TIBC µM	59.0 (1.8)	63.5 (1.8)	62.8 (1.8)
TIBC in z score	-0.205 (0.136)	0.122 (0.131)	0.072 (0.135)
UIBC in z score after sqr trans	0.691 (0.120) [B,C]	-0.164 (0.116) [A,C]	-0.505 (0.119) [A,B]
TS%	36.2 (2.0) [B,C]	60.7 (1.9) [A,C]	67.2 (2.0) [A,B]
TS% in z score	-0.954 (0.102) [B,C]	0.295 (0.099) [A,C]	0.625 (0.102) [A,B]
Tf mg/l	147.8 (4.6)	159.4 (4.5)	157.7 (4.6)
Tf in z score	-0.214 (0.135)	0.127 (0.131)	0.076 (0.135)
Ferritin in z score after sqr trans	-1.244 (0.069) [B,C]	0.496 (0.067) [A,C]	0.698 (0.069) [A,B]
TNF-α in z score after sqr trans	-0.861 (0.111) [B,C]	0.337 (0.107) [A]	0.489 (0.110) [A]
IL-1β in z score after Ln trans	-0.853 (0.110) [B,C]	0.227 (0.106) [A,C]	0.598 (0.109) [A,B]
CCL11 in z score after sqr trans	0.397 (0.131) [B,C]	-0.309 (0.127) [A]	-0.063 (0.131) [A]
IL-10 pg/ml	13.6 (4.1) [B,C]	45.9 (4.0) [A]	42.1 (4.1) [A]
IL-10 in z score	-0.621 (0.125) [B,C]	0.352 (0.121) [A]	0.238 (0.124) [A]
Comp1: zTNFα + zIL-1β	-1.011 (0.098) [B,C]	0.332 (0.095) [A,C]	0.641 (0.097) [A,B]
Comp2: z(zTNFα+ zIL-1β) - zIL-10	-0.393 (0.163) [C]	-0.022 (0.160)	0.401 (0.164) [A]

All results are shown as mean (SE). [A,B,C]: pairwise comparisons between group means

Fe: Iron; TIBC: Total iron binding capacity; UIBC: Unsaturated iron binding capacity; TS%: Transferrin saturation percentage; Tf: Transferrin;

TNF: Tumor necrosis factor; IL: Interleukin; CCL11: Eotaxin; Comp1 and Comp2: Two z unit-weighted composite scores indicating M1 macrophage activation (comp1) and M1/CIRS ratio (comp2).

Data that were Ln or square root (sqr) transformed (trans) are shown as mean (SE) z scores, while all other data are shown as mean (SE) values and z scores (mean, SE) as well.

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Table 4. Results of multivariate GLM analysis: association between diagnosis (3 groups: β-thalassemia major with and without depression and normal controls) and immune biomarkers.

Tests	Dependent variables	Explanatory variable	F	df	p	Partial η^2
#1. Multivariate GLM	TNF-α, IL-1β, CCL11, IL-10, Comp1, Comp2	Diagnosis	23.09	8 / 312	<0.001	0.372
		Sex	0.83	4 / 156	0.508	0.021
		Age	1.16	4 / 156	0.329	0.029
Tests for between-subjects effects	TNF-α	Diagnosis	44.99	2 / 159	<0.001	0.361
	IL-1β	Diagnosis	47.63	2 / 159	<0.001	0.375
	CCL11	Diagnosis	7.64	2 / 159	0.001	0.088
	IL-10	Diagnosis	18.47	2 / 159	<0.001	0.189
	Comp1	Diagnosis	81.67	2 / 159	<0.001	0.507
	Comp2	Diagnosis	5.84	2 / 159	0.004	0.068
#2. Multivariate GLM	TNF-α, IL-1β, CCL11, IL-10, Comp1, Comp2	Number of transfusions	0.75	4 / 104	0.559	0.028

TNF-α: tumor necrosis factor

IL: interleukin

Comp1: computed as zTNFα + zIL-1β indicating M1 macrophage activation

Comp2: computed as z(zTNFα + zIL-1β) - zIL-10 indicating the M1/CIRS ratio

 doi:10.20944/preprints201909.0033.v1

Table 5. Results of binary logistic regression analysis with β-thalassemia major with major depression (MDD) as dependent variable and no MDD as reference group.

Dependent variables	Explanatory variables	B	SE	Wald	df	p	OR	95% CI
#1. MDD vs. No-MDD	#Transfusions	1.073	0.294	13.45	1	<0.001	2.93	1.64-5.20
#2. MDD vs. No-MDD	#Transfusions	1.120	0.306	13.41	1	<0.001	3.07	1.68-5.59
	TS%	0.595	0.269	4.91	1	0.027	1.81	1.07-3.07
#3. MDD vs. No-MDD	#Transfusions	1.16	0.320	13.26	1	<0.001	3.20	1.71-5.60
	TS%	0.614	0.277	4.92	1	0.027	1.84	1.07-3.18
	IL-1β	0.631	0.280	5.10	1	0.024	1.88	1.09-3.25

#Transfusions: total number of blood transfusion units

TS%: Transferrin saturation percentage

IL: interleukin

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Table 6. Results of multiple regression analysis with the Children's Depression Inventory (CDI) as dependent variable and iron status and immune biomarkers, number of transfusions and use of vitamin C as explanatory variables.

Dependent variables	Explanatory variable	β	t	p	F model	df	p	R^2
#1. CDI in all subjects	Model				181.43	2/161	<0.001	0.559
	Ferritin	0.612	9.78	<0.001				
	Iron	0.208	3.30	0.001				
#2. CDI in all subjects	Model				100.38	3/160	<0.001	0.653
	#Transfusions	0.444	6.60	<0.001				
	Ferritin	0.329	4.68	<0.001				
	Iron	0.147	2.61	0.010				
#3. CDI in all subjects	Model				144.08	2/161	<0.001	0.642
	#Transfusions	0.569	10.10	<0.001				
	Comp1 (zTNF+ zIL-1β)	0.333	5.91	<0.001				
#4. CDI in all subjects	Model				84.29	4/159	<0.001	0.680
	#Transfusions	0.411	6.26	<0.001				
	Comp1(TNF+IL-1β)	0.217	3.63	<0.001				
	Ferritin	0.231	3.16	0.002				
	Iron	0.116	2.11	0.036				
#5. CDI in thalassemia major	Model				18.97	1/109	<0.001	0.148
	#Transfusions	0.367	4.37	<0.001				
	Comp1 (zTNF+ zIL-1β)	0.220	2.60	0.011				
	Folic acid	-0.204	-2.41	0.018				
Comp1 (zTNFα + zIL-1β) in all subjects	Model				62.99	2/161	<0.001	0.439
	Ferritin	0.514	7.00	<0.001				
	TS%	0.213	2.90	<0.001				
IL-1β in all subjects	Model				25.60	3/160	<0.001	0.324
	Ferritin	0.249	2.48	0.014				

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	TS% #Transfusions	0.206 0.206	2.52 2.20	0.013 0.030				
TNF-α in all subjects	Model Ferritin	 0.567	 8.77	 <0.001	76.95	1/162	<0.001	0.322
CCL11 in all subjects	Model Ferritin	 -0.24	 3.02	 0.003	9.14	1/162	0.003	0.053
IL-10 in all subjects	Model Ferritin	 0.433	 6.11	 <0.001	37.34	1/162	<0.001	0.187

Comp1: z unit-weighted composite score indicating M1 macrophage activation.

TNF: tumor necrsis factor

IL: interleukin

TS%: Transferrin saturation percentage